Nuclear Magnetic Resonance Studies of Two-Iron-Two-Sulfur Ferredoxins. 5. Hyperfine-Shifted Peaks in Hydrogen-1 and Carbon-13 Spectra†

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ABSTRACT: A number of hyperfine-shifted peaks are visible in 200- or 360-MHz ^1H NMR spectra and 50.3-MHz ^{13}C NMR spectra of oxidized and reduced two-iron-two-sulfur ferredoxins. The positions and intensities of the hyperfine-shifted ^1H NMR peaks are nearly identical in spectra of ferredoxins from spinach, *Anabaena variabilis*, and *Spirulina maxima*, indicating that the enviroment of the iron-sulfur cluster in each protein is highly conserved. The ^1H NMR peak at 42 ppm in the spectrum of reduced ferredoxin previously reported to have a two-proton intensity [Salmeen, I., & Palmer, G. (1972) *Arch. Biochem. Biophys. 150*, 767–773] has a one-proton intensity and is assigned to the C_α-H of one of the cysteine residues ligated to iron. The spectral properties of hyperfine-shifted ^{13}C NMR peaks of a plant-type ferredoxin are reported for the first time. Through the use of uniform enrichment to 20% ^{13}C of the ferredoxin II from *Anabaena variabilis*, 2 resolved hyperfine-shifted peaks for the oxidized protein and 11 hyperfine-shifted peaks for the reduced protein were observed; the temperature dependence of the chemical shift of each hyperfine-shifted ^{13}C peak of reduced ferredoxin was determined.

Hydrogen-1 NMR spectra of 2Fe·2S*[1] ferredoxins in either the oxidized or reduced state exhibit resonances that are shifted outside the diamagnetic region (0–10 ppm) by hyperfine interactions. These resonances have been studied in ^1H NMR spectra of spinach and parsley ferredoxins at 220 MHz (Poe et al., 1971), spinach ferredoxin at 60 MHz (Salmeen & Palmer, 1972), and ferredoxin from a cyanobacterium, *Synechococcus lividus*, at 220 MHz (Anderson et al., 1975). Poe and co-workers (1971) observed eight peaks, each of single proton intensity, in the 11–22 ppm region of the spectrum of reduced parsley ferredoxin. Four of these resonances, those shifted farthest downfield, had positive temperature coefficients (downfield shift with increasing temperature), and the other four resonances had negative temperature coefficients. The authors assigned these resonances to the eight C_β-H of the four cysteine ligands and attributed the differential temperature dependence to greater localization of the additional electron on one of the two iron atoms. Only six hyperfine-shifted resonances were observed in the spectrum of reduced spinach ferredoxin: four with positive temperature coefficients and the other two with negative temperature coefficients. Dunham and co-workers (1971) predicted on the basis of susceptibility, Mössbauer, and ^1H NMR data that, in reduced ferredoxin, the four C_β-H resonances of the two cysteine residues ligated to the Fe(II) atom should have positive temperature coefficients and chemical shifts around 20 ppm, while the four C_β-H of the two cysteine residues ligated to the Fe(III) atom should have negative temperature coefficients and chemical shifts around 170 ppm [well outside the spectral range of the experiments of Poe and co-workers (1971)]. Salmeen & Palmer (1972) observed a broad peak of 6 ± 2 proton intensity at 37 ppm (line width about 3.7 kHz) in the spectrum of oxidized spinach ferredoxin. This peak had a positive temperature dependence and was assigned to the eight C_β-H resonances of the cysteine ligands. In the spectrum of reduced spinach ferredoxin, they reported a peak at 42 ppm with a two-proton intensity. This peak had a negative temperature coefficient and was assigned to the two C_α-H resonances of the cysteine residues ligated to Fe(III). Although they did not resolve additional peaks, Salmeen & Palmer (1972) predicted that the four C_β-H resonances of the two cysteine residues ligated to Fe(III) should be observed near 468 ppm and that the two C_α-H resonances of the the two cysteine residues ligated to Fe(II) should be observed around 4–5 ppm. We have resolved and studied the temperature dependence of eight hyperfine-shifted resonances that are characteristic of the 2Fe·2S* center of reduced ferredoxins.

