Some properties of a blue copper protein 'plantacyanin' from cucumber peel

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Absorption, circular dichroism, electron spin resonance and resonance Raman spectra of a blue copper protein, plantacyanin from cucumber peel have been measured and these spectral properties compared with those of other blue copper proteins. From the spectral properties, amino acid analysis and redox potential, we discuss the active site and redox properties of this protein.

Plantacyanin *Blue copper protein* *Redox potential* *Amino acid analysis*
Circular dichroism spectrum *Resonance Raman spectrum*

1. INTRODUCTION

Although the structural features of the copper sites in plastocyanin [1] and azurin [2] have been reported in biological and spectroscopic studies and crystal structure analyses, less popular 'blue copper proteins' require further study. Blue copper centers differ structurally not only in their ligating groups but also in their coordination geometry around the copper ion. Here, we describe properties of plantacyanin, one of the blue copper proteins from cucumber peel. Though the function of plantacyanin has not yet been established, it is likely to take part in electron transfer in a system containing peroxidase, cytochrome *c* and ascorbate oxidase.

2. MATERIALS AND METHODS

Plantacyanin was obtained as in [3]. The final absorption ratio of A_{280}/A_{593} was 10, being equal to the reported value.

Optical and circular dichroism (CD) spectra were obtained with a Hitachi 323 spectrophotometer and JASCO J-500A spectropolarimeter attached with a data processor DP-500N, respectively, at room temperature in a cell of 1 or 10 mm lightpath. Electron spin resonance (ESR) spectra were measured on a JEOL JES-FE1X spectrometer and the field was calibrated by using Li-TCNQ ($g = 2.0023$) and MnO ($\Delta H_{3-4} = 86.9$ G).

Resonance Raman spectrum in the region 200–700 cm^{-1} was obtained on a JASCO R-800 spectrometer using He–Ne laser excitation into the ~600 nm electronic absorption band.

Copper content of plantacyanin was estimated by ESR spectroscopy using the Cu(II) complex of tris(2-benz[d]imidazolylmethyl)amine which affords a similar ESR spectrum to that of plantacyanin as a standard [4]. The molar extinction coefficient at 593 nm was determined to be 800 $M^{-1} \cdot cm^{-1}$, which agreed well with that in [3]. The reduction potential of the copper ion was determined by absorption spectroscopy utilizing the Fe(III)–Fe(II) redox couple, whose reduction potential had been measured by cyclic voltammetry on a Yanagimoto P-1000 voltammetric analyzer attached with an NF function generator. Amino acid analysis was performed by an Irica A-3300 amino acid analyzer for the hydrolyzed apo-plantacyanin in vacuo with 6 M HCl for 24 h at 110°C. The content of cysteine residues was determined for the pre-treated sample with performic acid. Tryptophan content was estimated from the absorption spectrum of plantacyanin denatured with 6 M guanidine–HCl. Absorption and CD spectra have received computer curve fittings and were divided into several Gaussian bands.

3. RESULTS AND DISCUSSION

Plantacyanin displayed a molar extinction co-

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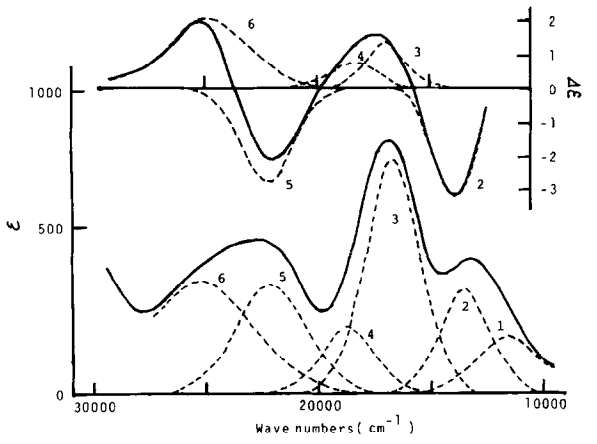

Fig.1. Absorption and CD spectra of plantacyanin (0.1 M phosphate buffer, at pH 7.2).

