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BLUE-COPPER PROTEINS: NUCLEAR MAGNETIC RESONANCE INVESTIGATIONS

ELDON L. ULRICH *

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907 (U.S.A.)

JOHN L. MARKLEY **

Department of Chemistry, Purdue University, West Lafayette, Indiana 47907 (U.S.A.)

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CONTENTS

ABBREVIATIONS

EPR: electron paramagnetic resonance
NMR: nuclear magnetic resonance
UV: ultraviolet
TMS: tetramethylsilane
DSS: 2,2-dimethyl-2-silapentane-5-sulfonate
TSP: 3-trimethylsilylpropionate-2,2,3,3-d_4
pH *: uncorrected pH meter reading of a ^2H_2O solution measured with a glass electrode standardized with ^1H_2O buffers

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A. INTRODUCTION

(i) Properties of blue-copper proteins

Copper containing proteins are involved in a variety of biological functions. These include electron transport, copper storage and many oxidase activities [1]. Several copper proteins are easily identified by their beautiful blue color and have been labeled "blue-copper" proteins. As shown in Table 1, the blue-copper proteins can be divided into two classes, the oxidases and the electron carriers. The oxidases are of high molecular weight and contain three forms of bound copper: the blue-copper chromophore (Type I), EPR detectable non-blue (Type II) and EPR non-detectable bound copper atoms (Type III) [1]. They have been heavily studied by EPR [1—5] and other spectroscopic techniques [2,6,7], but their high molecular weights have discouraged NMR investigation.

(ii) Chemical and physical studies

Of the six electron transport proteins listed in Table 1, the azurins and plastocyanins have received particular attention in structural studies. Plastocyanin is found in the chloroplasts of higher plants, algae, and in many cyanobacteria [13]. Its function is to pass an electron from membrane bound cyto-

TABLE 1

Two classes of blue-copper proteins

Protein	M.W.	No. of Cu	Types of Cu	Source	Ref.
Oxidases					
Laccase	60,000—141,000	4	I, II, III	*Rhus vernicifera* *Polyporous versicolor*	2
Ascorbate oxidase	140,000	8	I, II, III	Plants and bacteria	2
Ceruloplasmin	132,000	6	I, II, III	Human serum	2
Electron transport proteins					
Plastocyanin	10,500	1	I	Higher plants and cyanobacteria	2
Azurin	14,000	1	I	Bacteria	2
Stellacyanin	20,000 (107a.a.)	1	I	*Rhus vernicifera*	2, 8, 9
Rusticyanin	Unk.	Unk.	I	*Thiobacillus ferrooxidans*	10
Umecyanin	14,600	1	I	Horseradish	2
Plantacyanin	8,000	1	I	Cucumber and spinach	11, 12

chrome f to the P_{700} chlorophyll reaction center in the photosynthetic electron transport chain [14]. Azurin appears to be a component of the respiratory electron transport chain in several bacteria [15,16]. Both proteins are small (Table 1) and contain a single Type I copper atom [2,17,18]. The intense blue color of the purified proteins has stimulated many structural studies of the copper binding site. Katoh and coworkers first isolated plastocyanin [19] and in subsequent papers chemically identified a unique cysteine residue as a probable copper ligand [18,20]. Later, UV and visible spectroscopic studies by McMillin *et al.* suggested that a charge transfer between the cysteine ligand and the copper is responsible for the large visible extinction coefficient of the oxidized blue-copper protein [21]. The Type I bound copper atom has been found by NMR relaxation to be inaccessible to solvent water [22–24]. Both plastocyanin and azurin can be purified easily in milligram quantities and, because of their low molecular weight, many of these have been sequenced [25–34, 36–39]. The sequences reveal limited homologies [16] between the two blue-copper proteins azurin and plastocyanin (Fig. 1). A histidine (H), asparagine (N) and valine (V) are conserved in all sequences in the first half of the protein chains, and a tyrosine (Y), cysteine (C), proline (P), histidine (H), methionine (M) and glycine (G) are conserved in the second half of the protein. Because of the similar spectral properties of the proteins it is expected that the copper ligands should be among the conserved residues. The original sequence of plastocyanin from broad leaf dock gave residue 92, which is methionine in all other species, as a leucine [29]. This caused us and possibly others to discount methionine as a possible copper ligand [40–43]. The sequence has recently been amended and gives methionine as residue 92 [39].

(iii) NMR studies

High resolution NMR spectroscopy is a recent addition to the techniques that have been used to study the blue-copper proteins. The first ^1H NMR results were published in 1975 [43,44], and the work is now rapidly expanding. In the analysis of protein structure–function relationships, X-ray crystallography and NMR spectroscopy at present provide the most detailed information. NMR spectroscopy, because of the solution state of the protein sample, is quite versatile when one is designing experiments. However, X-ray crystallography is required at present to provide the detailed 3-dimensional orientation of protein groups. Owing to their small size, availability, and the large collection of background data including sequences, plastocyanins and azurins have been attractive candidates for NMR spectroscopy. The only published NMR studies of blue-copper proteins have concerned these proteins. In this review the two proteins will be discussed in turn, starting with plastocyanin. Conclusions drawn from the NMR data on plastocyanin and azurin will then be compared with information obtained from the plastocyanin X-ray crystal studies now in progress [45–48]. The possibilities for future NMR studies of blue-copper proteins in light of the recent publication of the complete plastocyanin X-ray structure will be discussed.

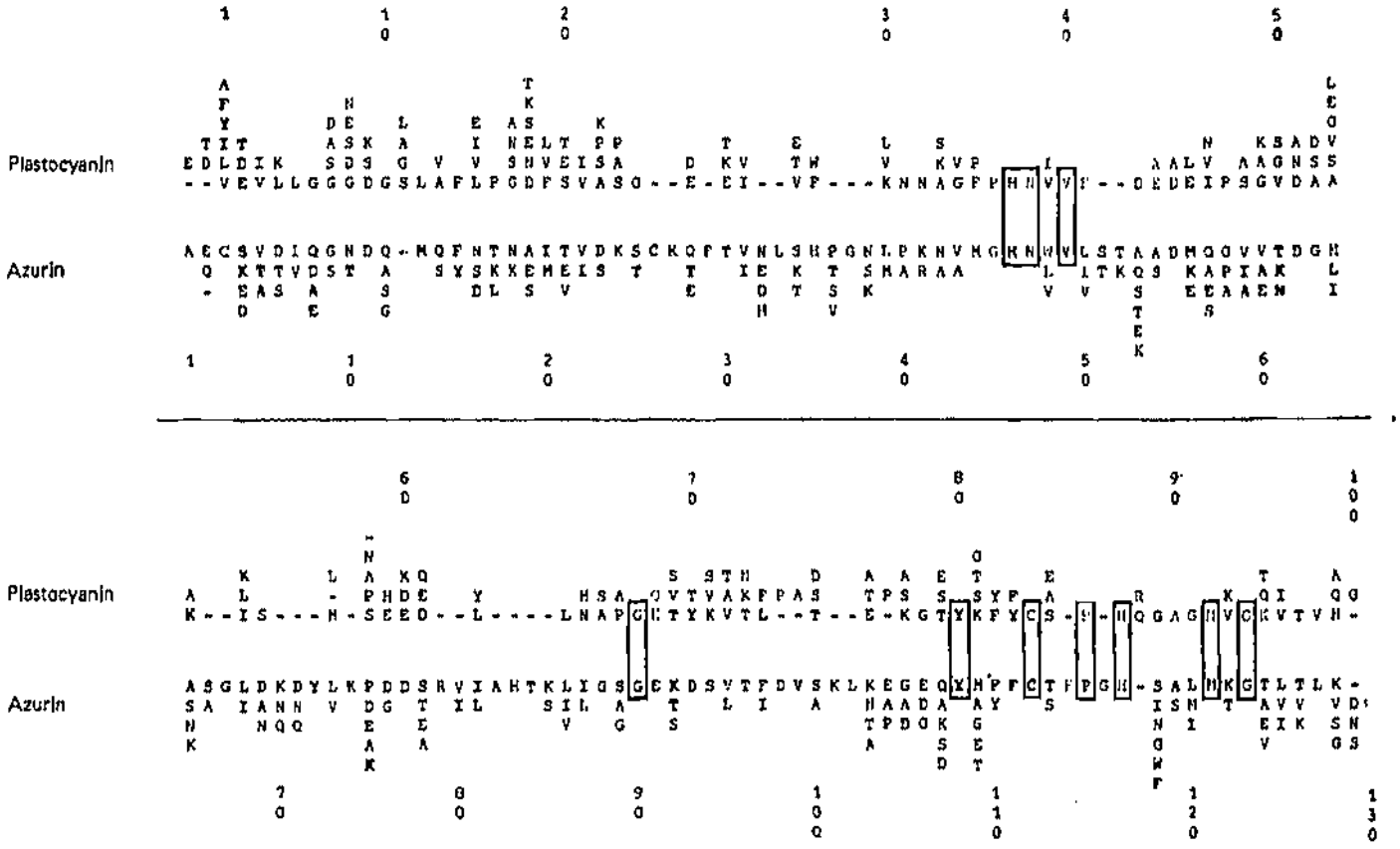


Fig. 1. Composite sequences for the blue-copper proteins plastocyanin (above) and azurin (below). The sequences are aligned essentially as described by Ryden and Lundgren [16] except that a deletion is added at position 86 in the plastocyanin sequence so that histidine-87 in plastocyanin would align with histidine-117 in azurin. Sequences are given in the one-letter code [35]. 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: spinach (*Spinacia oleracea*) [32]; french bean (*Phaseolus vulgaris*) [25]; broad bean (*Vicia faba*) [26]; potato (*Solanum tuberosum*) [27]; vegetable marrow (*Cucurbita pepo*) [28]; broad leaf dock (*Rumex obtusifolius*) [29,39]; elder (*Sambucus nigra*) [30]; green algae (*Chlorella fusca*) [31]; blue-green algae (*Anabaena variabilis*) [33]; and sequences of the first 40 amino acids of several plants from the family Compositae [34]. Azurins: *Pseudomonas aeruginosa* (P6009) [37]; *P. fluorescens* B (Stanier B-93; ATCC 17487) [37]; *P. fluorescens* C (Stanier C-18; ATCC 17400) [37]; *P. fluorescens* D (Stanier D-35; ATCC 17414) [37]; *P. fluorescens* (P 6009/1) [36]; *P. denitrificans* (NCIB 9498) [37]; *Alcaligenes* sp. [37]; *A. faecalis* (NCIB 8156) [37]; *A. denitrificans* (NCIB 8582) [37,38]; *Bordetella bronchiseptica* (NCTC 8344) [37].

B. PLASTOCYANINS

(i) Proton NMR spectroscopy

Plastocyanins from many sources, including french bean, spinach and the cyanobacterium *Anabaena variabilis* have been studied by high-resolution NMR spectroscopy [43,44,49,60]. Normal and resolution-enhanced ^1H NMR spectra of reduced plastocyanin in ^2H_2O are presented in Fig. 2; and the ^1H NMR spectra of the oxidized and reduced forms of french bean, spinach and *Anabaena* plastocyanins are compared in Fig. 3. The most striking features common to spectra of each species are the large number of high-field shifted resonances and non-exchanged N—H peaks. Peaks a—k in Fig. 2 disappear in ^2H_2O solution after removal and replacement of the copper (discussed later) and are assigned to slowly exchanging N—H groups in hydrogen-bonded or otherwise solvent-inaccessible regions of the protein. The unassigned high-field resonances (labeled with Greek letters in Fig. 2) are expected to arise from aliphatic protons located above or below the planes of aromatic rings. Those resonances found in reduced spinach plastocyanin have the following relative intensities using the methyl peak M1 as 3.0 protons: α, 1.0; β, 1.1; γ, 3.1; δ, 0.8; ϵ, 0.9; ζ, 0.9; η, 0.9 [43]. The presence of several high-field resonances combined with the solvent relaxation data mentioned earlier and the large number of unexchanged N—H protons suggest that plastocyanin is a compact protein with the copper buried in a solvent-inaccessible region.