To our knowledge no study of hyperfine-shifted ^{13}C resonances of a 2Fe·2S* ferredoxin has been reported in the literature. Packer and co-workers (1978) carried out a ^{13}C NMR investigation of a clostridial ferredoxin which contains two 4Fe·4S* clusters. In our laboratory, cells of *Anabaena variabilis* were grown with CO_2 (20% ^{13}C) as the sole carbon source. Hyperfine-shifted resonances were observed in spectra of the oxidized and reduced forms of the ferredoxin isolated from these cells.

Experimental Procedures

Samples were prepared, and ^1H and ^{13}C NMR spectra were obtained as described previously (Chan & Markley, 1983).

Results and Discussion

^1H NMR. The broad resonance around 37 ppm reported by Salmeen & Palmer (1972) was not observed at either 200 MHz or 360 MHz in spectra of the oxidized ferredoxins from spinach, *A. variabilis*, or *S. maxima*. The extreme width of

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[1] Abbreviations: Fd, ferredoxin; 2Fe·2S*, the iron-sulfur center consisting of two iron atoms and two inorganic sulfur atoms; LB, line broadening factor used in processing the free induction decay by exponential multiplication; FID, free induction decay; pH*, pH meter reading of a solution in ^2H_2O uncorrected for the deuterium isotope effect; ul, uniformly labeled.

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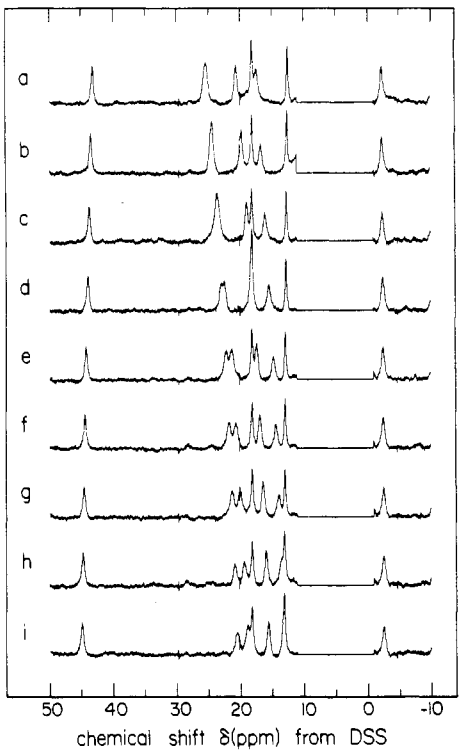

FIGURE 1: Hyperfine-shifted resonances in the ^1H NMR spectrum (360 MHz) of reduced *Anabaena variabilis* ferredoxin II as a function of temperature: (a) 30, (b) 26, (c) 22, (d) 20, (e) 16, (f) 14, (g) 12, (h) 10, and (i) 8 °C. The ferredoxin concentration was 3.3 mM in 0.5 mL of 0.05 M deuterated phospate buffer. The pH* of the solution was raised to 8.84, and solid sodium dithionite was added under an inert atmosphere to reduce the protein. The final pH* of the solution was not measured; it was determined to be 7.4 from the chemical shift of the histidine resonances (Chan & Markley, 1983). The region between 0 and 10 ppm was zeroed to base line for clarity.

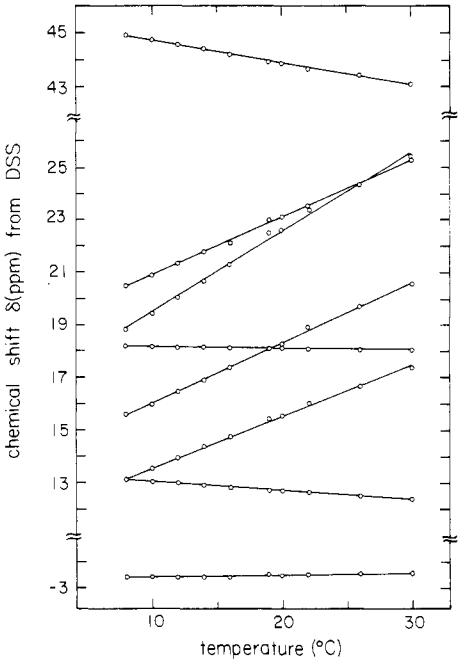

FIGURE 2: Temperature dependence of the hyperfine-shifted resonances in 360-MHz ^1H NMR spectra of reduced *Anabaena variabilis* ferredoxin II. The chemical shifts of the peaks from Figure 1 are plotted against temperature.

