

# Nuclear Magnetic Resonance Studies of Two-Iron-Two-Sulfur Ferredoxins.

## 1. Properties of the Histidine Residues<sup>†</sup>

Tze-Ming Chan<sup>‡</sup> and John L. Markley\*

**ABSTRACT:** The <sup>1</sup>H and <sup>13</sup>C NMR peaks from the imidazole rings of the two histidine residues in *Anabaena variabilis* ferredoxin II (histidine-16 and histidine-93) were assigned by reference to spectra of the homologous ferredoxin from *Spirulina maxima* which contains only one histidine residue (histidine-93). The conserved histidine-93 has unusual properties: its pK<sub>a</sub>' in the oxidized protein is abnormally low (5.3 in *A. variabilis* ferredoxin and 5.9 in *S. maxima* ferredoxin); its chemical shifts do not change with temperature but

do depend on the oxidation state of the protein. Histidine-16 of *A. variabilis* ferredoxin is normal: its pK<sub>a</sub>' in the oxidized protein is 7.0; its chemical shifts change with temperature as expected for a normal heat of ionization, and its chemical shifts are not affected by reduction of the protein. These results indicate that histidine-93 is influenced strongly by the tertiary structure of the protein but that histidine-16 is normal and exposed.

**T**wo-iron-two-sulfur ferredoxin is a component of the photosynthetic electron transport chain in cyanobacteria and in chloroplasts of higher plants (Trebst & Avron, 1977). The structure and functions of this type of ferredoxin have been reviewed (Lovenberg, 1973-1977). Recently a 2.5-Å resolution X-ray crystallographic structure of *Spirulina platensis* ferredoxin became available (Fukuyama et al., 1980; Tsukihara et al., 1981).

The few previous <sup>1</sup>H NMR studies of 2Fe-2S\* ferredoxins<sup>1</sup> (Poe et al., 1971; Salmeen & Palmer, 1972; Anderson et al., 1975), which only concerned the hyperfine-shifted resonances, were carried out with ferredoxins isolated from spinach, parsley, and a cyanobacterium, *Synechococcus lividus*, and were performed at 220 MHz or lower frequencies. We have used high-resolution <sup>1</sup>H and <sup>13</sup>C NMR to study structure-function relationships of several 2Fe-2S\* ferredoxins. Preliminary reports have been given (Chan et al., 1981, 1982; Chan & Markley, 1982). This paper describes the properties of the histidine residues, and the following papers present additional aspects of the research (Chan et al., 1983a,b; Chan & Markley, 1983a,b).

### Experimental Procedures

**Materials.** Ferredoxins from cyanobacteria, *Spirulina maxima* and *Anabaena variabilis*, were isolated in the laboratory. Cell paste of *S. maxima*, obtained from the commercial culture center of Sosa Texcoco Co., Lake Texcoco, Mexico, in the winter of 1977, was a gift from Dr. D. W. Krogmann of Purdue University. Cells of *A. variabilis* (both natural abundance and enriched in <sup>13</sup>C to 20%) were grown in our laboratory. Cell pastes were stored frozen and thawed just before purification of the proteins. Chemicals were from the following sources: <sup>2</sup>H<sub>2</sub>O (99.8% and 100% isotopic purity), Bio-Rad; carbon dioxide (20%, <sup>13</sup>C), Monsanto; Sephadex G-75 and DEAE-Sephadex (A-25), Pharmacia; Whatman

DEAE-cellulose (DE-52), Reeve Angell; <sup>2</sup>HCl and KO<sup>2</sup>H, Merck Sharp & Dohme; sodium dithionite (purified), J. T. Baker. All other chemicals used were reagent grade or the best commercially available.

**Growth of *Anabaena variabilis* Cells.** The procedure for growing *A. variabilis* was adapted from that used at the Los Alamos Scientific Laboratory (V. H. Kollman and C. T. Gregg, personal communication). The cells were grown in 80 L of medium C of Kratz & Myers (1955) in a water-cooled, rocking, Plexiglas tank (150 × 100 × 12 cm) with fluorescent lights above and below. Carbon dioxide (10% in air) was pumped into the tank through gas dispersion tubes. The passage of carbon dioxide was controlled automatically so as to keep the pH between 9.2 and 9.4. The pH of the culture increases when the cells carry out CO<sub>2</sub> fixation. When the pH rises above 9.4, a relay system turns on the flow of carbon dioxide which lowers the pH of the culture; when the pH falls below 9.2, the flow of carbon dioxide is shut off. The cells were harvested in mid-log phase by passing the culture through a continuous-flow rotor in a centrifuge. The cell paste was then frozen and stored. <sup>13</sup>C-Enriched cyanobacteria were grown in a similar manner with carbon dioxide (20% <sup>13</sup>C) as the sole carbon source.

