Spinach Plastocyanin: Comparison of Reduced and Oxidized Forms by Natural Abundance Carbon-13 Nuclear Magnetic Resonance Spectroscopy

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SUMMARY Differences between the reduced Cu(I) and oxidized Cu(II) forms of spinach plastocyanin were investigated by natural abundance carbon-13 nuclear magnetic resonance spectroscopy at 67.9 MHz using proton noise decoupling. The spectra confirm that histidines 38 and 91 are copper ligands and demonstrate that coordination is by the $N^{\delta 1}$ of both imidazole rings. Spectra of reduced plastocyanin yielded 128 separately resolved carbon resonances. Upon oxidation, 16 of these were observed to disappear; yet there was little change in the positions or intensities of other peaks. Those peaks which disappear are assigned to carbons near the metal. The protein evidently does not undergo a substantial change in conformation upon change of redox state.

INTRODUCTION There is as of yet no x-ray crystallographic structure available for a blue-copper protein. Plastocyanins and azurins from several species have been sequenced, and sequence homologies between these two classes of blue-copper proteins have been discussed recently (1). The function of azurin is still unclear, but plastocyanin (MW 10,000) is known to serve as a specific electron carrier which transports electrons from cytochrome f to P700 of photosystem I (2). A great deal of interest has been focused on the redox and spectral properties of plastocyanin and other blue-copper proteins (3-8). Ample evidence from chemical (9) and spectroscopic (6,7) studies demonstrates the involvement of a cysteine sulfur ligand to the copper . Proton NMR[a] studies indicated that two histidyl groups are coordinated to the copper in spinach and Anabaena variabilis plastocyanins (10) and Pseudomonas aeruginosa azurin (11). The involvement of two histidyls as copper

[a]Abbreviations used: NMR, nuclear magnetic resonance; ppm, parts per million.

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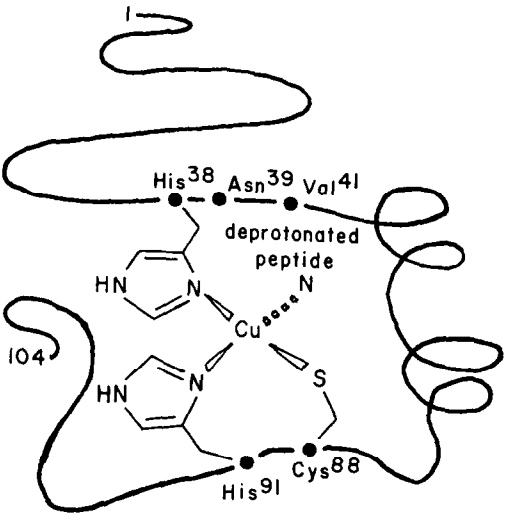

Figure 1. Model for the copper binding site of plastocyanins (see text).

ligands in Pseudomonas aeruginosa azurin was recently confirmed by ^{13}C NMR spectroscopy, which indicated further that each of the imidazole rings is coordinated by the $N^{\delta 1}$ (12). Published ^{1}H-NMR spectra of french bean plastocyanin (13) also are consistent with two histidyl groups serving as copper ligands. Infrared spectral studies of bean plastocyanin suggested that the fourth copper ligand is a deprotonated peptide nitrogen (5). Gray and co-workers have proposed a distorted tetrahedral structure (3) similar to that shown in Figure 1. We present here ^{13}C-NMR spectra of spinach plastocyanin which confirm the assignment of two histidyl ring nitrogens as copper ligands and demonstrate that the imidazole nitrogens involved are the same (both $N^{\delta 1}$) as in Pseudomonas aeruginosa azurin (12). The NMR data are consistent with a cysteine sulfur and a peptide nitrogen as the other two ligands.

MATERIALS AND METHODS Spinach plastocyanin was isolated and purified as previously reported (10). Solutions used for NMR spectroscopy contained 37 mg plastocyanin in 2 ml 0.15 M phosphate buffer in D_2O. Minimum quantities of solid $Na_2S_2O_4$ and ferricyanide were used respectively to reduce and oxidize the plastocyanin. 10 mm O.D. sample tubes were used. ^{13}C-NMR spectra were obtained at 67.9 MHz using a Bruker WH 270 spectrometer at Bruker Instruments, Inc., Billerica, Massachusetts. We thank Dr. Bruce Hawkins for assistance in obtaining the spectra.