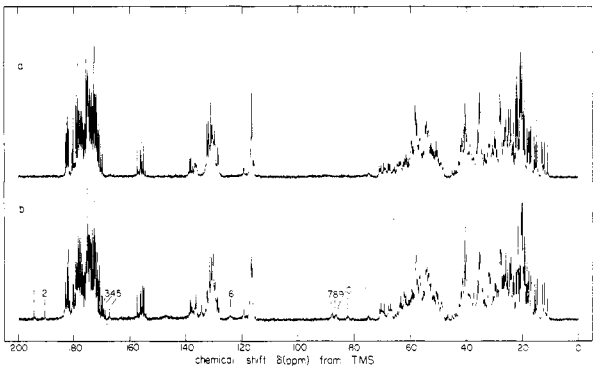

FIGURE 3: ^{13}C NMR spectra (50.3 MHz) of *Anabaena variabilis* ferredoxin II (ul 20% ^{13}C). (a) Oxidized ferredoxin. The protein concentration was 4.5 mM in 2.5 mL of 0.05 M deuterated phosphate buffer, pH* 7.32 at 24 °C; 4000 scans were taken. (b) Reduced ferredoxin. The protein concentration was 6.5 mM in 2.5 mL of 0.05 M deuterated phosphate buffer, pH* 7.31 at 24 °C; 3000 scans were taken. The hyperfine-shifted peaks are numbered 1–11.

the reported peak (about 3 kHz) may explain our failure to observe this resonance with high-field pulse spectrometers. A delay was inserted between the observed pulse and the beginning of data acquisition to allow recovery of the probe. This delay is usually set equal to one dwell time, which is 50 μs for a spectral width of ±10 000 Hz. For a very broad signal, a large part of the signal will be lost to transverse relaxation (T_2) during this delay. Residual probe ringing also leads to rolling base-line problems when a larger spectral width is used. These difficulties may have prevented us from resolving the expected broad peak.

With reduced *A. variabilis* Fd, eight hyperfine-shifted resonances, each of one-proton intensity, were observed in the region from −13 to 70 ppm. Spectra at nine different temperatures (8–30 °C) are shown in Figure 1, and the chemical shifts of these peaks are plotted as a function of temperature in Figure 2. Very similar results (not shown) were obtained with spinach Fd and *S. maxima* Fd (Chan, 1982). The four hyperfine-shifted resonances near 20 ppm, which have positive temperature coefficients, have been assigned to the four C_β-H of the two cysteines ligated to Fe(II) (Poe et al., 1971). The resonance near 42 ppm occupies the same position as the two-proton peak reported by Salmeen & Palmer (1972). Integration of the present spectrum clearly shows, however, that the 42 ppm peak has a one-proton intensity. The corresponding peak in the 360-MHz spectrum of reduced spinach ferredoxin also has a one-proton intensity. The spectrum of *S. lividus* ferredoxin obtained by Anderson et al. (1975) also contains a one-proton peak at this position. A single peak with nearly constant line width in this region was observed over the temperature range studied (8–30 °C). Hence, we assign the 42 ppm peak to the C_α-H of one of the cysteines ligated to Fe(III).

Two other peaks with negative temperature coefficients were observed between 10 and 20 ppm, and a third peak with a positive temperature coefficient was observed around −2 ppm. One of these three peaks probably arises from the C_α-H of the second cysteine ligated to Fe(III). Since the electronic ground state of high-spin Fe(III) is symmetric (to a first approximation), the other two resonances must arise from nuclei that are close to the Fe(II) site and experience pseudocontact effects. A pseudocontact field from the Fe(II) site may also explain the large difference in the chemical shifts of the two C_α-H of the cysteines ligated to Fe(III) if one of them is closer to the Fe(II) atom than the other. In other experiments, we observed no hyperfine-shifted proton resonances outside the 70 to −12 ppm region.

