ELECTRON SPIN RESONANCE OF Cu(II) IN COPPER-HEMOGLOBIN COMPLEXES.

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Summary. Electron spin resonance data indicate that two cupric ions bind to a molecule of hemoglobin at pH 5.4 to 8.9. The binding sites of both ions are identical and are characterized by the presence of resolved superhyperfine structure indicating a considerably covalent bond between the metal and most probably four equivalent nitrogens. Introduction of more than two Cu(II) ions produces precipitation of the protein accompanied by changes in the ESR spectra. The ESR spectra of Cu(II) are identical in oxygenated and methemoglobin. Fe(II) of the former oxidizes partly to Fe(III). Comparison with copper-myoglobin complexes indicates that the metal sites are different in the two proteins.

Hemoglobin and myoglobin present very convenient, relatively well understood systems for the studies of metal-protein interactions. Considerable amount of information concerning the copper-myoglobin complex has been obtained by means of chemical studies (1-4), X-ray diffraction (5) and electron spin resonance (ESR) (6-7).

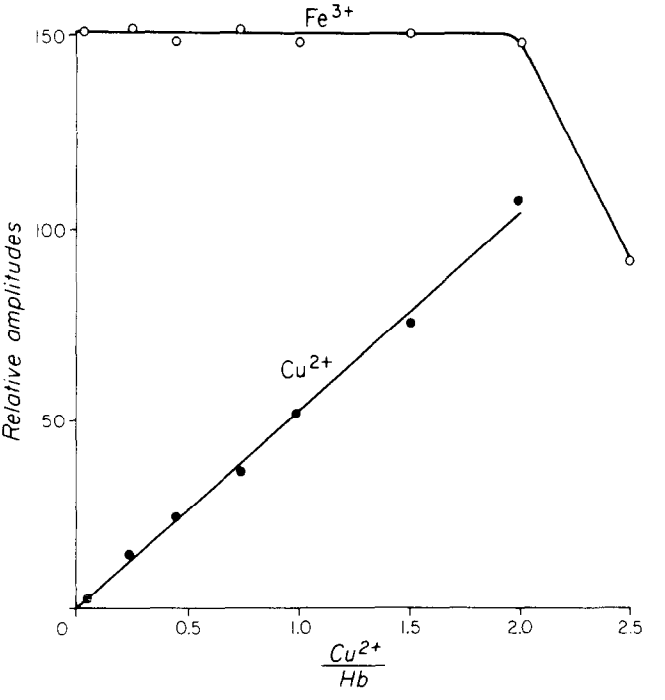
We have undertaken studies of the ESR spectra of Cu(II) bound to human hemoglobin. There is at the present time no information available concerning this system. In particular the X-ray diffraction data are as yet non existant. Since X-ray diffraction has shown at least in two cases, that the sites of foreign atoms in hemoglobin crystals are vastly different than in myoglobin (8-10), we were interested in comparing Cu(II) spectra in hemoglobin with the ones reported for Cu(II) in myoglobin (6-7). We expect furthermore that the proper understanding of binding of paramagnetic ions in proteins may permit their use as an additional marker, useful in investigating biologically significant transformations of the latter.

Experimental

Human hemoglobin was obtained from normal donors and hemolizates were prepared by the method of Drabkin (11). Bovine hemoglobin was obtained from Worthington Biochemical Corp. and from Sigma Chemical Co. Ferri (met) hemoglobin was prepared of stoichiometric amount of potassium ferricyanide to oxyhemoglobin, followed by dialysis against 0.25 M NaCl.

Copper (Cu(II)) was added as $CuCl_2$ to hemoglobin by direct mixing, followed by two hour incubation at 28°C. In some cases it was added by dialysis following procedure described by Breslow (1).

Electron spin resonance measurements were performed with a Varian V-4500 Spectrometer at 100 KHz modulation and temperature of 77°K.

