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RECIPROCAL FORMATION OF PLASTOCYANIN AND CYTOCHROME *c*-553 AND THE INFLUENCE OF CUPRIC IONS ON PHOTOSYNTHETIC ELECTRON TRANSPORT

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Summary

The green alga *Scenedesmus acutus* is able to synthesize plastocyanin and cytochrome *c*-553. The concentrations of plastocyanin and cytochrome *c*-553 vary inversely in response to the cupric-ion concentrations of the growth medium (Bohner, H. and Böger, P. (1978) FEBS Lett. 85, 337—339). Both proteins form a homogeneous donor pool to the reaction center of Photosystem I. This donor pool can be varied quantitatively and qualitatively by different growth conditions. These variations have no influence on algal growth or photosynthetic electron transport as measured in vivo by oxygen evolution, fluorescence induction and cytochrome *f*-553 and *c*-553 redox reactions using Cu^{2+} concentrations of less than 10 μM in the culture medium. At higher cupric-ion concentrations, which already retard algal growth, specific sites of the photosynthetic electron-transport chain are affected: the oxidizing side of Photosystem II and the reducing side of Photosystem I.

Introduction

Plastocyanin and algal cytochrome *c*-553 may replace each other functionally in vitro [2—5] and in vivo [1,6]. Both redox proteins act as electron carriers between cytochrome *f* and *P*-700, the reaction-center chlorophyll of Photosystem I. Some green algae allow for a variation of both plastocyanin and cytochrome *c*-553 content through the copper-ion concentration of the growth

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; TMPD, *N*-tetramethyl-*p*-phenylenediamine.

medium. Thereby, the electron donor pool preceding Photosystem I, i.e. plastocyanin plus cytochrome *c*-553, is variable quantitatively and qualitatively, whereas the amount of cytochrome *f* and *P*-700 remains more or less constant [1]. The variation of the plastocyanin/cytochrome *c*-553 ratio in *Scenedesmus acutus* as induced by different cupric-ion concentrations does, however, neither change the growth rate, the chlorophyll content of the cells, nor oxygen evolution. Moreover, it is shown by fluorescence yield and cytochrome *f*/*c* redox-reaction measurements that photosynthetic electron transport itself is not influenced by quantitative and qualitative variations of the Photosystem-I electron-donor pool (i.e. plastocyanin plus cytochrome *c*-553).

Materials and Methods

Cultivation of algae. Scenedesmus acutus (strain 276-3a, Algae Culture Collection, University of Göttingen) was grown autotrophically in nutrient solutions with different $CuSO_4$ concentrations [1,7] and harvested after 62 h. The cupric-ion induced changes in the plastocyanin/cytochrome *c*-553 ratio neither influence chlorophyll content of the cells nor growth if: (1) the copper concentration in the nutrient solution does not exceed 10 μM, (2) the inoculum is grown previously in a nutrient solution with 0.2 μM $CuSO_4$, and (3) the algae are harvested at a cell density not exceeding $3 \cdot 10^7$ cells/ml. In some cases, the algae were grown in the presence of N_2/5% CO_2, which also did not affect the growth rate (see Table I).

Quantitative determination of plastocyanin and cytochrome *c*-553 followed the procedure described in Ref. 1.

Chloroplast particles. The algae were harvested, washed first in a medium containing 0.9% NaCl and 10 mM Tris/HCl, pH 8.0, and then in the homogenizing medium consisting of: 40 mM Tricine/NaOH, pH 7.5; 450 mM sucrose; 10 mM NaCl; 5 mM $MgCl_2$; 2 mM sodium ascorbate; and 0.8% bovine serum albumin. An equal amount of wet algal cell paste (2.5 g) and homogenizing medium (2.5 ml) was mixed with 45 g of glass beads (0.5 mm diameter) and homogenized in a Vibrogen-Zellmühle (Bühler, Tübingen) for 1 min, with intervals of 5 s full speed and 5 s off. After removal of cell debris by low-speed centrifugation (1 min at $900 \times g$), the particles were sedimented by centrifugation at $1200 \times g$, 1 min. The pellet was resuspended in an equal volume of the homogenizing medium; the chlorophyll content was determined according to Arnon [8]. The assay systems are described in detail in the legend of Table II.

