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PARTICLES OF PHOTOSYSTEM 2 CONTAIN PLANTACYANIN

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PS2 particles prepared from chloroplasts of three plant species were shown to contain the basic blue copper protein, plantacyanin, which may be extracted from the particles by concentrated saline solutions containing Triton X-100. Antibodies to plantacyanin were found to inhibit the photosynthetic oxygen evolution performed by the particles. Thus, evidences were obtained for participation of this protein in the oxygen-evolving activity of PS2 particles.

The strongly basic blue monocopper protein, plantacyanin, was isolated from some plants [1-4], and it was investigated thoroughly during past years [5-12]. Although its physiological role remains unsettled, however it was observed that plantacyanin is unable to replace plastocyanin, the acidic blue monocopper electron-carrier protein mediating electron transfer between photosystems 1 and 2. Evidences have also been obtained that plantacyanin is mainly located in chloroplasts [4]. In order to establish functions of plantacyanin it would be useful to continue these studies in the direction of searches of the subchloroplast localization of the protein, and, in particular, to determine whether particles of photosystem 2 (PS2), which are known to be connected with the oxygen-evolving activity of chloroplasts, contain this protein. This problem is well-grounded because chemical assays of copper in PS2 preparations revealed that they contain approximately 2 copper atoms per reaction center of PS2, P-680 [13-16]. Besides, some plants grown under Cu deficiency were found to have the decreased level of the oxygen-evolving activity [16]. In the recent work [17] the

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protein resembling plantacyanin (auracyanin) was isolated from the green photosynthetic bacterium *Chloroflexus aurantiacus*.

Bearing in mind these observations we had an aim to detect plantacyanin in PS2 preparations isolated from different plants by conventional methods and, if it would be the case, to consider whether this protein had any role in the oxygen-evolving activity of PS2 particles. Results of these studies are reported in this communication.

MATERIALS AND METHODS. Intact chloroplasts were prepared from leaves of spinach (*Spinacea oleracea*) and goosefoot (*Chenopodium album*) as well as cucumber peelings (*Cucumis sativus*) essentially according to Nakatani and Barber [18]. PS2 particles from chloroplasts were prepared by the method of Ford and Evans [19] with one exception that for preparation of PS2 particles from goosefoot Triton/Chl ratio was 10:1 whereas for spinach and cucumber it was usually 25:1. Pellets of PS2 particles were suspended in 0.4M sucrose, 10mM NaCl, 5mM $MgCl_2$ and 40mM MES-NaOH, pH 6.5. The suspension was used immediately or stored at -70°C for some days until use. Under these conditions no marked changes of the oxygen-evolving activity were observed.

Plantacyanins in their electrophoretically homogeneous forms were prepared from cucumber peelings and spinach leaves essentially according to the method [12]. The spectral ratio, A_{278}/A_{595}, of completely oxidized states of the proteins (at pH 6.0 and 22°C) were 5.9 and 5.3 for cucumber and spinach plantacyanins, respectively. Reduced forms of plantacyanins and apoforms of these proteins were prepared as it has been described earlier [9].

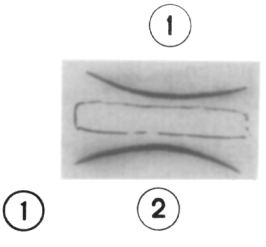
Rabbit antibodies to spinach and cucumber plantacyanins were induced and purified according to [12]. Enzyme immunoassays (EIA) of plantacyanin were carried out using goat antibodies to rabbit IgG:horseradish peroxidase conjugate ("Sigma") according to [20]. In these assays o-phenylenediamine was used as a substrate for peroxidase, and the product formed was followed by absorbance at 492nm. The equipment for the screening of EIA measurements was supplied by Flow Labs. Immunodiffusion experiments were carried out in 1.2% Agarose according to Ouchterlony [21].

