

Connectivity of proton and carbon spectra of the blue copper protein, plastocyanin, established by two-dimensional nuclear magnetic resonance

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NMR studies of plastocyanin have centered on the ligands to the copper atom at the active site, particularly histidines-37 and -87. Heteronuclear (^{13}C , ^1H) *J*-connectivity spectroscopy has enabled cross assignment of ^1H and ^{13}C NMR resonances from the two copper-ligated histidines. In addition to providing assignments of the ^{13}C resonances, the two-dimensional Fourier transform NMR results require the reversal of the original ^1H NMR assignments to the ring protons of histidine-37. The line widths of the ring protons of histidine-87 are field-dependent leading to determination of the reduced lifetime of the proton on the N_δ atom (about 400 μs).

2DFT-NMR Plastocyanin Electron transport Photosynthesis Histidine Cu-ligand

1. INTRODUCTION

Plastocyanin, a 'type I' or 'blue copper' protein involved in photosynthetic electron transport [1], consists of a single polypeptide chain of around 100 residues [2] and a single copper ion coordinated to the δ -nitrogen of 2 histidines [3,4], the sulfur of 1 methionine [4], and the sulfur of 1 cysteine [5]. Previous NMR investigations have revealed chemical and dynamic properties of higher plant plastocyanins and their sites of interaction with redox inhibitors [3,6–12]. Here, we use heteronuclear (^{13}C , ^1H) chemical shift-correlated two-dimensional Fourier transform (2DFT) NMR [13,14] and one-dimensional (1DFT) ^1H and ^{13}C NMR spectroscopy to analyze properties of the histidines of plastocyanin from the cyanobacte-

rium, *Anabaena variabilis*, as a function of redox state and pH.

2. EXPERIMENTAL

Heteronuclear (^{13}C , ^1H) chemical shift-correlated 2DFT NMR spectroscopy was initially applied to small molecules [13,15], including a series of carbohydrates [16]. Although the technique gives rise to polarization transfer from ^1H to ^{13}C which enhances the 2DFT signal, the inherently low sensitivity of natural abundance ^{13}C NMR has presented an obstacle to the extension of the method to large peptides and proteins. This limitation can be overcome either by uniformly enriching with ^{13}C as was done with ferredoxin (M_r 11 000) [14] or by using a high concentration of the peptide or protein with ^{13}C at natural abundance. Heteronuclear 2DFT studies of siomycin A (M_r 1600) [17] and turkey ovomucoid third domain (M_r 7014) [18] have been accomplished by making use of high sample concentrations (12 to 30 mM). We have followed the strategy of ^{13}C enrichment in order to study *A. variabilis* plastocyanin. Because

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Abbreviations: 2DFT-NMR, two-dimensional Fourier-transform nuclear magnetic resonance; TSP, sodium 3-(trimethylsilyl) propionic acid; TMS, tetramethylsilane; ppm, parts per million

there is no ^1H - ^{13}C coupling between the aromatic and aliphatic spectral regions, the resolution can be optimized by accumulating the heteronuclear 2DFT data of each region independently [14]. Results with the aromatic region are presented here; an analysis of the aliphatic region is in progress.

The 2DFT contour maps and ^{13}C 1DFT NMR spectra were obtained at a frequency of 50 MHz for ^{13}C and 200 MHz for ^1H . The ^1H 1DFT NMR spectra were obtained at 470 MHz or 200 MHz. Plastocyanin was isolated from *A. variabilis* grown on 20% ^{13}C -enriched CO_2 as the sole carbon source. The samples used for 2DFT experiments contained 165 mg of 20% ^{13}C plastocyanin in 2.5 ml D_2O . (The pH-values reported are the actual meter readings obtained with a glass electrode and were not corrected for the deuterium isotope effect; hence they are designated as pH*.) The 2DFT NMR data were accumulated in ~50 h using 800 or 860 scans/block (pH* 7.6 and 6.1, respectively), and 128 blocks of 1024 data points each. The ^1H decoupler was positioned at 6.0 or 5.5 ppm (pH* 7.6 and 6.1, respectively) in the ^1H frequency

region, with the delay set so as to cover a ^1H window of 3.5 ppm downfield of the decoupler.

