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Studies of the Metal Sites of Copper Proteins. Ligands of Copper in Hemocuprein*

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ABSTRACT: The electron paramagnetic resonance, optical absorption, and circular dichroism spectra of bovine erythrocuprein (hemocuprein) were studied under various conditions to obtain information on the copper binding site. When the pH of the protein solution was raised to pH 11.5, a superhyperfine pattern of nine lines with spacing of about 14 G was observed in the electron paramagnetic resonance spectrum of the protein-bound copper. This spectrum can be accounted for by the presence of three to four nitrogen atoms as ligands of copper. The original spectrum was fully recovered by lowering the pH. Also the parallel changes noticed in the absorption and circular dichroism spectra were reversible. The circular dichroism spectrum at pH 11.5 displayed the same multiplicity of ellipticity bands as at neutral pH, only the relative intensity of the peaks being changed; this strongly supports the idea that the copper site is still in its native state at the pH where the nitrogen hyperfine pattern is observed. On the other hand, raising the pH above pH 12 brought about another type of nitrogen hyperfine pattern in the electron paramagnetic spectrum of the protein and drastic changes in optical spectra, which are related to an irreversible denaturation of the protein.

Addition of cyanide was also able to induce a reversible appearance of nitrogen hyperfine pattern, but the modification of the copper site seemed more profound in this case, as indicated by the circular dichroism spectrum in the presence of cyanide. The effect of exchanging deuterium oxide for water in the electron paramagnetic resonance spectrum of hemocuprein suggests that the copper site is exposed to the solvent. Tryptophanyl, tyrosyl, and sulfhydryl residues seem not to be involved in the direct binding of the copper.

In a search for direct information on ligands of copper in copper proteins, we have extended our studies (Finazzi Agrò *et al.*, 1970) to hemocuprein (bovine erythrocuprein), the copper protein of ox red blood cells, for which an enzymatic function, *i.e.*, superoxide dismutase activity, has recently been claimed (McCord and Fridovich, 1969). This protein has a molecular weight of approximately 34,000 and contains 2 Cu(II)/molecule. Copper appears to be essential to the catalytic action, and cannot be replaced by other ions in this role (McCord and Fridovich, 1969). Thus hemocuprein is a new copper enzyme which lacks a detailed physicochemical characterization as yet. In spite of that, it seemed to us particularly suited for a study of the mode of binding of the copper ion, since its metal site appears to be intermediate, as far as can be argued from spectroscopic data, between the unusual copper coordination of "blue" copper proteins and the copper complexes of model peptides. In fact the electron paramagnetic resonance parameters of human erythrocuprein (Malmström and Vänngård, 1960), which is most probably very similar to the bovine protein, can be accounted for by a symmetry only slightly distorted from square planar. Moreover the protein is blue-green (maximum around 680 nm) with an extinction coefficient ($\epsilon_{680} \simeq 300$ cm^{-1} M^{-1}) which is significantly higher than that of low molecular weight copper complexes in this region (Brill *et al.*, 1964) but very much lower than in the case of blue copper proteins. In the present paper we report the results of spectroscopic studies, namely electron paramagnetic resonance, circular dichroism, and optical absorption spectra in different conditions, of hemocuprein as a first approach to the description of its copper site in terms of ligand atoms and in comparison with what is known about the far more deeply analyzed metal site of the blue copper proteins.

Material and Methods

All chemicals were reagent grade, and were used without further purification. Hemocuprein was purified from ox blood according to McCord and Fridovich (1969). Protein concentration was measured by a biuret procedure (Goa, 1953) or by optical absorption at 258 and 680 nm (McCord and Fridovich, 1969); the values obtained by different methods were in good agreement with each other.