Fluorescence assays were performed in an Aminco-Chance DW2 spectrophotometer with a temperature controlled and magnetically stirred cuvette holder, equipped for side illumination [9,10]. Fluorescence was excited by red light (657 nm, Balzers; light intensity 50 W/m^2) or blue light (K45, Balzers; light intensity 1100 W/m^2). The photomultiplier was operated at a constant voltage of 350 V and shielded against actinic light by a far-red cut-off filter (Schott, RG 715). Transient signals were detected and stored in a signal averager (Nicolet 1072), and the data plotted by an *X*-*Y* recorder (Hewlett Packard 7044A). The algae suspension was diluted to 5 μg chlorophyll/ml by nutrient solution and kept in dim light under magnetic stirring. Before each

measurement, the suspension was dark-adapted and equilibrated to 20°C in the cuvette holder.

Light-induced absorbance changes were measured by the same Aminco-Chance instrument, dual-wavelength mode [9,10]. Actinic light at an intensity of 50 W/m^2 was defined by two interference filters with maximum transmission at 657 nm and 713 nm (Balzers). Spectral bandwidth of the measuring (553 nm) and reference (561 nm) beams was 3 nm. Chlorophyll concentration was 50 μg of chlorophyll/ml, corresponding to $3.0 \cdot 10^7$—$3.5 \cdot 10^7$ cells/ml. Chlorophyll concentration in intact cells was determined according to Böger [11].

Results

The green alga *Scenedesmus acutus* is able to synthesize both plastocyanin and cytochrome *c*-553. Only plastocyanin is detected if the growth medium contains a sufficient amount of cupric ions (>0.1 μM), Fig. 1A. As noted before, there is an inverse correlation of the concentration of plastocyanin and cytochrome *c*-553 dependent on the cupric ion concentration of the growth medium [1,7]. During growth, cytochrome *c*-553 may appear in dense cultures due to exhaustion of copper from the nutrient solution. In addition, a low partial pressure of oxygen promotes cytochrome *c*-553 synthesis (see Table I, col. 1,7 and 5,8; comp. also Refs 6, 7.). Cultivation of algae with an N_2/CO_2 mixture led to very high amounts of soluble cytochrome *c*-553, up to 3.5 nmol per μmol of chlorophyll (Fig. 1A). Even at higher copper concentrations (>1 μM) some cytochrome *c*-553 is still detectable under these semianaerobic conditions.

In Fig. 1B the sum of plastocyanin and cytochrome *c*-553 content of *Scenedesmus* is plotted against the cupric-ion concentration of the growth medium, at the start of cultivation. Together with those of Table I, the data clearly show that not only the composition but also the size of this electron-carrier pool varies, showing a dip at low copper concentrations. The minimum pool size cannot be attributed exactly to a certain copper concentration, since it is also dependent on the partial pressure of oxygen. Anaerobic conditions apparently stimulate cytochrome *c*-553 synthesis independently of the copper concentration.

When the algae are grown with different cupric-ion concentrations, we may obtain cells containing only cytochrome *c*-553 or only plastocyanin or both electron carriers. However, both the growth rate and oxygen evolution are almost identical. This is demonstrated by the data of Table I. At low copper concentrations we generally observe one cytochrome *c*-553 per *P*-700, with almost no plastocyanin detectable [1]. This value is the minimum size of the electron donor pool. The rate of oxygen evolution, as mentioned, does not change. Limiting the electron transport rate by lowering the light intensity from 400 to 25 W/m^2 again has no influence on the photosynthesis of algae with different amounts of plastocyanin and/or cytochrome *c*-553. Obviously, the rate-limiting step in electron transport is not at the site of the Photosystem-I donor pool. An equimolar ratio of either cytochrome *c*-553 or plastocyanin per Photosystem-I reaction center appears to be sufficient for photo-

