Nuclear Magnetic Resonance Studies of the Copper Binding Sites of Blue Copper Proteins: Oxidized, Reduced, and Apoplastocyanin†

John L. Markley,* Eldon L. Ulrich, Steven P. Berg, and David W. Krogmann

ABSTRACT: Proton nuclear resonance spectra at 250 MHz of plastocyanins from spinach (*Spinacia oleracea*) and a blue green alga (*Anabaena variabilis*) are reported. Spectra of the reduced plastocyanins contain well-resolved peaks from slowly exchangeable N-H, histidine C_2-H tyrosine ring, peptide α-CH, and high-field protons. The widths of these peaks indicate that the plastocyanins are monomeric. When the plastocyanins are oxidized, several changes in the spectra are observed including disappearance of peaks assigned to two histidine side chains. The pK_a' values of the two histidine residues of reduced spinach plastocyanin are abnormally low (4.9 and <4.5). These pK_a' values become more normal in apoplastocyanin or plastocyanin inhibited by cyanide. The results suggest that the imidazole groups of the two histidine residues are liganded directly to the copper in plastocyanin. The displacement of copper by cyanide is reversed at low pH. Spectra of apo- and reduced plastocyanins show only minor differences. However, the slowly exchangeable protons of plastocyanin exchange more rapidly in the apoprotein. Copper binding apparently does not cause a major reorganization of the protein structure, but the presence of copper does stabilize this structure.

Plastocyanins are small blue copper proteins found in photosynthetic organisms (Katoh, 1960). The protein is the specific electron carrier that appears to transport electrons from cytochrome *f* to P700 of photosystem I (Wood, 1974). The single copper of plastocyanin may exist in either the oxidized Cu(II) or reduced Cu(I) form. Although the amino acid sequences of plastocyanins from various species are now available (Kelly and Ambler, 1974; Milne et al., 1974; Ramshaw et al., 1974a,b; Scawen and Boulter, 1974; Scawen et al., 1974, 1975; Haslett et al., 1974) very little is known about the copper binding site. Katoh and Takamiya (1964) presented evidence for a direct sulfur–copper bond from cysteine as has been found in all other known blue copper proteins. The copper is apparently liganded by other protein groups since denaturation of plastocyanin facilitates the removal of the metal (Katoh and Takamiya, 1964). Blumberg and Peisach (1966) determined by NMR[1] relaxation studies that the copper is inaccessible to solvent water. We report here proton NMR evidence for the participation of two histidine imidazole groups in copper binding. All plastocyanin sequences published to date contain one cysteine and two histidines in homologous and highly conservative regions.

Materials and Methods

Plastocyanins were isolated from *Anabaena variabilis* and spinach (*Spinacia oleracea*) by a moddification of previous methods (Katoh et al., 1962; Lightbody and Krogmann, 1967).[2] The purity of the plastocyanin preparations was determined by sodium dodecyl sulfate gel electrophoresis, Sephadex gel filtration, amino acid analysis, and by correlating the protein concentrations determined by the molar extinction coefficient of plastocyanin (Katoh et al., 1962) and ultraviolet absorption (Warburg and Christian, 1941). Sodium dodecyl sulfate gel scans of purified spinach plastocyanin indicated that the protein was at least 97% homogeneous. Gel filtration on G-50 Sephadex and subsequent protein assays of fractions showed a single protein peak. Amino acid analysis gave an amino acid profile which was substantially the same as expected from the amino acid sequence (Scawen et al., 1975). There was no detectable *N*-acetylglucosamine present. When the protein concentration of the purified plastocyanin was determined by both the molar extinction coefficient and by the methods of Warburg and Christian the concentrations were within 3% of each other.