Electron paramagnetic resonance spectra were recorded on a Varian 4502-14 spectrometer, equipped with 100-KHz field modulation within a Varian multipurpose cavity, and variable-temperature accessory. Frequencies were measured

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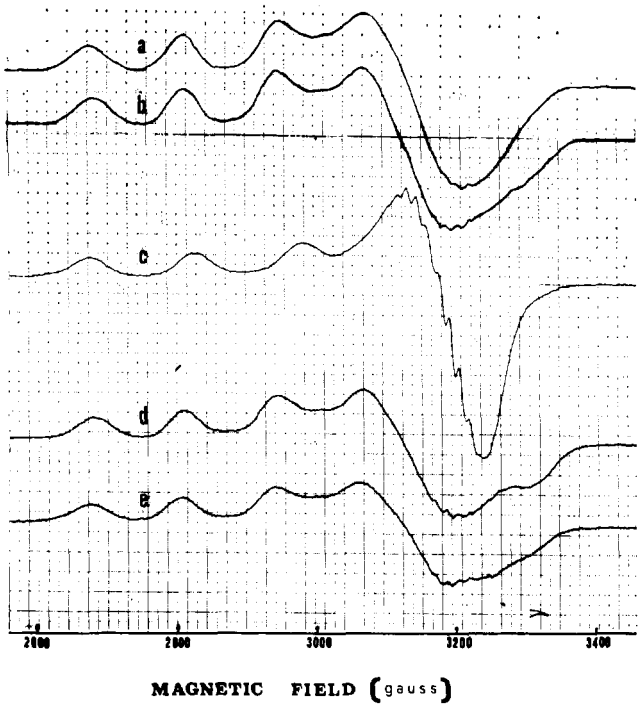

FIGURE 1: Electron paramagnetic resonance spectra of hemocuprein at various pH values. A 3.5% solution (0.3 ml) of hemocuprein in water (pH 6, curve a) was used. The pH of the solution was raised by small additions of KOH to pH 9 (curve b), pH 12 (curve c), and then readjusted with concentrated HCl to pH 10 (curve d) and finally to pH 8 (curve e). Microwave power: 6 mW; modulation amplitude: 5 G; temperature −150°. The amplification of the curves c, d, and e was 0.8 times that of the curves a and b.

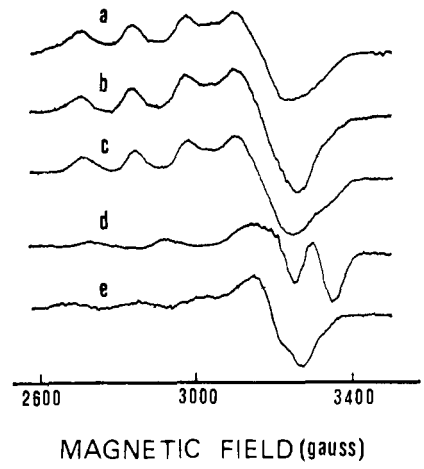

FIGURE 2: Electron paramagnetic resonance spectra of hemocuprein at various pH values. A 1.5% solution (3 ml) of hemocuprein in water (pH 6.0, curve a) was used. The pH of the solution was sequentially adjusted, as indicated in the legend to Figure 1, to pH 11.5 (curve b), 10.5 (curve c), 12.5 (curve d), 7.5 (curve e). Microwave power: 6 mW; modulation amplitude: 10 G; temperature −196°.

with a Hewlett Packard X 532 B frequency meter. g values (Malmström and Vänngård, 1960) were calculated by the Fieldial and the measured frequency to within 0.5%.