**Isolation of Ferredoxins.** Crude ferredoxin was isolated from *A. variabilis* cells by the procedure of Ho et al. (1979). The fractions with A<sub>420</sub>/A<sub>276</sub> ratios higher than 0.45 were loaded on a long DEAE-Sephadex column (2.5 × 90 cm) equilibrated with 10 mM phosphate buffer, pH 7.5, containing 0.1 M NaCl. The column was washed with the same buffer containing 0.45 M NaCl and then eluted with a linear gradient of NaCl (0.55-0.65 M; total volume 4 L). Two brown bands were observed on the column. The first one eluted was the major band and is termed ferredoxin II according to convention (Cammack et al., 1977). The second band contained about 10% of the total ferredoxin and is termed ferredoxin I. Fractions of ferredoxin II with A<sub>420</sub>/A<sub>276</sub> ratios higher than 0.57 were considered to be pure. NMR studies were carried out on ferredoxin II; in this and the following papers, it is

<sup>†</sup> From the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. Received March 9, 1983; revised manuscript received July 21, 1983. This work was supported partially by a grant from the U.S. Department of Agriculture Competitive Research Grants Office, Cooperative State Research Service, Science and Education. 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. This research was carried out in partial fulfillment of the requirements for the Ph.D. degree by T.-M.C. (Chan, 1982).

<sup>‡</sup> Present address: Schering-Plough, Bloomfield, NJ 07003.

<sup>1</sup> Abbreviations: 2Fe-2S\*, the iron-sulfur center consisting of two iron atoms and two inorganic sulfur atoms; Fd, ferredoxin; EDTA, ethylenediaminetetraacetic acid; A<sub>420</sub>/A<sub>276</sub>, absorbance at 420 nm/absorbance at 276 nm; FID, free induction decay; o.d., outer diameter; NOE, nuclear Overhauser effect; DEAE, diethylaminoethyl; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; pH\*, pH meter reading of a solution in <sup>2</sup>H<sub>2</sub>O uncorrected for the deuterium isotope effect.

Table I: pH Titration Parameters for the Histidine Residues of Oxidized *Anabaena variabilis* and *Spirulina maxima* Ferredoxins at 22 °C from 470 MHz NMR Measurements

ferredoxin	assignment	$pK_a'$ or $pH_{mid}^a$	Hill coefficient	chemical shift (ppm) from DSS	
				low pH* plateau	high pH* plateau
<i>A. variabilis</i>	His <sup>93</sup> C $\epsilon$ -H	5.32 $\pm$ 0.01	0.71 $\pm$ 0.01	8.814 $\pm$ 0.007	7.745 $\pm$ 0.001
	His <sup>93</sup> C $\delta$ -H	5.26 $\pm$ 0.02	0.61 $\pm$ 0.01	7.585 $\pm$ 0.005	7.134 $\pm$ 0.001
	His <sup>16</sup> C $\epsilon$ -H	6.95 $\pm$ 0.02	0.84 $\pm$ 0.02	8.778 $\pm$ 0.007	7.727 $\pm$ 0.007
	His <sup>16</sup> C $\delta$ -H	7.05 $\pm$ 0.03		7.12 $\pm$ 0.01	6.80 $\pm$ 0.01
		5.50 $\pm$ 0.13 <sup>a</sup>		7.03 $\pm$ 0.01 <sup>a</sup>	7.12 $\pm$ 0.01 <sup>a</sup>
<i>S. maxima</i>	His <sup>93</sup> C $\epsilon$ -H	5.87 $\pm$ 0.04	0.82 $\pm$ 0.04	8.49 $\pm$ 0.02	7.49 $\pm$ 0.02
	His <sup>93</sup> C $\delta$ -H	6.00 $\pm$ 0.03	0.93 $\pm$ 0.04	7.42 $\pm$ 0.01	7.12 $\pm$ 0.01

<sup>a</sup> Spectroscopic perturbation caused by the titration of one or more neighboring groups.

referred to as *A. variabilis* Fd for simplicity.

A similar procedure was used to isolate ferredoxin from *S. maxima*, except that fractions having  $A_{420}/A_{276}$  ratios higher than 0.4 were loaded on the DEAE-Sephadex column. Two ferredoxins were obtained. Ferredoxin II was the major band (80% of the total ferredoxin), and protein fractions having  $A_{420}/A_{276}$  ratios higher than 0.53 were considered pure. The sequence of the first 20 amino acid residues from the N-terminus was identical with the published sequence, and the overall amino acid composition was identical with the published result (Tanaka et al., 1975). Again, NMR studies were carried out only on ferredoxin II, referred to here as *S. maxima* Fd.

**Preparation of NMR Samples.** Ferredoxin samples were dialyzed against a dilute phosphate buffer (~2 mM). The final volume of the ferredoxin solution was adjusted by adding the same buffer to give a final phosphate concentration of 50 mM when the lyophilized ferredoxin sample was dissolved in 0.5 mL of 100% <sup>2</sup>H<sub>2</sub>O. Ferredoxin solutions to be used for <sup>1</sup>H NMR were lyophilized 3 times from 99.8% <sup>2</sup>H<sub>2</sub>O to minimize the water signal and to exchange labile N-H groups. The pH values of the samples were determined and adjusted by methods described previously (Markley & Porubcan, 1976). They were not corrected for the deuterium isotope effect and hence are labeled pH\*. All pH\* measurements were made at room temperature. For <sup>13</sup>C NMR studies, the phosphate concentration of each sample was adjusted to 50 mM by the same procedure, and the solution was lyophilized.