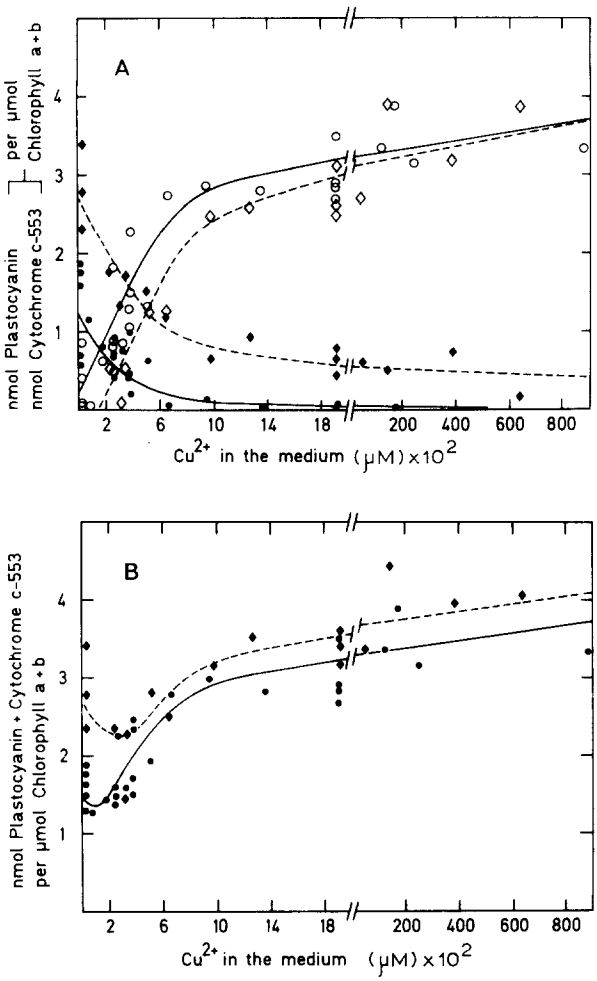

Fig. 1. A. Content of cytochrome *c*-553 (♦, ◆) and plastocyanin (○, ◇) of *Scenedesmus acutus* dependent on the $CuSO_4$ concentration of the nutrient solution. The cultures were grown in the presence of air/5% CO_2 (○, ●) or N_2/5% CO_2 (◇, ◆). B. Combined content of cytochrome *c*-553 plus plastocyanin of *Scenedesmus acutus* dependent on the $CuSO_4$ concentration of the nutrient solution. The cultures were grown in the presence of air/5% CO_2 (●) or N_3/5% CO_2 (◆).

synthetic electron transport. At very high concentrations of copper sulfate in the medium we noted an inhibition of oxygen evolution. This can, however, be attributed to a direct inhibition of photosynthesis by cupric ions and will be treated below in more detail.

To analyze the influence of size and composition of the Photosystem-I donor pool on photosynthetic electron transport in vivo we used the fluorescence assay as a second method.

Although interpretation of the details of the fluorescence induction curve is still subject to controversy, it is generally accepted that it mainly reflects the redox state of Q, the primary acceptor of Photosystem II (comp. review [12]). The withdrawal of electrons from Q and hence the activity of the electron-transport chain between both photosystems should influence variable fluo-

TABLE I

GROWTH AND OXYGEN EVOLUTION OF *SCENEDESMUS ACUTUS* WITH DIFFERENT CYTOCHROME *c*-553 AND PLASTOCYANIN CONTENT

Air was replaced by nitrogen (lowering the oxygen partial pressure) and the $CuSO_4$ concentration of the culture medium was varied to influence the content of (plastidic) cytochrome *c*-553 and plastocyanin. The algae described in lines 1 to 3 originated from the same inoculum; those in lines 4 to 8 are from another one.

Culture conditions		Cell density (cells per ml) $\times 10^7$	nmol formed per μmol total chlorophyll		μmol O_2 per mg Chl per h			
Grown under 5% CO_2 and	$CuSO_4$ concn. (μM)		Cytochrome *c*-553	Plastocyanin	Temperature (°C)	Light intensity (W/m^2) 400	200	25
(1) Air	0	2.35	1.55	0.1	30	—	262	26
(2) Air	0.03	2.35	1.07	0.2	30	—	267	26
(3) Air	0.16	2.25	0.12	2.9	30	—	269	31
(4) Air	0	2.7	1.85	0	25	216	—	—
(5) Air	0.4	2.6	0.05	4.3	25	220	—	—
(6) Air	55.0	1.0	0.05	4.9	25	94	—	—
(7) N_2	0	2.6	2.33	0	25	210	—	—
(8) N_2	0.4	2.6	0.88	2.0	25	224	—	—

rescence if one or more reaction steps become rate-limiting. Therefore, we studied fluorescence induction in algae grown with different cupric-ion concentrations. The data of Fig. 2 show that the fluorescence induction curves are not influenced, within experimental variations, by copper-sulfate concentrations ranging from 0 to 0.4 μM in the growth medium. Especially the maximum yield in fluorescence (*P*) and the rate of decay to the quasi-stationary level (*S*) were almost identical; The *P*-*S* decay is thought to reflect the rate of electron transport from Q to secondary acceptors of the electron-transport chain. Again, variations in the size and composition of the electron-donor pool to *P*-700 are without influence on fluorescence induction in *Scenedesmus*.

