Copper binding sites associated with photosystem 2 preparations

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[1]

Abstract

Some steps in the isolation method of photosystem 2 (PS2)-enriched particles (BBY) influenced the Cu content of the final preparation. In particular, the centrifugation at 10 000 x g used to remove starch after Triton X-100 treatment of the thylakoids, yielded starch-free BBY with a low copper content. This contrasted with the high Cu content of the starch-containing BBY. Differences in Cu levels of both preparations seemed thus to be related to the starch content of the sample. Four unidentified proteins were found in the starch fraction. They are probably new copper binding sites in the photosynthetic cell.

Introduction

The involvement of copper in PS2 has been demonstrated by using copper chelators (Trebst 1963, Katoh and San Pietro 1966, Renger et al. 1967, Ban- and Crane 1976), as well as by copper deficiency experiments in higher plants (Horvath et al. 1983, Droppa et al. 1984a,b, Lopez Gorge et al. 1985). However, the role of Cu in PS2 is far from being completely understood, perhaps due to the focus interest, in recent years, on the function ofMn in this photosystem.

Some authors have described Cu as a constituent of PS2 (see review of Droppa and Horváth 1990), but the reported Cu content in PS2 preparations varies greatly. This is probably due to species variability, and also to different PS2 preparation procedures (Anderson et al. 1964, Gol'dfel'd and Khalilov 1979, Droppa et al. 1984a, Sibbald and Green 1987a, Baron et al. 1990). Experiments carried out by different authors (Droppa et al. 1987, Baron et al. 1990) in the last ten years, have been conducted in order to clarify the molecular status of Cu in PS2, as well as to know the mechanism by which the depletion of this micronutrient affects PS2 efficiency.

In this sense Cu deficiency results in a higher degree of saturation of thylakoid hpid, which affects the function of the PS2 acceptor side (Droppa et al 1987) In addition, the pigment

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composition of LHC2 (major PS2 light-harvesting complex) appears disturbed (Baron Ayala *et al*. 1992).

Searching for a putative Cu-binding protein, Sibbald and Green (1987b) suggest that about 50% of Cu in PS2 is bound to the LHC2 complex, wim an unknown ftmction. The aim of our study was to elucidate the reasons for the reported differences of Cu-content in PS2, and to search for possible Cu-binding sites in this photosystem.

Materials and methods

Pea (Pisum sativum L. cv. Lincoln) plants were hydroponically grown in Hewitt full nutrient solution, in a growth chamber for 4 weeks as described in Baron Ayala and Sandmann (1988). Leaves of spinach (Spinacea oleracea L.) were obtained from the local market.

PS2 particles (BBY) were isolated according to Berthold et al. (1981) with the modifications introduced by Ford and Evans (1983). Leaves were ground in 50 mM sodium phosphate, pH 7.4, 5 mM $MgCl_2$, and 300 mM sucrose. The homogenate was filtered through nylon cloth and after centrifugation for 15 min at 5.000 x g (1^{st} chloroplast pellet), the sediment was washed in grinding medium and centrifuged again in the above conditions (2^{nd} chloroplast pellet). This pellet was dispersed in 5mM $MgCl_2$, and incubated for 2 min to remove the envelope chloroplast membranes. An equal volume of 50 mM Mes, pH 6.5, 400 mM sucrose, 5 mM $MgCl_2$ and 15 mM NaCI was added and the suspension centrifuged at 3000 x g for 10 min (1^{st} thylakoid pellet). The membranes were resuspended in a buffer containing 2 mM MES, pH 6.5, 5 mM $MgCl_2$ and 15 mM NaCl, pelleted under similar conditions and resuspended in the same buffer (2^{nd} thylakoid pellet) After addition of Triton X-l00 in a ratio 25:1 (detergent:chlorophyll, m:m), the suspension was left in the dark at 4ºC for 25 min, and then centrifuged for 4 min at 10000 x g m order to obtain a greenish starch pellet. The fine supernatant suspension was now centrifuged at 40000 x g for 25 min, and the resulting starch-free BBY pellet (BBY_{s-}) was resuspended in 50 mM MES, pH 6.5, 400 mM sucrose, 5 mM $MgCl_2$ and 15 mM NaCl (stock buffer). To obtain starch-containing BBY (BBY_{s+}) the centtifugation after the Triton treatment was omitted. To remove excess detergent, BBY particles were washed twice with the stock buffer.