**Reduction of Ferredoxin.** The ferredoxins as isolated were in the oxidized state. Samples were reduced by addition of solid sodium dithionite in the absence of oxygen. The reduction was performed in an anaerobic chamber manufactured by Plas-Labs, Lansing, MI. It consists of a large Plexiglas chamber and a small airlock compartment through which samples and equipment can be transferred in and out of the chamber. Residual oxygen in the chamber is removed by reaction with added hydrogen; the atmosphere in the chamber is circulated through three layers of palladium-coated alumina nuggets which are heated by the exothermic reaction. The water formed is removed by passing the atmosphere through silica gel and activated molecular sieve. Complete reduction of ferredoxin samples could not be achieved with dithionite at pH values below about pH 7, because dithionite has a reduction potential of about -300 mV at pH 7 whereas ferredoxin has a reduction potential around -400 mV. Therefore, the pH\* of the sample was raised to about 8.5, and excess solid dithionite was added. The pH\* of the solution was measured in the usual manner inside the anaerobic chamber.

**<sup>1</sup>H NMR Spectroscopy.** The Nicolet NT-360 spectrometer formerly in the Purdue University Biochemical Magnetic Resonance Laboratory was used to obtain 360-MHz Fourier-transform spectra. The standard one-pulse sequence was

used with a 90° pulse (10  $\mu$ s). Free induction decays (FIDs) were obtained by using quadrature detection and digitized into 8K data point arrays. A delay of 3 s followed the acquisition; 256 FIDs were averaged. The temperature of the probe was regulated by the computer ( $\pm 1$  °C), which adjusts the current passing through a heating filament in the probe, which in turn heats the stream of air/nitrogen passing through the probe. The temperature of the sample was determined to be the same as the probe temperature setting by an NMR thermometer (Van Geet, 1970). The Nicolet NT-470 spectrometer currently in the Purdue University Biochemical Magnetic Resonance Laboratory was used under similar conditions to obtain 470-MHz spectra.

**<sup>13</sup>C NMR Spectroscopy.** Spectra were obtained with a Nicolet NT-200 wide-bore spectrometer at a <sup>13</sup>C resonance frequency of 50.3 MHz. The sample consisted of a 2.5-mL solution in a spherical cell inside a thick wall 20 mm o.d. NMR tube. The spherical cell was surrounded by water which acts as a heat sink. Square-wave modulated, on-resonance <sup>1</sup>H decoupling (200 MHz) was used. The probe temperature was controlled as described above, but the actual sample temperature was higher than the probe temperature setting because of the dielectric heating effect of broad-band decoupling. The sample temperature was measured as the temperature of the surrounding water by a thermocouple. Bilevel decoupling was used routinely to prevent overheating of the sample: high power (10 W) was used during acquisition, and low power (2 W) was used during the rest of the time to maintain the NOE.

## Results

<sup>1</sup>H NMR spectra at 470 MHz of oxidized and reduced *A. variabilis* Fd are shown in Figure 1. The singlet peaks due to the histidine residues can be readily identified in the aromatic region. All of the ferredoxins sequenced to date have a conserved histidine at position 93 (Hase et al., 1978). *A. variabilis* Fd has an additional histidine at position 16 (Chan et al., 1983a).

**Oxidized Ferredoxins.** Spectra of oxidized *A. variabilis* Fd were obtained at pH\* values between 4 and 11. Representative spectra of the aromatic region at 22 °C are shown in Figure 2. The titration curves of the four histidine resonances are shown in Figure 3. The data were analyzed by using a nonlinear least-squares analysis program (Markley, 1973); the titration parameters for the transitions are summarized in Table I.

A similar titration study was carried out with *S. maxima* Fd, which has only the conserved histidine, His<sup>93</sup>. Two singlets can be resolved easily in the aromatic region of the spectra at pH\* values 4–10 (Figure 4). The titration curves for these resonances are shown in Figure 3. Their titration parameters are also listed in Table I.

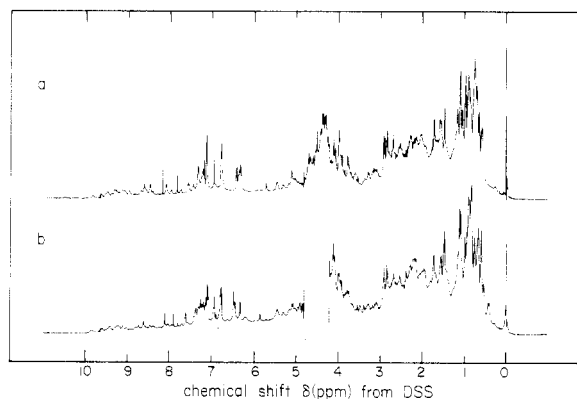


FIGURE 1:  $^1\text{H}$  NMR spectra (470 MHz) of oxidized and reduced *Anabaena variabilis* ferredoxin II. Ferredoxin concentrations were 3.3 mM. The  $\text{pH}^*$  of the oxidized ferredoxin sample (a) was 7.22; the  $\text{pH}^*$  of the reduced protein (b) was 7.26. The sample temperature was 22  $^\circ\text{C}$ . Each spectrum resulted from the sum of 256 transients. In the spectrum of the reduced protein, saturation of the residual  $^1\text{H}^2\text{O}$  left a large broad peak around 4.5 ppm; this peak was zeroed to simplify the plot. The peak at 0 ppm is from the internal standard, DSS.