Only very high cupric-ion concentrations, which already start to inhibit algal growth, show a clear effect on the variable fluorescence yield. With increasing copper concentrations (>10 μM), the maximum fluorescence level (*P*) decreases as compared to the control. Moreover, the rise from the initial fluorescence level (*I*) to the maximum level (*P*) is retarded and *P* is reached later. On the other hand, the (*I*)-rise itself is only weakly affected (Fig. 2, traces 6—10). This clearly indicates decreasing rates of water-splitting activity with increasing cupric-ion concentrations, since electron pressure from the Photosystem-II donor side becomes insufficient to reach the high transient level of reduced Q during illumination. The influence of high cupric-ion concentrations is clearly distinguishable from the effect caused by addition of DCMU (Fig. 2, trace 1). This control experiment shows an immediate rise to the maximum fluorescence level (*P*), since the electron-withdrawing pathway from the quencher Q is blocked.

Green algae like *Scenedesmus acutus*, where the cytochrome *c*-553 content is easily manipulated should be ideal objects for investigations on the function of this redox carrier by measuring light-induced redox reactions. Moreover, these

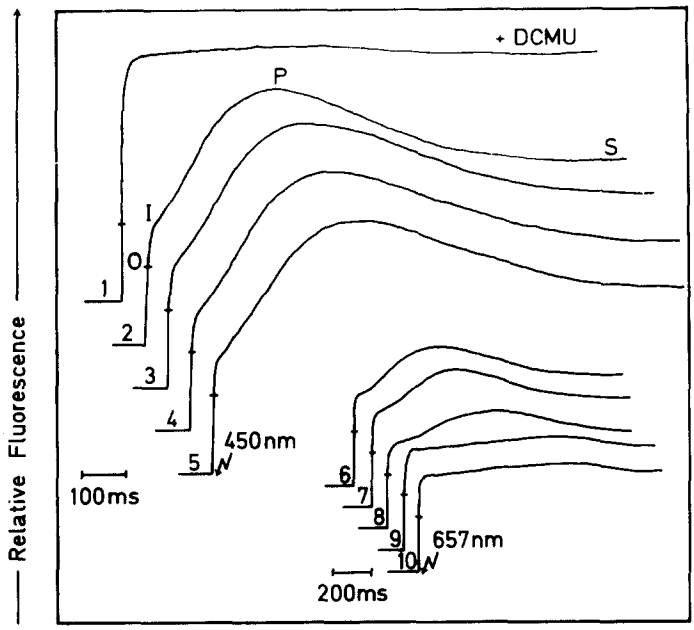

Fig. 2. Fluorescence induction in *Scenedesmus acutus* grown with different cupric-ion concentrations (in brackets). Samples 1—5 were diluted to 10 μg chlorophyll/ml and excited by blue light; samples 6—10: 5 μg chlorophyll/ml, actinic light (657 nm). The cytochrome (Cyt) *c*-553 plus plastocyanin (PC) content of the samples was (nmol/μmol chlorophyll): (1) = (2) (—Cu), 1.34 Cyt *c*-553, 0 PC; (3) (0.027 μM Cu), 0.88 Cyt *c*-533, 0.7 PC; (4) (0.04 μM Cu), 0.15 Cyt *c*-553, 2.5 PC; (5) (0.2 μM Cu), 0.05 Cyt *c*-553, 2.85 PC; (6) (—Cu), 1.85 Cyt *c*-553, 0 PC; (7) (0.4 μM Cu), 0.05 Cyt *c*-553, 4.3 PC; (8) (45 μM Cu), 0.05 Cyt *c*-553, 4.5 PC; (9) (55 μM Cu), 0.05 Cyt *c*-553, 4.9 PC; (10) (64 μM Cu), 0.05 Cyt *c*-553, 5.5 PC.

redox reactions are observable in the intact cell. It should be remembered in this context that *Scenedesmus acutus* contains two *c*-type cytochromes with an α-band maximum at 553 nm. The membrane-bound cytochrome *f*-553 and the soluble cytochrome *c*-553 have both been isolated, purified to homogeneity, and characterized biochemically. Both cytochromes may be distinguished on the basis of their spectroscopic properties (only the α-peak is identical), their isoelectric points, molecular weight and amino acid composition [13]. As shown in Fig. 3C, trace 1, light-induced absorbance changes of cytochrome *f*-553 are measurable in intact cells (due to the high copper-sulfate content of the growth medium no cytochrome *c*-553 was detectable). Both red-light and far-red light excitation oxidize cytochrome *f*-553 and cytochrome *c*-553; the resulting spectrum shows no participation of other cytochromes in the absorbance change under these conditions (Fig. 3A). Far-red light or red light plus DCMU oxidize cytochrome *f*-553 and cytochrome *c*-553 almost completely (Fig. 3B).