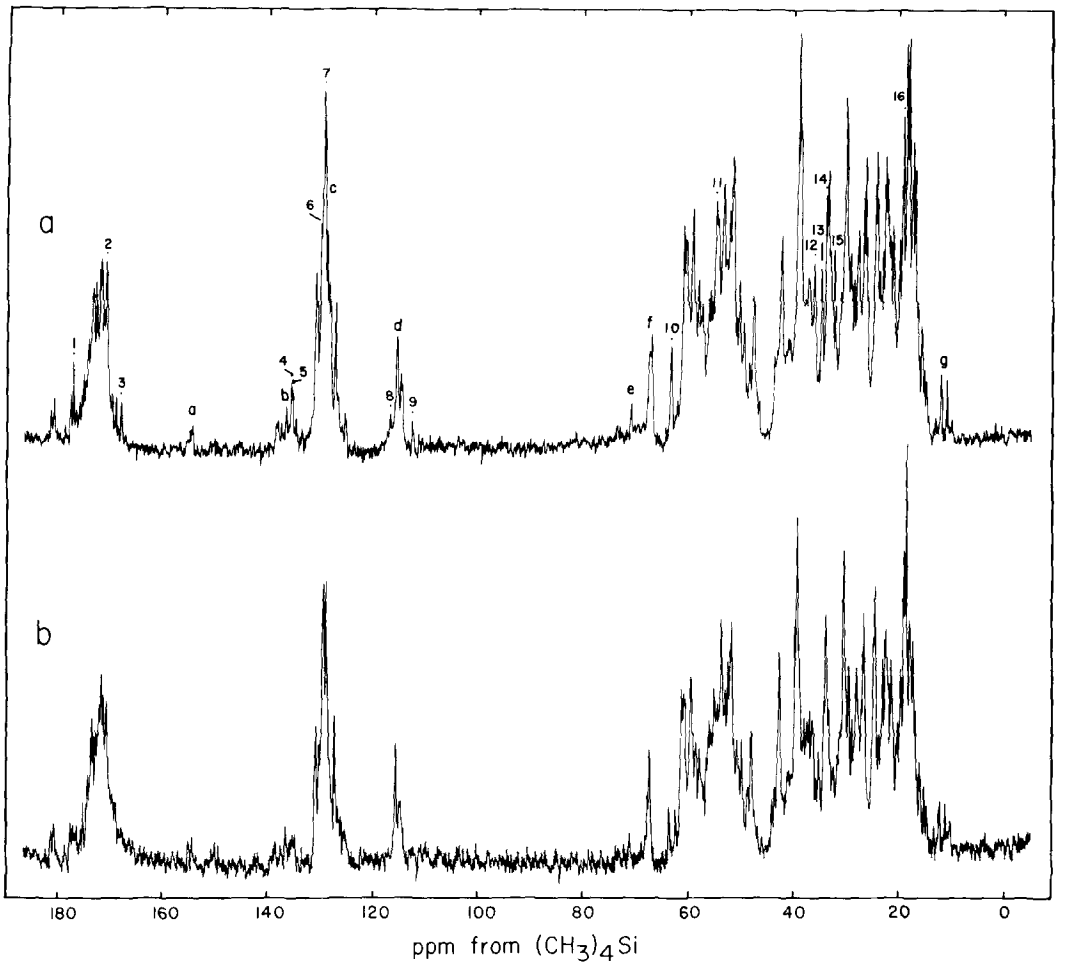

Figure 2. Natural abundance C-NMR spectra at 67.9 MHz of spinach plastocyanin. Each spectrum was obtained at 33°C using proton noise decoupling, deuterium lock, quadrature detection, 16,384 time domain addresses, and a recycle time of 0.54 s. A line broadening factor of 5 Hz was applied to the spectra. The protein concentrations were 1.7 mM in 0.15 M phosphate buffer in D O, pH* 7.00 (uncorrected meter reading). (c) Reduced plastocyanin after 96,940 accumulations. (b) Oxidized plastocyanin after 29,485 accumulations.

RESULTS AND DISCUSSION ^{13}C-NMR spectra of reduced and oxidized spinach plastocyanin at pH 7.00 are compared in Figure 2. 128 separate peaks are resolved in the spectrum of reduced plastocyanin. Certain of the peaks in Figure 2a can be assigned tentatively to particular kinds of amino acids on the basis of their characteristic chemical shifts (14). The peaks in region a correspond to the C^{ζ} nuclei of the 3 Tyr rings. Peaks in region b correspond to the C^{γ}

of the 6 Phe rings. Most of the intensity in region c may be attributed to Phe ring C^{δ}, C^{ε}, and C^{ζ} and Tyr C^{δ} and C^{γ} peaks. Peaks in region d correspond to the 6 C^{ε} carbons of the 3 Tyr rings; of these, two single-carbon peaks are resolved. C^{β} peaks from the 5 Thr residues are expected in region f. Peak e which has an abnormal chemical shift of 70.6 ppm which does not match the chemical shift of any free amino acid may correspond to a deshielded Thr C^{β}. Region g includes the chemical shifts expected for the C^{δ} nuclei of the 3 Ile residues. Additional intensity in this region may be attributed to shielded Ile C^{γ_2} and/or Met C^{ζ} nuclei.