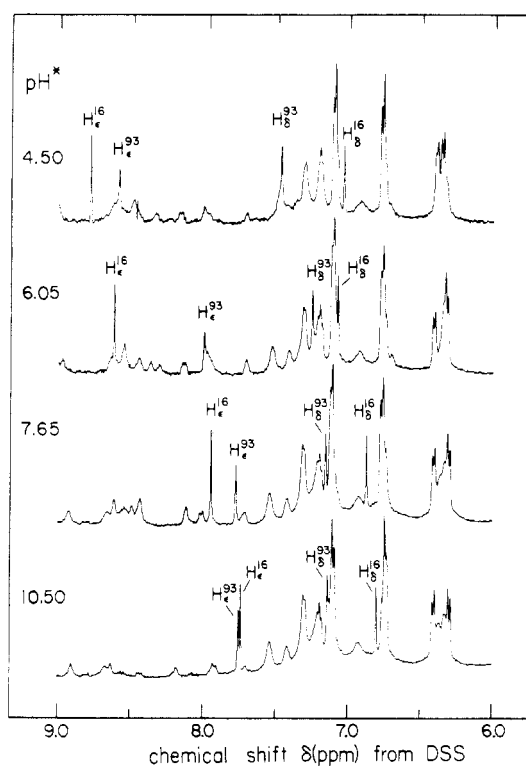


FIGURE 2: Aromatic region of  $^1\text{H}$  NMR spectra (470 MHz) of oxidized *Anabaena variabilis* ferredoxin II obtained at different  $\text{pH}^*$  values as indicated. The protein concentration was 3.3 mM, and the sample temperature was 22  $^\circ\text{C}$ . Each spectrum resulted from the sum of 256 transients.

Spectra of oxidized *A. variabilis* Fd were obtained at different temperatures from 8 to 28  $^\circ\text{C}$  at  $\text{pH}^*$  7.07; four of these spectra (aromatic region) are shown in Figure 5.

**Reduced Ferredoxins.** Partially reduced *S. maxima* Fd, obtained by adding sodium dithionite to a solution of ferredoxin at  $\text{pH}^*$  7.2, exhibited separate resonances due to  $\text{His}^{93}$  in the reduced and oxidized forms. The  $\text{C}_\alpha\text{-H}$  resonance of the reduced form is 0.15 ppm downfield from that of the oxidized form. There is no saturation transfer (at 360 MHz) between the two resonances. Since the  $T_1$  values of these resonances are about 0.5 s, lack of saturation transfer implies that the electron self-exchange rate constant is much less than  $1 \times 10^3$

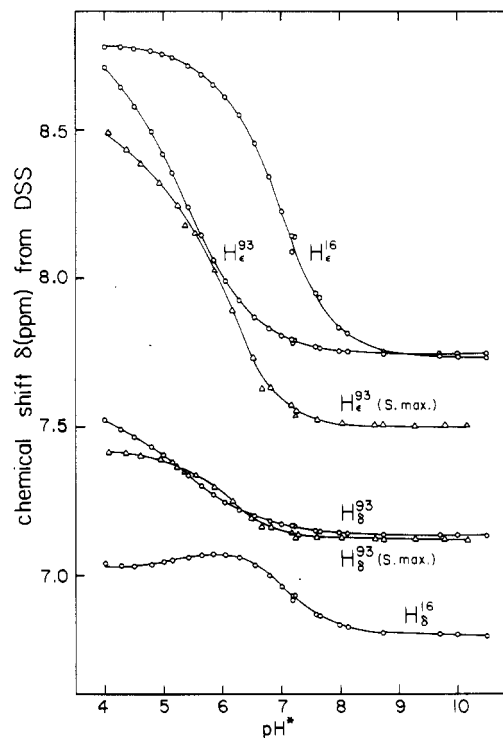


FIGURE 3:  $^1\text{H}$  NMR titration curves of the histidine peaks of oxidized *Anabaena variabilis* ferredoxin II (circles) and *Spirulina maxima* ferredoxin II (triangles). Experimental conditions are listed in the legends to Figures 2 and 4.

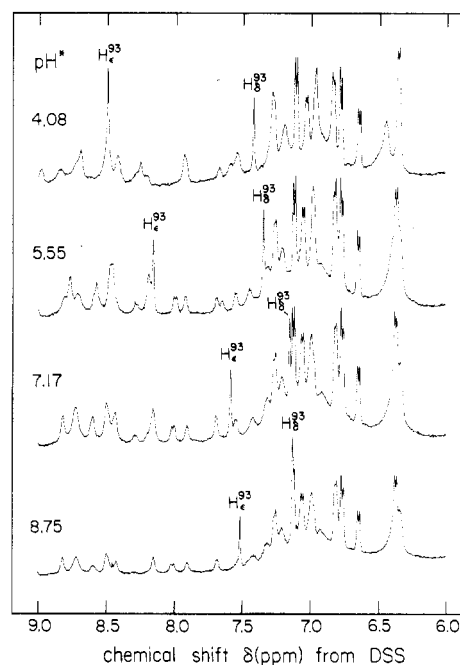


FIGURE 4: Aromatic region of  $^1\text{H}$  NMR (470 MHz) spectra of oxidized *Spirulina maxima* ferredoxin II illustrating the  $\text{pH}^*$  dependence of the histidine peaks. The ferredoxin concentration was 3.3 mM, and the sample temperature was 20  $^\circ\text{C}$ . Each spectrum resulted from the sum of 256 accumulations. The  $\text{pH}^*$  of each sample is indicated.