Circular dichroism spectra were recorded at 26° using a Cary 60 spectropolarimeter equipped with 6001 CD attachment. The measurements were made in 1-cm light-path cells. In the region between 600 and 750 nm it was necessary to program a slit width of 1 mm in order to keep the dynode voltage below 0.4 kV. The circular dichroism data were reported in terms of mean residue ellipticity, $[\theta]_\lambda$, in (deg cm^2) dmole^{-1}, where $[\theta]_\lambda = \theta_\lambda MRW/100cd$ and c is the concentration in grams per milliliter, d is the path length in decimeters, θ_λ is the observed ellipticity, and MRW is the mean residue weight, which was taken as 115. The calibration of the circular dichroism attachment was checked with a 100-mg/ml solution of 10d-(+)-camphorsulfonic acid (Merck AG) in water. Optical absorption spectra were performed in a Beckman DK2A ratio recording spectrophotometer. pH measurements were made at room temperature in a pH 4 Radiometer equipped with a G-200B glass electrode.

Results

Electron Paramagnetic Resonance Spectra of Hemocuprein. Figure 1a shows the low-temperature X-band electron paramagnetic resonance spectrum of hemocuprein dissolved in water. Electron spin resonance parameters were $g_{||} = 2.268$, $g_{max} = 2.087$, and $|A_{||}| = 0.014$ cm^{-1}. They are quite similar to those reported by Malmström and Vänngård (1960) except for the value of the hyperfine coupling constant which is significantly lower in the bovine protein. Also the line shape appears to be identical with that shown by Carrico and Deutsch (1969) for human erythrocuprein. The power at half-saturation was 30 mW.

When the pH of hemocuprein solution was raised up to pH 12, the electron paramagnetic resonance spectrum changed gradually as indicated in Figure 1b,c. A superhyperfine structure is evident on the high field line of the spectrum at pH 12, consisting of a set of nine lines regularly spaced (splitting of about 14 G) that cannot be ascribed only to partially resolved copper hyperfine structure in the $g_\perp$ region. Lowering the pH restored the original spectrum (Figure 1d,e) suggesting that the copper ligands do not change at pH 12.

In another series of experiments (Figure 2) the pH range of reversible appearance of the form with ligand hyperfine structure was more carefully studied. At pH 11.5 (Figure 2b) the nine hyperfine lines pattern is already present, but the spectrum differs from that at pH 12, as the electron paramagnetic resonance parameters are the same as those at neutral pH. This would be a rather improbable result in the case that the nature and geometry of ligands around copper had been grossly modified. Adjusting the pH only to pH 10.5 the neutral pH type of spectrum is restored (Figure 2c).

When, however the pH was raised above 12, a copper–biuret-type electron paramagnetic resonance spectrum appeared (Figure 2d; $g_{||} = 2.179$ and $|A_{||}| = 0.019$ cm^{-1}) and a completely new copper signal was observed on going back to neutral pH (Figure 2c; $g_{||} = 2.235$, $g_{max} = 2.056$, and $|A_{||}| = 0.0175$ cm^{-1}) indicating irreversible denaturation of the native site.

Visible Absorption and Circular Dichroism Spectra of Hemocuprein. The optical absorption spectrum of hemocuprein in water (Figure 3) has a broad peak in the visible range with a maximum around 680 nm. The corresponding circular dichroism spectrum (Figure 4) shows the presence of two major peaks almost identical in rotatory strength but opposite in sign. The maximum of the positive peak is centered at about 600 nm while that of the negative peak

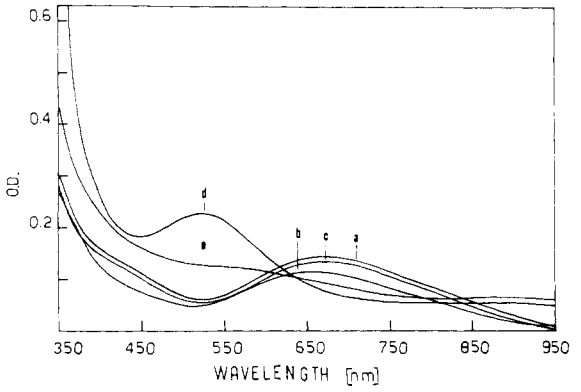

FIGURE 3: Optical spectra of hemocuprein at various pH values. The letters indicate the same pH values as in Figure 2.