Removal of polypeptides from the oxygen evolving complex (OEC) was performed as described in Ljungberg et al. (1986).

Chelator-treated particles were obtained by suspending spinach and pea BBY in 5 mM NaEDTA, pH 7.8, for 25 min. Particles were recovered by centrifugation at 40000 x g, resuspended in stock buffer and pelleted again.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 17.5% polyacrylamide gels containing 4 M urea, as described in Laemmli (1970).

For metal determinations, aliquots of the different suspensions of pea and spinach particles were digested with 0.1 HNO_3 and n-octyl alcohol added (as surfactant). The copper content was determined by atomic absorption spectrometry (Perkin-Elmer 503), using a hollow cathode lamp and a graphite furnace as atomization source (Perkin-Elmer HGA-400).
Oxygen evolution was measured with a dark type oxygen electrode. As electron acceptor, 0.4 mM *p*-benzoquinone was accepted.

Results

Table 1 shows the steps and the different types of particles obtained throughout the procedure outlined above and the Cu content of these preparations. The Cu found in the first chloroplast pellet represented only about 35% of total Cu in leaf homogenate, both in spinach and pea. This loss was due, at least in part, to removal of Cu proteins from the mitochondria and cytosol, such as cytochrome oxidase, superoxide dismutase, etc. (Sandmann and Bóger 1983, Corpas et al. 1991).

Table 1. Cu contents for the different preparations in a typical BBY isolation process. Total Cu is for BBY isolation from 100 g leaves. Every chloroplast (chlor) fraction was collected and volume, copper and chlorophyll (Chl) contents were measured. The second thylakoid (thyl) pellet was used (50 %) for BBY_{S+} and BBY_{S-} isolation.

	Spinach total Cu [mg]	atoms Cu per 300 Chl molecules	Pea total Cu [mg]	atoms Cu per 300 Chl molecules
Leaf homogenate	67.0	4.6	56.0	3.6
1st chlor pellet	26.0	2.7	21.0	2.4
2nd chlor pellet	24.0	2.6	18.0	2.3
1st thyl pellet	20.0	2.4	14.5	2.0
2nd thyl pellet	20.0	2.0	14.0	1.8
Triton superload from (BBY_{S+})	6.0	1.8	5.0	2.0
Triton superload from (BBY_{S-})	6.0	1.8	5.0	2.0
BBY_{S+}	3.0	2.3	2.0	2.0
BBY_{S-}	0.4	0.2	0.2	0.2
Starch fraction	2.5	-	1.5	-

In the chloroplast and thylakoid pellets, there was a decrease in the Cu content, probably related to the release of ribulose bisphosphate carboxylase (large subunit, LSU and small subunit SSU, see Fig. 1) (Walker and Webb 1981); in this case the Cu loss was approx. 20%. After Triton treatment and centrifugation to obtain the BBY pellets, 50-60% of the remaining Cu appeared in the supernatant, mainly in the PS 1 complex, and about 30% in the BBY pellet with starch (BBY_{S+}). The additional 10000 x g centrifugation used to

remove the co-isolated starch changed the Cu content of the finally obtained BBY. After the centrifugation for starch removal and further centrifugation to pellet BBY, the Triton supernatant had the same Cu concentration as above, but the starch-free BBY (BBY_{s-}) retained *ca*. 5% of the Cu present in the chloroplast. The extra copper not retained by BBYg. was found in the starch fraction. When the starch removal step was omitted, the Cu content of starch-containing BBY (here named BBY_{s+}) obtained from spinach was 20 Cu/300 Chl, which was higher than the 0.2 Cu/300 Chl in BBY_{s-} Similar results were obtained in pea samples.