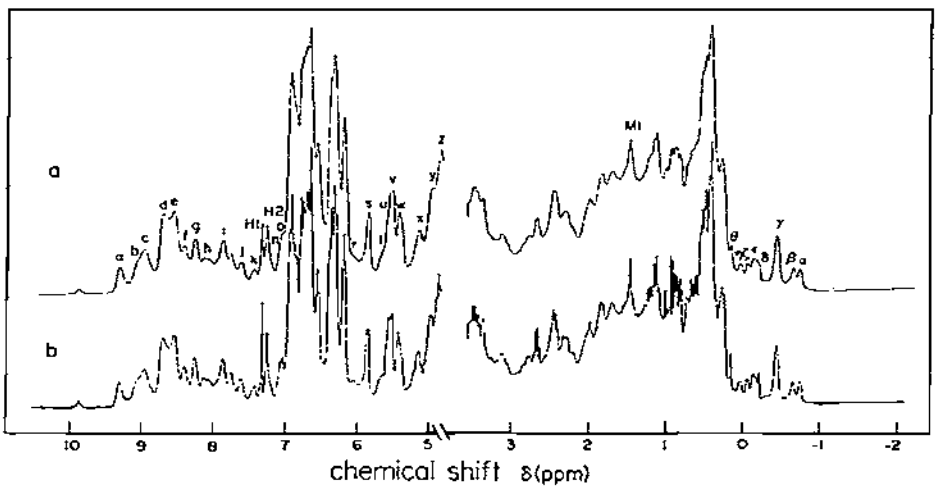


Fig. 2. ^1H NMR correlation spectra of reduced spinach plastocyanin at 250 MHz. Protein concentration was 4 mM at pH* 7.54 in 100 mM potassium phosphate buffer. (a) Normal spectrum, 500 scans accumulated, 1.5 Hz filtering factor. (b) Resolution-enhanced spectrum, 4. 4. 100. filtering factor. Chemical shifts are measured from an external 5% TMS standard. These shifts can be converted to an internal DSS standard by the equation δ 5% TMS + 0.33 ppm = δ DSS.

The aliphatic region of resolution-enhanced spectra of spinach plastocyanin (Fig. 2) exhibits many well-resolved single peaks. The resonance of three-proton intensity labeled M1 has been assigned to a methionine methyl group [43]. A similar resonance can be found in the spectrum of reduced *Anabaena* plastocyanin (Fig. 3c) and has been found in the spectra of almost all plastocyanins [43,49]. Several doublets representing methyl groups found in many aliphatic amino acids have been resolved by the resolution enhancement method. These resonances may be assigned in principle by double irradiation techniques [50].

In the aromatic region (6 to 10 ppm) of Fig. 2 the resonances labeled H1 and H2 have been assigned to C^{ϵ_1} ring protons of the two histidine residues of spinach plastocyanin [43]. The assignments are based on the chemical shift, single proton intensity and pH dependence of each peak. French bean and *Anabaena* plastocyanin spectra also contain two peaks with identical characteristics. H1 and H2 have been assigned to the homologous histidine residues 37 and 87 in each protein [43,44]. In addition, *Anabaena* has a third single proton resonance, H3, which has been assigned to the non-homologous histidine-59 found in this protein [43] (see Fig. 3c).

The aromatic region of the proton NMR spectrum (Fig. 2) contains several apparent doublets which may correspond to tyrosine ring protons, although the definitive decoupling experiments have not been completed. Titrations of tyrosine ring proton resonances were not observed over the pH range 7—12 [51] which is consistent with fluorescence data that has shown all three plastocyanin tyrosines to be inaccessible to solvent [52,53]. This region of the spectrum also contains the resonances from phenylalanine ring and histidine C^{δ_2} ring protons. To the high-field side of the aromatic region are found the peaks labeled r—z. These resonances are either abnormally shielded protons of aromatic rings or abnormally deshielded α-C protons.

Protein-bound metal ions in many cases can be used in NMR studies as internal probes of protein structure. In general, the unpaired electron of a paramagnetic metal ion may cause contact shifts, pseudocontact shifts, or dipolar and scalar line broadening [54—56]. One or more of these effects will be observed in the NMR spectra, depending upon the electronic relaxation time and anisotropy of the paramagnetic center. Contact and pseudocontact shifts have been well exploited in ^{1}H NMR structural investigations of cytochromes *c* [57—59]. Because of the relatively long relaxation time of Cu(II), contact and pseudocontact shifted resonances should be exceedingly broad [54,55].

In Fig. 3 the spectra of three plastocyanins in the oxidized and reduced forms are compared. Oxidation of reduced plastocyanin creates differences in all regions of the spectrum. Either a conformational change or the above-mentioned magnetic properties of the oxidized copper could produce the observed effects. For the moment, only differences attributed to the copper paramagnetism will be discussed. Proton resonances from amino-acid groups directly bound to the copper will be broadened by both scalar and dipolar mechanisms, but other protons should be relaxed only by the dipolar process.

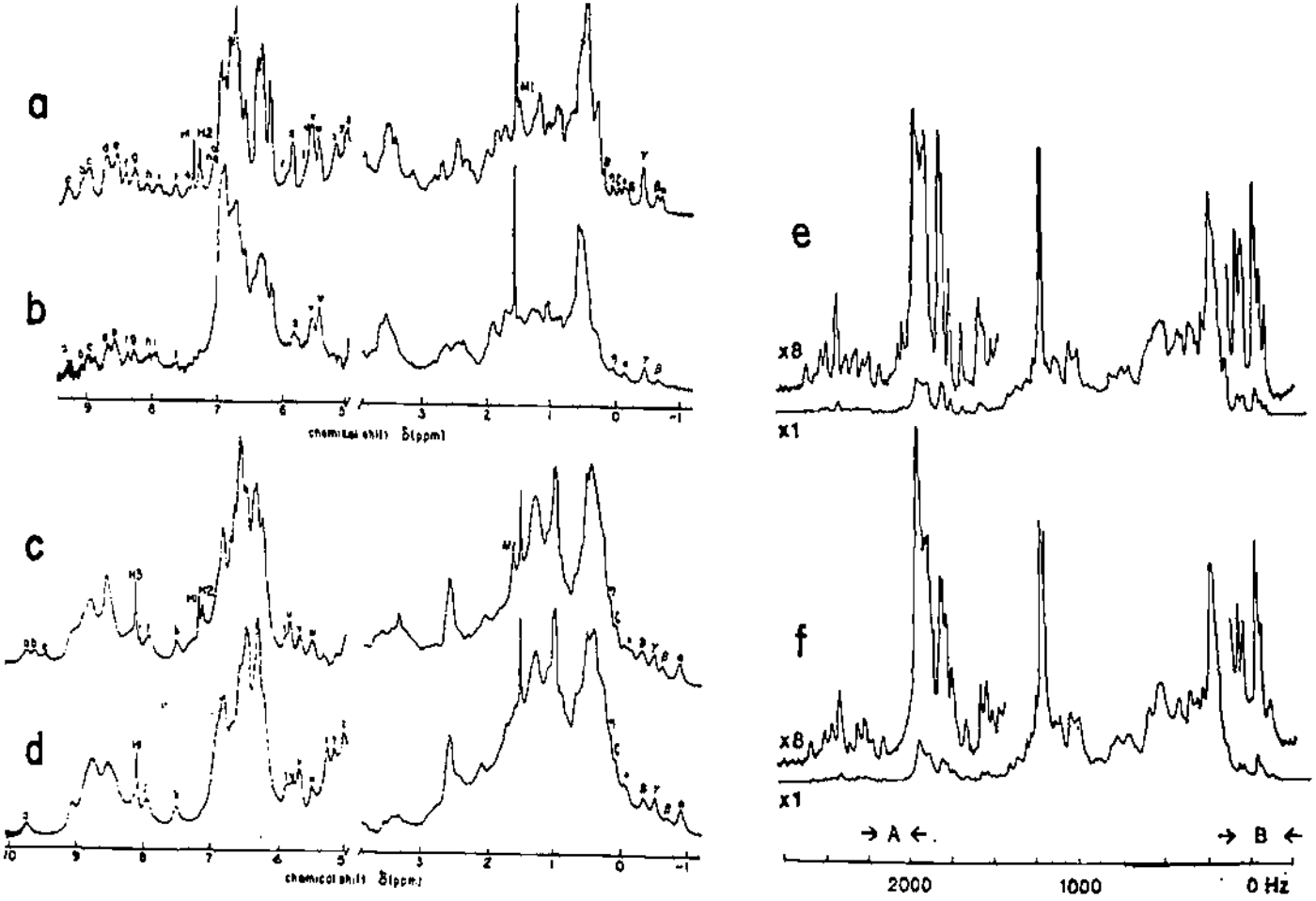


Fig. 3. Comparison of ^{1}H NMR spectra of reduced and oxidized spinach (a and b), *Anabaena variabilis* (c and d) and french bean (e and f) plastocyanins. Spectra a–d are correlation NMR spectra (250 MHz). Spectra e and f are 270 MHz pulsed NMR spectra. The sharp resonance at 1.5 ppm in the spectra of spinach and *Anabaena* plastocyanin was originally labeled M1 [43] but was later found to be a dialyzable impurity [51]. M1 is now used to designate a protein methyl resonance found in the spectra of the reduced plastocyanins. Spinach and *Anabaena* plastocyanin chemical shifts are measured as in Fig. 2. French bean plastocyanin chemical shifts are measured from a DSS standard. From refs. 43 and 44.

Dipolar relaxation depends on the inverse sixth power of the copper—proton distance, and thus protons whose resonances are broadened beyond detection must be located quite near the copper [54,55]. In all three plastocyanins, histidine peaks H1 and H2 and a few high-field and N—H peaks are lost. Significantly, the third histidine resonance, H3, in the *Anabaena* spectra is unaffected by copper oxidation, as are several high-field peaks in all three proteins. These data strongly indicate that two histidines are copper ligands, and that there are several other amino acid groups packed closely around the copper. Intensity is reduced in the tyrosine, phenylalanine and aliphatic regions of the spectra. Freeman et al. [60] have identified phenylalanine and tyrosine resonances which are paramagnetically broadened. The methyl resonance M1 at 1.8 ppm from DSS is also missing in spectra of oxidized plastocyanin (Fig. 3). Freeman et al. [60] recently found that the 3-proton resonance at 1.88 ppm is not present in spectra of reduced carrot and barley plastocyanins. Another singlet resonance is observed at 0.57 ppm from internal DSS in all reduced, but not oxidized, plastocyanins studied. They suggest that the latter peak at 0.57 ppm corresponds to the copper-liganded methionine [60].

