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^{1}H-NMR study of plantacyanin from spinach

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High-resolution ^{1}H-NMR studies of spinach plantacyanin in its oxidized, reduced and apo-forms revealed that two histidines, one tryptophan and at least one methionine are very close to copper. These amino acids seem to be considered as possible ligands of copper in the protein. Spinach plantacyanin, as well as the cucumber protein, has the hydrophobic core around its copper.

Plantacyanin; Basic copper protein; ^{1}H-NMR; (Spinach)

1. INTRODUCTION

Basic, blue copper-containing proteins, plantacyanins, were isolated from a number of plants [1–6]. Many properties of these proteins were already elucidated. In particular, optical and EPR spectra indicate that the surroundings of copper in plantacyanins are unique. Nevertheless, the structure of their copper centre (i.e. immediate ligand amino acids of copper, the symmetry of the ligand tie and distances between copper ion and ligand atoms, as well as the organization of the amino acid chain around the copper site) is far from resolved.

High resolution ^{1}H-NMR was used in studies of the copper's surroundings in plantacyanin from cucumber [7–9], and two models of the immediate ligand surrounding copper in the protein were developed. According to both models the ligands of copper are two nitrogen and two sulfur atoms (2N, 2S tie). However, in one model ligand amino acids are histidine, tryptophan and two methionines [7,8], whereas in the other model these are two histidines, methionine and cysteine [9]. The NMR data indicate that the copper core in cucumber plantacyanin is surrounded by hydrophobic amino acids. In connection with these results it was important to investigate using the NMR method plantacyanins obtained from other sources. In this communication we report some of the results of the NMR study of copper's surroundings in plantacyanin from spinach and compare them with the NMR data obtained for cucumber plantacyanin.

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2. MATERIALS AND METHODS

Plantacyanin from spinach leaves (*Spinacea oleracea*) was isolated essentially according to [2] with an additional step of chromatography on CM-Sephadex, C-50. The final preparation was electrophoretically homogeneous and had a spectral ratio, A_{280}/A_{595}, of 5.3. The reduced form of plantacyanin was prepared by addition of dithionite to the oxidized protein followed by gel-filtration through Sephadex G-25 (superfine) to remove the excess of the reductant. Apoprotein was prepared according to [4] using diethyldithiocarbamate as a chelator of copper.

For NMR experiments freeze-dried preparations of oxidized, reduced and apo-plantacyanins were dissolved in D_2O (100%) and incubated at 26°C for several days to exchange peptide NH protons. The rate of deutero-exchange of NH protons was measured at 30°C and pH 8.1.

The pH of protein solutions was adjusted to the desirable values by the addition of 0.3 N NaOD or DCl.

^{1}H-NMR spectra were obtained on Bruker Physics instruments WM-500 and WP-360 operating at 500 and 360 MHz, respectively. For higher resolution of the spectra, the method of Ferrige and Lindon [10] was used. The external standard was TSP (trimethylsilyl propionate).

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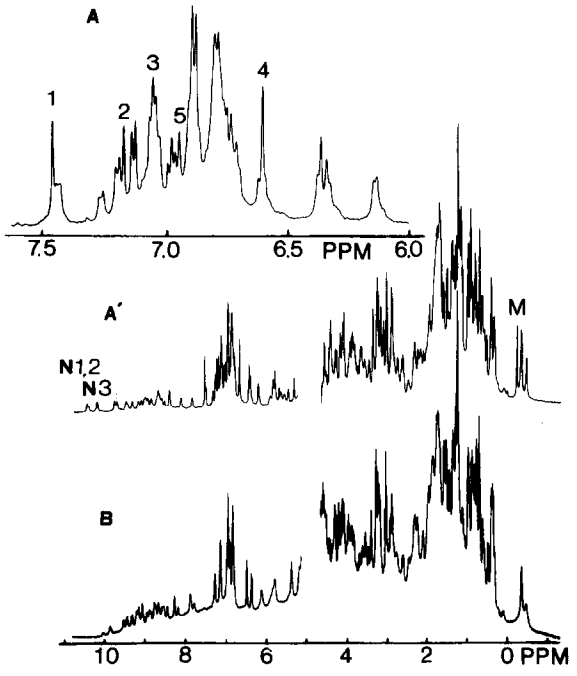

Fig.1. ^{1}H-NMR spectra of spinach plantacyanin in reduced (A,A') and oxidized (B) states. Spectra were obtained at pH 6.0 and 30°C.

