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FORMATION OF SILVER PLASTOCYANIN IN *SCENEDESMUS* *

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Silver ions up to 5 μM do not affect growth of the green microalga *Scenedesmus acutus*. They induce formation of protein species precipitable by an antibody specific against plastocyanin. The metal is incorporated into a part of the induced protein in competition with copper. Bismuth, lead and molybdenum had no effect. The amount of both silver- and copper-containing plastocyanins so formed apparently regulates concurrently inhibition of soluble plastidic cytochrome *c*-553. The silver-copper competition for the build-up of blue plastocyanin can be shown with intact cells, not with isolated algal plastocyanin.

Introduction

The copper-containing redox protein, plastocyanin, is found in some green [1,2] and blue-green algae [3] provided copper is present in the liquid nutrient in a concentration exceeding 0.05 μM. Otherwise, the function of plastocyanin in electron transfer to P-700 is taken over by cytochrome *c*-553 [3,4].

In cultures of *Scenedesmus acutus* grown in a copper-deficient medium, plastocyanin is not detectable by electron-spin resonance or spectrophotometric methods. Nevertheless, apoplastocyanin can be found by quantitative immunoelectrophoresis with an antibody prepared against pure holoplastocyanin [5,6]. After addition of copper, deficient cultures exhibit an induction of plastocyanin formation and a subsequent decrease of plastidic cytochrome *c*-553 synthesis [7].

The copper in the blue plastocyanin is liganded by one cysteine, one histidine, and two methionine residues [8] and can be removed in vitro by silver or mercury ions leading to decolorization [9]. The preparation of a cobalt(II) derivative from apoplastocyanin has been reported [10].

Consequently, we tried to substitute copper by silver and by those other cations having a high affinity for thiol groups, with regard to their ability to induce plastocyanin formation. This can be successfully performed using copper-deficient *Scenedesmus* cells and following the induction time of plastocyanin formation. Furthermore, the competition of copper vs. silver for insertion into the plastocyanin molecule was compared in vivo and in vitro.

Materials and Methods

Scenedesmus acutus (strain 276-3a, Algal Culture Collection, University of Göttingen) was grown as described [1,11]. Copper-deficient cells were cultured for several growth periods before use as described [7] using electrodialyzed water, but analytical grade chemicals with the omission of copper salts. Copper, silver, molybdenum, bismuth, and lead were added to deficient cells as $CuSO_4$, $AgNO_3$, $(NH_4)_6Mo_7O_{24}$, $BiO(NO_3)$, or $Pb(NO_3)_2$ (see Table I). Plastocyanin and cytochrome *c*-553 were determined in supernatants of cell homogenates by differential absorbance

* This paper is dedicated to Professor Dr. W. Menke on the occasion of his 70th birthday, in honour of his pioneering research on thylakoid composition and architecture.

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spectroscopy [7]. Determination of the quantity of metal-free plastocyanin forms together with copper plastocyanin (= holoplastocyanin) was performed by quantitative immunoelectrophoresis [5]. Silver plastocyanin was determined as bound silver in the antibody precipitate. For that purpose, the immunoprecipitates were obtained by mixing one aliquot of 0.3 M phosphate buffer, pH 7.8, two aliquots of an antiserum against pure plastocyanin, and two aliquots of the 200 000 × *g* supernatant of a crude cell homogenate prepared as reported [7]. After an incubation period of 72 h at 4°C, the precipitate was collected by centrifugation (5 min; 15 000 × *g*) and resuspended in 0.5 N HNO_3. Silver as well as copper were determined in an atomic absorption spectrometer (Varian, model AA 575) equipped with a carbon-rod atomizer. The ashing temperature was 700°C for 20 s.

Replacement of copper by silver in plastocyanin in vitro was carried out with *Scenedesmus* plastocyanin isolated and purified as described previously [12]. 8 nmol of oxidized plastocyanin, plus $AgNO_3$ and $CuSO_4$ at concentrations as indicated were added to 20 mM Tricine (*N*-tris(hydroxymethyl)methylglycine) buffer, pH 8.0, in a final volume of 1 ml. After 30 min, the absorbance at 597 nm was recorded. The samples were subsequently dialyzed overnight against 2 l water, then the silver and copper contents were determined.

Results and Discussion

In the experiment of Table I, various metal ions were added to copper-deficient cells in order to check for a possible incorporation of the metals into plastocyanin instead of copper. Without copper present (control), these algae contained no blue copper plastocyanin, but a low amount of copper-free immunoreactive plastocyanin. Silver effectively induced formation of this immunoreactive plastocyanin, silver has the highest affinity for thiol groups of all cations shown in Table I. The 'immunoreactive plastocyanin', as it is called here, comprises plastocyanin precursors [6] and apoplastocyanin (metal-free plastocyanin), as well as metal-containing plastocyanin. No inhibition of growth was observed with bismuth, lead and molybdenum in the concentrations indicated which cannot induce plastocyanin synthesis, since their affinity for SH-groups is less than copper and silver. As shown on the right side of the table, these metals were added together with moderate copper concentrations to ensure plastocyanin induction due to copper (see first line). Even then, the silver effect was additive when the total immunoreactive protein was determined. That is to say, both metals induce formation of immunoreactive apoplastocyanin-type molecules.