The oxygen evolution was measured at 22°C with a Clark type oxygen electrode. The saturating light was provided by two 150W projectors with a red filter placed on opposite sides of the reaction vessel. Both lamps were fitted with $CuSO_4$ filters. The medium for assays of the oxygen evolution (2ml) contained 70mM sucrose, 30mM Na,Na-phosphate buffer, pH 6.5, 30mM NaCl, 1mM phenyl-p-benzoquinone ("Sigma") and PS2 particles from spinach (60µgChl) or 400mM sucrose, 30mM MES, pH 6.5, 20mM NaCl, 5mM $MgCl_2$, 2mM phenyl-p-benzoquinone and PS2 particles from cucumber peelings (120µgChl). Under these conditions oxygen-evolving activities were routinely 120 and 55 $\mu molO_2 \times mgChl^{-1} \times h^{-1}$ for PS2 particles isolated from spinach and cucumber, respectively. Some experiments for measurements of the PS2 system activity were also carried out using 2,6-dichlorophenol indophenol ("Sigma") under conditions described in [22].

Extracts from PS2 particles used for EIA and immunodiffusion experiments were prepared by treatment of the particles (400mg Chl) with 1M NaCl containing 0.06% Triton X-100, 10mM MES, pH 6.5, with following centrifugation and ultrafiltration under conditions described in Results.

The content of chlorophyll in PS2 preparations was determined by the method of Arnon [23].

RESULTS AND DISCUSSION. First of all, the immunological approach was used to detect plantacyanin in PS2 particles prepared from *Spinacea oleracea*, *Chenopodium album* and *Cucumis sativus*. In experiments of this line the particles (routinely 400mg of chlorophyll) were extracted with 10mM MES-buffer, pH 6.5, containing 1M NaCl and 0.06% Triton X-100. The extracts were clarified by centrifugation at 15000xg for 1hr, concentrated 100 times by ultrafiltration through an Amicon UM-2 membrane, and then their 10µl aliquotes were assayed by the immunodiffusion technique using rabbit antibodies to plantacyanins from corresponding sources. Fig.1 shows the typical diffusion pattern obtained. Reactions were found to be positive in cases of all PS2 preparations. The availability of plantacyanins in PS2 particles was also demonstrated by more sensitive EIA method. As an example, Fig.2 shows results of the EIA-titration of cucumber plantacyanin. The linear dependence on the protein concentration was observed up to 2×10^{-7}M. It is of importance to note that similar data were also obtained for extracts isolated from PS2 particles of spinach, goosefoot as well as many other plants from which plantacyanin has not been purified yet. Thus,



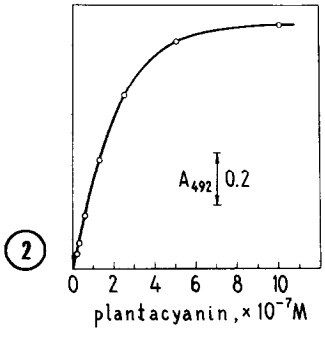


Fig.1. Immunodiffusion patterns observed with the extract from spinach PS2 particles (1) and purified spinach plantacyanin (2). The central ditch contains rabbit antibodies to spinach plantacyanin.

Fig.2. The EIA-titration curve of plantacyanin from cucumber.

Table 1. Effects of different exogenously added plantacyanin preparations from spinach on the oxygen-evolving activity of PS2 particles from spinach

Preparations	Activity ($\mu molO_2/mgChlxh$)
Control	120
+ apoplantacyanin, $7x10^{-5}M$	117
+ reduced plantacyanin, $7x10^{-5}M$	122
+ oxidized plantacyanin, $9x10^{-6}M$	120
+ oxidized plantacyanin, $7x10^{-5}M$	93

Activity was assayed using phenyl-p-benzoquinone.

both immunodiffusion and EIA data suggest that subchloroplast PS2 particles seem to contain plantacyanin.

Therefore, it was expectable that the exogenously added plantacyanin should have no activating effect on the rate of the oxygen evolution activity of PS2 particles. In this connection, special series of experiments were carried out to test possible effects of exogenousely added reduced, apo- and oxidized forms of plantacyanin on the oxygen-evolving activity using both phenyl-p-benzoquinone and 2,6-dichlorophenol indophenol methods. Typical results of these experiments are shown in Table1. They indicate clearly that exogenously added plantacyanin preparations had no activating effect on the oxygen-evolving function of PS2 particles. (Moreover, under high concentrations of the oxidized form of plantacyanin even a slight inhibition of the oxygen evolution was observed. This "paradoxical" effect will be considered in details in forthcoming communications).