(ii) Carbon-13 NMR spectroscopy

Natural abundance ^{13}C NMR spectra of oxidized and reduced spinach plastocyanin taken at 67.9 MHz have been published [40]. The experiments have recently been repeated at 90.5 MHz [51] with significantly better resolution. These spectra of reduced and oxidized spinach plastocyanin are shown in Fig. 4. The 90.5 MHz ^{13}C NMR spectrum of reduced spinach plastocyanin contains 136 resolved lines. There are 15 single-carbon peaks detected in spectra of the reduced Cu(I) plastocyanin which are absent in spectra of the oxidized protein. These peaks, numbered 1—15 in Fig. 4a, must correspond to carbons which are very close to the copper. All 15 peaks are given tentative assignments to amino acids which appear to be near the copper atom in the X-ray structure [48] (Table 2). These tentative ^{13}C assignments should be superior to the previous set [40] which was based on sequence homologies and on an incorrect model for the copper binding site [62,114]. The assignments of peaks 5—10 are most certain [40]; these correspond to the ring carbons of histidine-37 and -87 (see Fig. 1). From studies of metal binding to glutathione [61], chemical shifts of 61 ppm and 34 ppm are expected for the C^{α} and C^{β}, respectively, of cysteine-84. Peak 11 and peaks 13 or 14 may correspond to the cysteine carbons. Peaks 1, 2, 12 and 13 are possible candidates for the copper-liganded methionine C^{o}, C^{α}, C^{β} and C^{γ} resonances, respectively.

No ^{13}C resonances that shift position between oxidized and reduced plastocyanin were resolved at 67.9 MHz [40]. However, at 90.5 MHz five peaks labeled a—e in Fig. 4b were observed in the spectrum of oxidized plastocyanin but not in the spectrum of reduced plastocyanin. The presence of these resonances strongly suggests the existence of local conformational differences in the protein structure in oxidized and reduced plastocyanin as will be discussed later.

TABLE 2

Peaks of interest in 90.5 MHz ^{13}C NMR spectra of spinach plastocyanin

Peak number [a]	$\delta_{(CH_3)_4Si}$ (ppm)	Possible assignments [b]				
Peaks that vanish upon oxidation of Cu(I) spinach plastocyanin						
1	178.16	His C^0,	Cys^{84} C^0,	Met^{92} C^0,	Gly C^0,	Asn^{38} C^0, Tyr^{83} C^0,
2	171.89	(172.6)	(175.7)	(175.0)	(172.7)	(173.1) (176.0)
3	169.14	Phe^{14} C^0,	Leu^{12} C^0,	Ser^{85} C^0,	Pro^{86} C^0,	Val^{93} C^0
4	169.03	(176.0)	(175.9)	(172.6)	(175.2)	(174.9)
5	136.63	His^{37} $C^{\epsilon 1}$,	His^{87} $C^{\epsilon 1}$			
6		(135.2)	(135.2)			
7	131.17	His^{37} C^{γ},	His^{87} C^{γ}			
8	129.48	(130.3)	(130.3)			
9	118.00	His^{37} $C^{\delta 2}$,	His^{87} $C^{\delta 2}$			
10	115.66	(118.7)	(118.7)			
11	62.82	Pro^{86} C^{α},	Val^{93} C^{α},	Cys^{84} C^{α} [c]		
		(61.3)	(60.7)	(61)		
12	55.57	Phe^{14} C^{α},	His C^{α},	Asn^{38} C^{α},	Met C^{α}	
		(57.4)	(53.7)	(51.5)	(53.9)	
13	27.92	His C^{β},	Cys^{84} C^{β},	Gln^{88} C^{β},	Met C^{β},	Met C^{γ}
		(28.0)	(26.0)	(28.1)	(31.0)	(30.7)
14	21.08	Pro^{86} C^{γ},	Val^{93} C^{γ}			
15	20.61	(23.2)	(19.3)			
Peaks found in Cu(II) spinach plastocyanin spectra but not in Cu(I) plastocyanin spectra						
a	172.51	Any amino acid carbonyl carbon				
b	129.85	Phe $C^{\epsilon 1}$ + $C^{\epsilon 2}$, $C^{\delta 1}$ + $C^{\delta 2}$; Tyr $C^{\delta 1}$, $C^{\delta 2}$, C^{γ}				
c	50.74	Leu C^{α}				
d	30.73	Val C^{β}, Tyr C^{β}				
e	22.97	Pro^{16} C^{γ}, Val C^{γ}				

[a] As indicated in Fig. 4. [b] 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 Wüthrich [74]. [c] Calculated on the basis of metal—glutathione complexes using the data of Fuhr and Rabenstein [61].

Figure 5 shows the three possible histidine ring species with the atoms labeled by the crystallographic convention. At pH values well below the histidine pK_a only species A will exist, and the C^{γ}, $C^{\epsilon 1}$ and $C^{\delta 2}$ will all have single unique resonance positions. As the pH is raised the histidine ring may deprotonate at either $N^{\delta 1}$ or $N^{\epsilon 2}$. These two tautomers cannot be distinguished by ^1H NMR because the shift is upfield in both cases. On the other hand, by ^{13}C NMR spectroscopy, deprotonation at the $N^{\delta 1}$ position causes an upfield shift of the C^{γ} resonance of about 2.1 ppm, whereas deprotonation at the $N^{\epsilon 2}$

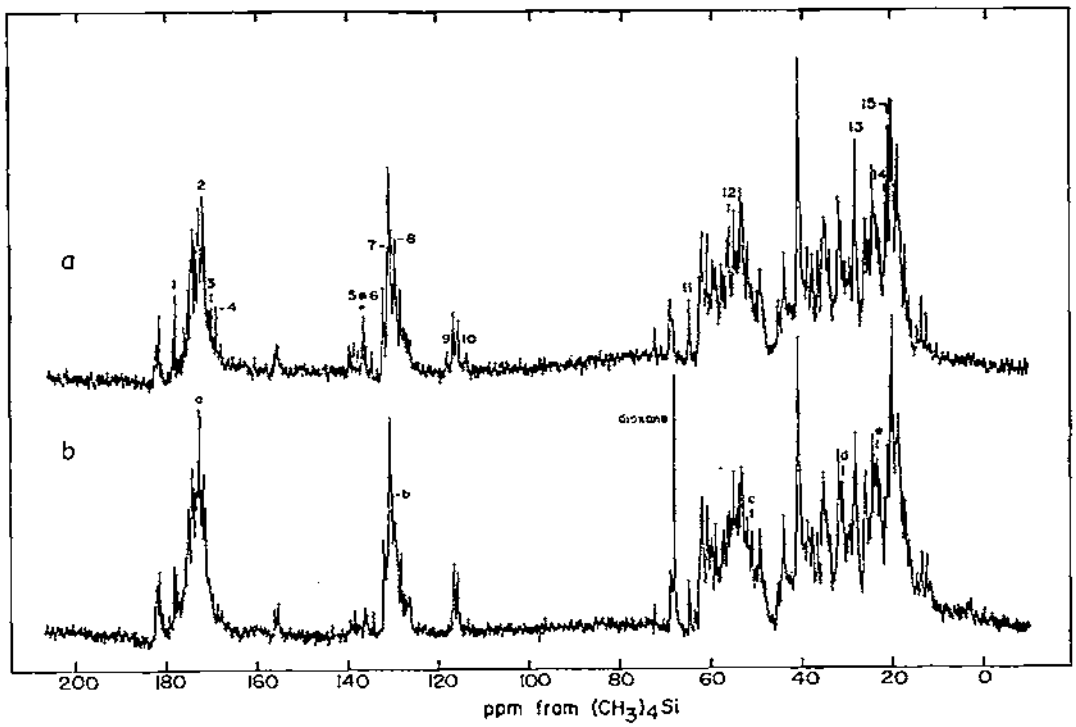


Fig. 4. ^{13}C NMR spectra (90.5 MHz) of reduced (a) and oxidized (b) spinach plastocyanin. The reduced protein spectrum is an accumulation of 41,984 pulses on a 10 mM protein sample at pH* 7.00, 0.135 M potassium phosphate buffer with an excess of dithionite. The oxidized protein spectrum is 81,920 45° Ernst angle accumulations on a 10 mM protein sample at pH* 7.00, 0.135 M potassium phosphate buffer and an excess of potassium ferricyanide. Chemical shifts are referenced to TMS.

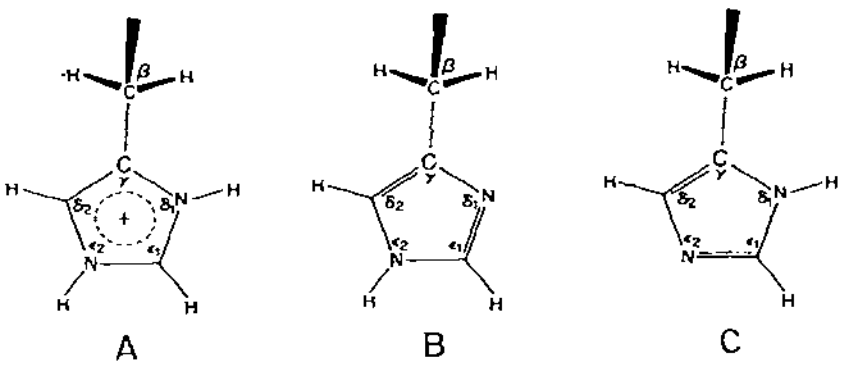
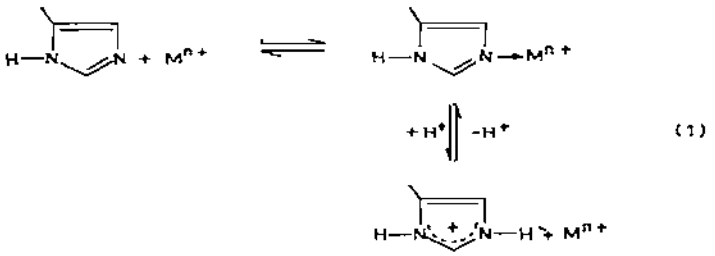


Fig. 5. Histidine side chain in the imidazolium form (A), $N^{\epsilon 2}$—H imidazole form (B), and $N^{\delta 1}$—H imidazole form (C).

causes a downfield shift of about 7.1 ppm [63,64]. The majority of histidine residues studied thus far are found in the $N^{\epsilon 2}$—H imidazole form at pH values above their pK'_a's. The chemical shifts of the C^{γ} resonances (peaks 7 and 8, Fig. 4a) for both histidines in spinach plastocyanin indicate that the $N^{\epsilon 2}$—H imidazole form is present. Copper ligation, therefore, must be to the $N^{\delta 1}$ of each histidine [40]. Copper binding to imidazole at neutral pH does not displace the lone N—H proton, but simply coordinates to the unprotonated nitrogen [65].



To protonate the imidazole ring at low pH the metal ion must first be displaced.

(iii) Histidine residue pH titration curves

The pH dependence of the chemical shifts of ^1H NMR peaks H1 and H2 (Fig. 2) [43] and ^{13}C NMR peaks 9 and 10 (Fig. 4) [51] has further identified these peaks as arising from histidine residues. The titration curves of the histidine ^1H NMR resonances of spinach plastocyanin are presented in Fig. 6. Very similar titration curves have been obtained for the french bean plastocyanin histidines [44]. A complete titration curve has not been determined for any plastocyanin because of protein denaturation below pH 4.5. The pK'_a's found for spinach plastocyanin, 4.9 and less than 4.5, [43] are lower than those of normal histidine residues ($pK'_a = 6.8$) [66]. Removing the copper from the protein by the addition of KCN shifts the pK'_a's of both histidines to more normal values between 6.5 and 6.8 (Fig. 6). The ^{13}C NMR data showed that the copper ion is coordinated to the $N^{\delta 1}$ position at the histidine ring. This is the same position which must be protonated at low pH for the histidine to show a pH dependent low-field shift in ^1H NMR spectra (see eqn. 1). Since the copper ion competes with the proton for the $N^{\delta 1}$ electron pair its presence should reduce the histidine pK_a as was found experimentally [43,44].