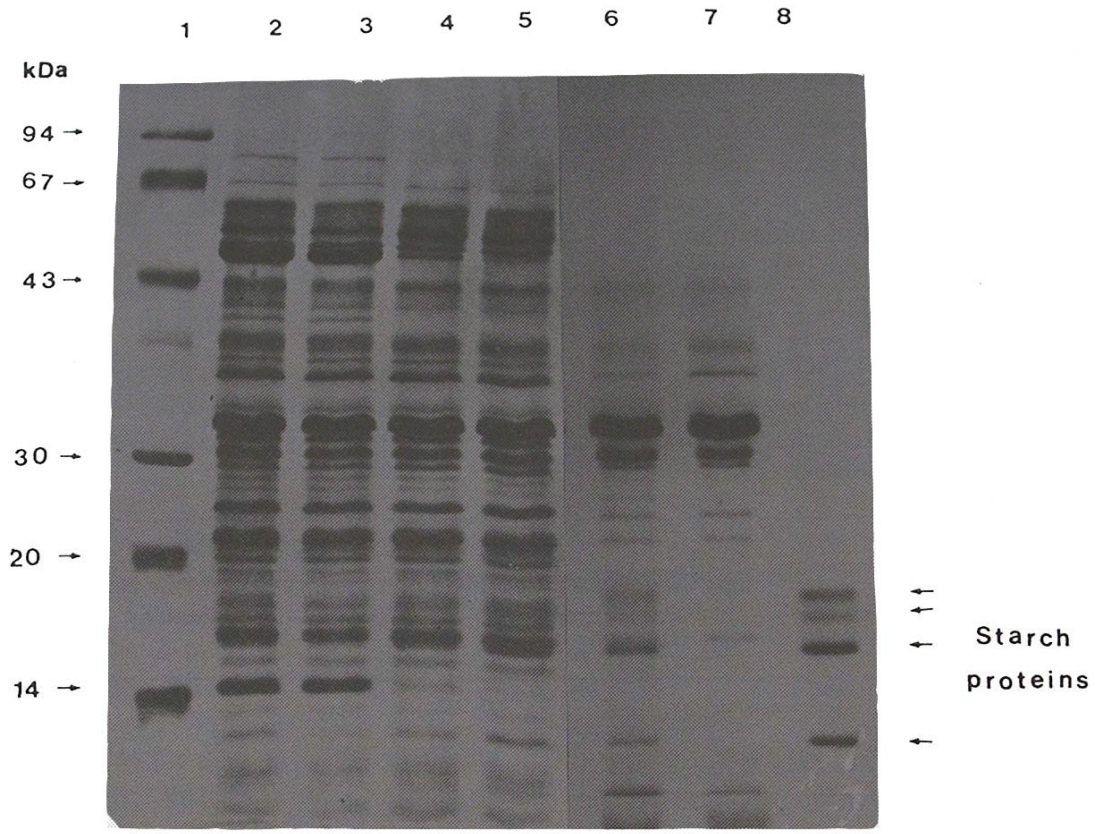

Fig. 1 . SDS-4 M urea PAGE of pea preparations in the BBY isolation process. (*1*) standards, (*2*) 1st chloroplast pellet, (*3*) 2nd chloroplast pellet, (*4*) 1st thylakoid pellet, (*5*) 2nd thylakoid pellet, (*6*) BBY_{s+}, (*7*) BBY_{s-}, (*8*) starch fraction.

