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REACTIONS OF PLASTOCYANIN AND CYTOCHROME 553 WITH PHOTOSYSTEM I OF *SCENEDESMUS*

KARL-JOSEF KUNERT, HERBERT BÖHME and PETER BÖGER

Fachbereich Biologie, Universität Konstanz, D-7750 Konstanz, Giessberg (G.F.R.)

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SUMMARY

Chloroplast material active in photosynthetic electron transport has been isolated from *Scenedesmus acutus* (strain 270/3a). During homogenization, part of cytochrome 553 was solubilized, and part of it remained firmly bound to the membrane. A direct correlation between membrane cytochrome 553 and electron transport rates could not be found. Sonification removes plastocyanin, but leaves bound cytochrome 553 in the membrane. Photooxidation of the latter is dependent on added plastocyanin. In contrast to higher plant chloroplasts, added soluble cytochrome 553 was photooxidized by 707 nm light without plastocyanin present. Reduced plastocyanin or cytochrome 553 stimulated electron transport by Photosystem I when supplied together or separately. These reactions and cytochrome 553 photooxidation were not sensitive to preincubation of chloroplasts with KCN, indicating that both redox proteins can donate their electrons directly to the Photosystem I reaction center. *Scenedesmus* cytochrome 553 was about as active as plastocyanin from the same alga, whereas the corresponding protein from the alga *Bumilleriopsis* was without effect on electron transport rates.

It is suggested that besides the reaction sequence cytochrome 553 → plastocyanin → Photosystem I reaction center, a second pathway cytochrome 553 → Photosystem I reaction center may operate additionally.

INTRODUCTION

The operational sequence of cytochrome *f* and plastocyanin within the photosynthetic electron transport chain was first suggested by studies with mutants of *Chlamydomonas* [1, 2] and with spinach subchloroplast particles [3]. Reconstitution experiments with cell-free systems have been done almost exclusively with chloroplasts from higher plants. In these organelles, cytochrome *f* remains firmly bound to the

Abbreviations: cytochrome 553, cytochrome *f* from *Scenedesmus acutus*; DAD, 3,6-diaminodurene (= 2,3,5,6-tetramethyl-*p*-phenylenediamine); DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; MV, methylviologen (1,1′-dimethyl-4-4′-dipyridylium dichloride); Tricine, *N*-tris(hydroxymethyl)-methylglycine buffer, adjusted with NaOH.

thylakoid membrane when plastocyanin is removed by treatment with detergents or sonification. This redox protein can then be added back, giving rise to a light-induced oxidation of cytochrome *f* (see ref. 4 for survey).

From unicellular algae, on the other hand, both plastocyanin and cytochrome *f* can be obtained in a soluble pure form (see ref. 5 for properties of this plastidic algal cytochrome). This leads to the expectation that algal thylakoid material might be prepared depleted of both redox proteins in an easier and more gentle procedure than with higher plants [6]. Accordingly, algae particles should be more suitable for reconstitution experiments, since both redox carriers from the same organism can be added back to the assay in exact concentration and reproducible manner.

However, investigations are rare dealing with reactions of these redox proteins in cell-free algae systems and are by no means conclusive [4, 7–9]. The main obstacle is to isolate chloroplast material yielding substantial electron transport rates. The experimental conditions for this achievement have to be carefully established for each organism and many of them fail to exhibit electron transport rates in vitro.

The algal cytochromes differ significantly in their physical properties from those of higher plant plastids. Comparative assays ought to be expanded in order to answer the question whether or not the function of redox carriers in algae is comparable to that in higher plant chloroplasts. This can be questioned even more strongly after lack of plastocyanin was demonstrated in the coccoid alga *Bumilleriopsis filiformis* (Xanthophyceae), where its role is taken over by cytochrome 553 [4]; also *Euglena* seemingly has no plastocyanin (own findings; [9]).

This paper reports some findings on redox protein interactions at the reducing site of Photosystem I using a cell-free assay of *Scenedesmus acutus*. Besides the *f*-type cytochrome this alga contains plastocyanin.