efficient of 800 $M^{-1} \cdot cm^{-1}$ at 593 nm, the weakest value for all blue copper proteins, of which the corresponding value sometimes reaches ~6000 $M^{-1} \cdot cm^{-1}$. Plantacyanin, nonetheless, exhibited all bands characteristic of the blue copper proteins as visualized on both the absorption and CD spectra in fig.1 (- - -, components). Components 2, 3 and 4 are reasonably assigned as charge transfer bands due to S(Cys)→Cu in comparison with the corresponding spectral features of other blue copper proteins [5,6]. As to bands 5 and 6, the assignment has been controversial; they are most probably due to charge transfer from histidine imidazoles to copper(II) ($\pi N \rightarrow d_x 2_{-y}2$). Band 1 is considered as one of d–d transition bands [7]. The fact that all corresponding bands agreed between plantacyanin and plastocyanin or azurin suggests the participation in ligation of one cysteine and two histidine residues. Of special interest is the fact that all bands (especially band 3) had rather greater values of Kuhn anisotropy factor ($\gamma = |\Delta\varepsilon/\varepsilon|$) comparing with those values of other blue copper proteins as summarized in table 1. This might be elucidated on the basis of an assumption that copper in plantacyanin lies in a somewhat different environment than the copper ions of plastocyanin and azurin.

The high rhombic character of copper(II) ion in plantacyanin, similar to that in rusticyanin [8], mavicyanin [9] and stellacyanin [10], is clearly recognized on the ESR spectrum (fig.2A). In contrast, plastocyanin [11], azurin [12] and umecyanin [13] exhibit the ESR signals with an axial symmetry. These facts may suggest that the stereochemical arrangement of ligating groups as well as the set of ligating groups differ from each other. In highly alkaline medium, we obtained a somewhat different ESR signal as indicated in fig.2B. To avoid keeping this protein for a long time in highly alkaline medium, the pH of the solution was lowered without delay, the original ESR signal being reproduced. Blue copper of stellacyanin [10] and

Table 1

Spectroscopic properties and redox potentials of blue copper proteins[a]

Protein	E° (V)	$\varepsilon_{\sim 600\ nm}$ /type I Cu	$\gamma(=\lvert\Delta\varepsilon/\varepsilon\rvert)$ Band 4	Band 3	Band 2	Symmetry of ESR signal	[Ref.]
Plastocyanin (cucumber)	0.35	4500	0.002	0.0005	0.002	Axial	[20]
Azurin (*Ps. aeruginosa*)	0.33	4800	0.0005	0.002	0.009	Axial	[6]
Stellacyanin (lacquer tree)	0.18	4100	0.0005	0.001	0.015	Rhombic	[6]
Plantacyanin (cucumber)	0.27	800	0.005	0.003	0.009	Rhombic	[Here]
Laccase (tree)	0.42	5700	0.005	0.0003	0.005	Axial	[18]
(fungal)	0.77	4900	0.002	0.0006	0.002	Axial	[18]
Ceruloplasmin (human)	0.49 0.54	5500	0.005	0.0003		Axial	[19]
Ascorbate oxidase (cucumber)	0.42	3200	0.003	0.0009	0.007	Axial	[20]

[a] E° value of mavicyanin and rice bran blue to exhibit a rhombic ESR signal has been reported to be 0.29 and 0.27 V, respectively

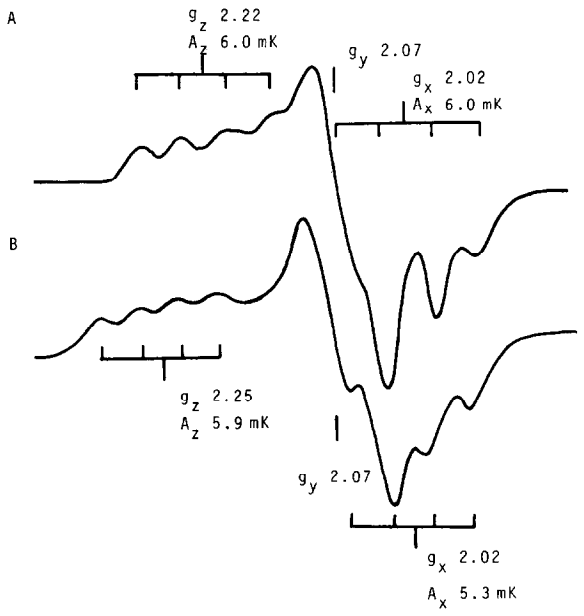

Fig.2. The X-band ESR spectra of plantacyanin at pH 7.2 (A) and pH 11 (B).