Plastocyanin samples were oxidized with ferricyanide if necessary to reach the fully oxidized Cu(II) form or reduced with sodium ascorbate to produce the Cu(I) form. Iron–cyanide complexes were removed from oxidized plastocyanin samples and ascorbate from the reduced plastocyanin samples by passing them through a Sephadex G-25 column. Solutions of plastocyanin (approximately 2 mg/ml) were prepared in 0.1 *M* phosphate buffer (pH 7.0). These solutions were lyophilized and then lyophilized twice again from 99.8% ^2H_2O (Bio-Rad Laboratories) to remove exchangeable protons. The lyophilized samples were stored at 5° and dissolved in ^2H_2O just before use for NMR spectroscopy. The plastocyanin concentration in these samples was approximately 10 mg/ml or 1 m*M*. The samples inhibited by KCN contained in addition 60 m*M* KCN. This concentration of KCN is sufficient to inhibit electron transport in in vitro chloroplast systems (Ouitrakul and Izawa, 1973).

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; ESR, electron spin resonance; ppm, parts per million; pH*, uncorrected pH meter reading of a ^2H_2O solution measured with a glass electrode standardized with H_2O buffers.

[2] Details of the improved isolation and purification procedures (S. P. Berg and D. W. Krogmann, unpublished results) will appear in a separate publication.

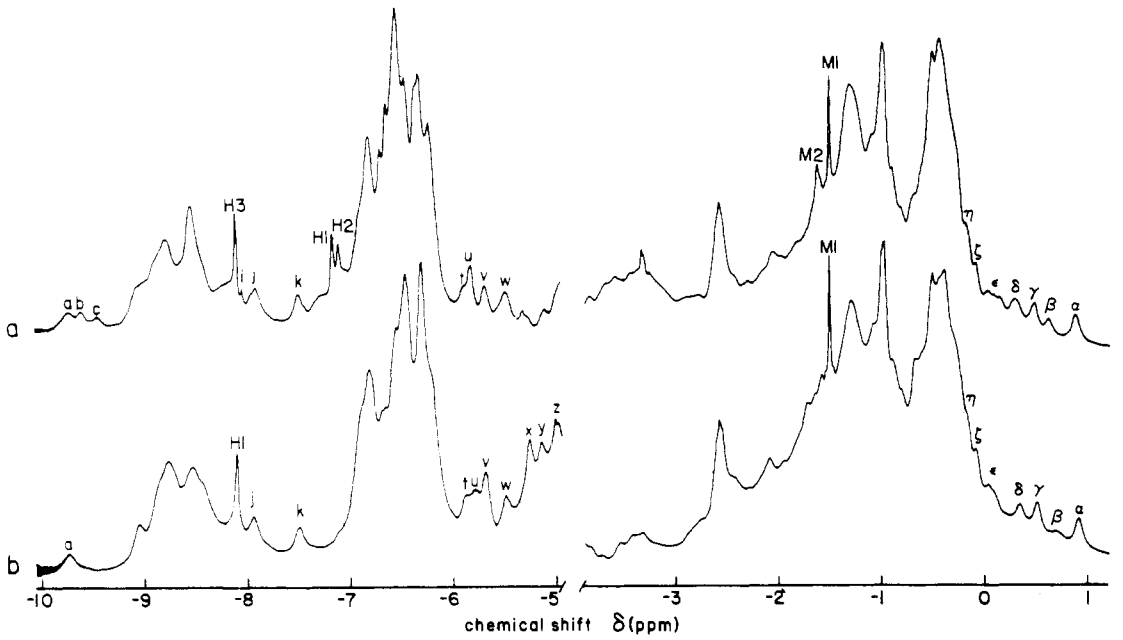

FIGURE 1: Proton correlation NMR spectra (250 MHz) of: (a) reduced anabaena plastocyanin, pH* 6.40; (b) oxidized anabaena plastocyanin, pH* 6.39; both samples in approximately 0.5 *M* phosphate in ^2H_2O. The amplitudes of the low-field spectra are approximately twice those of the high-field spectra.