In the polypeptide pattern on SDS-PAGE of the pea preparations (chloroplasts and BBYs), corresponding to those shown in Table 1, an outstanding feature is the difference between the starch-containing and the starch-free BBY particles in the 10-20 kDa region (Fig. 1). This moved us to further investigate the composition of the greenish starch pellet (Fig. 1,

line 7 and Fig. 2, line 4). The starch fraction contained four proteins with apparent molecular masses of 20, 19, 17 and 12.6 kDa, whereas the BBYs- particles appeared depleted in the starch-associated proteins. In contrast, these proteins were present in the BBY_{S+} Parallel results were obtained in pea and spinach (Figs. 1 and 2), as well as in

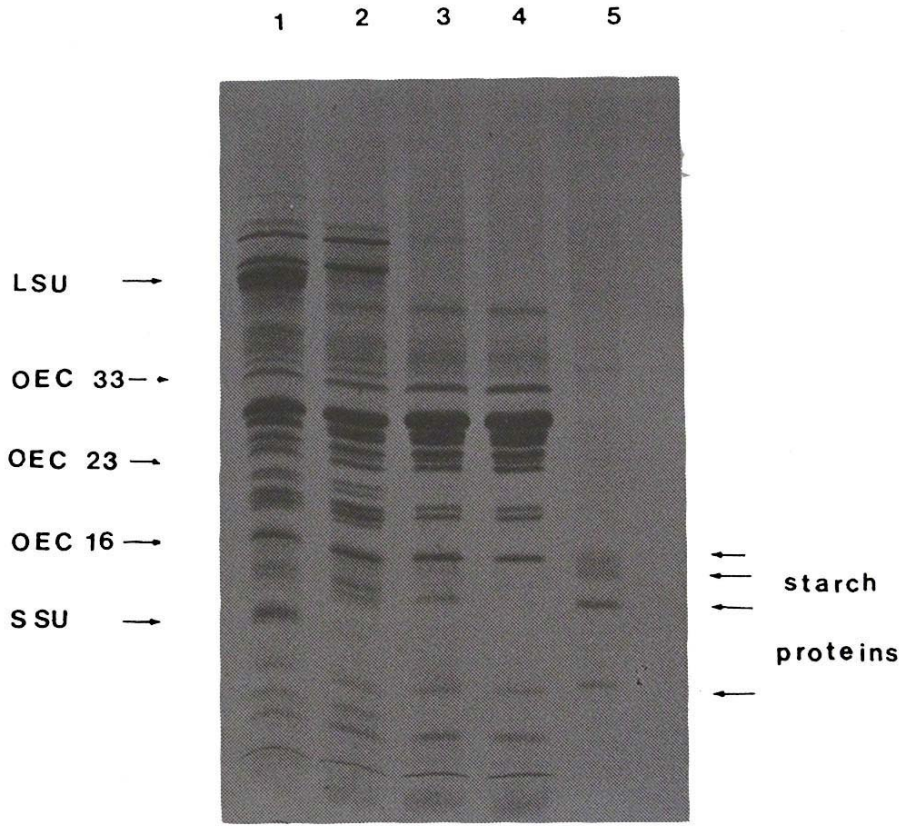

Fig. 2. SDS-4 M urea PAGE of spinach samples in the BBY isolation process. (*1*) 2nd chloroplast pellet, (*2*) 2nd thylakoid pellet, (*3*) BBY_{s+}, (*4*) BBY_{s-}, (*5*) starch fraction.

hydroponically grown spinach.

To investigate the crucial point of starch removal from the BBY preparation further, a gradual elimination was performed. The starch associated polypeptides were progressively released according to the increase in centrifugation speed (Fig. 3); a centrifugation at 10000 x g fully removed both starch and proteins. The release of these polypeptides paralleled a decrease in Cu content (Table 2). After a thorough starch removal, BBY_{s-} particles (*i.e.* 10000 x g centrifuged) had only 0.2 atoms Cu per 300 Chl; this amounted to *c*a. 5% of the total thylakoid Cu and was at the lower limit of detection. Comparing (in the same Table) oxygen evolution rates of BBY_{S+} and BBY_{S-}, no differences were observed.
Cu can interact with the oxygen-evolving complex (Ramaswamy and Nair 1978, Ono *et al.*

1984). To test this hypothesis, and in order to study the strength of Cu-binding to PS2, BBY_{s+} and BBY_{s-} particles were washed with 5 mM EDTA, 1 M NaCI, 1 M CaCl;, 3 M NaSCN/0.01% *Triton X-100* and 0.8 M Tris/HCl, pH 8.4. The last four treatments are known modifers of the oxygen evolving complex (OEC).