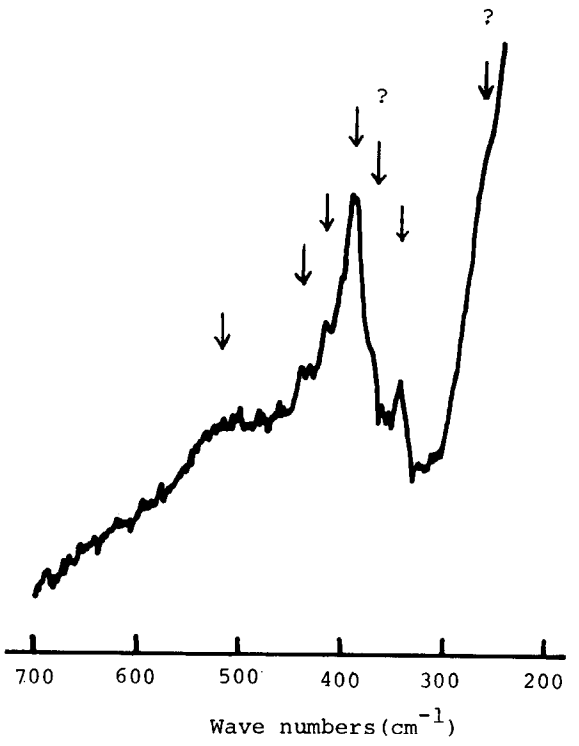

Fig.3. Resonance Raman spectrum of plantacyanin. The excitation wavelength was 6328 Å.

Table 2

Amino acid analysis of plantacyanin

Amino acid	Residues/9000 M_r protein
Asx	11.2
Thr	4.1
Ser	10.1
Glx	5.8
Pro	4.4
Gly	15.1
Ala	5.7
Cys	3.4
Val	3.9
Met	1.1
Ile	1.6
Leu	3.1
Thr	2.8
Phe	9.7
Lys	4.1
His	2.6
Arg	2.4
Trp	1.2

rusticyanin [8] have been reported to transform into type II copper in alkaline and neutral media, respectively. The above described existence of two modifications as a blue copper protein seems to be a unique character of plantacyanin. The metastable state of the protein conformation induced by an increase in pH ultimately would give rise to a slight perturbation of the steric arrangement of the ligating groups to afford the modified ESR signal in fig.2B. The structural change of this kind may be related to the fact that plantacyanin is a highly basic protein (isoelectric point = 10.6).

The amino acid content of plantacyanin is given in table 2. The protein contains three histidine and cysteine residues. The ligation of the former is confirmed by the presence of LMCT bands, 5 and possibly 6, and that of the latter by the presence of LMCT bands, 2, 3 and 4 and the prominent delocalization of a copper 3d electron which is reflected on the small copper hyperfine coupling in the ESR spectra in fig.2. However, the ligating group on the fourth position, which is occupied by

a methionine residue in plastocyanin and azurin, remains unknown.

Since stellacyanin which exhibits a rhombic ESR signal has no methionine residue [10], some other residue has been expected to be coordinated around the copper. This may be the disulfide group of cystine, which produces a similar ligand field strength with that furnished by a thioether group of a methionine residue [14]. However, a question arises as to why such a reactive group must ligate as a candidate for the fourth ligating group of copper ion which reversibly alters its oxidation state although the group can interact both with Cu(II) and Cu(I) ions. In addition, if a certain sulfur-containing group strongly interacts with Cu(II) ion and induces a considerable rhombic distortion to it, there must be at least two LMCT bands (σ(S)→Cu and π(S)→Cu) in the ultraviolet to visible regions [15]. However, no such bands were observed in the relevant regions. For plantacyanin the possibility of the ligation of some groups other than methionine or cystine residue are still retained.