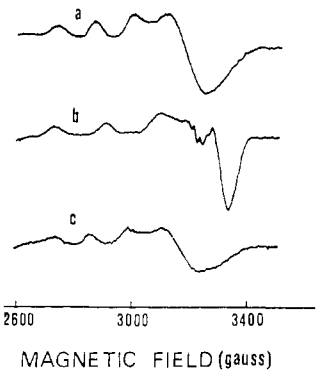

FIGURE 5: Effect of cyanide on the electron paramagnetic resonance spectrum of hemocuprein. Hemocuprein, 3%, dissolved in 3 ml of 0.1 M potassium phosphate buffer (pH 6.5). Curve a: as such; curve b: after addition of 0.1 M KCN (pH changed to 8.8); curve c: the solution of b after exhaustive dialysis against 0.1 M potassium phosphate buffer (pH 6.5). Microwave power: 6 mW; modulation amplitude: 10 G; temperature −196°.

can only tentatively be assigned around 750 nm because of the instrumental limitations in this region. Additional bands are evident in the circular dichroism spectrum in the regions around 430 and 350 nm.

At pH 11.5 small changes in the circular dichroism spectrum were observed. The shape of the band centered at 600 nm appeared to be slightly modified, suggesting that this peak results from the presence of at least two optically active bands of the same region. Furthermore, the ellipticity in the region around 430 nm increased, while the intensity of the shoulder at 350 nm was notably reduced. These modifications of the circular dichroism spectrum were reflected in concomitant shifts of optical absorption maxima. All these changes were fully reversed by lowering the pH of the solution. On the other hand, a further increase of the pH (>12) brought about dramatic changes in the optical properties of hemocuprein. The color of the protein solution turned from blue-green to pink. The native pattern of hemocuprein circular dichroism spectrum was completely abolished and replaced by one characterized by a positive peak at 580 nm and by two negative peaks at 510 and 325 nm. These changes could not be reversed by lowering the pH.

Effect of Cyanide. Hemocuprein was treated with cyanide, in 0.1 M phosphate buffer (pH 6.5), to avoid too elevated pH rise in the presence of high concentration of KCN. The electron paramagnetic resonance spectrum (Figure 5b) showed in fact that the appearance at a relatively low pH (pH 8.8) of a superhyperfine pattern at high field, but also of an entirely different set of electron paramagnetic resonance parameters ($g_{\parallel} = 2.205$ and $|A_{\parallel}| = 0.017\ cm^{-1}$).

Optical absorption and circular dichroism spectra after cyanide treatment are reported in Figures 6 and 7 and show the profound modifications of the optical properties of the protein in these conditions. Dialyzing out cyanide after short exposure to this ligand restored the original spectra of the protein, but some irreversible denaturation occurred, as demonstrated by precipitation of protein material during the treatment.