Whereas EDTA, Nad and $CaCI_2$ washes did not significantly change the Cu content, Tris and NaSCN/*Triton X-100* drastically diminished the Cu content in the BBY_{s+} (Table 3).

Table 2. Effect of different centrifugation speeds for starch removal on the Cu contents [atoms per 300 Chl molecules] and oxygen evolution rates of BBY particles. Oxygen evolution rates [mmol(O_2) kg^{-1}(Chl) s^{-1}] are in brackets.

	spinach	pea
BBY_{s+}	2.0 (111)	2.0 (167)
BBY (1 000 x *g*)	0.9	1.0
BBY (3 000 x *g*)	0.6	0.7
BBY (5 000 x *g*)	0.4	0.5
BBY_{s-} (10 000 x *g*)	0.2 (111)	0.2 (167)

Table 3. Effect of different washes on the Cu concentrations of spinach and pea BBY_{s+} and BBY_{s-}. Mn values are in brackets.

	spinach BBY_{s+}	BBY_{s-}	pea BBY_{s+}	BBY_{s-}
control	2.0 (4.5)	0.2 (4:5)	2.3 (4.0)	0.2 (4.0)
5 mM EDTA	1.5 (4.2)	0.2 (4.3)	2.0 (4.0)	0.2 (4.0)
1 M NaCl	1.9 (4.0)	0.2 (4.0(	2.2 (4.0)	0.2 (4.0)
1 M $CaCl_2$	1.4 (1.4)	0.1 (2.0)	1.4 (1.6)	0.1 (1.5)
3 M NaSCN, *Triton*	0.8 (0.1)	0.0 (0.1)	0.9 (0.2)	0.1 (0.2)
Tris HCl, pH 8.4	1.0 (0.3)	0.1 (0.2)	1.1 (0.2)	0.1 (0.2)

Discussion

The results of Table 1 indicate that the presence of Cu in PS 2 (BBY_{s+}) was linked to that of starch in the complex. Removal of starch from a BBY_{s+} preparation dramatically decreased the PS2 Cu content. After *Triton* treatment, followed by centrifugation to obtain BBY particles, about half of the thylakoid Cu appeared in the supernatant (from BBY_{s+} or BBY_{s-}) probably in the PSI-linked plastocyanin. The bulk of the second half of chloroplast Cu remains associated to the BBY_{s+}. But if *Triton*-treated thylakoids are subjected to a previous low-speed centrifugation to remove starch, the bulk of the Cu is retained by starch and the Cu content of the BBY particles is reduced to 0.2 Cu/reaction centre. The total Cu

content of the chloroplast is about 2-4 times higher than that of the plastocyanin-bound Cu (Droppa et al. 1990), and this extra Cu is considered to be PS2 associated. We found that approximately 50% of the thylakoid Cu remains in the PS2 complex, only if the starch removal is avoided in the isolation process. Centrifugation at 10000 x g, after the Triton treatment of thylakoids, appears to be the crucial step in the PS2 isolation method for determining the copper content of me BBY particles finally obtained. Moreover, me centrifugation speed for starch removal is also very important, because we have found (Table 2) that between 1000 and 10000 x g after 4 min, me decrease in copper content was progressive.