The plastocyanin samples were titrated with 1 *M* KO^2H or 1 *M* ^2HCl added by a micrometer syringe and Teflon needle. The pH was measured with a Corning Model 112 meter and Ingold combination electrode standardized with buffers in H_2O (Sargent-Welch) bracketing the measurement. The notation pH* is used to indicate the direct pH meter readings of ^2H_2O solutions uncorrected for the isotope effect at the glass electrode. Proton correlation NMR spectra (Dadok and Sprecher, 1974) were obtained at 250 MHz. The spectra are the result of 500 1.5-sec scans of a 1.5-kHz band width. A theoretical line of 1.5-Hz width was used for the cross correlation. Low-field and high-field spectra were obtained separately so that the residual water peak could be avoided. NMR titration data were analyzed by a nonlinear least-squares computer program (Markley, 1973a). Chemical shifts were obtained relative to the water peak used as a lock signal. These have been converted to parts per million [δ (ppm)] from 5% $(CH_3)_4Si$ in CCl_4 external to a diamagnetic protein sample for comparison with previous results from this laboratory.

Results

Comparison of Reduced and Oxidized Plastocyanins. The proton NMR spectra of these algal and higher plant plastocyanins exhibit numerous common features. Correlation NMR spectra (250 MHz) of the oxidized and reduced forms are compared in Figure 1 (anabaena plastocyanin) and Figure 2 (spinach plastocyanin). Many peaks from single protein groups are resolved at 250 MHz. Unassigned single peaks in the low-field region are identified by lower case Roman letters, and those in the high-field region by Greek letters. Peaks a–k disappear in ^2H_2O solution after removal and replacement of the copper (see below) and are assigned to slowly exchangeable N–H groups in hydrogen-bonded or otherwise solvent-inaccessible regions of the protein. Peaks r–z lie upfield of the normal aromatic region. These resonances correspond either to abnormally shielded aromatic rings of phenylalanine or tyrosine or possibly to abnormally deshielded α-CH protons. According to amino acid analyses anabaena plastocyanin contains three His, six Phe, three Tyr, and no Trp residues (Bishop, personal communication), and spinach plastocyanin contains two His, six Phe, three Tyr, and no Trp residues (Scawen et al., 1975). The histidine resonances H1, H2 and H3 (Figure 1) and H1 and H2 (Figure 2) are assigned on the basis of the magnitudes and pH dependences of their chemical shifts and unit proton intensities (see below) to histidine C_2–H nuclei. Peaks H1 and H2 of reduced anabaena plastocyanin and H1 and H2 of reduced spinach plastocyanin have similar chemical shifts, and all disappear when the plastocyanins are oxidized. Therefore, these peaks probably correspond to two homologous histidine residues in the plastocyanins. The amino acid sequence of anabaena plastocyanin is not available at present, but the sequence of another algal plastocyanin (chlorella) has been published. Chlorella plastocyanin (Kelly and Ambler, 1974) contains three histidine residues. Two are homologous with His-37 and -87 found in the plastocyanins of spinach (Scawen et al., 1975) and other higher plants (Milne et al., 1974; Ramshaw et al., 1974a,b; Scawen and Boulter, 1974; Haslett et al., 1974; Scawen et al., 1974). The third is an extra histidine not found in any of the plastocyanins from higher plants that have been sequenced. NMR peak H3 of anabaena plastocyanin may correspond to a histidine homologous to the extra histidine of chlorella plastocyanin.

In the aliphatic region, both anabaena and spinach plastocyanins exhibit two NMR peaks of three-proton intensity (M1 and M2, Figures 1 and 2) in the methionine S–CH_3 region around −1.5 ppm. One methyl peak is sharp (M1, $\Delta\nu_{1/2}$ = 4–6 Hz) and one methyl peak is broad (M2, $\Delta\nu_{1/2}$ = 11–15 Hz) in both anabaena and spinach reduced plastocyanins at pH* 6.4. The broad peaks are missing from spectra of both oxidized plastocyanins. The sharp methyl peak was absent in spectra of some plastocyanin samples and may correspond to a dialyzable impurity. The broad peak M2 is tentatively assigned to a Met S–CH_3 group.

