Volume 162, number 1 FEBS 0842 October 1983

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

Christopher L. Kojiro and John L. Markley*

Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA

Received 20 June 1983

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, ^{1}H) *J*-connectivity spectroscopy has enabled cross assignment of ^{1}H 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 ^{1}H 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,^{1}H) chemical shift-correlated two-dimensional Fourier transform (2DFT) NMR [13,14] and one-dimensional (1DFT) ^{1}H and ^{13}C NMR spectroscopy to analyze properties of the histidines of plastocyanin from the cyanobacterium, *Anabaena variabilis*, as a function of redox state and pH.

* To whom correspondence should be addressed

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

2. EXPERIMENTAL

Heteronuclear (^{13}C,^{1}H) 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 ^{1}H 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 11000) [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

Published by Elsevier Science Publishers B.V.

there is no $^{1}H-^{13}C$ 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 ^{1}H. The ^{1}H 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 ^{1}H decoupler was positioned at 6.0 or 5.5 ppm (pH* 7.6 and 6.1, respectively) in the ^{1}H frequency region, with the delay set so as to cover a ^{1}H 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 ^{1}H or ^{13}C 1DFT NMR spectrum (plotted at the sides). The ^{1}H 1DFT NMR spectrum contains peaks from N–H as well as C–H groups. Since N–H groups do not give rise to $^{13}C-^{1}H$ 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)

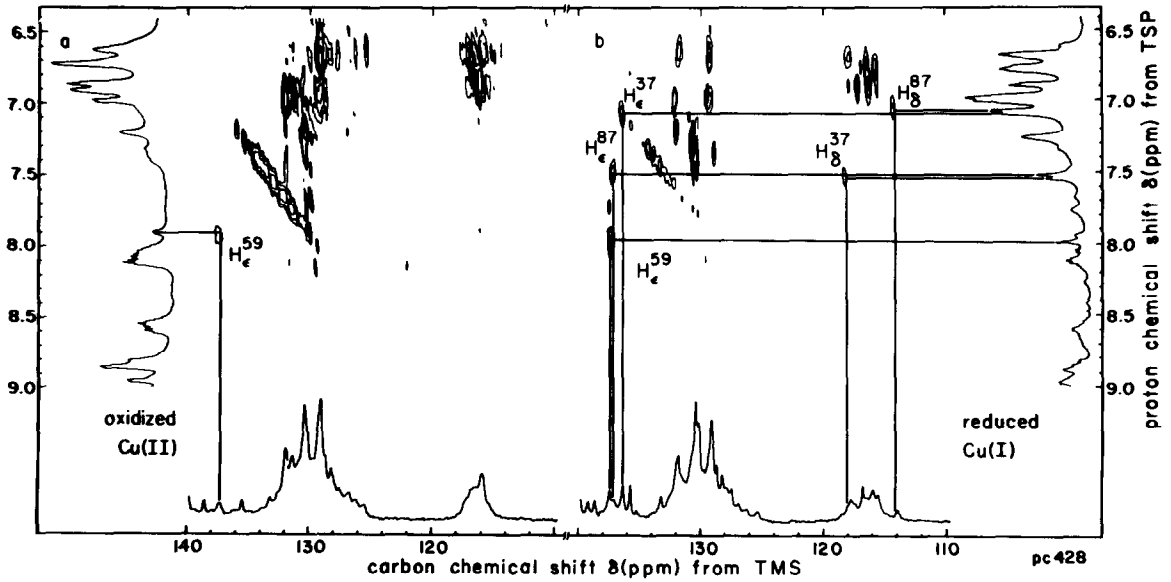

Fig.1. Comparison of one-dimensional (1DFT) and heteronuclear (^{13}C,^{1}H) chemical shift-correlated two-dimensional Fourier transform (2DFT) spectra of (a) reduced [Cu(I)] and (b) oxidized [Cu(II)] *A. variabilis* plastocyanin at pH* 7.6. The 2DFT contour map was obtained at a frequency of 50 MHz for ^{13}C and 200 MHz for ^{1}H. The ^{1}H 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 $C_{\epsilon}-H$ of histidine 37.