^{13}C NMR. ^{13}C NMR spectra of oxidized and reduced *A. variabilis* Fd are shown in parts a and b of Figure 3, respectively. In addition to resonances in the usual positions for

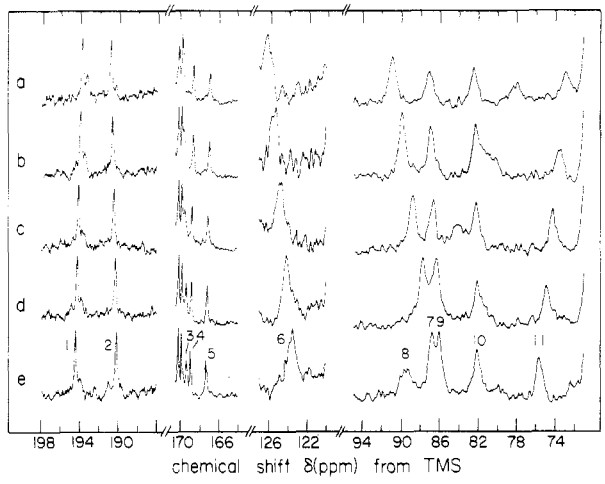

FIGURE 4: Temperature dependence of the hyperfine-shifted peaks in ^{13}C spectra of reduced *Anabaena variabilis* ferredoxin II (ul 20% ^{13}C). The ferredoxin concentration was 4 mM in 2.5 mL of deuterated phosphate buffer. The pH* of the solution was raised to 8.97, and solid sodium dithionite was added under an argon atmosphere to reduce the protein. The final pH* of the solution was 7.3. Only the spectral region containing the hyperfine-shifted resonances numbered 1–11 is plotted. The temperatures at which the spectra were taken were (a) 11.7, (b) 15.4, (c) 19.4, (d) 24.1, and (e) 28.3 °C.

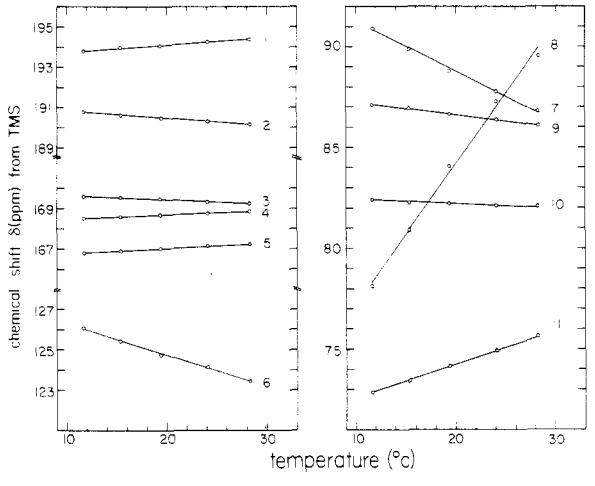

FIGURE 5: Temperature dependence of the hyperfine-shifted resonances in the ^{13}C spectrum of reduced *Anabaena variabilis* ferredoxin II. The chemical shifts of the hyperfine-shifted resonances (1–11, Figure 4) are plotted against temperature.

a diamagnetic protein, the spectrum of oxidized ferredoxin exhibits two broad peaks around 75 and 168 ppm. The spectrum of reduced ferredoxin contains 11 resonances that lie outside the normal diamagnetic regions. Five of these resonances (peaks 1–5 in Figure 3b) are relatively sharp, and the other six (peaks 6–11) are broader. Partial ^{13}C spectra showing these peaks at five different temperatures are shown in Figure 4.

The five hyperfine-shifted resonances around the carbonyl region (peaks 1–5) remain as sharp singlets in the proton-coupled spectrum; therefore, they are probably due to carbonyl carbons of the cysteines bonded to the 2Fe·2S* cluster or to carbonyl carbons that are very close to it. The other six peaks (peaks 6–11) split into broad multiplets in the proton-coupled spectrum. Because of the large line widths of these resonances, their exact multiplicities could not be determined. These six peaks must arise from C_α and C_β atoms of the cysteines or from protonated carbons that are very near the 2Fe·2S* center.

The chemical shifts of these hyperfine-shifted resonances are plotted as a function of temperature in Figure 5. The interpretation of the temperature coefficient is less straightforward for ^{13}C than for ^1H NMR. For example, the two resonances around 190–194 ppm are both shifted approximately the same distance downfield from their diamagnetic positions, but the plot of their chemical shifts vs. temperature yields two straight lines with slopes of similar magnitude but with opposite signs. Of the three resonances that are shifted upfield from the carbonyl region, two have positive temperature coefficients and the other has a negative temperature coefficient. With the present data we are unable to assign ^{13}C peaks to individual cysteines ligated to Fe(II) or Fe(III).

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