3. RESULTS AND DISCUSSION

NMR spectra of reduced and oxidized plastocyanins are compared in fig.1. The heteronuclear 2DFT contour plot provides higher resolution of the aromatic region than either the ^1H or ^{13}C 1DFT NMR spectrum (plotted at the sides). The ^1H 1DFT NMR spectrum contains peaks from N-H as well as C-H groups. Since N-H groups do not give rise to ^{13}C - ^1H cross peaks, the contour plot allows one to sort the peaks into these two categories of proton resonances. Numerous differences are observed between the spectra of the Cu(I) and Cu(II) forms of plastocyanin. Peaks present in the contour plot of Cu(I) but not Cu(II) plastocyanin probably arise from C-H groups near the metal ion.

The dependence of the spectra on pH (fig.2) helps establish which peaks correspond to histidine residues. The two normal titration curves (fig.3)

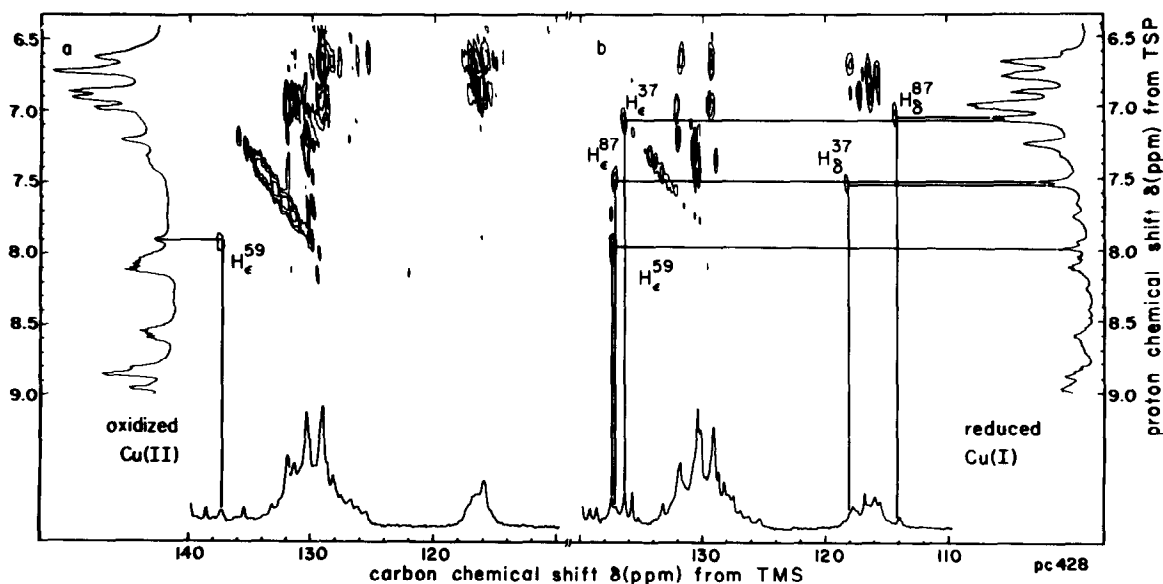


Fig.1. Comparison of one-dimensional (1DFT) and heteronuclear (^{13}C , ^1H) chemical shift-correlated two-dimensional Fourier transform (2DFT) spectra of (a) reduced [Cu(I)] and (b) oxidized [Cu(II)] *A. variabilis* plastocyanin at pH* 7.6. The 2DFT contour map was obtained at a frequency of 50 MHz for ^{13}C and 200 MHz for ^1H . The ^1H 1DFT NMR spectra shown at the sides were obtained at 470 MHz, and the ^{13}C 1DFT NMR spectra shown at the bottom were obtained at 50 MHz. Cross peaks from histidine ring protons are assigned in the figure: for example, peak H_ϵ^{37} corresponds to the C_ϵ -H of histidine 37.