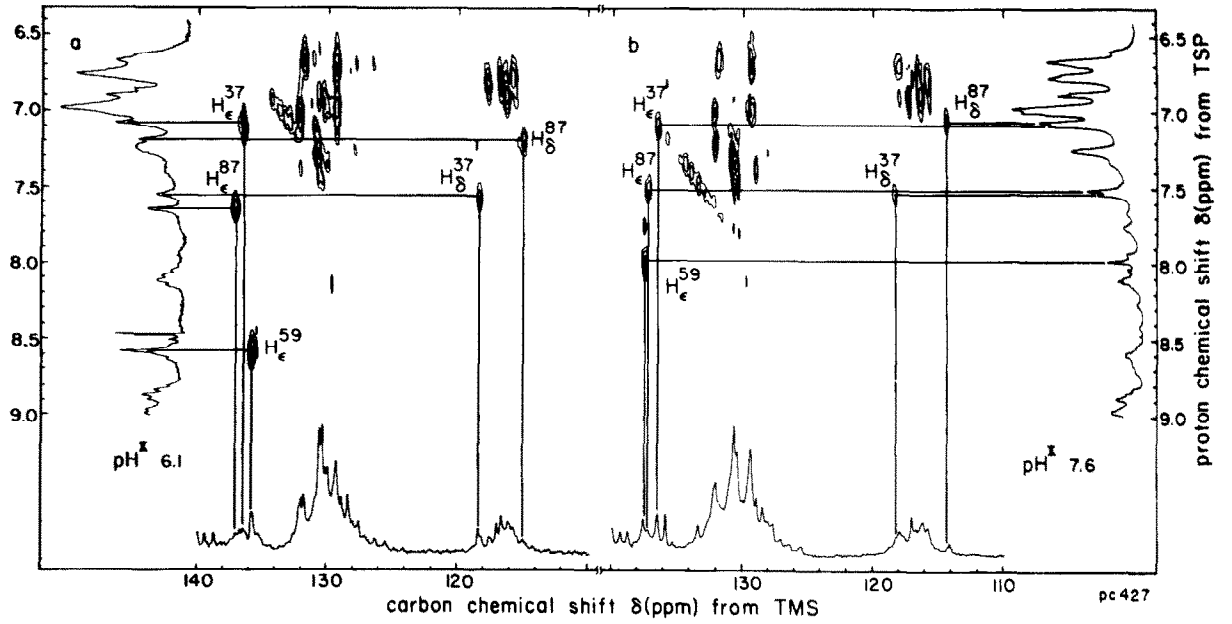

Fig.2. Comparison of one-dimensional (1DFT) and heteronuclear (^{13}C,^{1}H) chemical shift-correlated two-dimensional Fourier transform (2DFT) NMR spectra of [Cu(I)] *A. variabilis* plastocyanin at two pH*-values: (a) pH* 6.1 and (b) pH* 7.6. The 2DFT spectrum is represented as a contour plot, and the 1DFT spectra are plotted at the sides and bottom. The NMR spectral conditions were as in fig.1 except that the ^{1}H 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 $Cr(CN)_6^{3-}$ 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 pK'_a = 5.10 ± 0.01, and peak H_δ^{87} with pK'_a = 5.11 ± 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 ^{1}H NMR chemical shift difference between the histidine C_ϵ–H and the C_δ–H peak in model peptides is only 0.7 to 1.2 ppm [21], with the C_ϵ–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}C–^{1}H groups. Having assigned the ^{1}H-NMR resonances on the basis of their pH titration behavior, we can now cross assign the correspond-