At the present level of resolution, 16 single-carbon peaks are detected in the spectrum of reduced Cu(I) spinach plastocyanin which vanish when the protein is oxidized to the Cu(II) form. These peaks are numbered 1-16 in Figure 2a. The peaks correspond to carbons which are very close to the metal and whose resonances become broadened beyond detection by the paramagnetic Cu(II). The most certain assignments are for peaks 4-9 which correspond to all the ring carbons of His^{38} and His^{91}.[b] The other assignments in Table I are more speculative. From metal binding studies to glutathione (16), chemical shifts of 61 ppm and 34 ppm are expected for the C^{α} and C^{β}, respectively, of Cys^{88}. These carbons may correspond to peak 10 (63.37 ppm) and peak 13 or 14 (34.90/33.98 ppm). Possible assignments for the remaining peaks were made by considering only those residues which are conserved in all plastocyanin and azurin sequences and thus are likely copper ligands (1). These suggestions must be regarded only as working hypotheses for further studies.

An alignment of the sequences of azurins and plastocyanins near the presumed copper ligands is shown in Figure 3. This alignment differs from that of Ryden and Lundgren (1) in that His^{91} of plastocyanin and His^{117} of azurin are considered here to be homologous. Also residue 96 of plastocyanin is not conserved as Met in all sequences (17). There is some evidence for a

[b] The amino acid numbering system used here for plastocyanins is based on the sequence of Anabaena variabilis plastocyanin (15) which has 104 residues and is the longest known plastocyanin.

Table I. Peaks that vanish upon oxidation of Cu(I) spinach plastocyanins.

peak number*	$^{\delta}(CH_3)_4Si$ ppm	possible assignments†
1	177.0	
2	170.82	Asn^{39} C°, Asn^{39} C^{γ}, His C°, Cys^{88} C°, Val^{41} C°
3	167.95	(170.9) (171.2) (170.0) (173.5) (170.4)
4	135.62	His^{38} C^{ϵ_1}, His^{91} C^{ϵ_1}
5	135.28	(133.6) (133.6)
6	129.98	His^{38} C^{γ}, His^{91} C^{γ}
7	128.80	(130.3) (130.3)
8	116.76	His^{38} C^{δ_2}, His^{91} C^{δ_2}
9	112.56	(116.8) (116.8)
10	63.37	Cys^{88} C^{α}‡, Val^{41} C^{α}, Pro^{90} C^{α} (61) (61.1) (61.1)
11	54.86	Asn^{39} C^{α}, His^{38} C^{α}, His^{91} C^{α} (52.6) (53.7) (53.7)
12	36.15	Asn^{39} C^{β}, Tyr^{84} C^{β} (37.1) (37.0)
13	34.90	His^{38} C^{β}, His^{91} C^{β}, Cys^{88} C^{β}‡,
14	33.98	(27.6) (27.6) (34)
15	32.25	(Pro^{90} C^{β}, Val^{41} C^{β}, Tyr^{84} C^{β}, Asn^{39} C^{β}) (29.2) (29.7) (37.0) (37.1)
16	19.32	Val^{41} C^{γ}, Pro^{90} C^{γ} (17.4, 18.6) (23.9)

*As indicated in Figure 2a. †See text for rationale of assignments. The numbers in parentheses below the possible assignments are chemical shifts that are expected for random coil peptides as derived from studies of amino acids and peptides. Unless otherwise noted, these values are from Wuthrich (20). ‡Calculated on the basis of metal-glutathione complexes using the data of Fuhr and Rabenstein (16).

deprotonated amide nitrogen as the fourth ligand of plastocyanin (3-5). Since Asn^{39} is conserved in all plastocyanin sequences and since a homologuos Asn^{37} is present in all azurins, the ligand could be the side-chain amide of asparagine. Other possibilities include the backbone amide of the conserved