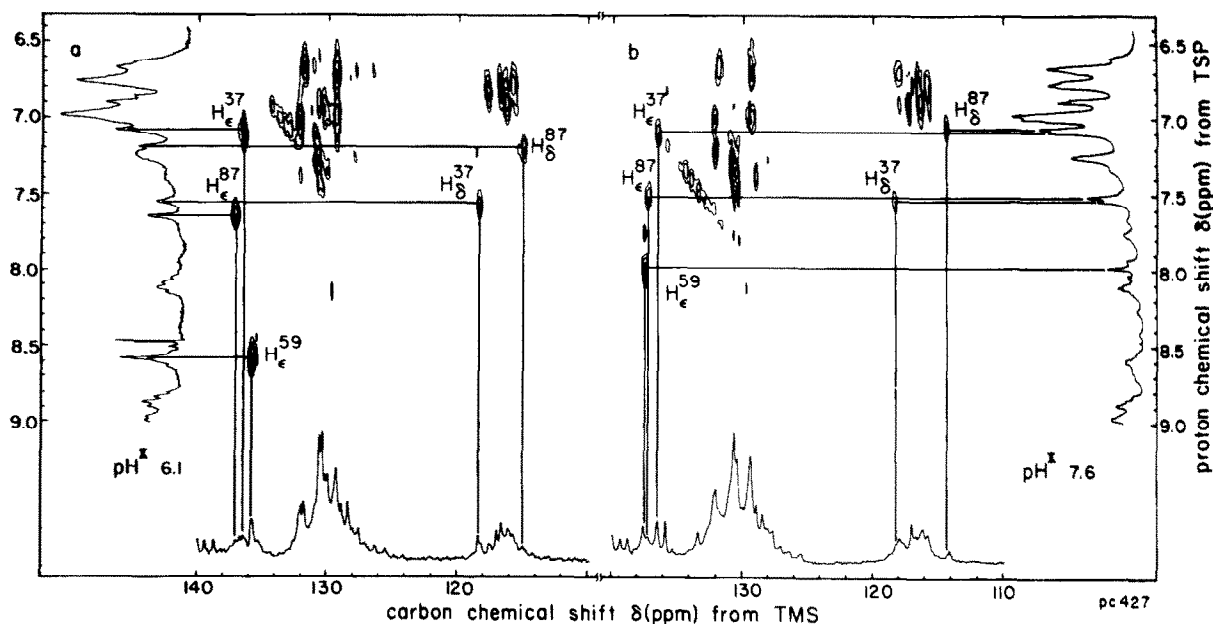


Fig.2. Comparison of one-dimensional (1DFT) and heteronuclear (^{13}C , ^1H) chemical shift-correlated two-dimensional Fourier transform (2DFT) NMR spectra of $[\text{Cu}(\text{I})]$ *A. variabilis* plastocyanin at two pH^* -values: (a) pH^* 6.1 and (b) pH^* 7.6. The 2DFT spectrum is represented as a contour plot, and the 1DFT spectra are plotted at the sides and bottom. The NMR spectral conditions were as in fig.1 except that the ^1H 1DFT NMR spectrum shown in (a) was obtained at 200 MHz. The notation used to designate peaks is explained in fig.1.

with pK'_a values of 7.26 ± 0.01 (peak H_ϵ^{59}) and 7.53 ± 0.33 (peak H_δ^{59}) are assigned [3] to histidine-59, the one histidine that is not a copper ligand. These peaks are observed in spectra of oxidized plastocyanin (fig.1) whereas the other histidine peaks are not. The paramagnetism of the copper(II) broadens the peaks from the ligated histidine rings beyond detection [3]. NMR studies of reduced spinach plastocyanin which has only two histidines have shown that one of the ligated histidines titrates with a pK'_a value of about 4.9 whereas the other does not titrate at all when the copper is present. The titratable peaks were assigned [19] to histidine-87 whose edge is exposed to solvent in the X-ray structure of poplar plastocyanin [4], and the non-titratable peaks were assigned to histidine-37 which is completely buried. This assignment was confirmed by an experiment involving interaction of $\text{Cr}(\text{CN})_6^{3-}$ with French bean plastocyanin [10,11]. The copper-ligated histidines of *A. variabilis* plastocyanin (fig.3) have titration properties that resemble those of spinach plastocyanin. We therefore assign the peaks by

homology to histidine-37 (non-titratable peaks H_ϵ^{37} and H_δ^{37}) and histidine-87 (peak H_ϵ^{87} with $\text{pK}'_a = 5.10 \pm 0.01$, and peak H_δ^{87} with $\text{pK}'_a = 5.11 \pm 0.02$).

In model peptides at neutral pH, the ^{13}C NMR peak from the C_ϵ of histidine appears at 135.2 ppm whereas that from the C_δ appears at 118.7 ppm [20]. The chemical shift difference of 16 ppm between the two is much larger than any environmental shift expected in a diamagnetic protein. Hence, the histidine C_ϵ and C_δ peaks in a protein spectrum can be distinguished unambiguously. On the other hand, the ^1H NMR chemical shift difference between the histidine $\text{C}_\epsilon\text{-H}$ and the $\text{C}_\delta\text{-H}$ peak in model peptides is only 0.7 to 1.2 ppm [21], with the $\text{C}_\epsilon\text{-H}$ resonance to lower field. In proteins, environmental shifts of this magnitude are commonly observed, and the assignment of histidine ring peaks may be in doubt. The heteronuclear 2DFT NMR technique allows the pairing of resonances from $^{13}\text{C}\text{-}^1\text{H}$ groups. Having assigned the ^1H -NMR resonances on the basis of their pH titration behavior, we can now cross assign the correspond-