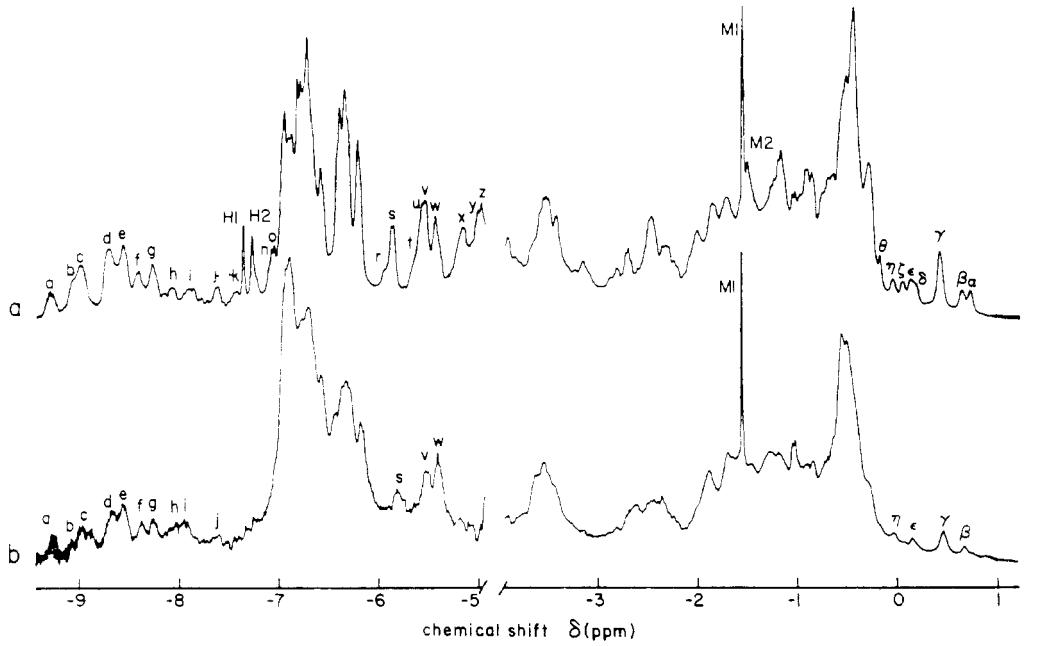

FIGURE 2: Proton correlation NMR spectra (250 MHz) of: (a) reduced spinach plastocyanin, pH* 6.48; (b) oxidized spinach plastocyanin, pH* 6.49; both samples in approximately 0.5 *M* phosphate in ^2H_2O. The amplitudes of the low-field spectra are approximately twice those of the high-field spectra.

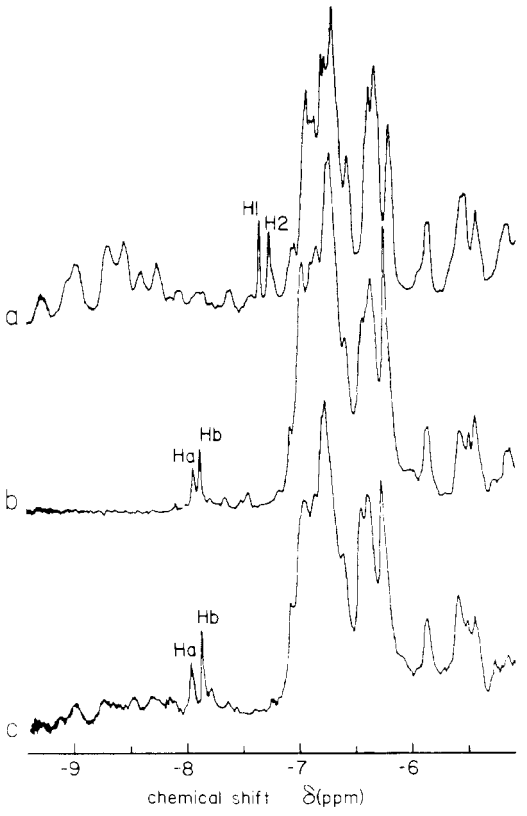

FIGURE 3: Comparison of the low-field proton NMR spectra in approximately 0.5 *M* phosphate in ^2H_2O of: (a) reduced spinach plastocyanin, pH* 6.48; (b) reduced spinach plastocyanin plus 60 m*M* KCN, pH* 6.50; and (c) spinach apoplastocyanin, pH* 6.50.