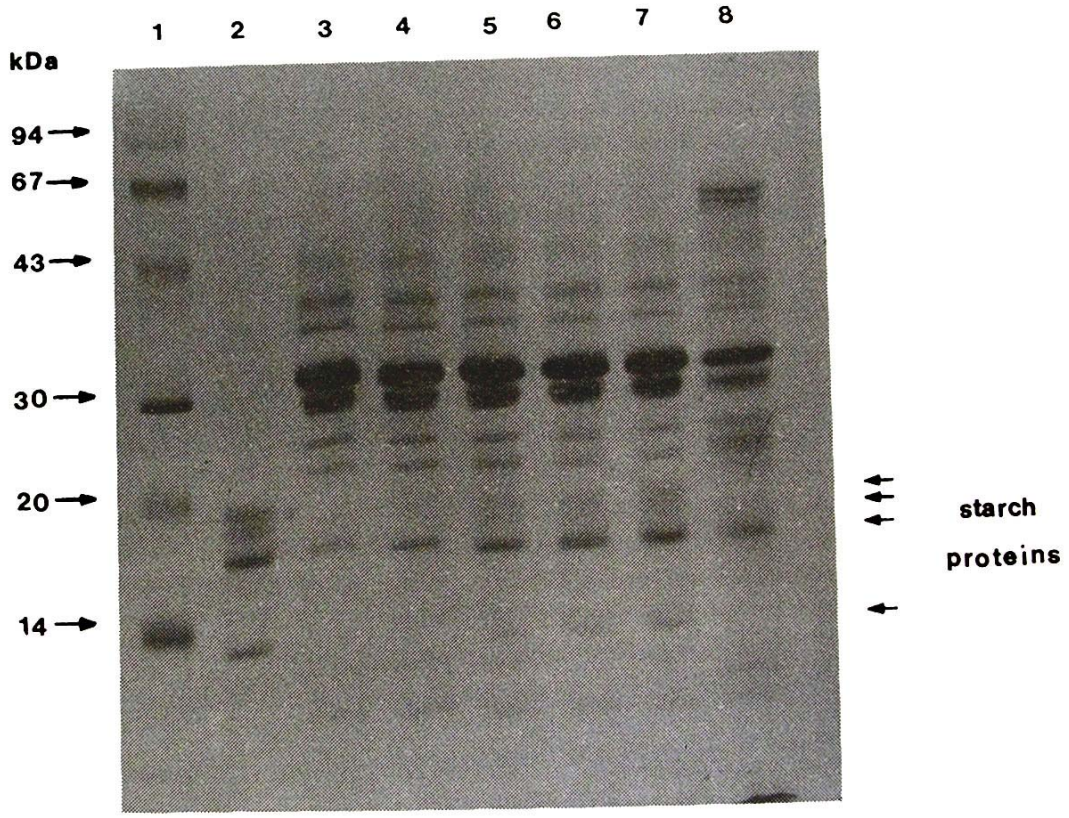

Fig. 3. Effect of different centrifugation speeds for starch removal on the polypeptide pattern of BBY. (*1*) standards, (*2*) starch fraction, (*3*) BBY_{s-}, 10 000 x *g*, (*4*) BBY, 5 000 x *g*, (*5*) BBY, 3 000 x *g*, (*6*) BBY, 1 000 x *g*, (*7*) BBY_{s+}, (*8*) 2nd thylakoid pellet.

The starch fraction in pea and spinach (Figs. 1 and 2) was associated with four proteins with molecular masses of 20, 19, 17, and 12.6 kDa, respectively. Consequently, it seems that Cu in the starch fraction is related to the presence of these polypeptides. Another result supporting this statement is the different polypeptide pattern between BBY_{s-}. and BBY_{s+} in the 20-10 kDa region, both in pea and spinach, which parallels changes in the copper

content of the PS2 complex.

The reported Cu content of PS2 preparations is variable, with Cu values per 300 Chl of 1.7 atoms Cu in a diatom (Holdsworth and Arshad 1977), 2.1 in *Vicia faba* (Gol'dfel'd and Khalilov 1979), 4.2 in barley (Sibbald and Green 1987b), and 3.3 in pea (Baron Ayala et al. 1992). Surprisingly, 5.7, 2.2, 2.5, 1.2 and 1.8 atoms of Cu per 300 Chl have been reported in spinach (Droppa and Horvath 1990).