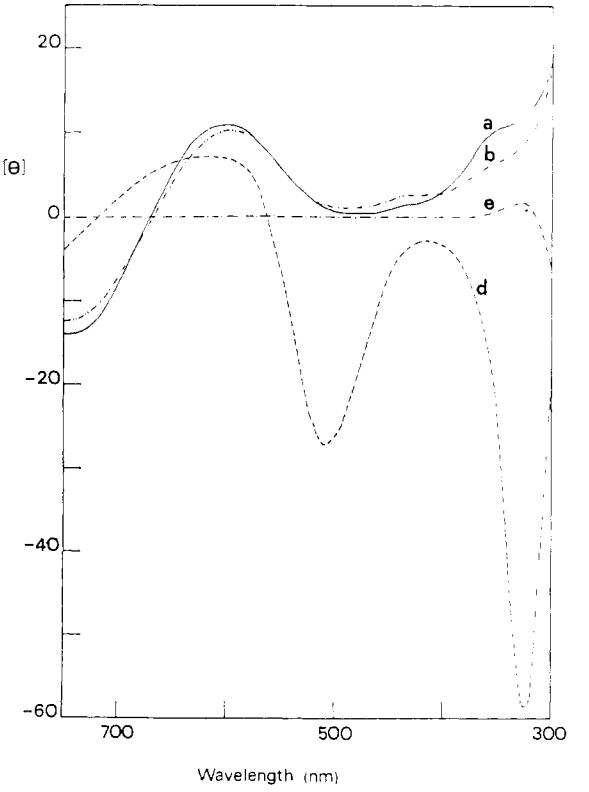

FIGURE 4: Circular dichroism spectra of hemocuprein at various pH values. The letters indicate the same pH values as in Figure 2.

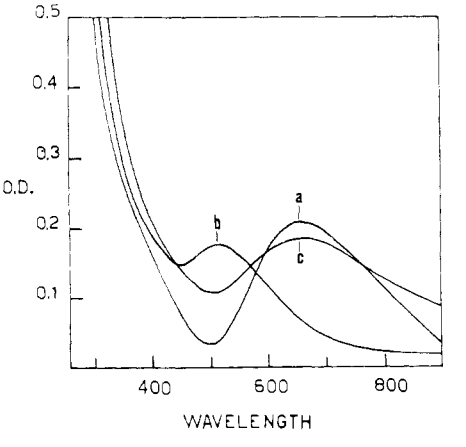

FIGURE 6: Effect of cyanide on the optical spectra of hemocuprein. Symbols as in Figure 5.

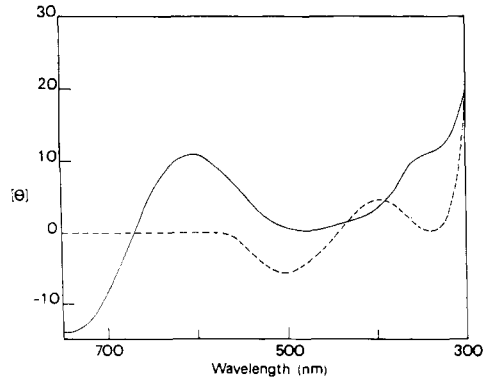

FIGURE 7: Effect of cyanide on the circular dichroism spectrum of hemocuprein. (——) as in Figure 5a; (---) as in Figure 5b.

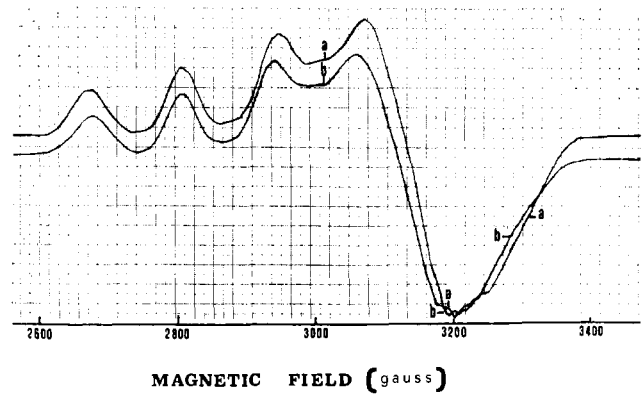

FIGURE 8: Effect of deuterium oxide on the electron paramagnetic resonance spectrum of hemocuprein. Curve a: hemocuprein, 4.5%, dissolved in 0.3 ml of water; curve b: the solution as in curve a was freeze-dried, dissolved in 1 ml of deuterium oxide (99.75%) and freeze-dried again. This procedure was repeated three times. The final solution for spectrum recording was in 0.3 ml of deuterium oxide. Microwave power: 6 mW; modulation amplitude: 8 G; temperature −150°. The amplification of curve a was 1.25 times that of curve b.