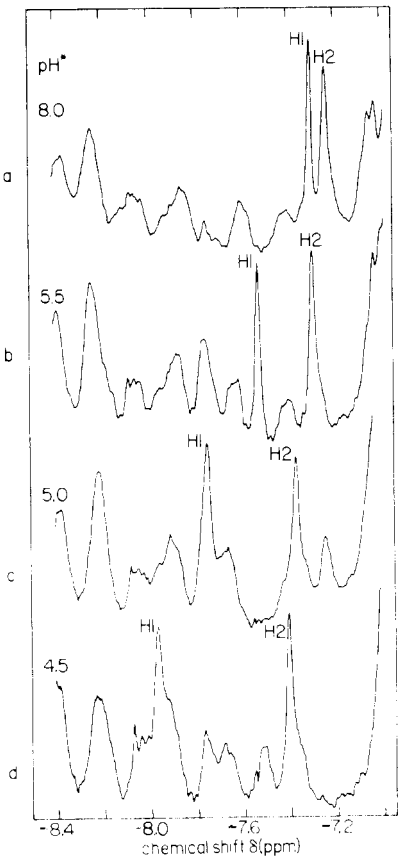

FIGURE 4: The histidine C_2–H NMR peaks of reduced spinach plastocyanin in approximately 0.5 *M* phosphate in ^2H_2O at various pH values: (a) pH* 8.01; (b) pH* 5.49; (c) pH* 5.01; (d) pH* 4.54.

The high-field peaks of anabaena and spinach plastocyanins are particularly well resolved. Using the methyl peak M2 as 3.0 protons the relative intensities of other aliphatic peaks of reduced spinach plastocyanins are: M1, 3.1; α, 1.0; β, 1.1; γ, 3.1; δ, 0.8; ϵ, 0.9; ζ, 0.9; η, 0.9. High-field peaks are generally attributed to protons of hydrophobic residues in contact with the hydrophobic shielding surfaces of aromatic rings. The assignments of these peaks must await further investigations.

Aromatic NMR Spectral Regions of Reduced, Cyanide-Inhibited, and Apoplastocyanins. The low-field NMR spec-

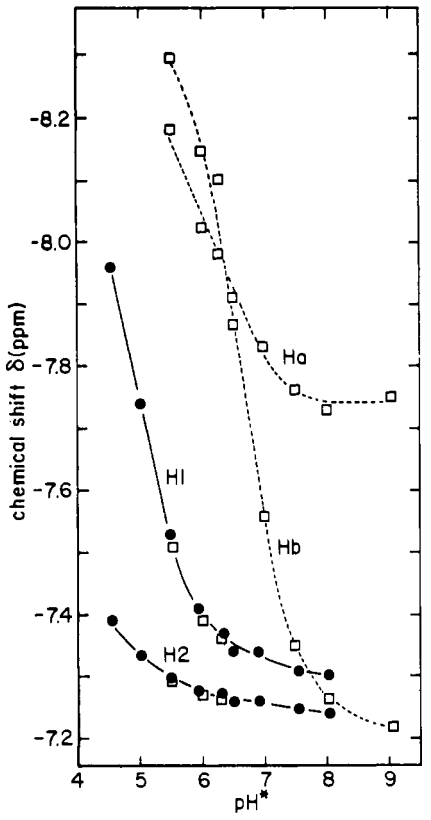

FIGURE 5: NMR titration curves of the histidine C_2-H peaks of reduced spinach plastocyanin (solid symbols, solid lines) and reduced spinach plastocyanin in 60 m*M* KCN (open symbols, dashed lines).