With increasing content of cytochrome *c*-553 and constant amounts of cytochrome *f*-553, the absorbance change induced by far-red light increases (Fig. 3C, traces 1, 2). Also in the extreme case, where cytochrome *c*-553 synthesis is stimulated by growth of the algae under semi-anaerobic conditions (gassing with N_2/CO_2), the far-red light response accounts for all of cytochrome

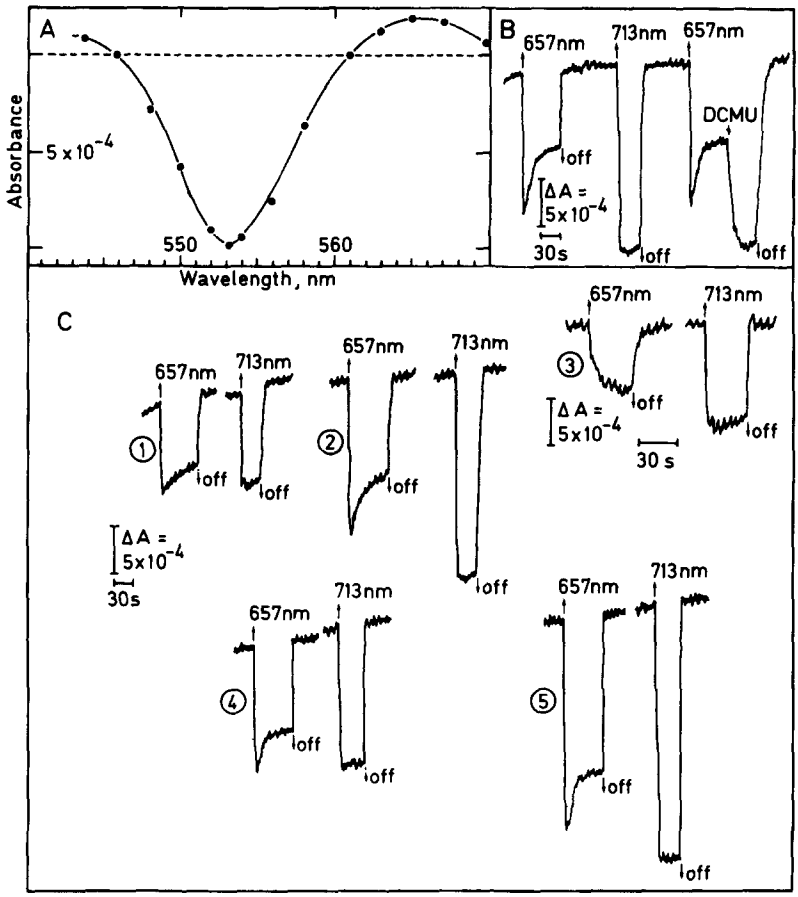

Fig. 3. Light-induced absorbance changes of cytochrome *f*-553 and cytochrome *c*-553 of *Scenedesmus acutus*. (A) Difference spectrum for the absorbance change induced by 713-nm light. The algae contained both cytochrome *f*-553 and cytochrome *c*-553 (1.2 nmol per 1 μmol chlorophyll). (B) Influence of DCMU (10 μM) on the light-induced absorbance change. The algae (40 μg chlorophyll/ml) contained 2.5 nmol cytochrome *c*-553 and 1 nmol cytochrome *f*-553 per μmol chlorophyll. (C) Absorbance changes of algae grown with different cupric-ion concentrations. The cytochrome *c*-553 and cytochrome *f*-553 content of the samples (nmol/μmol chlorophyll), and copper concentration of the nutrient solution in brackets, was: (1) (0.4 μM Cu), 0.05 Cyt *c*-553, 0.95 Cyt *f*-553; (2) (—Cu), 1.85 Cyt *c*-553, 1.05 Cyt *f*-553; (3) (64 μM Cu), 0.1 Cyt *c*-553, 1.05 Cyt *f*-553; (4) (0.4 μM Cu, with N_2/5% CO_2), 0.88 Cyt *c*-553, 0.95 Cyt *f*-553; (5) (—Cu, with N_2/5% CO_2), 1.85 Cyt *c*-553, 0.95 Cyt *f*-553.

f-553 and *c*-553 present (Fig. 3C, traces 4, 5).