MATERIAL AND METHODS

(1) *Biological material*

Scenedesmus acutus (strain 270-3a, Algae Culture Collection, University of Göttingen) was grown in sterile liquid mineral medium [10, 11], the iron complexed with EDTA (this medium was developed for short-time cultivation purposes). Fernbach flasks containing 1 l culture volume each were placed on a shaker in a temperature-controlled room (21–23 °C) as described [12, 13]. Light intensity was about 10 000 lux at the bottom of the vessels, supplied by a bank of fluorescent lamps which were composed equally of type L 65 W/32 and L 65 W/25 (Osram). Sterile air enriched with 5 % CO_2 (v/v) was constantly provided. The cultures were started with 10^6 cells/ml density with cells from a 2-days culture and harvested after a 48 h growth period (see Fig. 2).

(2) *Preparative methods*

(a) Plastocyanin from *Scenedesmus acutus*. The cell material was kindly provided by the Soeder group of Dortmund, Germany. There, the algae were grown in open flat containers for about three days in a greenhouse illuminated by sunlight; the medium was as mentioned above. It was important to harvest the cells from cultures of low density. This was done after the suspension had attained an optical density of about 0.5–0.6 at 560 nm (Medico photometer, Lange, Berlin; Dr. E. Stengel,

personal communication). The cultures were continuously checked for contamination by other algae and bacteria which was kept below 3 % of the algal material. Preparation was done as indicated in Fig. 1, line I. The wet paste of 400 g washed *Scenedesmus* cells was suspended in 0.02 M Tricine, pH 8.0, and adjusted to 2–3 mg chlorophyll per ml suspension. The material was sonified strongly (see legend), centrifuged for 30 min at 50 000 × *g* and the supernatant brought to 55 % saturation of ammonium sulfate. After centrifugation for 10 min at 15 000 × *g*, the supernatant was dialyzed vs. 0.02 M Tricine, pH 8.0, then oxidized with potassium ferricyanide ($5 \cdot 10^{-4}$ M), placed on a DEAE column (2 × 5 cm) and the protein eluted with an NaCl gradient of 0.06 to 0.13 M in 0.02 M Tricine, pH 8.0. The enzyme was further purified by gel filtration (Sephadex G-75) as described (4). For the yield see Results. The concentration was calculated on the basis of the blue copper chromophore using an extinction coefficient of 4.9 ($mM^{-1} \cdot cm^{-1}$) at 597 nm.

Disc gel electrophoresis (see below) exhibited one band although sometimes less than 1 % cytochrome 553 could be detected by its absorbance at 417 nm. In reconstitution experiments this impurity had no influence on electron transport activity as was checked with controls of pure cytochrome 553. However, when cells with higher cytochrome content were used (see Results), it was difficult to obtain a plastocyanin preparation without considerable cytochrome 553 contamination. In an elaborate step it could be removed by disc gel electrophoresis (7 × 100 mm columns; 16 % polyacrylamide gel according to ref. 14) and collected by eluting plastocyanin in a small dialysis bag at the bottom of the gel.

(b) *Scenedesmus* cytochrome 553. 3–4 l of culture suspension were harvested after a 48 h growth period (see Fig. 2), washed, and 16 g wet cell paste suspended in a buffered sucrose-containing homogenization medium, as described for the preparation of *Bumilleriopsis* chloroplasts [4], but omitting polyvinylpyrrolidone. Homogenization was done successively with 4–5 g of algae paste (equivalent to 20–25 mg of chlorophyll) added to 25 ml homogenization medium and using 42 g glass beads of 0.5 mm diameter; the volume of the homogenization vessel was approx. 100 ml. The instrument used throughout was the homogenizer of Braun, Melsungen, Germany; homogenization time was 1 min with 4000 rev./min. The homogenate was centrifuged for 30 min at 50 000 × *g* (Fig. 1, line II), and the yellow-green supernatant was brought to 55 % saturation with solid ammonium sulfate and centrifuged again for 10 min at the same *g* number. The brownish supernatant was then saturated with ammonium sulfate to 90 %, stirred for 1 h in an ice-bath and centrifuged again as mentioned just before. The pellet was suspended in about 5 ml of 0.02 M Tricine, pH 8.0, and dialyzed vs. the same buffer. The cytochrome 553 (its content at this step was about 1 per 600 chlorophylls; mol/mol), was placed on a DEAE-column (2 × 5 cm) and eluted with a linear gradient of 0.06 to 0.13 M NaCl as done for the corresponding preparation from *Bumilleriopsis* [4]. Very little ferredoxin is observed on top of the column. The yield of pure cytochrome 553 was about 30 nmol. No contamination with plastocyanin could be detected by EPR spectroscopy according to [18].