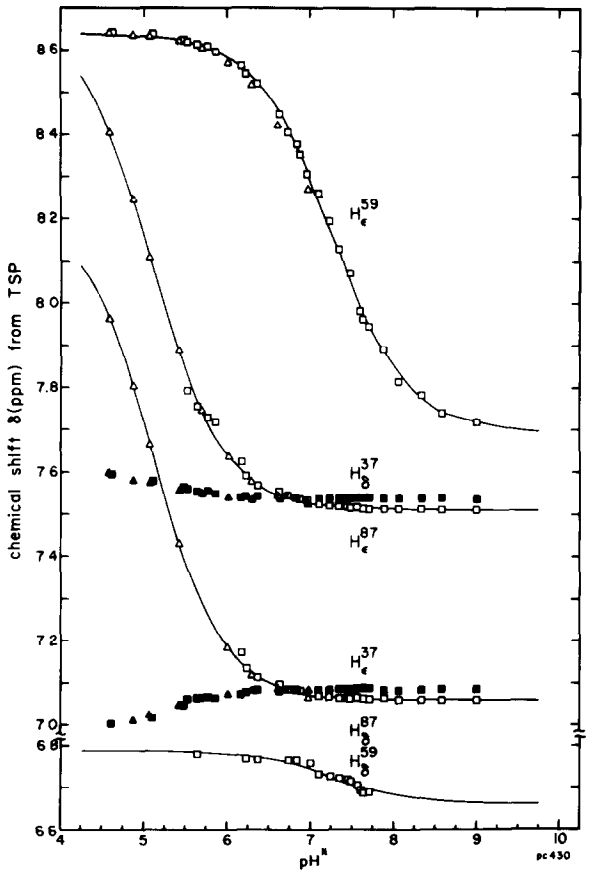

Fig.3. ^1H NMR pH titration curves of the histidine C_δ–H and C_ϵ–H peaks of reduced [Cu(I)] *A. variabilis* plastocyanin: (□,■) 470 MHz data; (△,▲) 200 MHz data. Plastocyanin was 3 mM in 20 mM phosphate buffer in ^2H_2O. The data were fit to a modified form of the Hill equation [21]. The notation used 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 C_ϵ–H peak to lower field than the C_δ–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 C_ϵ–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 $|\delta_{H^+} - \delta_{OH^-}|$ 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.

ACKNOWLEDGEMENTS

This research was supported by the US Department of Agriculture Competitive Research Grants Office, Cooperative State Research Service, Science and Education. NMR spectroscopy was carried out at the Purdue Biochemical Magnetic Resonance Laboratory supported by grant RR 01077 from the Biotechnology Resources Program of the Division of Research Resources, National Institutes of Health.

REFERENCES

[1] Wood, P.M. and Bendall, D.S. (1975) Biochim. Biophys. Acta 387, 115–128.
[2] Boulter, D., Haslett, B.G., Peacock, D., Ramshaw, J.A.M. and Scawen, M.D. (1977) Int. Rev. Biochem. 13, 1–40.
[3] Markley, J.L., Ulrich, E.L., Berg, S.P. and Krogmann, D.W. (1975) Biochemistry 14, 4428–4433.
[4] Colman, P.M., Freeman, H.C., Guss, J.M., Murata, M., Norris, V.A., Ramshaw, J.A.M. and Venkatappa, M.P. (1978) Nature 272, 319–324.
[5] Katoh, S. and Takamiya, A. (1964) J. Biochem. (Tokyo) 55, 378–387.
[6] Beattie, J.K., Fensom, D.J., Freeman, H.C., Woodcock, E., Hill, H.A.O. and Stokes, A.M. (1975) Biochim. Biophys. Acta 405, 109–114.

[7] Markley, J.L., Ulrich, E.L. and Krogmann, D.W. (1977) Biochem. Biophys. Res. Commun. 78, 106–114.
[8] Hill, H.A.O. and Smith, B.E. (1978) Biochem. Biophys. Res. Commun. 81, 1201–1208.
[9] Freeman, H.C., Norris, V.A., Ramshaw, J.A.M. and Wright, P.E. (1978) FEBS Lett. 86, 131–135.
[10] Cookson, D.J., Hayes, M.T. and Wright, P.E. (1980) Nature 283, 682–683.
[11] Cookson, D.J., Hayes, M.T. and Wright, P.E. (1980) Biochim. Biophys. Acta 591, 162–176.
[12] King, G. and Wright, P.E. (1982) Biochem. Biophys. Res. Commun. 106, 559–565.
[13] Maudsley, A.A. and Ernst, R.R. (1977) Chem. Phys. Lett. 50, 368–372.
[14] Chan, T.-M. and Markley, J.L. (1982) J. Am. Chem. Soc. 104, 4010–4012.
[15] Freeman, R. and Morris, G.A. (1979) Bull. Mag. Res. 1, 5–26.
[16] Morris, G.A., Hall, L.D.J. and Jonsson, N.B.-H. (1980) J. Mag. Res. 40, 377–380.
[17] Tokura, K. and Miyazawa, T. (1982) Eur. J. Biochem. 123, 127–131.
[18] Westler, W.M. and Markley, J.L. (1982) Xth Int. Conf. Magnetic Resonance in Biological Systems, Stanford CA, Aug. 29–Sept. 3, p.119.
[19] Ulrich, E.L. and Markley, J.L. (1978) Coord. Chem. Rev. 27, 109–140.
[20] Wüthrich, K. (1976) in: NMR in Biological Research: Peptides and Proteins, p.176, Elsevier, Amsterdam, New York.
[21] Markley, J.L. (1975) Acc. Chem. Res. 8, 70–80.
[22] Sudmeier, J.L., Evelhoch, J.L. and Jonsson, N.B.-H. (1980) J. Mag. Res. 40, 377–380.