If PS2 preparations indeed contain plantacyanin and this protein has any role in the oxygen-evolving activity of PS2 particles, it

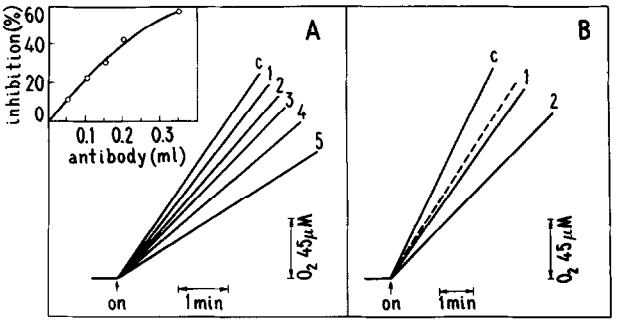


Fig.3. The inhibition of the oxygen-evolving activity of PS2 particles prepared from spinach (A) and cucumber (B) by antibodies.
(A) - Kinetic curves 1-5 were obtained in the presence of increasing amounts (0.05-0.35ml) of antibodies to spinach plantacyanin added to spinach PS2 particles. The dependence of the inhibition on amounts of antibodies is shown in the inset.
(B) - Kinetic curves obtained with antibodies to cucumber plantacyanin added to cucumber PS2 particles (1-0.1ml; 2-0.3ml). The dotted line shows the kinetic curve obtained when spinach antibodies (0.1ml) were added to cucumber PS2 particles.
C - controls (without antibodies).
ON - light was turned on.

would be expected that antibodies to plantacyanin added to these particles should inhibit the evolution of oxygen. Fig.3 shows results of these studies. As it follows, rabbit antibodies to spinach and cucumber plantacyanins inhibit the oxygen-evolving activity of PS2 particles in a dose-dependent manner. In particular, ~60% inhibition was observed with 0.35ml of spinach antibodies. It is necessary to note that in the presence of the same amount of nonspecific rabbit immunoglobulin G no inhibition was observed. Thus, the inhibition of the oxygen-evolving activity is due to the specific interaction of antibodies to plantacyanin with plantacyanin located in PS2 particles.

Summing up, the results described show that particles of PS2 prepared from chloroplasts contain plantacyanin, the very basic blue copper protein which was purified earlier from a number of plants and studied in details [1-12]. This protein may be extracted from the subchloroplast particles by concentrated buffer solutions containing Triton X-100. The immunological evidences have been obtained that the

protein is involved in the main function of the PS2, i.e. its oxygen-evolving activity. Thus, this work is the first demonstration that the certain copper-containing protein (plantacyanin) is connected with PS2 particles and this protein participates in the photosynthetic oxygen evolution.

Particles of PS2 were known to contain a number of polypeptides with M_r-values from 4 to 47kD. Roles each of these polypeptides in the oxygen evolution are subjects of many current studies (see, e.g. review [24]). The extraction of the polypeptides brings about the drop of the oxygen-evolving activity of PS2 particles. It is of interest, that there is one polypeptide among all these ones, which resembles plantacyanin in some respect (M_r of 10-13kD, isoelectric point, polarity index, amino acid composition). Antibodies to polypeptide with M_r of 11kD were found to inhibit the PS2 activity measured with 2,6-dichlorophenol indophenol [25]. However, no metals, and in particular copper, was reported to present in this polypeptide [26-28]. The possible reason of the metal absence in the polypeptide may be connected with hard conditions which are generally used for extractions of polypeptides from PS2 particles. These conditions cause inactivation (Zwitterionic detergents) and reduction (mercaptoethanol) of these polypeptides as well as chelation of metals (high concentrations of Tris) from their. Under these conditions, copper which is necessary for redox transformations, may be removed from polypeptides and, moreover, other physico-chemical properties of polypeptides may be also changed essentially. However, if even polypeptide with M_r of 10kD cannot be considered as inactivated plantacyanin, the data obtained show that plantacyanin participates in the oxygen evolution. Hence, it would be reasonable to search plantacyanin or related proteins among other protein components of PS2 particles. In any case, the results explain naturally the dependence of the PS2 system activity on the copper content in PS2 particles [13-16, 29].

It is of interest that, besides plantacyanin, in plants there is an other blue copper protein, so-called stellacyanin-like protein, which is also available in the homogeneous and native state [30]. It has some similarities with 24kD polypeptide which is reported to be connected with the oxygen-evolving activity of PS2. It is expected, therefore, that the investigation of the role of plantacyanin and this stellacyanin-like protein in the photosynthetic oxygen evolution may become the promising direction.