Low-field spectra of plastocyanin treated with KCN and apo-plastocyanin produced by the method of Katoh and Takamiya [20] are shown in Fig. 7. The histidine peaks have not been correlated specifically to those in the holo protein and so are labeled Ha and Hb. Note the reduced number of peaks between 7.5 ppm and 9.0 ppm in both spectra due to the rapid exchange of N—H protons with the solvent. The apo-proteins must have a more flexible structure which allows solvent ^2H_2O to penetrate regions of the protein inaccessible in the presence of bound copper.

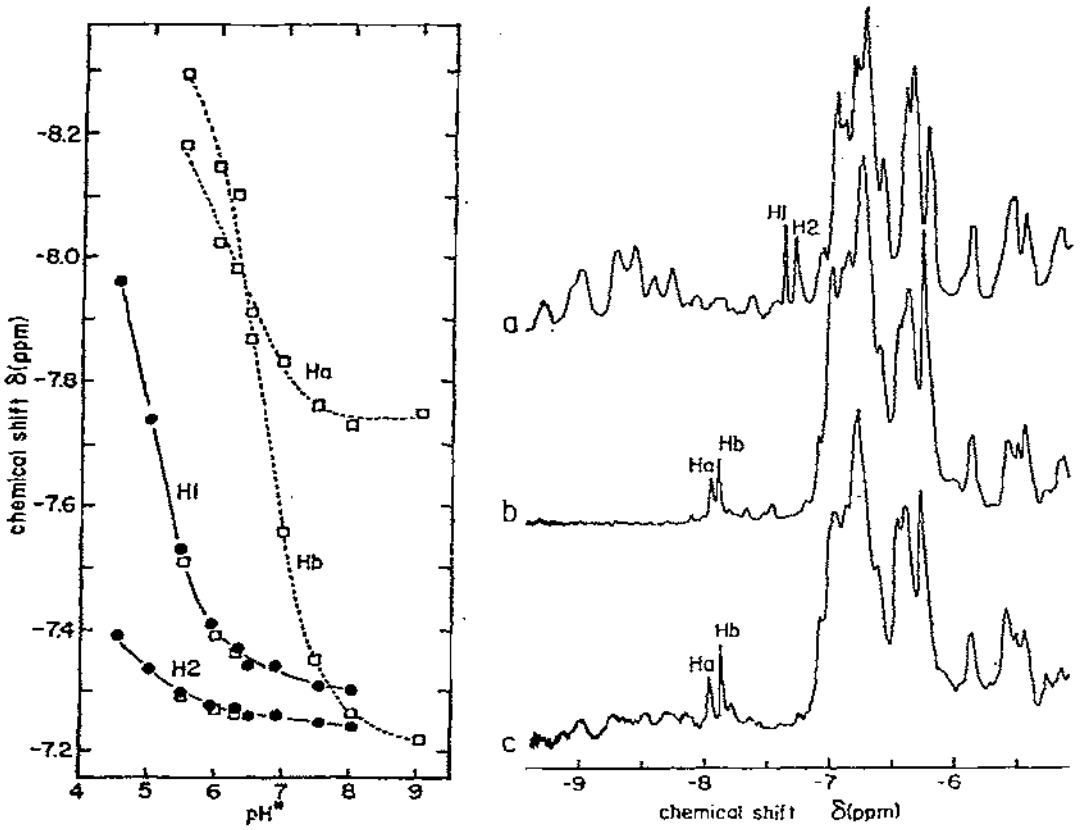


Fig. 6. ^{1}H NMR titration curves (250 MHz) of the histidine $C^{\epsilon 1}$—H peaks of reduced spinach plastocyanin (solid symbols, solid lines) and reduced spinach plastocyanin in 60 mM KCN (open symbols, dashed lines). From ref. 43.

Fig. 7. Comparison of the low-field ^{1}H NMR spectra (250 MHz) in approximately 0.5 M phosphate in $^{2}H_2O$ of: (a) reduced spinach plastocyanin, pH* 6.48; (b) reduced spinach plastocyanin plus 60 mM KCN, pH* 6.50 and (c) spinach apo-plastocyanin, pH* 6.50. From ref. 43.

(iv) Conformational differences between oxidized and reduced plastocyanins

The resonances of protons and carbon atoms intimately involved with the copper cannot be used to determine possible conformational changes at the copper binding site resulting from changes in the oxidation state. They are not resolved in spectra of Cu(II) plastocyanin, and even if they were, it would be impossible to distinguish conformational effects from hyperfine effects. NMR evidence for conformational changes between the redox states comes from sharp peaks not directly affected by the paramagnetic copper. Five ^{13}C resonances have been resolved in the 90.5 MHz spectrum of oxidized spinach plastocyanin which do not have counterparts in the spectrum of reduced plastocyanin (Fig. 4). These evidently represent carbons whose environments are

different in oxidized and reduced plastocyanin. The existence of a limited conformational change is supported by ^{1}H NMR difference spectra between oxidized and reduced spinach plastocyanin (Fig. 8). As expected, the preponderance of negative intensity observed is from peaks present in the spectrum of reduced plastocyanin but absent owing to hyperfine interactions in the spectrum of oxidized protein. Resonances found above the baseline can only arise from peaks found in the oxidized protein spectrum which shift upon reduction of the copper. Peaks 1—10 in Fig. 8 correspond to positive resonances found adjacent to negative peaks.

Beattie et al. [44] have documented a peak in the high-field region of french bean plastocyanin whose chemical shift is dependent on the redox state. Spectra of pure partially oxidized plastocyanin (Fig. 9) exhibit resonances for both oxidized and reduced protein, yet protein samples partially oxidized with ferricyanide showed a weighted average of the two peaks. On the NMR time scale the pure protein has a slow rate of self exchange, but the ferricyanide acts as an intermediary, increasing the speed of electron transfer to a relatively fast rate. An upper limit for the self exchange rate constant k_{ex} was found to be $k_{ex} << 2 \times 10^4\ M^{-1}s^{-1}$ when $[Cu(I)] = [Cu(II)] = 2.5 \times 10^{-3}$ M. The rate constant k for the plastocyanin—ferricyanide reaction was found to be greater than $1 \times 10^5\ M^{-1}\ s^{-1}$ when $[Cu(I)] = 3.0 \times 10^{-3}$ M and $[Fe(CN_6)^{3-}] = 2.0 \times 10^{-3}$ M. The upfield shift of this peak in the oxidized form indicates a local conformational change between the two protein forms. In a titration of reduced spinach plastocyanin with ferricyanide a resonance with characteristics similar to the titrating french bean plastocyanin high-field peak was not observed [51]. This indicates that the resonance possibly corresponds to a non-

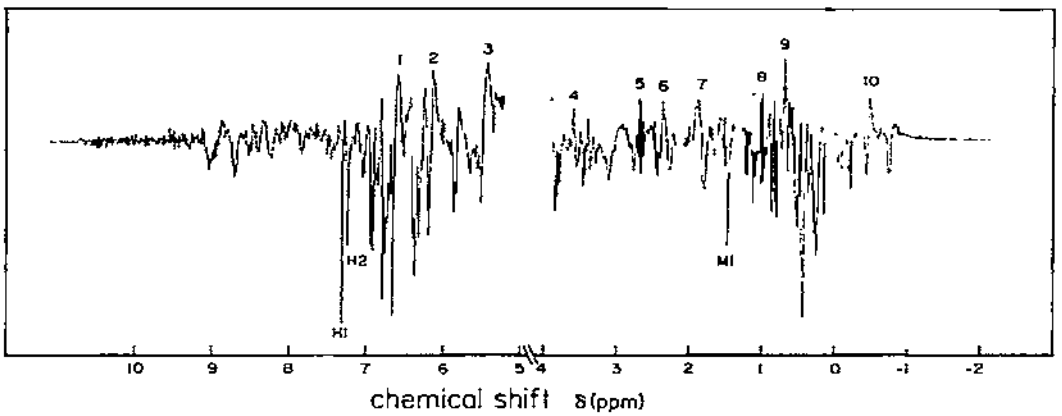


Fig. 8. 250 MHz ^{1}H NMR difference spectra of oxidized minus reduced spinach plastocyanin. The aliphatic spectra were obtained with 4 mM protein samples, 2 mM potassium phosphate, pH* 7.0. The aromatic spectra were obtained with 3.1 mM protein samples, 100 mM potassium phosphate, pH* 7.3. All spectra are resolution-enhanced correlation spectra. Excess potassium ferricyanide and sodium dithionite were used to oxidize and reduce the protein samples.

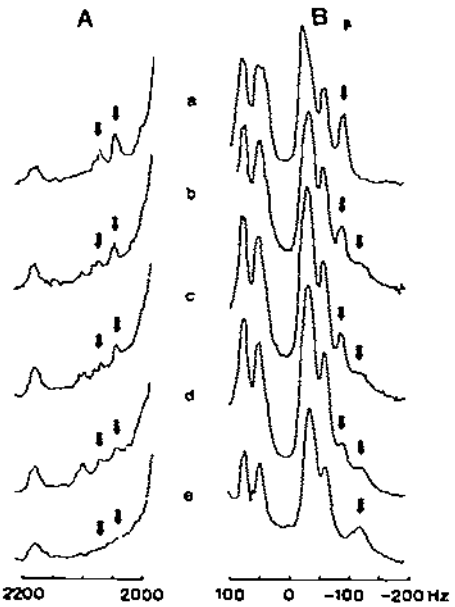


Fig. 9. Histidine $C^{\epsilon 1}$—H (A) and high-field (B) regions of the 270 MHz pulsed ^1H NMR spectra of mixtures of Cu(I)-plastocyanin and Cu(II)-plastocyanin in 100% ^2H_2O at pH* 7.4. Spectra a—e correspond to 0%, approx. 38%, approx. 45%, approx. 50% and 100% Cu(II)-plastocyanin, respectively. (The variable structure of the spectra near 2100 Hz was caused by incomplete deuterium exchange in some specimens). Chemical shifts are mea-measured from a DSS standard. From ref. 44.

conserved amino-acid residue. The differences between spectra of oxidized and reduced plastocyanin discussed above may be accounted for by small changes in the chemical shifts of a few amino-acid residues. Hence the conformational differences must be localized and limited. Crystals of oxidized plastocyanin can be reduced without cracking [46,48] which indicates again that conformational changes must be limited. Whether or not the conformational changes are functionally important must await further investigations.

C. AZURINS

Published NMR investigations of azurin have concentrated almost exclusively on the protein purified from *Pseudomonas aeruginosa*. As in the early NMR work on plastocyanin, research has focused on assigning the histidine resonances and determining their relationships with the copper. Both ^1H NMR and ^{13}C NMR data are available [41,42,67,68].