However, a competition between copper and silver is evident with regard to their incorporation into the apoplastocyanin which is being formed (Fig. 1). In this experiment, the copper concentration added to the deficient cells was adjusted to a value that allowed for some formation of cytochrome *c*-553 and for a substantial formation of plastocyanin. Addition of increasing Ag^+ concentrations decreased the content of copper-containing plastocyanin; concurrently,

TABLE I

EFFECT OF METALS ON THE FORMATION OF IMMUNOELECTROPHORETICALLY DETECTABLE (TOTAL) PLASTOCYANIN AND PLASTIDIC CYTOCHROME *c*-553 (Cyt. *c*-553) IN INTACT CELLS OF *SCENEDESMUS* WITH COPPER IONS ABSENT OR PRESENT IN THE CULTURE MEDIUM

Concentration of proteins expressed as nmol/μmol chlorophyll.

Addition	Absence of Cu^{2+}		(+) 0.05 μM Cu^{2+}	
	Cyt. *c*-553	Immunoreactive plastocyanin	Cyt. *c*-553	Immunoreactive plastocyanin
No addition (control)	3.7	<0.6	2.8	3.1
Ag (5 μM)	1.9	2.3	2.2	3.9
Bi (5 μM)	3.4	<0.6	2.1	2.3
Pb (5 μM)	3.6	<0.6	2.5	2.6
Mo (5 μM)	3.4	<0.6	2.3	2.8

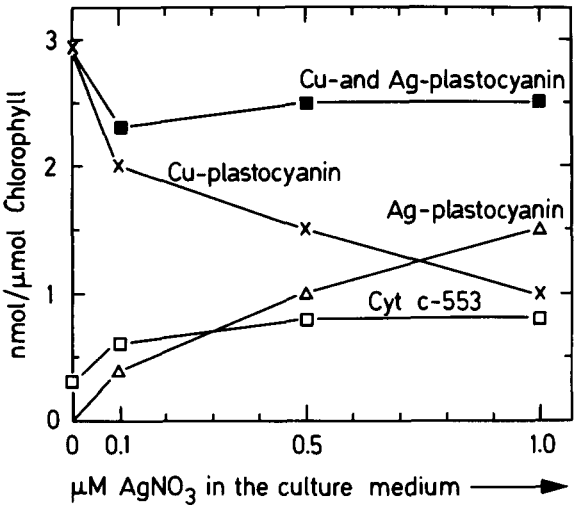

Fig. 1. Reciprocal formation of copper plastocyanin, silver plastocyanin, and plastidic soluble cytochrome *c*-553 in *Scenedesmus* cells cultivated in the presence of 0.2 μM $CuSO_4$ and variable amounts of silver nitrate.

the amount of silver bound to immunoprecipitable plastocyanin increased. The sum of these two plastocyanin species was approximately constant for the three silver concentrations applied (0.1, 0.5 and 1.0 μM Ag^+). As measured by sodium dodecyl sulfate gel electrophoresis [6], the molecular weight of the silver protein induced by silver was the same as that of copper plastocyanin (unpublished results).

By adding silver to copper-deficient cells, the silver-induced formation of immunoreactive plastocyanin resulted in a decrease of cytochrome *c*-553 content when no copper was added simultaneously (Table I). This is analogous to our previous finding where copper was added to copper-deficient cells instead of silver [1]. As demonstrated in Fig. 1, a parallel decrease of copper plastocyanin, determined by its optical absorbance at 600 nm, was evident with increasing silver concentration. The sum of holoplastocyanin (= copper plastocyanin) and silver plastocyanin followed a pattern reciprocal to the cytochrome content. Some cytochrome *c*-553 was formed in the presence of silver ions, however, an increase vs. chlorophyll content of the cell was observed only up to 1 μM silver. Referred to chlorophyll, the silver-dependent formation of cytochrome was reciprocal to the concurrent decrease of copper- and silver-plastocyanin (upper curve of Fig. 1). This finding indicates that silver ions apparently do not unspecifically suppress the concentration of the redox proteins.

These findings support the conclusion from our previous kinetic studies [7] that it is not the copper (or silver) ion which inhibits plastidic cytochrome *c*-553 synthesis during the induction period of plastocyanin formation, but that a compound near the end of the plastocyanin biosynthesis pathway is responsible. The data of Fig. 1 indicate that the level of silver and copper plastocyanin together is responsible for inhibiting cytochrome *c*-553 synthesis.