The pellet obtained after the first centrifugation (see above) can be mixed with a "chloroplast suspension medium", sonified and precipitated with ammonium sulfate as mentioned above, yielding most of the ferredoxin and plastocyanin besides very little additional cytochrome 553 (Fig. 1, line III).

(c) Isolated chloroplasts and their treatment. *Scenedesmus* cells were broken as mentioned in the foregoing chapter. The dark green homogenate was centrifuged for 7 min at 17 000 × *g* and the pellet resuspended in the "chloroplast suspension medium" (see Fig. 1).

Where indicated, this material was diluted to 0.2 mg chlorophyll/ml and 5 ml sonified weakly with the microprobe of a Branson sonifier (mod. J-17 A, setting at 2–3 of a 10 interval power scale) for 4 min in an aluminium beaker cooled by an ice-bath to keep the temperature of the sample below +10 °C. After centrifugation for 1 h at 250 000 × *g* ((Beckman L2-65B), the pellet was resuspended in the suspension medium and the chlorophyll adjusted to 2 mg/ml.

KCN preincubation of chloroplast material at 0 °C was done as reported [4] using a modified method of the Good group [15]. Plastocyanin was tested enzymatically with digitonin-treated photosystem-I particles according to (28).

(3) *Instruments*

Light-induced O_2-uptake was measured polarographically in a thermostated chamber at 20 °C in a 2 ml reaction volume, the light defined by a 610 nm cut-off filter as described [16]. The reaction medium is mentioned in the legend of Fig. 3. The set-up was built by the University workshop. A change of oxygen concentration down to 2 nmol/10 s per reaction chamber could be measured. For determination of cellular O_2 production the cells were diluted 1 : 2 with nutrient medium. Light-induced absorbance changes were determined with the dual-wavelength spectrophotometer DW2 (Aminco, Silver Spring, Md., U.S.A.). The assay was performed in open 3-ml cuvettes, stirred magnetically in a temperature-controlled cuvette holder at 20 °C. Actinic light intensity of wavelength 713 nm (Balzers, Filtraflex) was 90 $J/m^2 \cdot s^{-1}$. Cytochrome absorbance changes were measured at 553 nm with 540 nm as reference, with a bandwidth of 2 nm; amplifier time constant was set at 100 ms.

(4) *Chemicals*

Reagents for algae cultures and Tricine were purchased from Merck AG, Darmstadt, p.a. grade. DAD was from Fluka, Buchs, Switzerland; 1,5-diphenylcarbazide from Eastman-Kodak; other chemicals were supplied by Serva, Heidelberg, Germany.

RESULTS

Cytochrome 553; plastocyanin

The first protein was already briefly described as a cytochrome *c*-552 by Powls et al. [8], the absorbance peaks of the reduced form in the visible part of the spectrum being 417, 521 and 552 nm. Those from our preparation are 318, 417, 523 and 553/4 nm. These data, the ratio of the Soret to the α-band and the asymmetry of the latter strongly suggest that our preparation is a cytochrome-*c* of a (plastidic) *f*-type comparable to the cytochrome-552 mentioned above. More details of this protein will be reported elsewhere.