$\text{M}^{-1} \text{s}^{-1}$ . A value of  $1.7 \times 10^{-3} \text{M}^{-1} \text{s}^{-1}$  was obtained for the electrostatic corrected self-exchange rate constant for spinach ferredoxin based on the reaction with  $\text{Fe}(\text{EDTA})^-$  (Rawlings, 1977).

A solution of *A. variabilis* Fd was reduced completely in the anaerobic chamber, and spectra of the reduced protein were taken at different  $\text{pH}^*$  values. Four of these spectra (aromatic region) are shown in Figure 6. When the  $\text{pH}^*$  of the protein

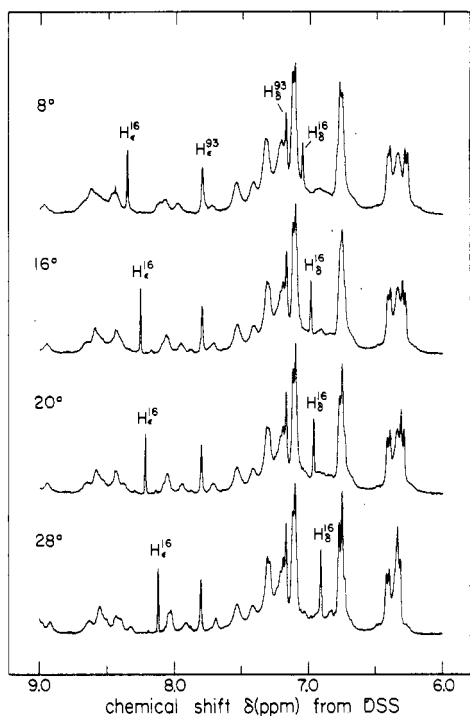


FIGURE 5: Aromatic region of  $^1\text{H}$  NMR (360 MHz) spectra of oxidized *Anabaena variabilis* ferredoxin II illustrating the temperature dependence of the histidine peaks. The protein concentration was 3.3 mM, and the  $\text{pH}^*$  was 7.07 at 22 °C. Each spectrum resulted from the sum of 256 transients. The temperatures at which the spectra were taken are indicated.

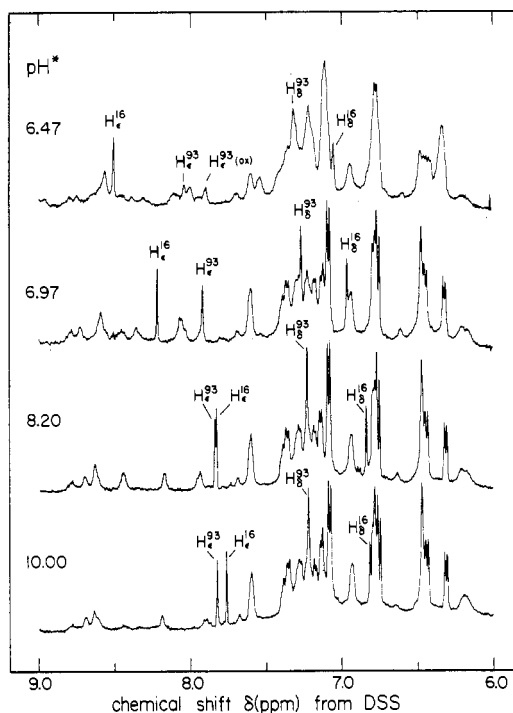


FIGURE 6: Aromatic region of  $^1\text{H}$  NMR (470 MHz) spectra of reduced *Anabaena variabilis* ferredoxin II illustrating the  $\text{pH}^*$  dependence of the histidine resonances. The ferredoxin concentration was 3.3 mM; the sample temperature was 22 °C. Each spectrum resulted from the sum of 256 transients. The  $\text{pH}^*$  value of each sample is indicated. The sample at  $\text{pH}^*$  6.47 was partially oxidized.

solution was below 7, a resonance due to the  $\text{His}^{93}$   $\text{C}_\alpha\text{-H}$  of the oxidized protein began to appear, i.e., the ferredoxin sample became partially oxidized. The ratio of oxidized to reduced protein became larger at lower  $\text{pH}^*$ . Partial titration curves for the histidine resonances are shown in Figure 7. The

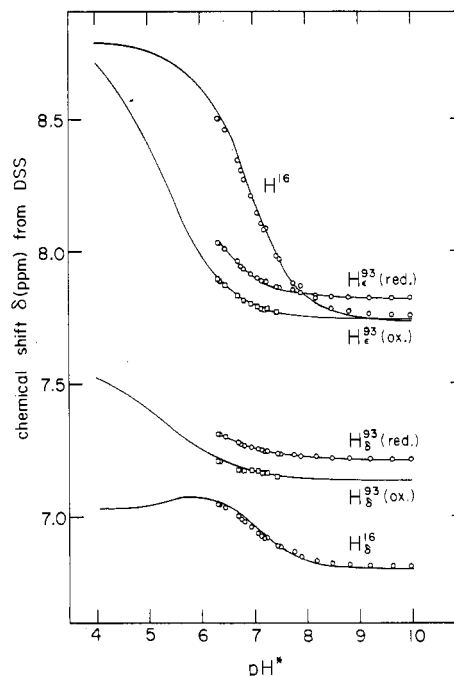


FIGURE 7:  $^1\text{H}$  NMR (470 MHz) titration curves of the histidine residues of reduced *Anabaena variabilis* ferredoxin II. The circles indicate the chemical shifts of the histidine peaks of reduced *A. variabilis* Fd at different  $\text{pH}^*$  values. The squares are the chemical shifts of the histidine peaks of the oxidized ferredoxin in a partially reduced protein sample. The four complete ( $\text{pH}^*$  4–10) titration curves are those of fully oxidized ferredoxin (from Figure 3).