Table II shows the effects of copper and silver on different 'types' of plastocyanin in more detail. Both silver and copper enhance the formation of immunoreactive plastocyanin, copper being more efficient than silver (line 3). At high copper concentration, the major part of this immunoreactive plastocyanin could

TABLE II

EFFECT OF SILVER NITRATE ON THE FORMATION OF PLASTOCYANIN AND PLASTIDIC CYTOCHROME *c*-553 IN *SCENEDESMUS* IN THE ABSENCE OR PRESENCE OF 1 μM COPPER SULFATE

No.	Protein or silver (nmol/μmol chlorophyll)	(A) Addition of $AgNO_3$ (μM) in the absence of copper				(B) Addition of $AgNO_3$ (μM) in the presence of copper			
		0	0.1	0.5	1.0	0	0.1	0.5	1.0
1	Cytochrome *c*-553	2.0	1.0	1.5	1.3	0.2	0.3	0.5	0.2
2	Copper plastocyanin	0.3	0.4	tr	0	3.9	3.7	3.2	3.3
3	Total immunoreactive plastocyanin(s)	tr	3.4	3.2	3.0	6.8	6.9	6.9	5.7
4	Ag in the antibody precipitate (silver plastocyanin)	0	0.4	1.1	1.1	0	0.5	0.7	1.2

tr, traces.

TABLE III

MOLAR RATIO OF SILVER-PLASTOCYANIN TO TOTAL IMMUNOREACTIVE PLASTOCYANIN IN THE PRESENCE OF DIFFERENT SILVER AND COPPER COMBINATIONS IN THE CULTURE MEDIUM

Ag^+ (μM)	Cu^{2+} (μM)		
	0	0.2	0.3
0	0	0	0
0.1	0.12	0.07	0.07
0.5	0.34	0.20	0.10
1.0	0.37	0.32	0.21

be found as copper plastocyanin independent of silver application. The effect of copper and silver is antagonistic as shown for the formation of copper plastocyanin. To inhibit cytochrome *c*-553 synthesis, copper could be substituted for by silver at low copper supply (part A, line 1). The silver incorporation into plastocyanin increased with increasing silver concentrations in the nutrient medium up to 0.5–1 μM. Higher silver concentrations had no additional effect. The absolute amount of silver found in the immunoprecipitate at different silver concentrations was but slightly influenced by simultaneously added copper (cf. parts A, B, line 4, of Table II). However, when referring the silver plastocyanin to the total immunoreactive plastocyanin species, this ratio increased when more silver was offered and decreased in the presence of higher copper concentrations (Table III). The maximum silver incorporation into apoplastocyanin of about 35% of the immunoreactive protein present could be achieved using 1 μM $AgNO_3$ without additional copper in the medium. When silver was absent from the culture, the level of silver impurity was so low that no silver plastocyanin was detectable.

The antagonistic effect of copper on the silver incorporation into apoplastocyanin in whole cells (Fig. 1) differed from experiments with isolated plastocyanin. As demonstrated by Fig. 2, application of silver ions resulted in decolarization of blue copper-containing plastocyanin with a concurrent release of copper from the protein. In this replacement experiment, copper ions in excess (up to 200 μM) had no protective effect (data not shown). A 3–4-fold excess

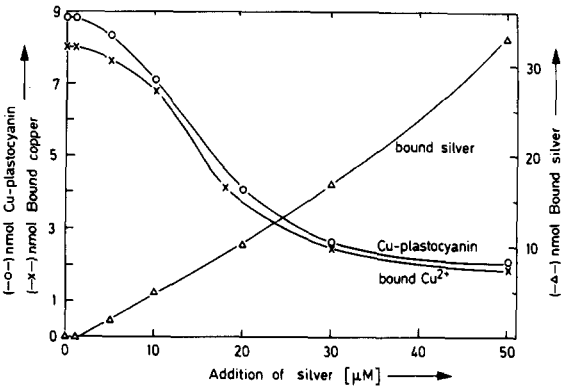

Fig. 2. Replacement of the plastocyanin copper by silver ions at different silver concentrations using isolated *Scenedesmus* plastocyanin. Parameters measured were decolorization of plastocyanin, remaining copper content of plastocyanin, and tightly bound silver after dialyzation. Copper ion concentrations from 0 to 200 μM given together with silver ions did not affect the decolorization of copper-plastocyanin (see Methods for details).

of silver vs. copper released from the protein was found when the silver bound to plastocyanin was determined after silver treatment. This differs from the in vivo experiment where a silver-containing plastocyanin is induced within the cell (Table III). There, only one-third of immunoreactive plastocyanin contained one silver atom and excess copper could decrease silver incorporation into plastocyanin. Furthermore, there is a competition between silver and copper for plastocyanin, demonstrated by the decreasing copper-plastocyanin content in silver-treated *Scenedesmus* cells (Fig. 1).

These findings indicate the absence of free silver ions in the chloroplast, and we assume the presence of a silver or silver/copper carrier protein that may act as (enzymatic) metal donor for an apoplastocyanin.

A low-molecular weight copper-chelating protein was found in copper-treated *Phaseolus aureus* [13]. Two proteins, one of them a 'copper-thionein', were recently reported to be present in a copper-resistant clone of *Agrostis gigantea* [14].

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