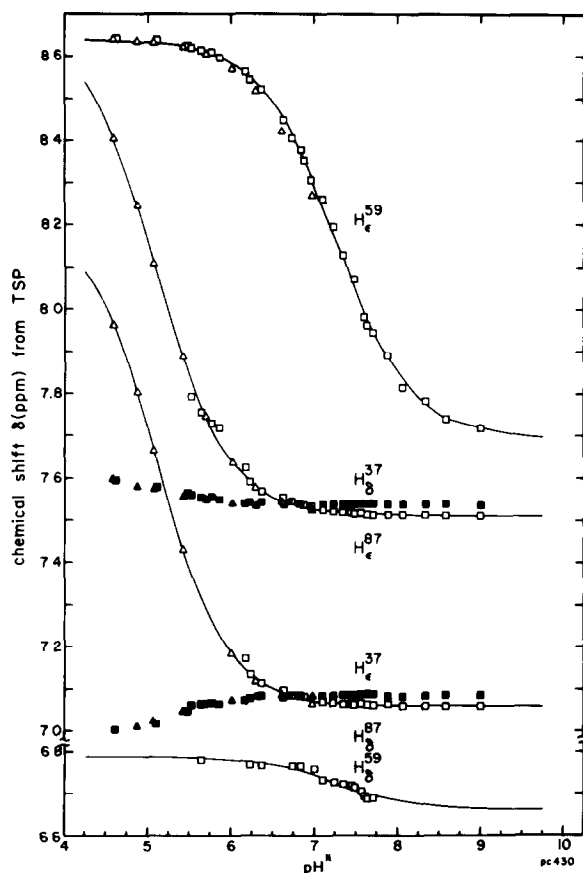


Fig.3. ^1H NMR pH titration curves of the histidine $\text{C}_\delta\text{-H}$ and $\text{C}_\epsilon\text{-H}$ peaks of reduced $[\text{Cu(I)}]$ *A. variabilis* plastocyanin: (\square, \blacksquare) 470 MHz data; ($\triangle, \blacktriangle$) 200 MHz data. Plastocyanin was 3 mM in 20 mM phosphate buffer in $^2\text{H}_2\text{O}$. The data were fit to a modified form of the Hill equation [21]. The notation used to identify titration curves is explained in fig.1.

ing ^{13}C resonances. This is illustrated in fig.2 for two pH^* -values that were chosen in order to establish whether the ^1H titration curves cross over (fig.3). Histidines 59 and 87 of *A. variabilis* plastocyanin follow the normal ^1H NMR pattern in having the $\text{C}_\epsilon\text{-H}$ peak to lower field than the $\text{C}_\delta\text{-H}$ peak. However, the heteronuclear 2DFT NMR results reveal that this normal order is reversed for the ring protons of histidine-37. All previous ^1H NMR studies of plastocyanins had assumed the downfield peak of histidine-37 to be the $\text{C}_\epsilon\text{-H}$.

Histidine peaks H_ϵ^{87} and H_δ^{87} of *A. variabilis* plastocyanin broaden as the pH is lowered, resulting in disappearance of the peaks at pH^*

< 5.5 . We found similar broadening of the histidine-87 ^1H NMR peaks of spinach plastocyanin at low pH^* -values at 470 MHz. We did not observe the broadening with either protein at 200 MHz. The dependence of linewidth on spectrometer frequency indicates the presence of a chemical exchange process at an 'intermediate' rate on the 470 MHz NMR time scale. Since the titration shifts $|\delta_{\text{H}^+} - \delta_{\text{OH}^-}|$ for the ring protons of histidine-87 of *A. variabilis* plastocyanin are 1.18 ppm for H_ϵ^{87} and 1.16 ppm for H_δ^{87} , exchange broadening at 470 MHz indicates that the reduced lifetime of a proton on the N_δ of histidine-87 is about 400 μs , longer than the normal value of around 100 μs for an exposed imidazole [22]. The longer lifetime or slow exchange of this hydrogen results either from decreased solvent accessibility to the site or a rate-determining conformational change required for protonation/deprotonation of the N_δ .

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