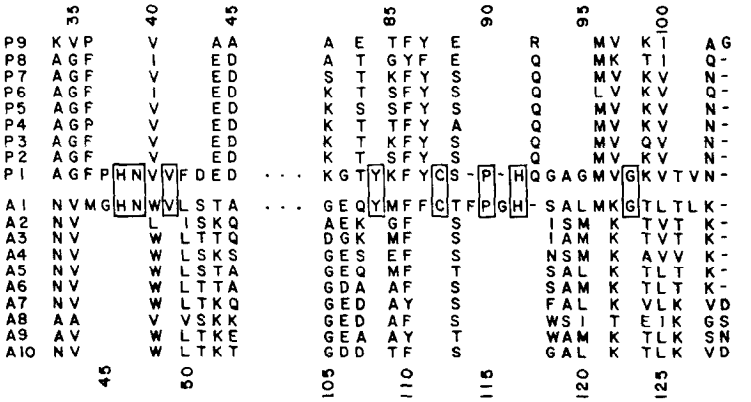

Figure 3. Amino acid sequences for 9 plastocyanins and 10 azurins around the presumed copper ligands as collected from the literature. Sequences are given in the one-letter code (21). Blanks indicate that the sequence is the same in all plastocyanins or azurins. Dashes indicate gaps in the alignment or, at the end, missing residues. Sources and references are as follows. Plastocyanins: P1, spinach (Spinacia oleracea) (22); P2, french bean (Phaseolus vulgaris) (23); P3, broad bean (Vicia faba) (24); P4, potato (Solanum tuberosum) (25); P5, vegetable marrow (Cucurbita pepo) (26); P6, broad leaf dock (Rumex obtusifolius) (17); P7, elder (Sambucus nigra) (27); P8, green alga (Chlorella fusca) (28); P9, blue-green alga (Anabaena variabilis) (15). Azurins: A1, Pseudomonas aeruginosa (P6009) (29); A2, P. fluorescens B (Stanier B-93; ATCC 17467) (29); A3, P. fluorescens C (Stanier C-18, ATCC 17400) (29); A4, P. fluorescens D (Stanier D-35; ATCC 17414) (29); A5, P. fluorescens (P 6009/1) (30); A6, P. denitrificans (NCIB 9496) (29); A7, Alcaligenes sp. (29); A8, A. faecalis (NCIB 8156) (29); A9, A. denitrificans (NCIB 8582) (29,31); A10, Bordetella bronchiseptica (NCTC 8344) (29).

Asn, the conserved Val, or one of the histidines. In order to investigate these possibilities, the C^{13}-NMR spectra of triglycylamide and its Ni(II) complex were investigated (W. M. Westler and J. L. Markley, unpublished). It was found that the terminal amide shifts 12.0 downfield on deprotonation. and that the two peptide amides shift 7.2 and 8.9 ppm downfield on deprotonation Peak 1 (177.08 ppm) thus could correspond to a deprotonated backbone amide carbonyl. The lack of a peak farther downfield, present in Cu(I) plastocyanin and absent in Cu(II) plastocyanin, apparently rules out the Asn^{39} side chain as a copper ligand.

Ugurbil et al. (12) in their ^{13}C-NMR study of Pseudomonas aeruginosa azurin reported 7 peaks in the aromatic region of the reduced protein

which vanish on oxidation. These peaks have chemical shifts of 166.7, 138.5, 137.6, 137.2, 135.7, 134.6, and 130.7 ppm. The peak at 166.7 ppm is very similar to peak 3 of the present study (167.9 ppm). Ugurbil et al. attributed the peak to an abnormal amide carbonyl. Specific assignments were not made for the peaks of the 2 histidyl rings coordinated to Cu in azurin; however, the His C^{γ} peaks were assumed to lie between 138.1 and 135.7. The liganded histidyl ring C(2)-H groups of Pseudomonas aeruginosa azurin and spinach plastocyanin have very similar proton NMR chemical shifts (J. L. Markley and D. R. McMillin, unpublished); therefore, the carbon peaks may also be expected to have similar chemical shifts.

It is noteworthy that at the present level of resolution only minor changes are noted in the chemical shifts of all other carbon peaks on conversion of plastocyanin from the Cu(I) to the Cu(II) form. This suggests that the copper binding site is fairly rigid and that the protein molecule does not undergo a significant conformational change when the copper is oxidized or reduced. It has been found that crystals of plastocyanin from pea leaves can be oxidized or reduced without cracking (32).

The present results support the ^{1}H-NMR evidence for two histidyl ligands to copper in plastocyanins (10). Furthermore, the ^{13}C chemical shifts (18-19) indicate that the liganding nitrogens are the $N^{\delta 1}$ of His^{38} and His^{91}. This is one advantage of ^{13}C over ^{1}H-NMR for studies of histidyl residues in proteins (12).

The 3-dimensional structure of plastocyanin was recently predicted (33) by applying 4 methods of secondary structure prediction to 8 sequences including spinach plastocyanin. The copper ligands predicted by this analysis were: Cys^{88}, Asp^{45}, Glu^{46}, and His^{38}. His^{91} was found to be fixed rigidly 9 Å from the binding site. This predicted structure almost certainly is in error. Residues 45 and 46 in Anabaena variabilis plastocyanin are Ala and Leu, respectively (15). Yet proton NMR studies indicate that the copper binding sites of Anabaena and spinach plastocyanins are very similar (10).

Furthermore, if His^{88} were 9 Å from the Cu(II) its ^{13}C signal would not be broadened and undetectable as the present results indicate.

The prospects appear bright for making definite assignments of many of the carbon resonances of plastocyanins. The strategies to be employed involve comparisons of spectra of plastocyanins from closely related species, pH titration studies, and double resonance NMR spectroscopy. These studies are underway.

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