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# THE $^{1}$H-NMR-STUDY OF THE COPPER SURROUNDING IN PLANTACYANIN

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The 300-MHz proton NMR spectra of oxidized, reduced and apo-forms of plantacyanin were studied. The data obtained show that one of two histidines is far from copper whereas the other is a ligand of the metal. Ligands of copper are also two methionines and, possibly, tryptophan. Although the surrounding of copper in plastocyanin consists of two sulfur and two nitrogen atoms, only histidine and methionine are invariant ligand amino acids of the metal in these two copper proteins from plants.

Methods of $^{1}$H and $^{13}$C NMR-spectroscopy were used successfully for determination of the copper surrounding in the number of copper-containing proteins, e.g. the enzyme, superoxide dismutase /1,2/ and electron-carriers of microorganisms (azurins) /3-5/ and plants (plastocyanins) /6-8/. It is of importance to note that in all these cases data of NMR-studies are in accordance with results of X-ray investigations of these proteins in crystaline states /9-11/.

Some years ago a novel copper-containing protein was isolated from plants. This protein designated as plantacyanin, in contrast to plastocyanin, was basic /12/. Recently the primary structure of the basic copper protein isolated from cucumber seedlings has been determined /13/. Optical and EPR spectra of plantacyanin indicate that copper in this protein, as in plastocyanin, belongs to type I (or "blue"), nowever the shape of the EPR spectrum of plantacyanin is rhombic whereas plastocyanin has an axial shape. This is evidence that the copper surroundings in these two plant proteins are not similar. NMR and X-ray data show that ligands of copper in plastocyanin are two histidines,

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one cystein and one methionine /8,10/. In order to obtain more detailed information on the copper surrounding in plantacyanin, we carried out the $^{1}$H-NMR-study of this protein.

## MATERIALS AND METHODS

Plantacyanin from cucumber peelings was obtained essentially as described earlier /14/. The preparation obtained was electrophoretically homogeneous and had in its oxidized state the spectral index, $A_{280}/A_{593}$, of 6.3. The reduced form was obtained by addition of a small crystal of sodium dithionite to the oxidized protein. Apo-plantacyanin was prepared according to the work /14/, using diethyldithiocarbamate as a chelator of copper. Samples of the protein were dialised against water to remove the buffer and then freeze-dried. The protein powder was dissolved in $^{2}H_2O$ and incubated at 20°C for several days. The procedure of freeze-drying was repeated and the powder was finally dissolved in $^{2}H_2O$. Desirable pH-values (uncorrected direct readings) of protein solutions were regulated with 0.3N $NaO^{2}H$ and $^{2}HCl$.

$^{1}$H-NMR spectra at 300 MHz were obtained using a Varian SC-300 instrument with 620L/100 computer (Varian/16K/) in the pulse Fourier Transform mode. In some cases spectral resolution was improved by the treatment of the free-induction decays using the triangular weighing function /15/. TSP (trimethylsilyl propionate) was used as an internal reference for determination of chemical shifts.

## RESULTS AND DISCUSSION

The $^{1}$H-NMR-spectra of plantacyanin in oxidized, reduced and apo-forms are shown in Fig.1. After complete deuteration of samples the proton resonances of 4 phenylalanins, 3 tyrosines, 1 tryptophan and 2 histidines were observed in the aromatic region (6.2 - 8.0 ppm) of NMR spectra. This region is presented in Fig.2.

First of all, the resonances of histidines were considered in detail because histidine is known to be an invariant ligand of the metal in many copper-containing proteins, and plantacyanin contains two histidines /4,7,14/.

It was found that the width and the chemical shift of the resonance 1 (7.68 ppm) were unchanged in the three forms of the protein, whereas resonance 2 (7.34 ppm) is slightly shifted to the down-field region after the reduction (0.03 ppm) and the removal of copper