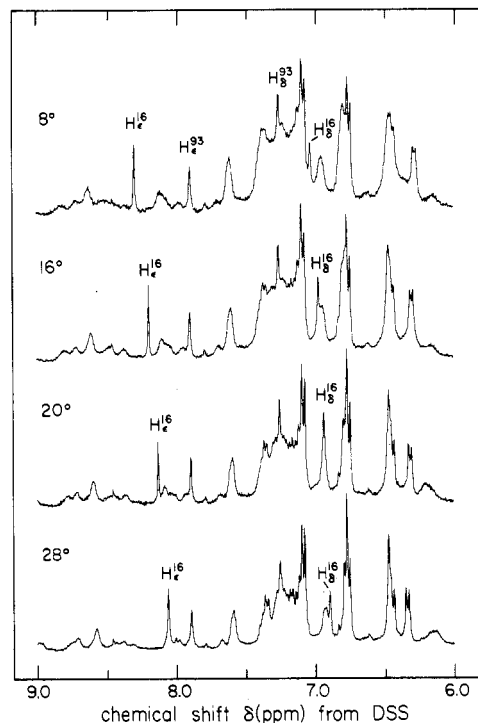


FIGURE 8: Aromatic region of  $^1\text{H}$  NMR (360 MHz) spectra of reduced *Anabaena variabilis* ferredoxin II illustrating the temperature dependence of the histidine resonances. The protein concentration was 3.3 mM; the  $\text{pH}^*$  was 7.2 at 22 °C. Each spectrum resulted from the sum of 256 transients. The temperatures at which the spectra were taken are indicated in the figure.

resonances of  $\text{His}^{93}$  in the reduced protein are downfield (about 0.08 ppm) from the corresponding resonances in the oxidized protein. By contrast, the chemical shifts of  $\text{His}^{16}$  do not change with the oxidation state of the protein. Spectra (aromatic region) of a sample of reduced *A. variabilis* Fd at  $\text{pH}^*$  7.2

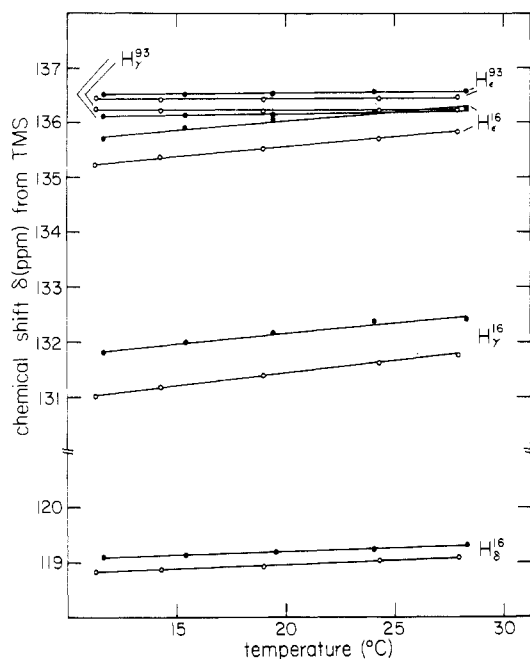


FIGURE 9: Temperature dependences of the  $^{13}\text{C}$  resonances of the histidine residues of *Anabaena variabilis* ferredoxin II in the oxidized (open circles) and reduced (closed circles) states. The  $\text{pH}^*$  of the oxidized ferredoxin sample was 7.14 at 22  $^{\circ}\text{C}$ ; the  $\text{pH}^*$  of the reduced protein sample was 7.26 at 22  $^{\circ}\text{C}$ . The resonances of  $\text{His}^{16}$  are temperature dependent, while those of  $\text{His}^{93}$  are not. The  $\text{C}_{\beta}$  resonance of  $\text{His}^{93}$  was not resolved.

at four different temperatures are shown in Figure 8.

**$^{13}\text{C}$  Resonances.** The  $\text{C}_{\epsilon}$  and  $\text{C}_{\delta}$  resonances of  $\text{His}^{93}$  and  $\text{His}^{16}$  of oxidized *A. variabilis* Fd have been assigned by two-dimensional heteronuclear ( $^{13}\text{C}$ ,  $^1\text{H}$ ) chemical shift correlation spectroscopy (Chan & Markley, 1982). The non-protonated carbon ( $\text{C}_{\gamma}$ ) resonances, of both histidines, were resolved by a  $J$ -modulated spin-echo technique (Chan et al., 1982). The corresponding resonances for the reduced ferredoxin have been assigned with the same techniques. These assignments will be discussed further in the third paper of this series (Chan & Markley, 1983a). The effect of temperature on the chemical shifts is shown in Figure 9.