Effects of Deuterium Oxide. To test if the effects of alkaline pH and of cyanide could be explained by a direct action on immediately adiacent ligands of copper and not only by long-range conformational influences, the effects of exchanging deuterium oxide for water on the electron paramagnetic resonance spectrum of the protein were analyzed. Thus a preliminary information on the accessibility of the copper site to the solvent should be obtained. The results of this experiment are shown in Figure 8, where significant changes of the spectrum after deuterium oxide treatment are evident. On the other hand, exposure of the protein to 6 M guanidine hydrochloride had no effect on the electron paramagnetic resonance spectrum.

Spectrophotometric Titration of Tyrosyl Residues. A single slope was observed with a pK' of about 11. The total optical density change accounted for two residues, which corresponds to the value obtained by amino acid analysis.[1] The titration curve is time independent and fully reversible if the experiment is carried out at 20° or less.

Discussion

It might appear an obvious and expected result to obtain direct evidence for the presence of nitrogen atoms as ligands of copper in the metal binding sites of copper proteins. However, direct demonstration of that is missing so far, and the concept that nitrogen plays a major role in binding copper to proteins rests mostly on general assumptions and model systems. Thus optical absorption maxima around 600 nm, as shown by many copper proteins, are considered to be due predominantly to nitrogen ligands (Brill *et al.*, 1964). On the other hand, studies of Cu(II) complexes of small peptides have indicated terminal amino, amido, and imidazole nitrogen atoms as preferential donors for Cu. In many instances the observation of a well-defined ligand hyperfine structure in the electron paramagnetic resonance spectra of such complexes (Wiersema and Windle, 1964; Bryce, 1966; Gould and Mason, 1967) has given direct demonstration of the presence and of the number of nitrogen Cu(II) bonds in the complex. Also in artificial Cu(II)–protein complexes (Windle *et al.*, 1963; Aasa *et al.*, 1963; Gurd *et al.*, 1967; Aasa and Aisen, 1968) nitrogen hyperfine structure has frequently been observed. Unfortunately, native copper proteins do not show that structure. In fact the nitrogen hyperfine structure detected by Ehrenberg and Yonetani (1961) in cytochrome oxidase has been assigned to a denatured form of the protein (Beinert *et al.*, 1962; Beinert and Palmer, 1964). Mondovì *et al.* (1967) have described a pattern of superhyperfine lines in pig kidney diamine oxidase, which becomes more evident in the presence of substrate. However, no conclusive evidence is available about the native state of the copper site in this protein thus far, and an unequivocal assignment of such a spectral feature to the native copper site is presently premature.

On the other hand, nitrogen hyperfine structure in copper proteins usually arises in their electron paramagnetic resonance spectra, when protein solutions are brought to high pH (*i.e.*, above pH 12); in this case the spectrum appears indistinguishable from that of the biuret–copper complex in alkaline solution and therefore can be attributed to binding of copper by four peptide nitrogen. However this spectral change is irreversible, and it is to be expected that a replacement of copper ligand atoms can easily be brought about by an irreversible change of protein structure (Peisach *et al.*, 1967; Malmström *et al.*, 1970). This irreversible appearance of nitrogen hyperfine structure at high pH is thus irrelevant for a direct information on the metal coordination in a native protein. Beside raising pH another treatment which can produce the appearance of ligand hyperfine structure is the binding to the protein-bound copper of various chemical reagents. This structure can be obviously due to the ligand atoms of the chemical reagent itself, as nitrogen hyperfine structure in NO-treated hemocyanin (Mason, 1966) or ^{13}C and F splitting in fungal laccase treated with CN^- and F^- (Malkin *et al.*, 1968). But it is also possible that nitrogen hyperfine structure from protein ligand atoms can be observed in the spectrum of the altered copper site, as is the case for cyanide treated laccase (Malmström *et al.*, 1968). If it can be presumed, by the usual criterium of reversibility, that ligand atoms of copper in the native site are not displaced by the addition of the chemical reagent, the hyperfine structure can reliably be used as a proof of the presence of nitrogen ligands in the copper binding site.