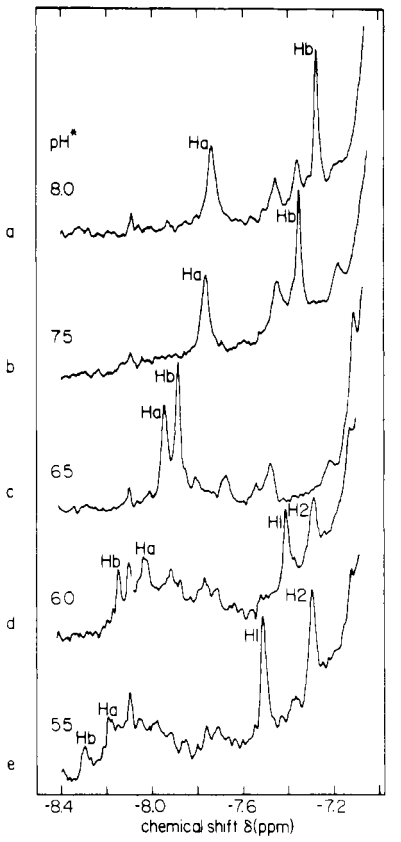

FIGURE 6: The NMR peaks of reduced spinach plastocyanin in the presence of 60 m*M* KCN in approximately 0.5 *M* phosphate in ^2H_2O at various pH values. Note the reversal of the cyanide effect at low pH (cf. Figure 4): (a) pH* 8.00; (b) pH* 7.49; (c) pH* 6.50; (d) pH* 6.00; (e) pH* 5.50.

tra of reduced, cyanide-inhibited, and apo- spinach plastocyanins at pH 7.5 are compared in Figure 3. The spectrum of cyanide-inhibited plastocyanin is almost identical with that of apoplastocyanin. The spectrum shown (Figure 3b) is that of reduced spinach plastocyanin to which KCN (60 m*M*) has been added. A very similar spectrum is obtained when KCN is added to oxidized plastocyanin. These results are in agreement with the conclusion of Katoh and Takamiya (1964) that excess cyanide removes copper completely from the protein. The most noticeable changes produced by removing the copper are enhanced deuterium exchange of the slowly exchangeable N-H peaks (−9 to −7.5 ppm), a downfield shift of the two histidine C_2-H peaks, and small changes in the tyrosine and phenylalanine region (−7 to −6 ppm) which should also contain the histidine C_4-H peaks.

NMR Titration Curves. NMR spectra of the histidine C_2-H region of reduced spinach plastocyanin at various pH* values are shown in Figure 4. The histidine titration data are plotted as the solid curves in Figure 5. It was not possible to follow the titration below pH* 4.5 because of precipitation of the protein. Both histidine residues of reduced spinach plastocyanin have abnormally low p*K'* values for histidine residues in proteins (Markley, 1975). The p*K'* value of histidine H1 is 4.9 ± 0.1, and the Hill coefficient for the transition is 0.86 ± 0.08. It was not possible to calculate a p*K'* for histidine H2, but its p*K'* value must be below 4.5.

The pH dependences of the histidine peaks of cyanide-inhibited reduced spinach plastocyanin were also followed (Figures 5 and 6). Since we are unable at present to correlate the histidine peaks of cyanide inhibited (or apo-) plastocyanin with those of Cu(I) plastocyanin, the histidine peaks are labeled Ha and Hb. In the presence of 60 m*M* KCN, both histidine residues have more normal p*K'* values of around 6.5-6.8. The chemical shifts of the histidines in their deprotonated forms are −7.20 ppm for Hb and −7.75 ppm for Ha. The chemical shift of a normal deprotonated histidine is around −7.4 ppm so that Ha is in a strongly deshielded environment and Hb is in a slightly shielded environment. At low pH, the titrating peaks Ha and Hb disappear, and peaks appear in the positions of H1 and H2 of uninhibited reduced plastocyanin (Figures 4 and 5). The midpoint for this transition occurs around pH* 6. Intermediate states are apparently present because the sum of the intensities of peaks Ha, Hb, H1, and H2 is less than two protons in the transition region. The apparent reason for the reversal of the cyanide effect at low pH is protonation of bound cyanide which would allow the protein to compete more favorably for the copper in solution.