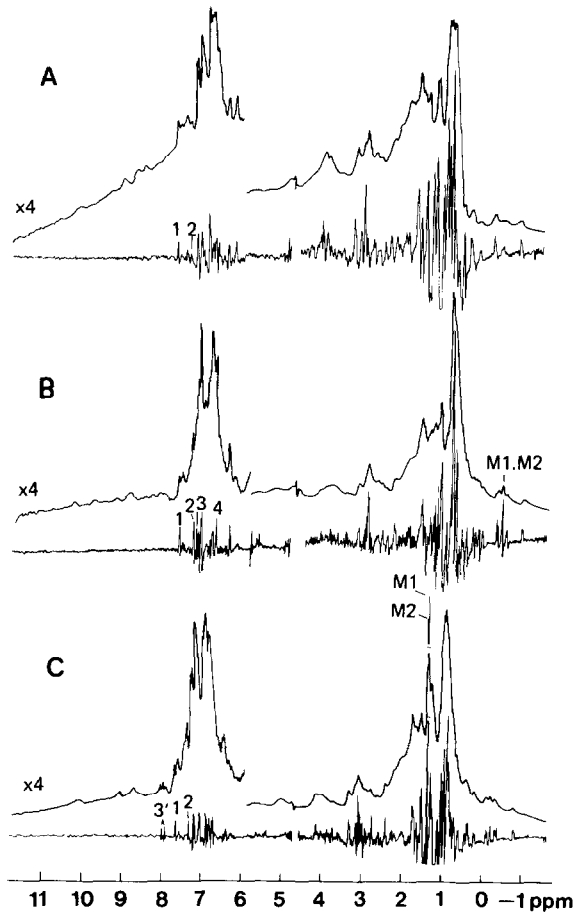


Fig.I. $^{1}$H-NMR-spectra at 300 MHz of plantacyanin in oxidized (A), reduced (B) and apo(C)-forms recorded at 32°C and pH* 5.60. Spectra of improved resolution are shown below each spectrum.

(0.04 ppm). In the oxidized protein resonance 2 is broadened to I4 Hz. Singlet signals with these chemical shifts were assigned to C2 (resonance I) and C4 protons (resonance 2) of one of histidines (His a). It is of importance to note that chemical shifts of resonances I and 2 were pH-independent for all three forms of the protein. Generally, the rate of deuterium exchange of C2 protons is significantly higher than that of C4 protons, and it is sharply decreased if histidine is coordinated to the metal /I6/. However we observed that resonance I in the three forms of the protein has the very low exchange rate (> I0 days). This property is characteristic for histidine localized in the hydro-

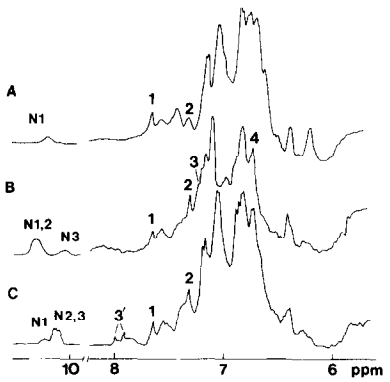


Fig.2. Downfield regions of NMR-spectra of oxidized plantacyanin (A), reduced plantacyanin (B) and apo-plantacyanin (C).

phobic region /I/. Indeed, histidines of plantacyanin were not accessible to the histidyl modifying reagent, diethyl pyrocarbonate /I4/. Further, the resonance NI (Fig.2) was assigned to NH-protons of the same histidine on the basis of its chemical shift, the rate of exchange and the correlation its behaviour with resonances I and 2. Although the resonance of C4 protons of His a was slightly broadened in the oxidized protein the possibility for His a as an immediate ligand for the copper may be excluded on the basis of the data considered above. Slight shifts of resonances I and 2 after the reduction and the removal of copper may be connected with small concomitant conformation changes.

On the other hand, the results obtained for second histidine (His b) indicate clearly that it is a direct ligand of the copper in plantacyanin. This conclusion is based on the following assignments and findings. Thus, in the spectrum of the reduced protein, two sharp resonances at 7.27 ppm and 6.78 ppm (3,4 in Fig.2B) were observed. These resonances were absent in oxidized and apo-forms of the protein. Chemical shifts of these resonances were unchanged in the pH-range of 3.0-9.0. Furthermore, two sharp singlets at 7.90 and 7.96 ppm in the spectrum of apo-protein (3' in Fig.2C) were assigned to C2 protons of His b on the basis of the typical pH-dependence of the shift, the rate of the exchange and the difference between chemical shifts of proto-