(i) Proton NMR spectroscopy

Azurins are larger proteins than plastocyanins (~129 vs. 100 amino-acid residues) and, therefore, exhibit more complex NMR spectra. The 220-MHz

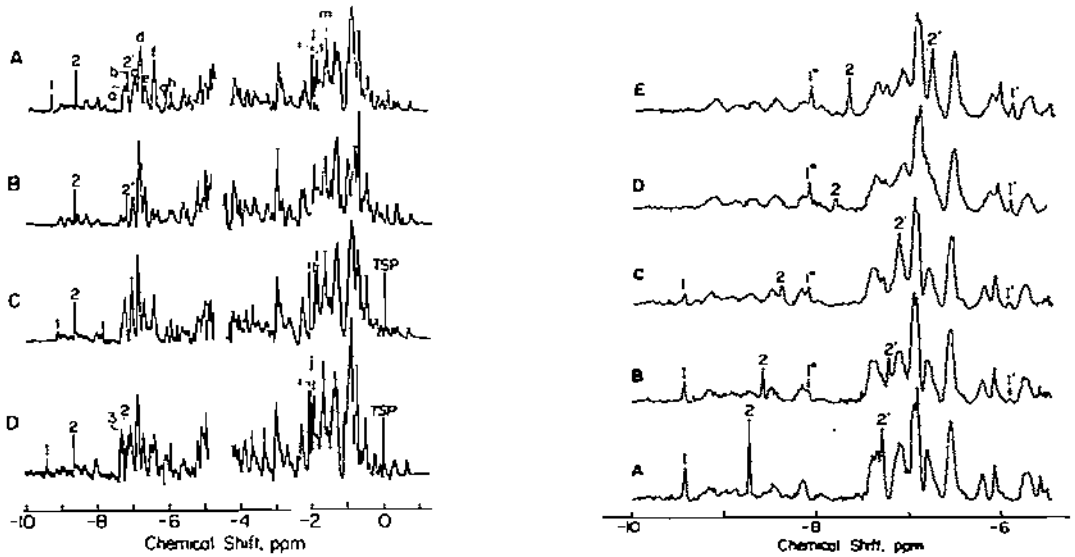


Fig. 10. 220 MHz convolution-difference ^{1}H NMR spectra of various forms of *P. aeruginosa* azurin, 0.1 M NaCl, at pH* 5.0 (measured at 24°C and 34°C), τ_1 and τ_2 (as defined by Campbell et al. [76]) are 0.64 and 0.03 s, respectively, for all spectra; K is given for individual spectra. (A) Reduced azurin; K = 0.9; (B) oxidized azurin, K = 0.9; (C) apo-azurin, K = 0.67; (D) Hg(II)-substituted azurin, K = 0.83. Amplitudes of the low-field spectra are twice that of the high-field spectra. Chemical shifts are measured from a TSP standard. From ref. 41.

Fig. 11. Effect of pH* on the aromatic region of the reduced *P. aeruginosa* azurin convolution-difference ^{1}H NMR spectrum, 220 MHz, 24°C, in 0.1 M NaCl; K = 0.83, τ_1 = 0.4 s, τ_2 = 0.03 s; (A) pH* 4.90; (B) pH* 6.95; (C) pH* 7.30; (D) pH* 8.40; (E) pH* 9.0. TSP was used as a standard for measuring chemical shifts. From ref. 41.

convolution difference ^{1}H NMR spectra of reduced, oxidized, apo and Hg(II) substituted *P. aeruginosa* azurins are shown in Fig. 10. As with plastocyanin, the azurin spectra contain many high-field shifted aliphatic resonances and non-exchanged N—H peaks, suggesting a compact globular protein.

In the aromatic region of the ^{1}H NMR spectra titrating resonances have been assigned to the $C^{\epsilon 1}$ protons of two of the four histidines of azurin. Hill et al. [42] found that only two azurin histidines titrate over the pH range 4—11. On the basis of peak position and titration behavior, resonances 1 and 2 (Fig. 11a) were assigned to the $C^{\epsilon 1}$ protons of two histidines. Peak 1 decreases in intensity with the corresponding appearance of peak 1* as the pH is increased. This indicates that histidine-1 has a slow rate of exchange between the imidazolium (peak 1) and imidazole (peak 1*) forms [42]; the pH_{mid} for the transition has been estimated at 6.4 ± 0.6. Ugurbil and Bersohn [41] found that resonance 1′ (Fig. 11) showed the same pH behavior as peak 1* and tentatively assigned this peak to the $C^{\delta 2}$ proton of histidine-1. All three ^{13}C resonances from the ring of histidine-1 are absent in spectra of oxidized azurin. This

places the residue near the copper ion; but the nearly normal pH_{mid} eliminates it as a copper ligand.

Peaks 2 and 2′ in Fig. 11 are related to the C^{ϵ_1} and C^{δ_2} protons, respectively, of the second titratable histidine identified by Hill et al. [42]. Histidine-2 shows a continuous shift with increasing pH and is present in spectra of both oxidized and reduced azurin [41,42]. The pK'_a for histidine-2 was found to be 7.57 in reduced azurin and 7.35 in oxidized azurin [41,42]. Ugurbil and Bersohn [41] noted that resonance 2 decreased in intensity and broadened at pH values between the two extremes of the pH titration profile for both oxidized and reduced protein.

Spectra of apo-azurin and Hg(II)-substituted azurin are shown in Fig. 10. Titration studies of both samples have allowed resonances for histidines-1 and -2 to be identified [41,42]. The peak positions of the histidine-2 C^{ϵ_1} and C^{δ_2} protons are unchanged in both of the modified azurin proteins, but the position of the histidine-1 C^{ϵ_1} proton is affected by modification [41]. As pointed out by Ugurbil and Bersohn, [41] these data support the conclusion that histidine-2 is far from the copper ion and that histidine-1, although not a copper ligand, is near enough to the copper that its environment is affected by modifications in the copper binding site. The pK'_a of 7.8 for a new peak that appears in the spectra of apo-azurin is higher than that of a normal histidine, and the chemical shift difference between imidazolium and imidazole forms was found to be smaller than normal [42]. The presence of a third histidine titrating in apo-azurin but not in azurin indicates that this histidine may be a copper ligand. Assuming that the two pH-insensitive histidines in holo azurin are copper

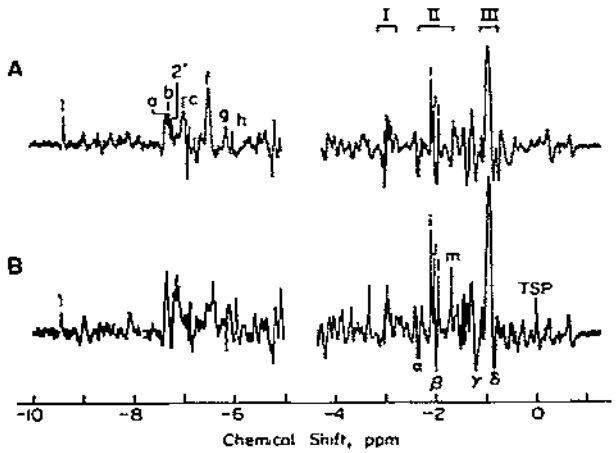


Fig. 12. Difference of the convolution-spectra difference ^1H NMR spectra of native-reduced (diamagnetic), Hg(II)-substituted (diamagnetic) and native-oxidized (paramagnetic) *P. aeruginosa* azurin. Convolution-difference spectra used are those shown in Fig. 10. (A) Reduced azurin CDS minus 0.98 times the oxidized azurin CDS. (B) Hg(II)-substituted azurin CDS minus 0.98 times the oxidized azurin CDS. The amplitudes of the low-field spectra (aromatic region) are twice those of the high-field spectra (aliphatic region). From ref. 41.

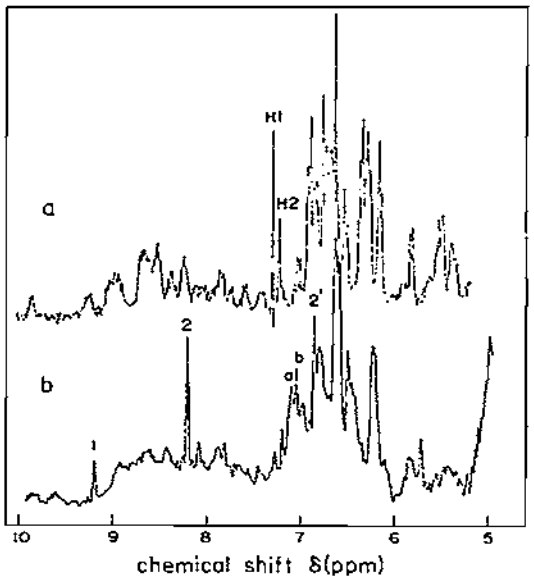


Fig. 13. ^{1}H NMR correlation spectra at 250 MHz of the aromatic region of reduced spinach plastocyanin (a) and *P. aeruginosa* azurin (b).

ligands, their $C^{\epsilon 1}$ proton resonances should be present in reduced minus oxidized azurin difference spectra and located downfield from the aromatic ring proton peaks. Ugurbil and Bersohn [41] on these assumptions assigned peaks a and b in Fig. 12 to the non-titrating histidine residues. Figure 13 compares the spectra of reduced spinach plastocyanin and *P. aeruginosa* azurin. The chemical shifts of peaks a and b in the azurin spectrum are quite similar to those of resonances H1 and H2 assigned to the $C^{\epsilon 1}$ protons of the histidine copper ligands in plastocyanin [69].

Resonances c, f, g and h in Fig. 10 were also characterized as peaks which are broadened beyond detection in spectra of oxidized azurin [41]. These resonances may correspond to copper ligand histidine $C^{\delta 2}$ protons or ring protons from the tyrosine, phenylalanine and tryptophan residues which have been shown by ^{13}C NMR to be near the copper [68].

Two difference spectra are shown in Fig. 12 [41]. Figure 12a is the spectrum of reduced azurin minus oxidized azurin. The aliphatic region contains several positive resonances which suggests that many aliphatic protons are in the copper environment. Resonances i, j and l have been assigned to methyl groups from 2 or 3 methionines based on their chemical shift, three-proton intensity and narrow line width [41]. The large positive peak at 0.95 ppm contains several resonances which were related by their chemical shift to leucine, isoleucine and valine CH_3 and possibly isoleucine CH_2 groups [41].

Spectra of the low-field N—H region of reduced and oxidized azurin in $^{1}H_2O$ have been obtained [41]. Protons whose resonances are resolved in this

region must be exchanging relatively slowly with the solvent and are likely to be hydrogen bonded or otherwise buried in the protein matrix. Given the hydrophobic and solvent-inaccessible nature of the copper binding site, several of the observed resonances correspond to copper ligands or other amino-acid residues in the copper environment. Many resonances found in the low-field spectral region of reduced azurin were missing in similar spectra of oxidized azurin. The absence of these lines could be caused by paramagnetic broadening, but could also arise from a more rapid proton—solvent exchange rate in the oxidized azurin conformation. Temperature and pH titration studies have been carried out, and the spectra of *P. fluorescens* azurin, a tryptophanless protein, and *P. aeruginosa* azurin were compared in attempts to make resonance assignments [41]. The lack of sensitivity either to pH or temperature changes hampered peak assignments to specific residues. Spectral comparison of the same protein purified from different species can be useful in making resonance assignments. This method has been used to great advantage in assigning resonances in hemoglobin spectra [70—73]. There, several proteins differing only by one amino acid could be compared and assignments made with the confidence that few conformational differences would exist between the proteins. Many amino acid substitutions exist between *P. aeruginosa* and *P. fluorescens* azurin besides the missing tryptophan [16]. This may have complicated the interpretation of the spectra, and a definite assignment of the tryptophan N—H resonance could not be made. Several very speculative assignments were made for resonances in the spectra of reduced azurin, but much more work will be required to make detailed assignments.