Discussion

It was originally proposed that spinach plastocyanin is a dimer of mol wt 21,000 containing two copper atoms per molecule (Katoh and Takamiya, 1964). The ESR results with *Chenopodium albumin* plastocyanin indicated equivalent coppers (Blumberg and Peisach, 1966), and Milne and Wells (1970) reported that plastocyanin from *Phaseolus vulgaris* is a monomer of mol wt 10,690 containing a single copper. The sharp NMR peaks observed here for spinach and anabaena plastocyanins are consistent only with monomeric structures. A mol wt of 11,000 for spinach plastocyanin has been determined recently by gel filtration studies (S. P. Berg, unpublished results).

In comparing spectra of Cu(I) and Cu(II) plastocyanins, changes that are common to both the algal and higher plant proteins are probably most noteworthy. The spectral changes on oxidation of Cu(I) plastocyanin to Cu(II) plastocyanin may result from (1) a possible conformational change in the protein structure and (2) the altered magnetic properties of the copper. Cu(I) is diamagnetic whereas Cu(II) is paramagnetic. In general, paramagnetic centers may cause contact shifts, pseudocontact shifts (Eaton and Phillips, 1965), and dipolar and scalar line broadening (Solomon, 1955; Bloembergen and Morgan, 1961). Because of the long electronic relaxation time of Cu(II), contact and pseudocontact shifted resonances should be exceedingly broad. Espersen et al. (1974) have recently reported that paramagnetic broadening of proton NMR spectra of ligands by fractional amounts of Cu^{2+} in fast exchange is frequently dominated by scalar effects rather than dipolar effects which have a dependence on r^{-6}. In the present data, there are no clear-cut examples of peaks broadened by Cu(II) but still resolvable. Rather, a number of peaks are unaffected by Cu(II) (including the histidine H3 and high-field methyl peaks α, γ, and δ of anabaena plastocyanin), and a number of peaks are broadened beyond detection (including histidine peaks H1 and H2 and methyl peak M2 of both plastocyanins). Although the regions are not well resolved, intensity is also lost in the tyrosine (−6.3 ppm), β-CH_2 (−2 ppm), and methyl (−0.5 ppm) spectral regions of both plastocyanins on oxidation. These results suggest that nonspecific dipolar broadening is of limited importance. Those peaks of Cu(I) plastocyanin that are missing in spectra of Cu(II) plastocyanin probably correspond to groups that are quite close to the copper. It is not possible at present to evaluate the relative importance of dipolar and scalar effects on these peaks.

A strong case may be made for direct coordination of the two histidine residues H1 and H2 to the copper. Direct coordination would explain the disappearance of the peaks in oxidized plastocyanin, their abnormally low pK' values in Cu(I) plastocyanin, and the normalization of these pK' values on removal of copper. One could instead postulate that the histidines are near the copper but not coordinated to it, but this would require that both histidines be affected by a conformational change on copper binding that reduces their pK' values by 1.6 or more pH units. The simpler interpretation at present appears to be direct coordination. There is indirect evidence for the involvement of one or more histidines as ligands in several other copper proteins (see references in Sundberg and Martin, 1974), and the recent X-ray crystal structure of superoxide dismutase reveals that its copper is liganded by four histidine imidazoles (Richardson et al., 1975).

Katoh et al. (1962) observed that the redox potential of spinach plastocyanin is constant at 370 mV from pH 10 down to pH 5.4, but that below pH 5.4 the redox potential increases with a slope of 60 mV/pH unit. The breakpoint is just above the pK' of histidine H1, and the slope may be explained by titration of one Cu-coordinated histidine. In fact, just on the basis of the pH dependence of the redox potential of plastocyanin Brill et al. (1964) previously suggested that imidazole might be a copper ligand. The increase in redox potential at low pH indicates that the Cu(I) form of plastocyanin is stabilized after displacement of histidine H1. This may be explained by two effects. First, the Cu(II) state is favored in model complexes having a maximum number of nonaquo ligands, so that removal of one of these should raise the redox potential. Second, a sulfur group such as cysteine stabilizes the Cu(I) state so that removal of one imidazole ligand should intensify this sulfur effect (Brill et al., 1964). According to this interpretation, the pK' of histidine H1 should be even lower than 4.9 in oxidized spinach plastocyanin. At lower pH both histidines H1 and H2 become protonated, and two copper ligands are displaced. This may explain why the copper can be removed so readily at low pH (Katoh and Takamiya, 1964).