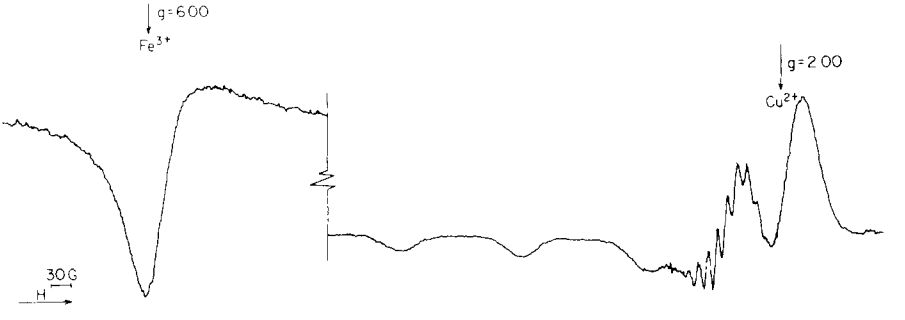


1. Relative amplitudes of the ESR signals of Fe(III) and Cu(II) in native methemoglobin as a function of concentration of Cu(II). The amplitudes of Fe(III) should not be compared with those of Cu(II). Hb concentration 50 mg/ml pH = 7.0, T = 77°K.

Results and discussion

ESR spectra of Cu(II) and of Fe(III) in 1.5 mM human native, methemoglobin have been obtained at pH 6.5 in 0.1 M KCl as a function of added Cu(II) concentration. The amplitude of the g=6 signal, characteristic of Fe(III) in methemoglobin (high spin), remains constant up to concentrations of two equivalent copper ions per mole of hemoglobin (Fig. 1); above this concentration a decrease in the amplitude of the Fe(III) signal is noticeable and is accompanied by a decrease in the amplitude of the absorption bands at 405, 540 and 576 mu, and by a partial precipitation. A comparison of ESR absorption signals of Fe(III) in the presence and absence of Cu(II) shows no difference in line width and shape indicating absence of spin-spin interaction, hence a separation of more than 5Å between ferric and cupric ions.

The amplitude of Cu(II) signal characteristic of the copper-hemoglobin complex (Fig. 2) increases linearly with added copper concentration, indicating that all added Cu(II) is bound to hemoglobin up to a concentration of two equivalent copper ions per molecule (Fig. 1). Above this concentration the Cu(II) signal changes considerably and we have, for the time being, not pursued



2. ESR spectrum of Fe(III) and Cu(II) in oxyhemoglobin. One molar equivalent of Cu(II) per mole of hemoglobin, pH = 7.0, Hb concentration 50 mg/ml, T = 77°K, 0.1M KCl.

TABLE I

PARAMAGNETIC RESONANCE PARAMETERS OF Cu(II) IN VARIOUS COMPLEXES

Copper complex	$g_{\parallel}$	$g_{\perp}$	$\lvert A_{Cu}\rvert$ (cm^{-1})	$\lvert A_{N}\rvert$ (cm^{-1})	pH	Ref.
Native hemoglobin*:						
(Cu/Hb $\leq$ 2)	2.208	2.054	0.0201	0.00149	5.4-8.9	this work
(Cu/Hb > 2)	2.27	2.057	0.0182	----	7.0	" "
Lyophilized hemoglobin:						
(Cu/Hb $\leq$ 2)	2.279	2.059	0.0171	----	5.1	this work
(Cu/Hb $\leq$ 2)	2.262	2.054	0.0182	----	6.7	" "
Myoglobin:						
	2.273	2.054	0.0175	----	6.4	6
	2.186	2.046	0.0194	----	10.4	6
Phtalocyanine:						
	2.179	2.050	0.0202	0.00145	----	13
Etioporphyrin II:						
	2.169	2.061	0.0188	0.00144	----	12

* Identical values for originally met or oxyhemoglobin.

the experiments in this concentration range (see however Table 1). Cu(II) spectrum is characterized by the presence of a well resolved superhyperfine structure superposed on the large copper absorption line. This superposition does not permit a very reliable comparison of the relative amplitudes of the lines. The best fit is obtained assuming a distribution of amplitudes 1:4:10:16:19:16:10:4:1, expected from an interaction of the Cu(II) electron with four magnetically equivalent nuclei, each of spin one. This and the observed spacing of 14.5 G between the individual superhyperfine lines identifies copper's neighbors as nitrogens. The structure is readily observed at a microwave power of 1mwatt. Higher powers produce saturation of the structure.