Using Co(II)-substituted azurin, Hill et al. [67] have been able to study several proton resonances shifted beyond the main protein spectral envelope. The paramagnetic Co(II) ion has an exceedingly fast electronic spin—lattice relaxation time ($T_{1e} < 5 \times 10^{-12}$ s) which permits the observation of large hyperfine contact and pseudocontact shifts of neighboring nuclei [74]. In

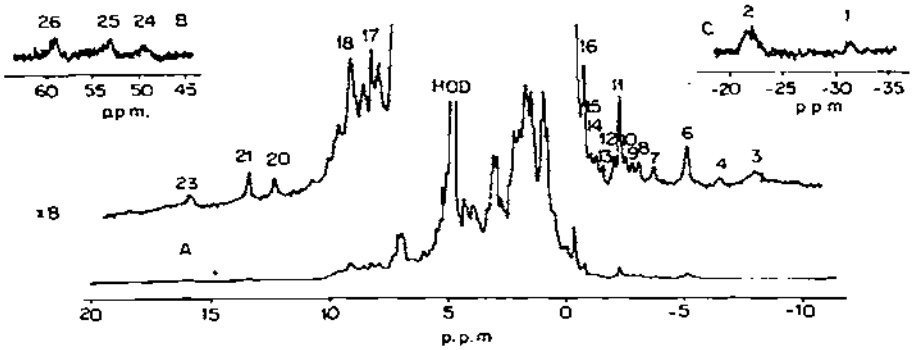


Fig. 14. The 270 MHz ^{1}H NMR spectrum of Co(II) azurin, pH* 4.7. (1A) 10 kHz spectral width, 21°C; (1B) low-field lines, 23.8°C; (1C) high-field lines, 26.5°C. Resonances are numbered 1—26 from high-field to low-field. Chemical shifts are measured from a DSS standard. From ref. 67.

Fig. 14 peaks which in copper azurin lie within the range 2—10 ppm are now spread over a range of −35 to 60 ppm. The pH behavior of peaks 17 and 18 is very similar to those of peaks 2 and 2′ of the copper protein and have been assigned to histidine-2. By similar criteria peaks 19 and 21 correspond to proton resonances 1 and 1* of histidine-1 in Fig. 11. Paramagnetic shifts caused by a predominantly dipolar mechanism will be temperature dependent [75] and fit the equation

$$\Delta_i = A_i(T^{-1} + BT^{-2}) + C_i \qquad (2)$$

where Δ_i is the observed chemical shift, C_i is the diamagnetic chemical shift, A_i is a constant for a particular resonance and B is a constant for all dipolar shifted resonances. By estimating B using the known diamagnetic chemical shifts of peaks 1 and 1* in apo and Cu(I) azurin spectra, C_i could be calculated for other resonances by extrapolating the observed variation of chemical shifts to infinite temperature. The extrapolated diamagnetic chemical shifts for peaks 17, 18, 19 and 21 are in reasonable agreement with the shifts observed in the apo and Cu(I) proteins. As can be seen in Table 3 most of the paramagnetic shifted resonances originate from aliphatic protons. This is consistent with the reduced minus oxidized azurin difference spectrum (Fig. 12), and indicates that the copper is located in a hydrophobic pocket. No assignments were made to resonances of copper ligands.

(ii) Carbon-13 NMR spectroscopy

A thorough ^{13}C NMR study of the azurin copper binding site has been

TABLE 3

Diamagnetic shifts calculated for the paramagnetically shifted resonances of Co(II) azurin. From ref. 67

Resonance	Calculated diamagnetic shift (p.p.m.)		Resonance	Calculated diamagnetic shift [a] (p.p.m.)	
	pH* 4.7	pH* 6.7		pH* 4.7	pH* 6.7
3	0.7		13	1.1	
4	0.8	0.6	14	1.6	1.3
5		2.1	15	1.7	1.5
6	0.2	0.1	16	0.3	
7	3.7	4.5	17	7.1(7.3)	
8	0.4	1.0	18	8.8(8.7)	
9	1.8	2.0	19		8.0(8.1)
10	3.6		20	5.8	5.5
11	0.8	1.0	21	9.4(9.3)	
12	0.6	0.8	22		7.3
			23	6.6	5.2

[a] Observed values in parentheses.

made by Ugurbil et al. [68]. These authors studied ^{13}C resonances in the region 170—110 ppm downfield from TMS. Upfield shifted carbonyl carbons, the C^{ζ} of arginine, and all of the aromatic ring carbons occur in this region. With the combination of off-resonance decoupling and convolution-difference spectroscopy, methine carbons which give broad lines are removed from the spectra whereas non-protonated carbons yield relatively narrow resonances [76,77]. Figure 15 compares the convolution-difference spectra obtained for oxidized and reduced azurin. Sixteen lines are clearly resolved in the reduced spectrum. Table 4 gives the assignments for each peak [68]. At pH 7.15 peak 4 has an intensity of three carbons and peak 7 is of two carbon intensity. The amide carbonyl (peak x) and arginine C^{ζ} (peak 1) resonances were assigned on the basis of their chemical shifts. Peak x has a rather large high-field chemical shift for an amide carbonyl resonance and is absent in the spectrum of oxidized azurin [68]. Peak x was assigned to the amide carbonyl copper ligand predicted by the model of Hare et al. [62]. Since the methionine which is the fourth ligand in plastocyanin [48] is conserved in all azurins (see Fig. 1), peak x must be reassigned. The ^{13}C NMR result indicates that this carbonyl group is near the copper and is in a very unusual environment as its chemical shift is at least 3 ppm upfield from the highest field position reported for a non-terminal amide carbonyl resonance in a diamagnetic protein [77]. Ugurbil et al. argued against assigning peak x to the N-terminal alanine residue on the basis of the

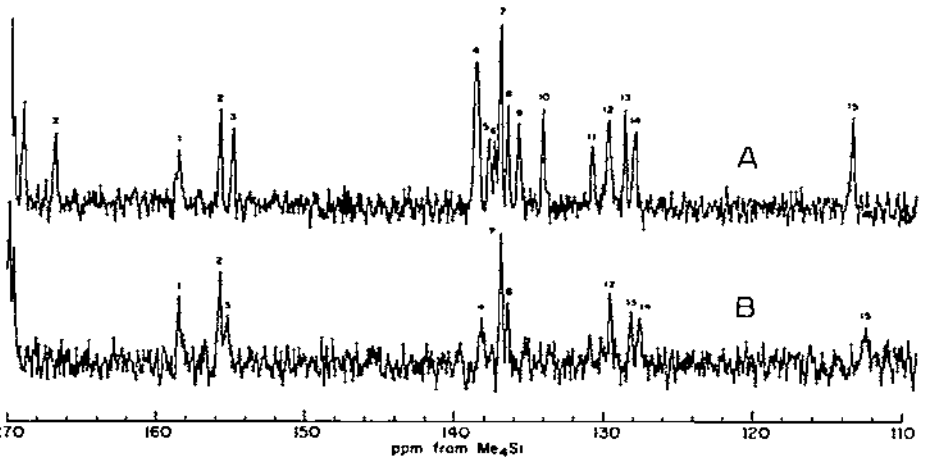


Fig. 15. Aromatic carbon region (and C^{ζ} of arginine) and upfield edge of the carbonyl region in convolution-difference natural-abundance ^{13}C NMR spectra of reduced and oxidized *P. aeruginosa* azurin in H_2O (0.05 M ammonium acetate, 31°C). Each spectrum was recorded at 15.18 MHz under conditions of noise-modulated off-resonance proton decoupling with 8192 time domain addresses and a recycle time of 1.105 s. (A) Reduced azurin (7.4 mM, pH 5.2) after 65,536 accumulations. Peaks assigned to C^{ζ} of arginine-79 and the 17 non-protonated aromatic carbons are numbered consecutively. (B) Oxidized azurin, (5.8 mM, pH 5.3) after 131,072 accumulations. Peak designations are discussed in the text. Chemical shifts are referenced to TMS. From ref. 68.

TABLE 4

Chemical shifts of some ^{13}C resonances of *P. aeruginosa* azurin at pH 5.2

Assignment	Peak designation [b]	Chemical shift [a]	
		Reduced	Oxidized
Amide carbonyl	x	166.7_5	
Arg-79 C^ζ	1	158.5_0	158.4
Tyr C^ζ	2 [c]	155.7_0	155.7
Tyr C^ζ	3 [d]	154.8_4	155.2
	4 [e,f]	138.5_2	138.1
	5	137.6_2	
6 Phe C^γ	6	137.2_4	
2 His C^γ	7 [f,g]	136.8_8	136.9
Trp-48 $C^{\epsilon 2}$	8 [f]	136.3_6	136.4
	9	135.6_6	
Tyr C^γ	10 [d]	133.9_8	
His C^γ	11	130.6_9	
Tyr C^γ	12 [c]	129.6_1	129.6
Trp-48 $C^{\delta 2}$	13	128.5_0	128.1
His C^γ	14	127.8_3	127.6
Trp-48 C^γ	15	113.2_7	112.5

[a] Values for reduced and oxidized azurin were obtained from the spectra of Figs. 15A (pH 5.2) and 15B (pH 5.3), respectively. [b] Peak designations are those of Figs. 15 and 16. [c] Peaks 2 and 12 arise from the same tyrosine residue. [d] Peaks 3 and 10 arise from the same tyrosine residue. [e] Three-carbon resonance in the spectrum of reduced azurin (see Fig. 15A); single-carbon resonance in the spectrum of the oxidized protein (Fig. 15B). [f] One-to-one connections between peaks 4, 7 and 8 of oxidized azurin and the corresponding peaks of the reduced protein are tentative. [g] Two-carbon resonance in Figs. 15A and 15B. From ref. 68.

high-field resonance position and the small observed pH titration shift (0.4 ppm) [68]. They suggested that the pH titration behavior may relate to the ionization of one of the titratable histidine residues. A resonance similar to peak x is found in the ^{13}C NMR spectrum of reduced spinach plastocyanin [40] (see Fig. 4, peak 4). Analysis of the plastocyanin X-ray structure may help in assigning these resonances and characterizing their peculiar environment.