Illumination with red light oxidizes up to 90% of cytochrome *f* (Fig. 3C, trace 1). Algae containing cytochrome *c*-553 in addition to cytochrome f show a transient oxidation only. Upon illumination, both cytochromes first become oxidized by Photosystem I until Photosystem-II electrons create a more reduced steady state during illumination. This steady state is more or less constant and corresponds roughly to the cytochrome *f*-553 content of the cell. The size of the transient depends on the amount of cytochrome *c*-553 present. This is not observed with N_2/CO_2-grown algae, where up to 35% of cytochrome *c*-553 may stay oxidized during illumination with red light.

As far as the kinetics of light-induced absorbance changes are concerned, we

find no significant differences within the time resolution of the dual-wavelength method. When the algae are cultivated in the presence of 60 μM copper sulfate or more, which leads to substantial inhibition of growth and oxygen evolution (Table I, col. 6), only 60% of cytochrome *f*-553 is photooxidizable by red light and the rate of oxidation is considerably slower (Fig. 3C, trace 3). This result indicates a second site of inhibition on the Photosystem-I electron-accepting pathway by high cupric-ion concentrations. We believe that this kind of inhibition of electron transport depends on the concentration of free cupric ions in the cell. This is variable to a certain extent, depending on physiological state of the algae and external concentration of $CuSO_4$. It should be emphasized that *Scenedesmus* is able to accumulate substantial amounts of copper [14].

The results so far showed that plastocyanin and cytochrome *c*-553 replace each other perfectly. Changes in the pool size, which appear on a changeover from one redox protein to the other, have no influence on photosynthesis in vivo. However, with chloroplast particles isolated from algae grown with different cupric-ion concentrations, different electron-transport activities are measurable.

As a general rule, we find that the electron transport rates, either with water or *N*-tetramethyl-*p*-phenylenediamine/ascorbate as electron donors and methylviologen as acceptor, are higher the more plastocyanin plus cytochrome *c*-553 is present in the cell before homogenization. Since *Scenedesmus* is able to synthesize more plastocyanin than cytochrome *c*-553 on a molar basis, chloroplast particles isolated from algae grown in the presence of cupric ions (>0.2 μM) are generally more active (Table II). This is further substantiated by an experiment where algae containing cytochrome *c*-553 were homogenized in

TABLE II

ELECTRON TRANSPORT RATES IN *SCENEDESMUS ACUTUS* CHLOROPLAST PARTICLES

The effect of different $CuSO_4$ concentrations in the culture medium and the effect of adding cytochrome *c*-553 during homogenization of the algae, or to the assay medium. Measurements at 25°C. The assay medium was: 50 mM Tricine/NaOH, pH 8.0; 400 mM sucrose; 20 mM NaCl; 4 mM $MgCl_2$; 10 mM K_2HPO_4; 0.2 mM sodium azide; and 0.1 mM methylviologen. For the system *N*-tetramethyl-*p*-phenylenediamine (TMPD)/ascorbate → methylviologen (MV) the assay included in addition: 0.1 mM TMPD; 1 mM sodium ascorbate; and 1 μM DCMU.

Addition of copper to the culture medium	Addition of cytochrome *c*-553 to		μmol O_2 per mg chlorophyll per h	
	Homogenization medium	Assay medium	$H_2O \rightarrow MV$	TMPD/ ascorbate → MV
minus $CuSO_4$	—	—	80 ± 26	132 ± 37 *
0.03 μM $CuSO_4$	—	—	145 ± 30	200 ± 45 *
0.20 μM $CuSO_4$	—	—	165 ± 38	235 ± 56 *
minus $CuSO_4$ **	0.2 mM ***	—	145	—
minus $CuSO_4$ **	0.2	0.2	200	—
minus $CuSO_4$ **	—	—	78	—
minus $CuSO_4$ **	—	0.2	168	—

* Average of 5 different preparations.