REFERENCES

1. Markossian, K.A., Aikazyan, V.Ts. and Nalbandyan, R.M. (1974) Biochim. Biophys. Acta **359**, 47-54.
2. Colman, P.M., Freeman, H.C., Guss, J.M., Murata, M., Norris, V.A., Ramshaw, J.A.M., Venkatappa, M.P. and Vickery, L.E. (1977) J. Mol. Biol. **112**, 649-650.
3. Aikazyan, V.Ts. and Nalbandyan, R.M. (1979) FEBS Lett. **104**, 127-130
4. Aikazyan, V.Ts. and Nalbandyan, R.M. (1981) Biochim. Biophys. Acta **667**, 421-432.
5. Sakurai, T., Okamoto, H., Kawahara, K. and Nakahara, A. (1982) FEBS Lett. **147**, 220-224.
6. Murata, M., Begg, G.S., Lambrou, F., Leslie, B., Simpson, R.J., Freeman, H.C. and Morgan, F.J. (1982) Proc. Natl. Acad. Sci. USA **79**, 6434-6437.
7. Babayan, M.A., Sarkissian, L.Kh., Nersissian, A.M., Sarukhanian, E.G. and Nalbandyan, R.M. (1983) Biochem. Biophys. Res. Commun. **117**, 385-391.
8. King, G., Andary, T.A., Freeman, H.C., Gavrilovic, L. and Wright, P.E. (1984) FEBS Lett. **166**, 288-292.
9. Nersissian, A.M., Babayan, M.A., Sarkissian, L.Kh., Sarukhanian, E.G. and Nalbandyan, R.M. (1985) Biochim. Biophys. Acta **830**, 195-205.
10. Sakurai, T., Sawada, S. and Nakahara, A. (1986) Inorg. Chim. Acta **123**, L21-L22.
11. Sakurai, T. (1986) Biochem. Biophys. Res. Commun. **139**, 961-966.
12. Nersissian, A.M. and Nalbandyan, R.M. (1988) Biochim. Biophys. Acta **957**, 446-453.
13. Holdsworth, E.S. and Arshad, J.H. (1977) Arch. Biochem. Biophys. **183**, 361-373.
14. Ramaswamy, N.K. and Nair, P.M. (1978) Plant Sci. Lett. **13**, 383-388.
15. Goldfeld, M.G. and Khalilov, R.I. (1979) Biophizika (Russ.) **24**, 762-764.
16. Droppa, M., Terry, N. and Horvath, G. (1984) Proc. Natl. Acad. Sci. USA **81**, 2369-2373.
17. Trost, J.T., McManus, J.D., Freeman, J.C., Ramakrishna, B.L. and Blankenship, R.E. (1988) Biochemistry **27**, 7858-7863.
18. Nakatani, H.Y. and Barber, J. (1977) Biochim. Biophys. Acta **461**, 510-512.
19. Ford, R.C. and Evans, M.C.W. (1983) FEBS Lett. **160**, 159-164.
20. Campbell, A.M. (1984) in Monoclonal antibody technology (Burdon, R.H. and van Knippenberg, P.H., eds.), Elsevier, Amsterdam.

21. Ouchterlony, O. (1968) in Handbook of Immunodiffusion and Immunoelectrophoresis, Ann Arbor-Humphrey Science Publishers, Ann Arbor, MI.
22. Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533-539.
23. Arnon, D.I. (1949) Plant Physiol. 24, 1-15.
24. Govindjee, Kambara, T. and Coleman, W. (1985) Photochem. Photobiol. 42, 187-210.
25. Koenig, F., Schmid, G.H., Radunz, A., Pineau, B. and Menke, W. (1976) FEBS Lett. 62, 342-346.
26. Ljungberg, U., Akerlund, H-E. and Andersson, B. (1984) FEBS Lett. 175, 255-258.
27. Ljungberg, U., Akerlund, H-E., Larsson, C. and Andersson, B. (1984) Biochim. Biophys. Acta 767, 145-152.
28. Ljungberg, U., Akerlund, H-E. and Andersson, B. (1986) Eur. J. Biochem. 158, 477-482.
29. Sibbald, P.R. and Green, B.R. (1987) Photosyn. Res. 14, 201-209.
30. Sarkissian, L.Kh. and Nalbandyan, R.M. (1983) Biosci. Rep. 3, 915-920.