Resonance Raman spectrum of plantacyanin (fig.3) displays peaks at 345, 393, 420 and 440 cm^{-1} which are attributable to Cu-S (cysteine) and possibly to Cu-N(imidazole) [6,16]. The spectral feature of plantacyanin is rather similar to that of stellacyanin but not to plastocyanin, again suggesting that plantacyanin resembles stellacyanin both in the set of ligating groups and their steric arrangements. A very weak shoulder is noticeable at ~265 cm^{-1} where ν(Cu-(thioether)) band emerges [17], but the shoulder is too obscured to be assigned definitely.

The oxidation reduction potential of plantacyanin was evaluated to be 0.27 V (*vs* NHE). Except rusticyanin (0.68 V) which shows blue color only in an acidic medium, blue copper proteins including plantacyanin which exhibit a rhombic ESR signal generally have lower reduction potentials (table 1). The rhombicity would make the copper ion change its oxidation state more easily. This might indicate that the redox property of blue copper proteins is not exclusively governed by the set of ligating groups; other factors such as protein conformational effects during the alteration of oxidation state of the copper ion and the polarity around the active site seems to be also important factors.

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REFERENCES

[1] Colman, P.M., Freeman, H.C., Guss, J.M., Murata, M., Norris, V.A., Ramshaw, J.A.M. and Venhatappa, M.P. (1978) Nature 272, 319–324.
[2] Adman, E.T., Stenkamp, R.E., Sieker, L.C. and Jensen, L.C. (1978) J. Mol. Biol. 123, 35–49.
[3] Aikazyan, V. Ts. and Nalbandyan, R.M. (1979) FEBS Lett. 104, 127–130.
[4] Sakurai, T., Narisako, K. and Nakahara, A. (1981) Chem. Lett. 104, 127–130.
[5] Lappin, A.G. (1981) in: Metal ions in biological systems (Sigel, H. ed) vol. 13, pp. 15–71, Marcel Dekker, New York.
[6] Gray, H.B. and Solomon, E.I. (1981) in: Copper proteins (Spiro. T.G. ed) pp. 1–39, Wiley Interscience, New York.
[7] Solomon, E.I., Hare, J.W., Dooley, D.M., Dawson, J.H., Stephens, P.J. and Gray, H.B. (1980) J. Am. Chem. Soc. 102, 168–178.
[8] Cox, J.C., Aasa, R. and Malmström, B.G. (1978) FEBS Lett. 93, 157–160.
[9] Marchesini, M., Minelli, H., Merkle, H. and Kroneck, M.H. (1979) Eur. J. Biochem. 101, 77–84.
[10] Peisach, J., Levine, W.G. and Blumberg, W.E. (1967) J. Biol. Chem. 242, 2847–2857.
[11] Penfield, K.W., Gay, R.R., Himmelwright, R.S., Eickman, N.C., Norris, N.C., Freeman, H.C. and Solomon, E.I. (1981) J. Am. Chem. Soc. 103, 4382–4388.
[12] Broman, L., Malmström, B.G., Aasa, R. and Vanngård, T. (1963) Biochim. Biophys. Acta 75, 365–376.
[13] Brill, A.S., Bryce, G.F. and Maria, H.J. (1968) Biochim. Biophys. Acta 154, 342–351.
[14] Lundeen, M. (1981) Inorg. Chim. Acta 56, 149–156.
[15] Sakurai, T., Suzuki, S. and Nakahara, A. (1981) Bull. Chem. Soc. Jpn. 54, 2313–2316.
[16] Siiman, O., Young, N.M. and Carey, P.R. (1976) J. Am. Chem. Soc. 98, 744–748.

[17] Ferris, N.C., Woodruff, W.H., Rorabacher, D.B., Jones, T.E. and Ochrymowycz, L.A. (1978) J. Am. Chem. Soc. 100, 5939–5942.
[18] Falk, L.-E. and Reinhammar, B. (1972) Biochim. Biophys. Acta 285, 84–90.
[19] Dawson, J.D., Dooley, D.M., Clark, R., Stephens, P.J. and Gray, H.B. (1979) J. Am. Chem. Soc. 101, 5046–5053.
[20] Sakurai, T., Okamoto, H., Kawahara, K. and Nakahara, A. (1982) unpublished.