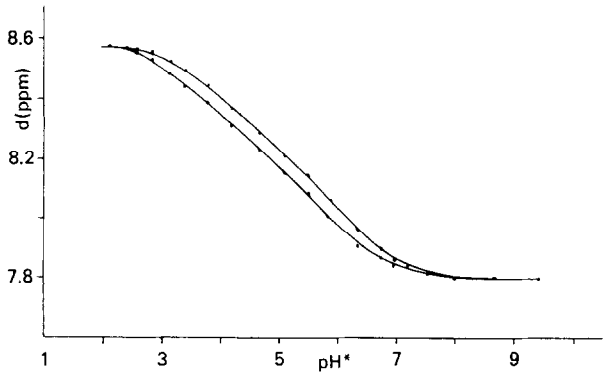


Fig.3. Titration of C2-protons of His b (the resonance 3′ of Fig. 2C) of plantacyanin. The resonance 3′ is titrated as two conformers with pK 4.9 and 5.2. The difference of chemical shifts was 0.76 ppm.

nated and deprotonated forms of His b (Fig.3). Also, certain changes were observed in the region of NH-protons (Fig.2). The removal of copper sharply accelerates (more than 12 times) the deuterium exchange of these protons. Resonance at 10.31 ppm was assigned to NH-protons of His b by the method of difference nuclear Overhauser effect spectroscopy /17/. When NH-protons with 10.31 ppm were selectively saturated, the responses of C2 and C4 protons of His b with 7.27 and 6.76 ppm were observed. Thus, His b almost certainly may be considered a ligand of the metal.

The reduction and removal of copper in plantacyanin bring about modifications also in other regions of NMR-spectra. In the spectrum of the reduced protein the singlet resonance at -0.26 ppm was observed (M1, M2 in Figs. 1B and 4A′). This resonance was absent in spectra of oxidized and apo-forms of plantacyanin (Figs. 1A and 4B′). In the difference spectrum "oxidized minus reduced" this signal is seen as a positive peak (Fig. 4C′). Two sharp resonances at 1.32 and 1.34 ppm in the spectrum of the apoprotein were assigned to $\delta CH_3$-protons of two methionines on the basis of the chemical shift and the intensity. In the reduced protein these protons have identical chemical shift at -0.26 ppm but in the spectrum of the oxidized protein the resonances are absent. It follows from these data that two methionines are ligands of the metal.

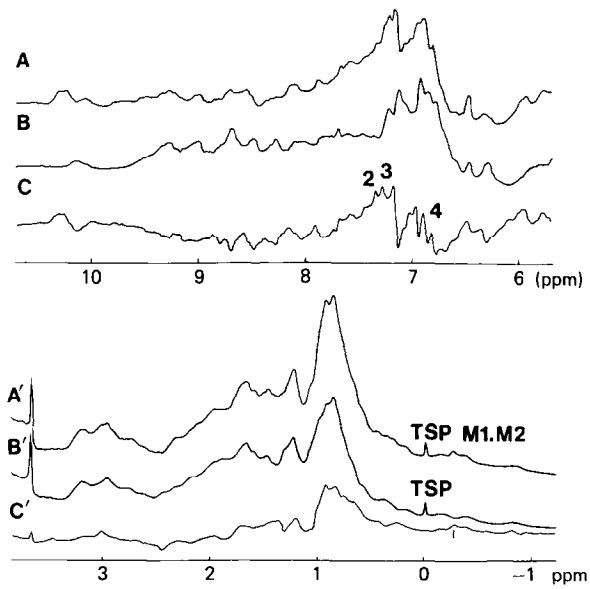


Fig.4. Changes in $^{1}$H-NMR-spectra of plantacyanin in regions 5.5 - 10.5 ppm and -1 - 3.5 ppm at 32°C and pH 6.0. (AA′) - reduced protein, (BB′) - oxidized protein, (CC′) - difference spectrum "reduced minus oxidized". Low-field spectra amplitude is twice as that of the upfield region.

The resonance N3 (Fig. 2B) was assigned to NH-protons of tryptophan /18/. This resonance is significantly broadened in the oxidized protein. Besides, the rate of exchange of these protons is increased more than 6 times in the apoprotein as compared with the reduced protein. Thus, tryptophan may be considered a ligand amino acid. It should be noted that previous fluorescent, spectrophotometric and EPR studies of plantacyanin also have indicated that tryptophan is close to the metal /14/.

Assignments of other resonances and dynamic changes in the structure of plantacyanin will be considered later.

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