Tyrosine C^γ, C^ζ and two histidine C^γ lines were identified by their pH titration behavior and chemical shifts [68]. A plot of all sixteen peak positions as a function of pH is shown in Fig. 16. The tyrosine residues have unusually high pK'_a values of about 11.4 and 12.5, which indicate solvent-inaccessible or hydrogen-bonded locations for these residues. As in the proton NMR studies, one histidine titrates continuously with a pK'_a of 7.5 (peak 14); another histidine titrates in a discontinuous fashion with a pH_{mid} of about 6.8—7.8 (peak 11); and two histidines do not titrate. By analysis of the C^γ titration curves it was found that histidine-1 deprotonates at the $N^{\delta 1}$ position with a slow rate

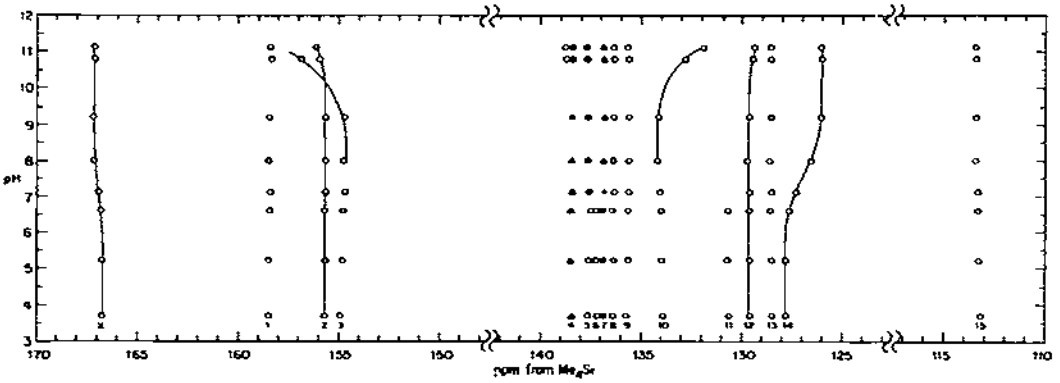


Fig. 16. Effect of pH on the ^{13}C NMR chemical shifts of some non-protonated carbons of reduced *P. aeruginosa* azurin in H_2O (0.05 M ammonium acetate, 31°C). Peak designations are those of Fig. 15a and Table 5. Open circles, closed circles and triangles indicate peaks that arise from 1, 2 and 3 carbons, respectively. The symbol + indicates a peak with an intensity intermediate between 2 and 3 carbons. Protein concentrations were in the range 6.6—7.4 mM. Each spectrum was obtained at 15.18 MHz under conditions of noise-modulated off-resonance proton decoupling, with 30,000 or more accumulations, a recycle time of 1.105 s, and a digital broadening of 0.88 Hz. The solid lines are best-fit titration curves, assuming a single pK_a in each case. (The chemical shifts of C^{ζ} and C^{γ} of the phenoxy form of each tyrosine residue were constrained to values 10.4 ppm downfield and 6.2 ppm upfield, respectively, of the corresponding values of the phenolic form of the residue.) From ref. 68.

of exchange, while histidine-2 undergoes the unusual deprotonation at the $N^{\epsilon 2}$ position [68]. Hydrogen bonding of the $N^{\delta 1}$ proton as is the case for histidine-12 in bovine pancreatic ribonuclease *A* [82] was suggested to explain the histidine-2 behavior [68].

The chemical shift of peak 15 in Fig. 15 immediately distinguished it as the C^{γ} carbon of the single tryptophan residue in *P. aeruginosa* azurin. By elimination, peak 13 was assigned to the tryptophan $C^{\delta 2}$ carbon. Resonances 4—9 were insensitive to pH and were assigned on the basis of chemical shift to the remaining phenylalanine C^{δ}, tryptophan $C^{\epsilon 2}$ and the C^{γ} of the two non-titrating histidine residues. The chemical shifts of the two non-titrating histidine C^{γ} resonances are diagnostic for the $N^{\epsilon 2}$—H imidazole tautomer. Therefore, the two histidines believed to be copper ligands are coordinated at the $N^{\delta 1}$ position [68].

The spectrum of fully oxidized azurin (Fig. 15b) contains fewer narrow resonances than that of the reduced protein. Peaks in the oxidized spectra are numbered to indicate the assignments to corresponding resonances in the reduced protein spectrum. The chemical shift of each resonance and its pH dependence were the criteria used to correlate peaks between the oxidized and reduced spectra. Peaks 4, 7 and 8 in the oxidized spectrum were assigned only on the basis of chemical shift coincidence with similar peaks in the reduced protein spectrum and hence the assignments are only tentative [68].

(iii) Distances of protein groups from the copper

The absence of peaks 5, 6, 9, 10, 11 and the decrease in intensity of peak 4 in the ^{13}C NMR spectrum of oxidized azurin suggests that the single tryptophan, one tyrosine, three or four phenylalanines and one histidine in addition to the two liganded histidines are near the copper atom [68].

Several of the resonances present in the convolution-difference ^{13}C NMR spectrum of oxidized azurin are broader than the corresponding resonances in the reduced protein spectra. Resonances of carbons from copper ligands are expected to be broadened beyond detection as discussed previously. Carbon resonances present in the oxidized spectra but broadened must arise from amino acids in the copper vicinity whose line broadening is predominantly dipolar and proportional to the inverse sixth power of the copper—carbon distance. Instrumental limitations and poor signal-to-noise ratios restricted line width measurements to lines between 2 and 6 Hz. Since a fast exchange between oxidation states is catalyzed by ferricyanide [68], the paramagnetic contribution to the line width could be controlled by varying the ratio of oxidized to reduced azurin in the presence of ferricyanide. In this way the line widths of resonances affected by the paramagnetic copper were kept within the measurable range, and eqn. (3) could be used to calculate the line widths of resonances in the fully oxidized protein [83].

$$W_A - W_A^0 = P_B(\pi\tau)^{-1}(Q + R^2)(Q^2 + R^2)^{-1} \quad (3)$$

W_A is the line width of the resonance in the dominant site (Hz), P_B is the mole fraction of species B, τ is the lifetime between exchange (s), $Q = 1 + (\pi\tau W_B^0)^{-1}$, $R = 2\delta_{AB}/W_B^0$, δ_{AB} is the chemical shift difference between the sites (Hz) and W_A^0 and W_B^0 are the line widths (Hz) of sites A and B. Assuming that for a significantly broadened peak in the presence of 1% oxidized azurin, $W_B^0 >> \delta_{AB}$ and thus $R^2 << Q$, eqn. (3) reduces to

$$W_A - W_A^0 = P_B W_B^0(1 + \pi\tau W_B^0)^{-1} \quad (4)$$

Further, the rates of exchange between oxidation states of azurin were assumed to be fast enough that $\pi\tau W_B^0 << 1$. For peaks x, 6 and one component of peak 4, W_B^0 was estimated to be greater than 800 Hz. The extreme broadening of these peaks would suggest that they originate from copper liganded amino acids; therefore, resonance 6 and one component of peak 4 were assigned to the C^γ of two histidines. Peak x, assigned to the peptide carbonyl carbon was also suggested as a copper ligand, because of the extreme line broadening [68]. This assignment appears to be eliminated by the recent plastocyanin X-ray results [48]. From the fully oxidized protein spectra paramagnetic broadening of $\lesssim 2$ Hz and ~ 5 Hz were measured for the $C^{\delta 2}$ and C^γ resonances, respectively, of the tryptophan residue. These rather small changes in line width would place the tryptophan residue near the copper but not directly bonded as a copper ligand in agreement with its photoemissive properties [84].

In a later paper Ugurbil and Bersohn [41] used the line broadening values

TABLE 5

Structural features of azurin; from ref. 41

Amino acid	Structural data	Source of information
Cys-112	Cu ligand	PMB binding [a] and metal replacement studies; [b] analogies with plastocyanin [c]
Cys-3—Cys-26	Buried disulfide bridge	Not reduced by CO_2^- [d]
His-35, -83	C^γ of one $\lesssim$7.5 Å from Cu, other is remote from Cu	^1H [e] and ^{13}C NMR [f]
His-46, -117	Cu ligand, imidazole $N^{\delta 1}$ coordination	^{13}C NMR [f]
Trp-48	$C^\gamma \lesssim 8.4$ Å, $C^{\delta 2} \lesssim 9.8$ Å from Cu; located in a hydrocarbon-like environment	^{13}C NMR [f]; ODMR and phosphorescence [g]
Tyr-72	Remote from Cu; $\gtrsim$20 Å from Tyr-108, inaccessible to the solvent, not adjacent to the disulfide bridge, and not hydrogen bonded	^{13}C NMR [f]; fluorescence [h] and phosphorescence [g]
Tyr-108	$C^\gamma \lesssim 7.5$ Å from Cu, C^ζ further away; inaccessible to the solvent, not adjacent to the disulfide bridge, and not hydrogen bonded	^{13}C NMR [f]; fluorescence [h] and phosphorescence [g]
Phe-110, -111, -114	Close to Cu	^{13}C NMR [f]
Arg-79	Remote from Cu	^{13}C NMR [f]
Two methionines, probably Met-44 and -109	S—CH_3 $\lesssim$8 Å from Cu	^1H [e] and ^{13}C NMR [f]
Unknown amide moiety	Cu ligand	^{13}C NMR [f]
Several Ile, Leu, and/or Val	Methyl groups close to Cu	^1H NMR [e]

[a] Finazzi-Agró et al. [78]. [b] Finazzi-Agró et al. [79]; McMillin et al. [21]; Ugurbil and Bersohn [41]. [c] Katoh and Takamiya [20]; Graziani et al. [53]; McMillin et al. [21]. [d] Fukuda and Bersohn [81]. [e] Ugurbil and Bersohn [41]. [f] Ugurbil et al. [68]. [g] Ugurbil et al. [84]. [h] Ugurbil and Bersohn [80].

for the tryptophan carbons and the inverse sixth power relationship for dipolar paramagnetic broadening to calculate upper limits of $C^\gamma \leqslant 8.4$ Å and $C^{\delta 2} < 9.8$ Å for the copper—carbon distances. By stretching the data even farther, carbon—copper distances for several other amino-acid residues were calculated. Table 5 summarizes all of the distance calculations and other structural features of the azurin molecule deduced from ^1H NMR, ^{13}C NMR and other spectroscopic techniques [41].

(iv) Conformational heterogeneity

Stopped-flow kinetic data [85,86] and tryptophan fluorescence studies

[87] have indicated a heterogeneity in the reduced azurin population. The ^{1}H and ^{13}C NMR pH studies suggest structural variations in both the Cu(I) and Cu(II) azurins which affect histidines-1 and -2. It may be possible to explain the discontinuity of the titration curve of histidine-1 and the broadening of histidine-2 by a single mechanism. Since histidine protonations are generally rapid on the NMR time scale, a discontinuous titration curve usually implies the existence of a slow conformational step [66]. If the pK'_a of histidine-2 is different in the two conformations, this could explain the broadening of the proton peak near the pK'_a. A pK'_a difference of as little as 0.05 could explain the results. The $^{13}C^{\gamma}$ peak of histidine-2 does not show broadening, therefore, it must be in rapid exchange. The relative titration shifts of the $C^{\epsilon 1}$—$H/^{13}C^{\gamma}$ of histidine-2 are 242 Hz/28 Hz. Thus, if this mechanism is correct, the exchange rate between the two forms of azurin must lie roughly between the ^{13}C and ^{1}H perturbation. If the conformational equilibrium affects the pK'_a by 0.05 pH units, the rate of exchange between the two conformational forms of azurin must lie between 0.7 and 6 s^{-1}. This is in fair agreement with the values of 17 s^{-1} [86] and 40 s^{-1} [85] found by kinetic methods.

Structural differences between oxidized and reduced azurin have been noted from analysis of reduced minus oxidized azurin difference spectra and a pK'_a shift for histidine-2 [41]. Histidine-2 has a pK'_a of 7.57 in reduced azurin and a pK'_a of 7.35 in the oxidized protein [41]. This pK'_a shift suggests that the environment of histidine-2 is different in the two oxidation states. In the reduced minus oxidized difference spectrum (see Fig. 12a) there are many resonances of negative intensity which are adjacent to resonances of positive intensity. Ugurbil and Bersohn [41] interpreted these as reflecting local differences between oxidized and reduced azurin. Also, a smaller number of negative peaks were found in the Hg(II) azurin minus Cu(II) azurin difference spectrum, than in the Cu(I) azurin minus Cu(II) azurin difference spectrum which indicates that the Hg(II) complex has a structure more like Cu(II) azurin than does Cu(I) azurin.