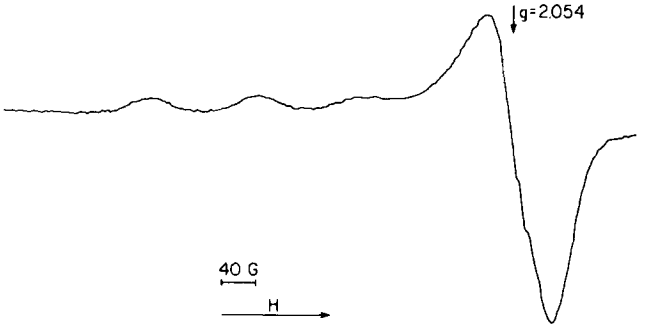
The superhyperfine structure is indicative of a strongly covalent bond between the cupric ion and the nitrogen ligands, very similar to the situation reported for Cu(II) in phtalocyanine and etioporphyrin II (12). Neiman and Kivelson have analyzed the reasons for the appearance of two large Cu(II) lines where in frozen solutions normally a single one is expected (13). They have shown that the structure containing absorption line corresponds to $\Delta M_s = \pm 1$, $\Delta M_{Cu}=0$ transition for the $M_{Cu} = 3/2, 1/2$ hyperfine states of molecules with symmetry axes perpendicular to the magnetic field, and for the $M_{Cu} = -1/2$ state corresponding to an angle somewhat smaller than 90°. $M_{Cu}= -3/2$ is responsible for the second large peak, corresponding to molecules at about 45° to the applied field. The much smaller peaks at lower fields are due to molecules with symmetry axes parallel to the applied magnetic field. We believe that the absence of resolved superhyperfine structure on the high field absorption line is due to anisotropy in the hyperfine splitting constant of copper which is considerably larger at 45° than at 90° and produces a smearing of the lines arising from the interaction with nitrogens.

The results of the present experiments as well as those of the pertinent previous studies are listed in Table 1.

The cupric ions can be removed from hemoglobin with EDTA. Addition of 3 mM of EDTA to 1.5 mM hemoglobin with copper obliterates the above discussed signal and produces one which corresponds to the Cu-EDTA complex, without affecting the Fe(III) absorption at g=6.

A similar series of experiments has been performed with human, native oxyhemoglobin. The iron in this case is ferrous and diamagnetic. On addition of Cu(II) one notices appearance of a signal at g=6, indicating partial oxidation of Fe(II). Addition of two equivalent cupric ions oxidizes approximately one ferrous ion and reduces a corresponding fraction of cupric ions. The details of Cu(II) ESR spectra are identical to those of Cu(II) in methemoglobin. This signal is unchanged between pH 5.4 and 8.9. Ferric ions undergo a high to low spin transition with the same pK of 7.8 as in the absence of copper (14).

Addition of copper to lyophilized bovine, or human hemoglobin results in a Cu(II) signal different than in native protein. The superhyperfine structure is very poorly resolved (Fig. 3), and the signal is similar to the one observed in precipitated Cu-Hb complex in native hemoglobin.



3. ESR spectrum of Cu(II) in lyophilized, human hemoglobin. One molar equivalent of Cu(II) per mole of hemoglobin, pH 7.0, Hb concentration 50 mg/ml, T = 77°K, 0.1M KCl.

Our data suggest that there is only one type of binding site for the complexing of the first two copper ions by native hemoglobin, in contrast to the single copper site in myoglobin (5). This site involves most probably four nitrogens which are magnetically equivalent and covalently bound to copper, in contrast to the results of the X-ray diffraction studies on copper-myoglobin crystals (5). These studies, at a resolution of 2.8 Å, have shown the presence of histidine, lysine and asparagine in the environment of Cu(II). The ESR results of Gurd et al. on the Cu(II)-myoglobin complexes (6) when compared with the present ones at a similar pH show differences both in the g values and in the absence of well resolved superhyperfine interaction in myoglobin.

Further experiments are needed to decide among the possible protein nitrogen ligands bound to copper.

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