## Discussion

**Assignments.** *S. maxima* Fd has only one histidine residue, the conserved  $\text{His}^{93}$ . The NMR titration study of the oxidized protein yielded a  $\text{pK}_a'$  value of 5.9 for  $\text{His}^{93}$  which is low compared with the value of 6.8 obtained for the model peptide, Gly-His-Gly (Markley, 1973). NMR peaks are resolved from the two histidine residues of *A. variabilis* Fd,  $\text{His}^{93}$  and  $\text{His}^{16}$ . The peaks exhibiting the lower  $\text{pK}_a'$  value of 5.3 are assigned to the homologous  $\text{His}^{93}$  because of their resemblance to those of  $\text{His}^{93}$  of *S. maxima* Fd, and the peaks yielding a normal  $\text{pK}_a'$  value of 7.0 are assigned to  $\text{His}^{16}$ . The difference in the  $\text{pK}_a'$  of  $\text{His}^{93}$  in the ferredoxins from that of the two species may be attributed to a change in the charge of the adjacent residue 94, which is lysine in *A. variabilis* Fd (Chan et al., 1983a) and glutamine in *S. maxima* Fd (Tanaka et al., 1975). The assignments to  $\text{C}_{\delta}$ -H and  $\text{C}_{\epsilon}$ -H positions (Table I) were verified by heteronuclear ( $^1\text{H}$ ,  $^{13}\text{C}$ ) two-dimensional chemical shift correlation spectroscopy (Chan & Markley, 1982).

**Effect of Reduction.** Because of the very slow electron self-exchange rate of ferredoxin, mixtures of oxidized and reduced ferredoxin exhibit separate resonances in the two oxidation states. The relative areas of the peaks provide a measure of the relative concentrations of the oxidized and reduced forms. The  $\text{C}_{\epsilon}$ -H and  $\text{C}_{\delta}$ -H resonances of  $\text{His}^{93}$  of

reduced ferredoxin are shifted downfield from the corresponding resonances of the oxidized protein. The distance of the  $\text{C}_{\epsilon}$  and  $\text{C}_{\delta}$  of  $\text{His}^{93}$  from the iron atoms in the  $2\text{Fe-2S}^*$  center, which is about 13–14 Å (T. Tsukihara, personal communication), is too far for the change in the chemical shift to be of pseudo-contact origin. The change in oxidation state of the iron-sulfur center causes a change in charge, which in turn may cause a change in the conformation around the iron-sulfur cluster and affect the environment of  $\text{His}^{93}$ . Titration parameters could not be obtained for the histidines of reduced ferredoxin, because of the failure of dithionite to reduce the protein at low pH.

The chemical shifts of the  $\text{His}^{16}$  resonances (*A. variabilis* Fd) do not change with the redox state of the protein. Partially reduced samples (e.g., Figure 6, top trace) yield separate resonances for  $\text{His}^{93}$  in each state, but only single sharp resonances are observed for  $\text{C}_{\epsilon}$ -H and  $\text{C}_{\delta}$ -H of  $\text{His}^{16}$ . The X-ray structure shows that  $\text{His}^{16}$  is about 20 Å from the iron-sulfur center. The insensitivity of the NMR parameters of  $\text{His}^{16}$  to reduction suggests that there is no conformational change in this region of the protein upon oxidation or reduction.

**Temperature Effect.** The  $^1\text{H}$  resonances assigned to  $\text{His}^{93}$  show little or no temperature dependence (the change in chemical shift is smaller than 0.01 ppm for  $\text{C}_{\delta}$ -H over a 20  $^{\circ}\text{C}$  change in temperature), whereas the resonances assigned to the  $\text{C}_{\epsilon}$ -H and  $\text{C}_{\delta}$ -H of  $\text{His}^{16}$  (in both oxidation states) change by 0.1 ppm or more over the same temperature range. Preliminary titration results at three temperatures indicate that the  $\text{pK}_a'$  of  $\text{His}^{93}$  does not change with temperature within experimental error, whereas the  $\text{pK}_a'$  of  $\text{His}^{16}$  varies linearly with  $1/\text{temperature}$ . The change in  $\text{pK}_a'$  of  $\text{His}^{16}$  with temperature is attributed to its relatively normal heat of ionization ( $\Delta H$ ). The  $\text{pK}_a'$  and its temperature dependence both indicate that  $\text{His}^{93}$  is influenced more strongly by the tertiary structure of the protein than  $\text{His}^{16}$  and that it has an anomalously low  $\Delta H$ .