D. DISCUSSION

Blue-copper proteins have been isolated from a wide variety of organisms ranging from the bacterium *Pseudomonas* to man. All of these proteins contain at least one Type I copper atom and are characterized by their deep blue color, small EPR hyperfine coupling constant, and high midpoint potential relative to the Cu(I)/Cu(II) couple in water. For the single copper proteins, azurin from *P. aeruginosa* and plastocyanins found in cyanobacteria and higher plants, the NMR data indicate a common structure for the copper binding site.

The unusually low pK'_a values for two histidine residues and the broadening of the $C^{\epsilon 1}$ proton resonances in oxidized plastocyanin and azurin ^{1}H NMR spectra has clearly established two histidines as copper ligands [41—43,49]. The ^{13}C NMR data demonstrated that ligation occurs at the histidine $N^{\delta 1}$ posi-

tion [40,68]. Several authors, from studies of model copper complexes [88—91] and blue-copper protein sequence homology [92,93], had suggested methionine as a copper ligand. The paramagnetic line broadening of apparent methionine methyl resonances in ^{1}H NMR spectra of azurin [41] and plastocyanins [43,60] has added support to these suggestions. Substantial spectroscopic and chemical evidence [20,21,53,94—102] has been found for a copper-liganded cysteine residue. Although other ligands such as tyrosine [103] or a deprotonated peptide nitrogen [62] have been proposed, solution of the plastocyanin structure by X-ray crystallography [48] has eliminated these possibilities for plastocyanin.

The structure of oxidized poplar plastocyanin as determined by X-ray crystallography is shown in Fig. 17. Histidines-37 and -87, cysteine-84 and methionine-92 are identified as the copper ligands. A distorted tetrahedron is indicated for the copper binding site geometry, but determination of the actual bond angles awaits refinement of the X-ray data. Figure 18 shows a detailed picture of the copper binding site [48]. In agreement with the ^{13}C NMR data [40,68] the histidines are shown to be ligated at the $N^{\delta 1}$ position. Small differences have been noted in the structures of oxidized and reduced plastocyanin

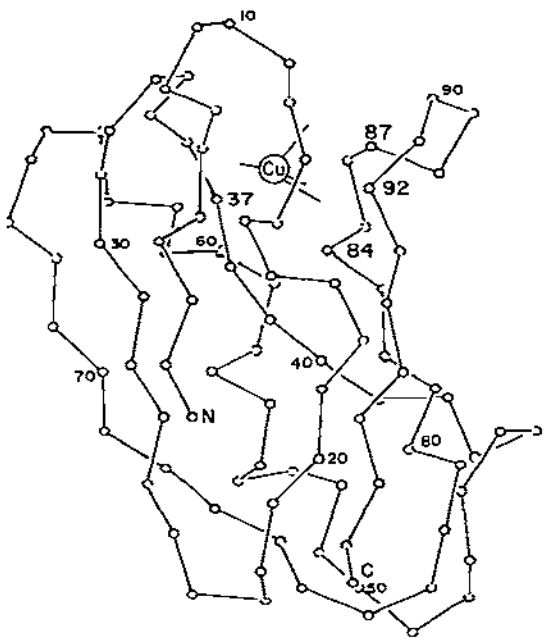


Fig. 17. The polypeptide chain in poplar plastocyanin. The circles represent α-carbon positions derived by applying the model-fitting procedure of Diamond [115] to atomic coordinates measured on the Watson—Kendrew model. Every tenth residue and the four copper-binding residues are numbered following the scheme of Boulter et al. [116] for higher plant plastocyanin sequences. The letters N and C denote the amino-terminal and carboxyl-terminal residues, respectively. The approximate positions of the Cu-ligand bonds are indicated. From ref. 48.

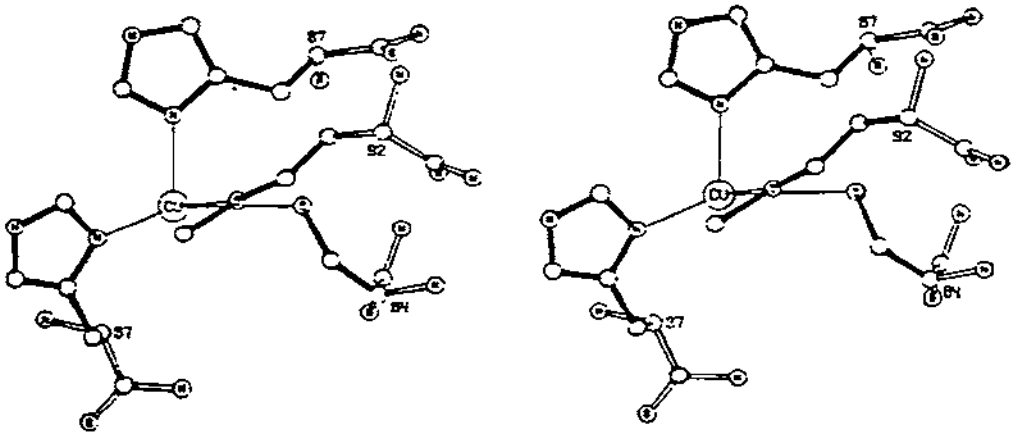


Fig. 18. Stereo-drawing of the copper-binding site in plastocyanin, showing the four ligand residues (histidine-37, cysteine-84, histidine-87 and methionine-92). From ref. 48.

with NMR spectroscopy. The crystal structure of reduced poplar plastocyanin is being solved [48] and comparison with the oxidized structure will soon be possible. The geometry of the metal ligands in the Cu(I) and Cu(II) proteins will be very interesting since it pertains to the mechanism of protein oxidation—reduction.

Assignment of the methyl ^{1}H NMR resonances of the liganded methionine has not been settled. A methyl resonance at 1.8 ppm in spectra of several plastocyanins and in *Pseudomonas* azurin is known to broaden upon copper oxidation [41,43], yet this methyl resonance has not been found in ^{1}H NMR spectra of all plastocyanins [60]. A second methyl resonance located at 0.5 ppm has been found by using a Carr—Purcell pulse sequence. The peak is present in spectra of several plastocyanins [60] and is also broadened in Cu(II) plastocyanin. This resonance is in a very difficult region of the spectrum for assignments to be made. The X-ray data show that only methionine-92 is within 6 Å of the copper atom [48] and theoretically capable of having its methyl resonance broadened [83]. It is difficult to explain how both methyl resonances can be broadened when one is quite a long distance from the copper atom. NMR studies of *Chlorella fusca* plastocyanin, where only methionine-92 is present [32], should help to resolve this inconsistency.

The X-ray structure of plastocyanin reveals that the copper is separated from the solvent by the imidazole ring of histidine-87. Resonance H1 is associated with the first histidine to be displaced from the copper at low pH and has a narrower line width than the other liganded histidine. These properties suggest that peak H1 corresponds to histidine-87. Histidine-37 is buried in the protein matrix and would be expected to have a broadened $C^{\epsilon 1}$ proton resonance as is found for peak H2.

Azurins show a large degree of amino acid sequence homology with plastocyanins in the regions surrounding the 4 residues providing the copper ligands of plastocyanin. From this homology Hill et al. [42] proposed histidines-46

and -117 and cysteine-112 as azurin copper ligands. Extending this argument, methionine-121 would be a logical choice as the fourth copper ligand [41]. The ^{13}C NMR data for *P. aeruginosa* azurin indicate that three histidines, three phenylalanines, a tryptophan and a tyrosine are near the copper, while an arginine and a histidine are far from the copper. Tryptophan-48 and arginine-79 are the only such residues found in *P. aeruginosa* azurin and therefore must, respectively, be placed near and far from the copper. As suggested by Ugurbil et al. [41,68] tyrosine-108, phenylalanine-110, -111 and -114 and histidine-35 are likely to be near the copper. Tyrosine-72 and histidine-83 may be far from the copper site. The arguments for these assignments are strengthened if one stretches the azurin sequence over the structure for plastocyanin seen in Fig. 17 by following the azurin—plastocyanin sequence homology developed by Ryden and Lundgren [16]. Colman et al. [48] have suggested that the azurin structure is quite similar to the plastocyanin structure with a few added amino acids in regions located far from the copper center, and the preceeding demonstration supports this argument. Further studies of the refined X-ray data should allow the assignment of many resonances in the ^{1}H and ^{13}C NMR spectra of plastocyanin and azurin.

There are still intriguing differences between the blue-copper protein structures which need to be understood. First, for example, the liganded histidine imidazoles of azurin have much lower pK_a''s than the corresponding ligands of plastocyanin [41—43]. Gray and coworkers [104,105] have proposed, based on kinetic data, that the azurin copper center is more buried than the corresponding plastocyanin site. Secondly all Type I copper binding sites must not be the same, for the amino acid sequence of stellacyanin is now known [8,9] and there are no methionines present. Recent studies of small copper complexes [103] suggest that an oxygen may substitute for a sulfur ligand and maintain many of the Type I copper characteristics. Stellacyanin when compared to other blue-copper proteins [2,106] has a low midpoint potential (+0.18 v vs. +0.3 v), lacks considerable sequence homology [8,9] and has quite different EPR [107] and resonance Raman spectra [98]. These variations may be explained by some unidentified change in copper ligation.

The structures of plastocyanin and azurin are beginning to come into focus, but structure—function relationships have been little probed. The parsley plastocyanin—cytochrome f electron exchange reaction is exceedingly fast [108], being thirty times more rapid than any other known plastocyanin—cytochrome reaction and orders of magnitude faster than the plastocyanin—small molecule reactions studied [108—110]. The observed rate and specificity of the plastocyanin—cytochrome f reaction argues strongly for involvement of a defined protein—protein interaction. At present, technical problems in purifying large quantities of cytochrome f have hindered NMR investigations of this reaction. Two theories, electron tunnelling and "outer sphere" interactions, are being investigated in attempts to explain the redox reactions of metalloproteins [58]. Colman et al. [48] have argued that the distorted ligand symmetry and two nitrogen—two sulfur ligand distribution of the plastocyanin copper is well

suited for an outer-sphere electron transfer mechanism. Such a mechanism has been proposed for plastocyanin reactions with inorganic oxidants and reductants [6]. These theories may be examined by NMR spectroscopy in the case of a protein—protein reaction with azurin. The natural reaction partner for azurin is believed to be a small protein, cytochrome c_{551}. Two research groups have published initial ^1H NMR studies of this cytochrome [111,112], and a low resolution X-ray map of the molecule is available [113]. Moore et al. [112] have promised some interesting NMR results on the azurin—cytochrome c_{551} complex.

These studies will be greatly enhanced when assignments can be made to many of the resolved lines in ^1H and ^{13}C NMR spectra of azurin and plastocyanin. With the assignment of resonances related to the copper ligands, the effects of protein binding on the geometry of the copper site can be investigated. Co(II) and Hg(II) substituted proteins might be of particular use in these experiments. It may also be possible to identify peripheral protein functional groups which are important for protein—protein interactions.

This review has been an attempt to present the recent progress made by NMR spectroscopy in elucidating the blue-copper protein structure. Other fields have provided much information on this problem and the reader is directed to the following review articles, and references therein for further information: [1,2,5—7,16,58,88,95,116,117]. With the structural knowledge which is now available on the blue-copper proteins and the tremendous amount of energy being directed to their study, the field of blue-copper protein structure and function promises to be quite exciting in the near future.

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