The considerable attention that has been paid to the possibility of detecting ligand hyperfine structure in electron paramagnetic resonance spectra of native copper proteins

[1] Unpublished data from this laboratory.

comes mainly from the fact that other kinds of direct information on the metal coordination are lacking, since no copper protein has been analyzed by X-ray crystallography, and electron nuclear double resonance studies (Rist *et al.*, 1970), are at their first steps in this field. The results presented above are primarily concerned with the appearance of a superhyperfine pattern on the high field line of the electron paramagnetic resonance low-temperature spectrum of hemocuprein in two conditions which gave reliable evidence for maintenance of the native ligands by the copper binding site. In fact the spectrum at pH 11.5 has nearly the same electron paramagnetic resonance parameters as that at neutral pH; furthermore, it was completely reversible by lowering the pH. It could be argued that raising the pH to 11–12 relaxes the rhombic distortion present in the copper site at neutral pH since the spectrum becomes more axial (Figure 1c) at pH 11–12. This produces the appearance of the ligand hyperfine structure, which is not observable in the rhombic spectrum at neutral pH because of the overlapping of its components in the x and y directions. Another indication that the copper site at pH 11.5 has not changed the ligands atoms is the circular dichroism spectrum, which shows the same ellipticity bands as at neutral pH, only the relative intensity of the bands being changed. It is improbable that substitution of ligands in the four planar positions does not affect also the multiplicity and the sing of the bands (Tang *et al.*, 1968). The circular dichroism spectrum above pH 12 is in fact completely different in terms of number, sign, and rotatory strength of ellipticity band. The small changes observed at pH 11.5 could reflect a minor perturbation of the ligand field, as a negative charge in fifth position produced by ionization of a water molecule. The suggestion that water is a weak apical ligand is supported by the modifications of the electron paramagnetic resonance spectrum in the presence of deuterium oxide. On the other hand, cyanide as apical ligand produces more substantial changes in the ligand field around copper, as indicated by the profound modifications of electron paramagnetic resonance and circular dichroism spectra. However, if the exposure of the protein to this ligand is not too long, the changes are reversible, suggesting that the superhyperfine structure shown by the cyanide-treated hemocuprein can be assigned to the native ligand distribution.

The splitting of these superhyperfine lines (~14 G) strongly suggests that they arise from nitrogen hyperfine coupling to copper. However, it should be recalled that when they are observed in the $g_\perp$ region, their number cannot be used with absolute reliance to determine the number of magnetically equivalent nitrogen atom the unpaired copper electron interacts with. In fact, since the value of the copper hyperfine splitting constant on the perpendicular direction, $|A_\perp|$, can be of the same order of magnitude as that of the ligand hyperfine splitting, overlap of copper hyperfine structure can produce a greater number of lines than the number present on a single copper hyperfine line. In this situation, it would be incorrect to relate the number of superhyperfine lines to the numbers of coordinating nitrogen atoms. Unfortunately, in the case of hemocuprein, no clear superhyperfine pattern was observed in the $g_\parallel$ region, where this restriction does not hold any more. On the other hand, no additional information was obtained from the liquid state electron paramagnetic resonance spectra,[1] which give an identical result as in the frozen state, due to the slow rate of rotation of the protein in solution. It is possible to make an assignment of three to four nitrogens as planar ligands for hemocuprein on the basis of the similarity between the spectrum of cyanide-treated protein and that of the Cu(II)–triglycylglycine complex in the same pH range (Falk *et al.*, 1967). However, these results do not give any indication about the nature of the individual amino acid residues that furnish these nitrogen atoms. Moreover, even a nitrogen hyperfine structure that may be explained on the basis of magnetically equivalent nitrogen ligands, does not prove that they are also structurally equivalent. As for other possible ligands, phenolic hydroxyls seem to be ruled out by the results of the spectrophotometric titrations, which showed that both tyrosyl residues ionize in a normal pH range, indicating that they are both accessible to the solvent and not involved in metal coordination (Warner and Weber, 1953; Wishnia *et al.*, 1961). Tryptophanyl residues are excluded since tryptophan determination[1] gave a tryptophan to copper ratio of about 0.5. On the other hand, the hemocuprein copper is not reduced at alkaline pH, and this speaks against the presence of sulfhydryl groups as ligands of the metal ion.