** Identical preparation treated differently, as indicated.

*** Due to centrifugation and resuspension of the particles added cytochrome *c*-553 was diluted to a final concentration of approximately 0.02 mM.

the presence of added cytochrome *c*-553. After removing excess of cytochrome *c*-553 by washing, the resulting particles were twice as active as the control. Apparently, a sufficient amount of cytochrome *c*-553 must remain trapped within the thylakoid lumen (comp. Ref. 4). It should be noted that addition of cytochrome *c*-553 to the assay medium stimulated electron transport further. Obviously, the Photosystem-I reaction centers of this chloroplast preparation are accessible to a certain extent to externally added cytochrome *c*-553, thereby restoring electron transport [3].

Discussion

It has been shown that plastocyanin and cytochrome *c*-553 may replace each other in certain algae [1,6]. Both redox proteins form the electron-donor pool to Photosystem I. Quantitative determinations have demonstrated that these water-soluble proteins are generally present in several-fold excess over their neighbouring redox-reaction partners, i.e. cytochrome *f*-553 and *P*-700 [1,15]. Physiological variation of this pool size by different growth conditions (see Fig. 1) did neither influence growth, nor oxygen evolution, nor other parameters of photosynthetic electron transport as measured by fluorescence-yield changes and photoreactions of cytochromes.

From the data presented it appears that an approximate equimolar ratio between cytochrome *f*-553, cytochrome *c*-553 or plastocyanin, respectively, and *P*-700 is sufficient for cellular growth.

The reason why plastocyanin or cytochrome *c*-553 are produced in excess over their immediate reaction partners may be related to their water solubility. There is increasing evidence that both proteins are located inside the thylakoid-membrane lumen and the rate of electron transport may be diffusion-controlled at this site [17]. A certain electron-buffering capacity appears to be of some advantage in any case. Under our growth conditions, however, such 'advantages' are not apparent for algal photosynthesis in vivo. Further, the data do not answer the question why plastocyanin is preferred to cytochrome *c*-553 during evolution of photosynthesis.

In an effort to determine the lower limit of the Photosystem-I donor pool size, which is sufficient for photosynthesis, we extended the investigation to the alga *Dunaliella parva*. This alga is able to synthesize plastocyanin only. Under cupric-ion deficiency, *Dunaliella* responds by decreasing plastocyanin content. Values below 1 nmol plastocyanin per μmol of chlorophyll are obtainable with no compensation by cytochrome *c*-553 synthesis. Under these conditions, copper deficiency leads to retardation in growth and lower rates of oxygen evolution are observed (details will be presented elsewhere; Sandmann, G. et al., in preparation).

Another influence of cupric ions on photosynthesis of *Scenedesmus* could be found with high concentrations of this ion in the nutrient solution. Copper sulfate is a well known algacide. Above a certain concentration of $CuSO_4$, a destruction of the photosynthetic apparatus is observed in *Chlorella* (1 mM, [18]) and *Scenedesmus* (50 μM, [14]). Specific sites of inhibition of the photosynthetic electron-transport chain were not determined. With *Scenedesmus* we observed an inhibition of growth and oxygen evolution (up to 60%,

Table I) at concentrations above 40 μM $CuSO_4$. Comparison of the fluorescence-rise curves and cytochrome *f*-553 photooxidation showed that two sites of inhibition are apparent. High cupric-ion concentrations specifically inhibit the oxidizing site of the Photosystem-II reaction center, but also the reducing pathway of Photosystem I. These in vivo results are in accordance with data obtained in vitro with isolated spinach chloroplasts. Low concentrations of $CuSO_4$ (2 μM for 50% inhibition) inactivated ferredoxin and the water-splitting reaction [19].

A third point is raised in connection with light-induced cytochrome absorbance changes. The data show that in vivo all of the cytochrome *f*-553 present in the cell is photooxidizable. The same holds true for soluble cytochrome *c*-553, which contributes to the total absorbance change measured. Since its amount is extremely variable by different culture conditions, different signal heights of the measured absorbance changes are expected. These are, of course, not due to variations of electron transport rates.

In this context, it should be pointed out that the total plastocyanin pool of *Scenedesmus* is photooxidizable as well; this was shown by quantitative EPR measurements [7]. These data, together with those reported here, suggest that both plastocyanin and cytochrome *c*-553, replacing each other perfectly, form a homogeneous donor pool to Photosystem I.

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