3. RESULTS

In general, NMR spectra of cucumber and spinach plantacyanins were found to have many analogies. In fig.1 the spectra of oxidized and reduced forms of spinach plantacyanin are shown. As it follows from fig.1, the change of the redox state of copper results in distinct modifications in the ranges 10–11, 5.5–8.0 and 0.5–1.0 ppm. Five singlet resonances clearly observed in the spectrum of reduced plantacyanin (fig.1A) are absent in the spectrum of the oxidized protein. Using methods of assignments described for cucumber plantacyanin [7,8], signals 1 (7.46 ppm) and 2 (7.18 ppm) were found to belong to C2 and C4 protons of one of histidines (His *a*), whereas signals 3 (7.05 ppm) and 4 (6.61 ppm) are due to C2 and C4 protons of the other histidine (His *b*). Chemical shifts of signals 1–4 were pH independent. It was shown also that signal 5 (6.95 ppm) belongs to C2 protons of tryptophan. The signal M (−0.22 ppm) with the integral intensity of three proton units was assigned to ϵCH_3 of methionine. Broad signals N1, N2,

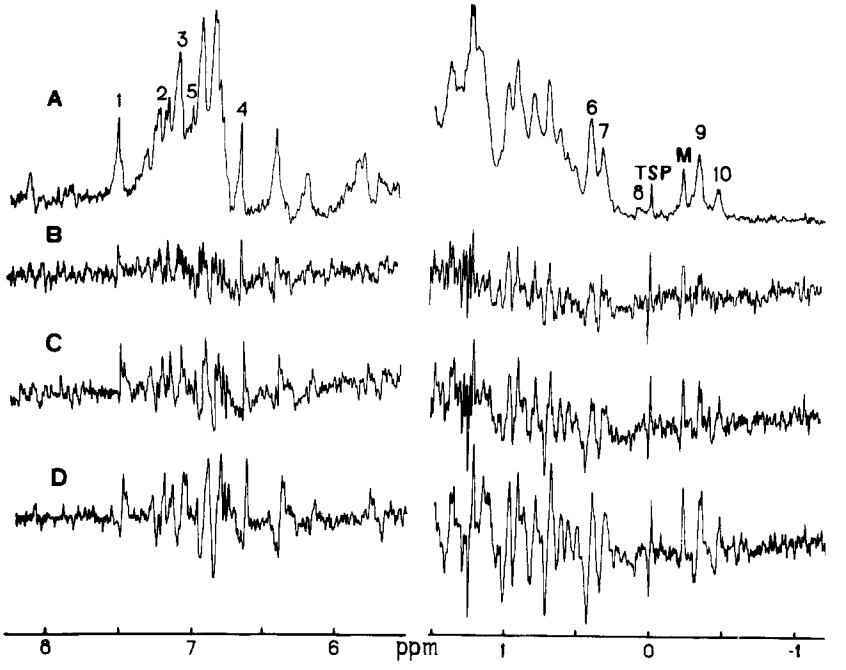

Fig.2. The titration of the reduced plantacyanin by $K_3[Fe(CN)_6]$. Spectra were recorded at pH 7.0 and 28°C. (A) Fully reduced plantacyanin and difference spectra, reduced minus oxidized; (B) 1% of the oxidized form; (C) 12% of the oxidized form; (D) 25% of the oxidized form.

N3 seem to be assigned to NH protons of imidazole and indol rings.

The oxidation of the protein was found to result in the selective broadening of some resonances or the increase of positive signals in difference spectra, reduced minus oxidized. Fig.2 shows the effect of oxidation by ferricyanide on NMR spectra of spinach plantacyanin. As it follows from difference spectra, the most sensitive to oxidation are resonances 1–5 and resonance M. During oxidation the appearance of positive signals of aromatic and methyl protons in difference spectra were noted. Using the paramagnetic broadening as the criterion for the proximity of protons to copper it may be concluded that many hydrophobic residues (Val, Leu, Ile, Phe) surround the copper site of the protein.