The multiplicity of ellipticity bands has been claimed to be the very distinguishing feature of the blue copper proteins, more than the rotatory strengths of the transitions, which are of the same order of magnitude both in the proteins and in Cu(II)–peptide complexes, as the glycyl-L-valine complex (Tang *et al.*, 1968). The circular dichroism spectrum of hemocuprein shows the same multiplicity of ellipticity bands in the visible range as in blue copper proteins in contrast with the much simpler spectrum of the Cu(II)–glycyl-L-valine. Thus the copper chromophore of hemocuprein could probably be accounted for by a molecular orbital model related to that of blue copper proteins. In this context, it has been pointed out (Tang *et al.*, 1968) that the number of transitions above 300 nm in the proteins suggest that the set of orbital involved is more complex than that expected on the basis of pure d–d transitions, although with the proper distortions of a copper complex four d–d transitions become magnetically allowed. It has been often suggested that the extra transition of copper proteins arise from charge transfer involving ligand orbitals. In this case the nature and the environment of the ligands would play a major role in producing the very different oscillatory strengths of the visible transitions in various copper proteins, rather than different distortions from the square-planar symmetry of the low molecular weight complexes. Thus, in *Pseudomonas fluorescens* azurin, the presence of copper ligands as cysteine and tryptophan buried in a strongly hydrophobic site (Finazzi Agrò *et al.*, 1970) gives rise to a strong absorption in the visible rangle. In the case of hemocuprein a charge-transfer transition, if present, is expected to be at higher frequencies, owing to the more hydrophilic environment of the copper (Kosower, 1968). Furthermore also the absorption intensity is dependent on the electron-donating or -attracting power of ligands, and on the dielectric constant of the microenvironment.

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Chromatographic Isolation and Characterization of Isolated Chains from Hemoglobin after Regeneration of Sulfhydryl Groups*

Kaspar H. Winterhalter† and Alfredo Colosimo

ABSTRACT: The chromatographic separation of isolated hemoglobin α^{SH} from α^{PMB} chains and of β^{SH} from β^{PMB} chains is reported.

The purity of the separated compounds was tested by electrophoresis, spectrophotometry, and atomic absorption analysis. Their isoelectric points and the isoelectric points of a number of other hemoglobin derivatives has been determined by ion focusing; the results are consistent with expectations based on the chemical differences between the various hemoproteins.

Isolated chains from hemoglobin in their native state are commonly obtained by reacting hemoglobin at pH 6 with excess PMB[1] (Bucci and Fronticelli, 1965). This method leads to dissociation of hemoglobin into α and β chains which have their sulfhydryl groups reacted with PMB. They can be isolated chromatographically. Regeneration of free SH groups in position 104 on α chains and positions 93 and 112 on β chains has been achieved by several methods (Bucci and Fronticelli, 1965; Tyuma *et al.*, 1966; De Renzo *et al.*, 1967; Geraci *et al.*, 1969); however, none of these methods removes PMB completely without partial denaturation of the protein. Since in many instances it may be crucial to obtain a homogeneous material, chromatographic separation of chains with free SH groups from PMB chains appeared to provide the best solution to the problem.

The procedure is particularly valuable for the detection of small differences in the functional properties of α and β chains, when minor contamination with PMB-containing chains may distort results in view of the rather large effect that the presence of mercurials may have on affinity and reaction rates in the combination with ligands (Antonini

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[1] Abbreviation used is: PMB, *p*-mercuribenzoate.