These differences were attributed to Cu^{2+} loss during preparative procedures. Alternatively, assuming differences between plant species, these divergences could be explained in terms of unequal starch removal, that promotes differences in the starch content and in the starch-polypepdde association.

Atomic absorption analyses of LHC2 have shown a large proportion of Cu in the major antenna of PS2 (Sibbald and Green 1987b). On the basis of our results, it is difficult to corroborate this. The polypepdde pattern of BBY_{s-} does not change in me range of molecular masses corresponding to the LHC2 polypeptides. Moreover, me Cu content of the BBY_{s-} preparation (insignificant), does not correspond to the Cu content expected in the antenna. We have also demonstrated the absence of a cross reaction between antibodies raised against LHC2, and the polypepddes from the starch fraction (unpublished) which retain almost 80 of the PS2 Cu.

Washes with EDTA of the BBY_{s+} particles demonstrate that Cu is not loosely associated with the complex (Table 3). Among the different washing agents reported by Ljungberg et al. (1986) to remove some polypeptides from the oxidizing side of PS2, Cu content is only significantly affected by Tris and NaSCN but unfortunately both also release about 95% of the Mn of the complex, as well as some related polypeptides. Some authors assume that there is an action site for Cu in the oxygen evolving complex (Ramaswamy and Nair 1978, Ono *et al*. 1984), and associate the release of polypeptides from the complex with the loss of Cu and with the decrease in the oxygen-evolving activity. Since these washes also led to a loss of manganese, which plays a crucial role in oxygen evolution, it is difficult to correlate changes in the Cu content with polypeptide release in the washed preparations.

To clarify the role of Cu on the oxidizing side of PS2, a treatment had to be found that could remove Cu without affecting Mn. One way we have found was by starch removal. BBY_{s+} and BBY_{s-} have similar Mn contents and oxygen-evolving activities, showing that the involvement of Cu in water-splitting needs to be reevaluated. Moreover, earlier experiments of some authors on Cu deficiency did not show the oxygen evolving complex as a Cu target site in PS2 (Droppa et al. 1987, Baron Ayala et al. 1992). Rather, they point to an influence of Cu on the lipid and pigment composition of PS2.

Our results suggest that the starch fraction polypeptides may be new copper binding sites in

the photosynthetic cell. We have demonstrated, by immunoassays with antibodies raised against some PS2 proteins, that there is no relationship between the starch associated proteins and the well-known PS2 proteins of similar molecular size (16 and 23 kDa proteins of the oxygen-evolving complex, 10 kDa phosphoprotein and 10 kDa polypeptide of Ljungberg et al. 1986). hi experiments with Cu-deficient plants, the loss of two polypeptides with a molecular mass of 28-29 kDa and 12.6-13.5 kDa (Droppa et al. 1984a, 1987) was described in PS2 particles isolated from spinach, barley and sugar beet. The 29 kDa polypeptide probably corresponds to the pigment-protein complex CP29 (Henrysson et al. 1989). The function and localization on thylakoid membranes of the 12.6-13.5 kDa polypeptide is not known. Droppa et al. (1984a) suggested similarities between this protein and the blue-copper protein, plantacyanin, found in chloroplasts, with a molecular mass of 9-10 kDa and optical and EPR spectra which differ considerably from those of plastocyanin (Aikazyan and Nalbandyan 1975, Sakurai et al. 1982).

The physiological function of the plantacyanin remains unknown. Our smallest "starch protein" migrates in a SDS gel also within this molecular size range.

We consider that the main goal of the future research is to identify and characterize the starch-associated proteins. Very unlikely these polypeptides are PS2-associated, because they can be easily eliminated from the complex by starch removal N-terminal sequencing experiments of the fragments from the trypsintreated proteins are in progress, mununogold localization in leaves and immunodetection of these proteins in other kind of preparations - “inside-out, rightside-out", *etc*. - may help. Further experiments are also necessary to clarify their function in relation to Cu binding.

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