The apoform of spinach plantacyanin was found to be less stable than that of cucumber plantacyanin. The effect of copper removal on the NMR spectrum of spinach plantacyanin is shown in fig.3. The removal of copper results in the modification of the magnetic surroundings of C2 protons of tryptophan (signal 5′), His *b* (signal 3′) and His *a* (signal 1′). Thus, low-field shifts of these signals were 0.25 ppm, ~0.5 ppm and 0.31 ppm, respectively. Interestingly, in spinach apoplantacyanin, as well as in the cucumber protein, C2 proton of His *b* is manifested as two slow

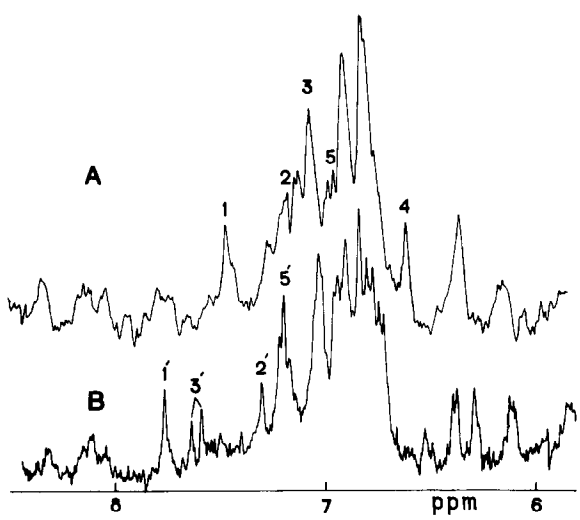

Fig.3. The low-field regions of NMR spectra of reduced (A) and apo- (B) plantacyanin.

exchanging conformers [7]. Further, after the removal of copper the signal of ϵCH_3 of methionine is shifted significantly to lower fields (from −0.22 ppm in the reduced protein to 1.96 ppm in the apoprotein). Other modifications which accompany the copper removal are insignificant. Thus, high-field signals 6–10 (figs 1 and 2) are shifted by less than 0.04 ppm. Signals of aromatic groups are also slightly shifted.

4. DISCUSSION

Plantacyanins from both spinach and cucumber were found to contain two histidines and one tryptophan. According to the NMR results described here, these three amino acids may be considered as potential ligands of copper in spinach plantacyanin. However, previous NMR studies of cucumber plantacyanin revealed that the copper in the protein is connected with two nitrogen and two sulfur ligands [7–9]. Since the copper sites in both plantacyanins have the same optical and EPR properties, the ligand tie around the copper in the proteins seems to be of the 2N,2S-type. Therefore, from three amino acids considered to be very close to the copper in spinach plantacyanin, only two are expected to be ligand amino acids. Thus, at present there are two alternative sets of nitrogen ligands to the copper in spinach plantacyanin: His and Trp or two His.

In previous NMR studies of cucumber plantacyanin evidence was also obtained for one [9] or two [7,8] methionines as sulphur ligands to copper. Thus it follows from studies of spinach plantacyanin, that one methionine is certainly the ligand amino acid of copper in this protein. However we were unable to follow signals of the other methionine residue because of a strong overlapping of many resonances in corresponding regions. Thus, the question of the second methionine ligand in spinach plantacyanin has to be resolved with additional experiments.

Difference spectra presented in fig.2 show that the copper site in the spinach protein is surrounded by many hydrophobic aliphatic and aromatic residues. A similar hydrophobic core was found in cucumber plantacyanin [8]. In general, according to NMR data there is a significant similarity in the structure of copper-bearing parts of both plantacyanins. Thus, chemical shifts of ligand amino

acid signals as well as signals of ring-current shifted methyl groups in these proteins have similar values. In both proteins the rate of deutero-exchange of NH protons of indole and imidazole rings is very slow ($K \ll 3600\ s^{-1}$). Also, the two histidines in plantacyanins are not titrated in the pH range 3.5–9.0. At the same time, some differences in the structure of both proteins should be expected because amino acid compositions and isoelectric points of these proteins are not similar, and preliminary observations indicate that antigenic properties of two plantacyanins are also different.

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