The results with apo- and cyanide-inhibited plastocyanins are interesting because they reveal the environments of the postulated ligands in the absence of the metal. Vallee and Williams (1968) have proposed that amino acid side chains that bind metals in metalloproteins may have abnormal properties in the absence of the metal that make them particularly well suited for their biological function. NMR spectroscopy clearly provides a way to investigate this idea. The reversal of cyanide binding to plastocyanin at low pH is significant in the context of recent studies of cyanide inhibited chloroplasts that reveal reversal of the cyanide effect below pH 6 (S. P. Berg, unpublished results).

The azurins comprise another class of blue copper proteins that have strong sequence homologies with the plastocyanins. The copper binding ligands of plastocyanins and azurins may be similar. It should be noted that the published sequences of plastocyanins and azurins (see Dayhoff, 1972, 1973) may be aligned so that one cysteine and two histidine residues match up.

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Steroid–Receptor Quantitation and Characterization by Electrophoresis in Highly Cross-Linked Polyacrylamide Gels†

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ABSTRACT: Conditions for discontinuous polyacrylamide gel electrophoresis have been defined in which progesterone receptors of chick oviduct cytosol and a variety of steroid-binding proteins from other sources are stable and amenable to quantitative analysis. The essential modifications from standard procedures include the use of (1) separation gels in which the cross-linking agent/acrylamide monomer = 15:85, (2) glycerol (10% v/v) in all phases of the Tris-glycine-HCl buffer system (pH 10.2 in the separation phase during electrophoresis at 0°), and (3) a layer of a charged reducing agent, thioglycolate, beneath the sample layer. Electrophoresis of untreated oviduct cytosol labeled with [^{3}H]progesterone ± competing steroids revealed a heterodisperse slow peak and a sharp fast peak. Both peaks displayed the steroid-binding specificity and saturability that are characteristic of intracellular receptors. Recovery of steroid from both the slow and fast components increased linearly with sample load up to 60 μl of cytosol (1.2 mg of protein)/gel (6 mm diameter). The specific progesterone binding detected by this technique was comparable to that detected by charcoal-dextran treatment or ion exchange filtration. Relative electrophoretic mobilities (R_f) of globular protein standards and steroid–protein complexes in cytosol and chick serum were measured in separation gels with total gel concentrations (T) systematically varied from 5 to 15% (w/v). Data were processed by computer programs to obtain weighted linear regressions of log R_f on T (Ferguson plots) and the joint 95% confidence limits of the slopes ($-K_R$) and intercepts of these plots. Molecular radii ($\bar{R}$) of the binding components and apparent molecular weights (M) were calculated from the linear correlation of $\bar{R}$ with $K_R^{1/2}$ for the standards. The value of M ~158,000 obtained for the cytosol fast component was independent of the length of the separation gel, the presence of a stacking gel or prior exposure of the cytosol to KCl. It was higher than expected from the sedimentation coefficient of 4.2 S in the same pH 10.2 buffer. Electrophoresis in 170-mm separation gels without stacking gels revealed that KCl extracts of protamine-precipitated cytosol contain a different receptor form, of lower net negative charge than the cytosol fast form. The results demonstrate the utility of electrophoresis in highly cross-linked gels of several concentrations to discriminate between various receptor forms and steroid-binding components of serum. This method may lead to overestimates of M for highly asymmetric receptor forms.

Attempts to purify steroid hormone receptors and to investigate their modes of action are complicated by the structural similarities of the receptors for different steroids, their tendencies to aggregate or form subunits, and the lability of the steroid–receptor complexes. In theory, receptor analysis should be facilitated by the technique of quantitative polyacrylamide gel electrophoresis, in which mobility is measured as a function of total gel concentration. This technique, developed by Chrambach and Rodbard (1971), permits rapid fractionation and characterization of large numbers of samples on the basis of both net charge and size. In practice, gel electrophoresis has been of limited utility in research on steroid–receptors, primarily because of the restricted mobility of large macromolecules, e.g., 8 S–10 S, in

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