The  $^{13}\text{C}$  chemical shift of the  $\text{C}_{\gamma}$  and  $\text{C}_{\epsilon}$  peaks of  $\text{His}^{93}$  of *A. variabilis* Fd at neutral pH are temperature independent as expected for the low  $\text{pK}_a'$  of the residue and its temperature insensitivity. The  $\text{His}^{93}$   $\text{C}_{\delta}$  peak was resolved only in the heteronuclear 2D chemical shift correlation experiment; its temperature dependence was not determined. The  $\text{His}^{16}$   $^{13}\text{C}$  peaks show temperature-dependent chemical shifts with the sample at neutral pH. The observed effect is explained by the  $\Delta H$  of  $\text{His}^{16}$ . The different carbons of  $\text{His}^{16}$  give different slopes because the magnitudes of their titration shifts are different; i.e., the  $\text{C}_{\epsilon}$  and  $\text{C}_{\gamma}$  resonances have larger  $\Delta\delta$ 's than the  $\text{C}_{\delta}$  resonance and hence larger chemical shift changes for a given  $\Delta\text{pK}_a'$ . In general,  $^{13}\text{C}$  and  $^1\text{H}$  resonances should shift in opposite directions with temperature because the  $\Delta\delta$ 's have opposite signs: proton resonances have negative temperature dependences while carbon resonances normally have positive temperature dependences. The single exception is the histidine  $\text{C}_{\gamma}$  for which  $\Delta\delta$  is positive if the  $\text{N}_{\epsilon}$  is protonated in the neutral form but negative if  $\text{N}_{\delta}$  is protonated (Deslauriers et al., 1974; Reynolds et al., 1973). The positive slope of the  $\text{C}_{\gamma}$  of  $\text{His}^{16}$  indicates that the  $\text{N}_{\epsilon}$ -H tautomer of the neutral imidazole predominates. The chemical shift of the  $\text{His}^{93}$   $\text{C}_{\gamma}$  resonance (136 ppm at  $\text{pH}^*$  7.3) is also close to the expected value for the  $\text{N}_{\epsilon}$ -H tautomer in a peptide (135 ppm). Correlation of the temperature dependence of  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts could be used as a strategy for cross assigning histidine  $^1\text{H}$  and  $^{13}\text{C}$  resonances.

## Acknowledgments

We thank Dr. D. W. Krogmann for providing the cell paste

of *Spirulina maxima* and the culture of *Anabaena variabilis*, Dr. T. Tsukihara for the revised X-ray coordinates of oxidized *Spirulina platensis* ferredoxin prior to their deposition in the Brookhaven Data Bank, Dr. D. Teagarden for constructing the rocker tank used to culture cyanobacteria, Dr. R. E. Santini for assistance with NMR instrumentation, and Drs. Eldon L. Ulrich and William M. Westler for helpful discussions.

**Registry No.** Histidine, 71-00-1.

## References

- Anderson, R. E., Dunham, W. R., Sands, R. H., Bearden, A. J., & Crespi, H. L. (1975) *Biochim. Biophys. Acta* **408**, 306-318.
- Cammack, R., Rao, K. K., Barger, C. P., Hutson, K. G., Andrew, P. W., & Rogers, L. J. (1977) *Biochem. J.* **168**, 205-209.
- 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* (third paper of five in this issue).
- Chan, T.-M., & Markley, J. L. (1983b) *Biochemistry* (fifth paper of five in this issue).
- Chan, T.-M., Ulrich, E. L., & Markley, J. L. (1981) in *Photosynthesis II. Electron Transport and Photophosphorylation* (Akoyunoglou, G., Ed.) pp 697-704, Balaban International Science Series, Philadelphia.
- Chan, T.-M., Westler, W. M., Santini, R. E., & Markley, J. L. (1982) *J. Am. Chem. Soc.* **104**, 4008-4010.
- Chan, T.-M., Hermodson, M. A., Ulrich, E. L., & Markley, J. L. (1983a) *Biochemistry* (second paper of five in this issue).
- Chan, T.-M., Ulrich, E. L., & Markley, J. L. (1983b) *Biochemistry* (fourth paper of five in this issue).
- Deslauriers, R., McGregor, W. H., Sarantakis, D., & Smith, I. C. P. (1974) *Biochemistry* **13**, 3443-3448.
- Fukuyama, K., Hase, T., Matsumoto, S., Tsukihara, T., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K., & Matsubara, H. (1980) *Nature (London)* **286**, 522-524.
- Hase, T., Wakabayashi, S., Matsubara, H., Roa, K. K., Hall, D. O., Widmer, H., Gysi, J., & Zuber, H. (1978) *Phytochemistry* **17**, 1863-1867.
- Ho, K. K., Ulrich, E. L., Krogmann, D. W., & Gomez-Lojero, C. (1979) *Biochim. Biophys. Acta* **545**, 236-248.
- Kratz, W. A., & Myers, J. (1955) *Am. J. Bot.* **42**, 282-287.
- Lovenberg, W., Ed. (1973-1977) *Iron-Sulfur Proteins 1-3*.
- Markley, J. L. (1973) *Biochemistry* **12**, 2245-2250.
- Markley, J. L., & Porubcan, M. P. (1976) *J. Mol. Biol.* **102**, 487-509.
- Poe, M., Phillips, W. D., Glickson, J. D., McDonald, C. C., & San Pietro, A. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 68-71.
- Rawlings, J., Wherland, S., & Gray, H. B. (1977) *J. Am. Chem. Soc.* **99**, 1968-1971.
- Reynolds, W. F., Peat, I. R., Freedman, M. H., & Lyster, J. R., Jr. (1973) *J. Am. Chem. Soc.* **95**, 328-331.
- Salmeen, I. T., & Palmer, G. (1972) *Arch. Biochem. Biophys.* **150**, 767-773.
- Tanaka, M., Haniu, M., Yasunobu, K. T., Roa, K. K., & Hall, D. O. (1975) *Biochemistry* **14**, 5535-5540.
- Trebst, A., & Avron, M., Eds. (1977) *Photosynthesis I, Photosynthetic Electron Transport and Photophosphorylation*, Springer-Verlag, New York.
- Tsukihara, T., Fukuyama, K., Nakamura, M., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K., Hase, T., & Matsubara, H. (1981) *J. Biochem. (Tokyo)* **90**, 1763-1773.
- Van Geet, A. L. (1970) *Anal. Chem.* **42**, 679-680.