Cells were harvested after a 48 h growth period, yielding a molar ratio of 600–800 chlorophylls to 1 cytochrome 553 when the latter was determined in the supernatant of Fig. 1, II; the thylakoids (of the pellet) still contained this cytochrome in

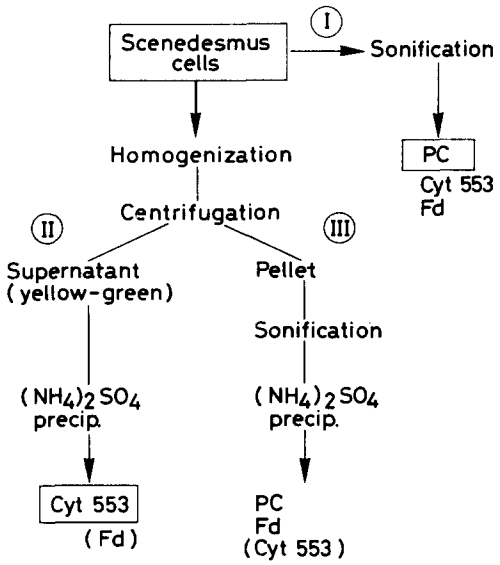

Fig. 1. Preparation of cytochrome 553 and plastocyanin from *Scenedesmus acutus*. Both sonification steps for protein preparation were done in 40 ml portions with the macroprobe of a Branson sonifier, mod. J-17 A, power setting at 6 of a 10-interval scale, for 4 min under continuous cooling [4]. In procedure (I) cells were washed and resuspended in 0.02 M Tricine, pH 8.0. The pellet (III) was suspended in 0.4 M sucrose; 50 mM Tricine, pH 8.2; 20 mM NaCl; 4 mM $MgCl_2$ and 10 mM K_2HPO_4 (="chloroplast suspension medium"). In both cases the chlorophyll content was adjusted to 3 mg/ml.

TABLE I

LIGHT-INDUCED OXYGEN UPTAKE

For reaction mixture (2 ml) see legend of Fig. 3, for homogenization see Methods

System	μmol O_2 · mg^{-1} chlorophyll · h^{-1}	System	μmol O_2 · mg^{-1} chlorophyll · h^{-1}
Chloroplasts, *Scenedesmus*:		Chloroplasts, *Scenedesmus*:	
$H_2O \rightarrow$ MV	25	Photosystem I assay	
+0.8 mM DPC	23	Ascorbate (4 mM)	33
+0.7 μM cytochrome 553	23	+2 μM cytochrome 553	97
+2 μM PC	24*	+2 μM PC	95
+DCMU 0.1 μM	5	DAD/ascorbate (0.6 mM/4 mM)	60
+0.6 mM DAD/4 mM ascorbate (±DCMU, 0.1 μM)	48	+2 μM cytochrome 553	127
Sonified chloroplasts:	12	+2 μM PC	126
+0.6 mM DAD/4 mM ascorbate (±DCMU)	24		

* The same rate was determined when 1,5-diphenylcarbazide (=DPC) and plastocyanin (=PC) were added together.

about the same ratio as determined by difference spectra (hydroquinone minus ferricyanide). The cytochrome 553 retained in the isolated thylakoids by our homogenization procedure was only solubilized to some additional percent by weak sonification (see Methods, Section 2b, c). When the cells were homogenized with a higher cell/glass bead ratio the chlorophyll to soluble cytochrome 553 ratio rose to about 1000 : 1. It should be noted that less than 10 % of the cytochrome 553 was lost from *Bumilleriopsis* chloroplast material after this homogenization procedure (comp. ref. 4). Prolonged growth resulted in denser cultures with cells of higher chlorophyll [13] and cytochrome content [25].

After homogenization with glass beads according to Methods, Section a, and subsequent sonification of chloroplast material by the procedures indicated in Fig. 1 (parts III or II), proper detection of the copper protein (after the ammonium sulfate step) is possible by e.g. EPR technique [4]. The supernatant, but not the chloroplast particles contained plastocyanin. Due to the low plastocyanin content of the *Scenedesmus* chloroplast material (below 10 % when compared with spinach chloroplasts) a quantitative estimation of possibly bound plastocyanin after sonification cannot be performed at present. The chlorophyll to plastocyanin ratio depends on cultivation age. It increases with prolonged cultivation. This problem is currently under investigation. Some biochemical properties of *Scenedesmus* plastocyanin have been described [18]. It should be noted that the *P*-700 content of our cell material was estimated to be about 1 to 300 chlorophylls (comp. legend of Fig. 4).

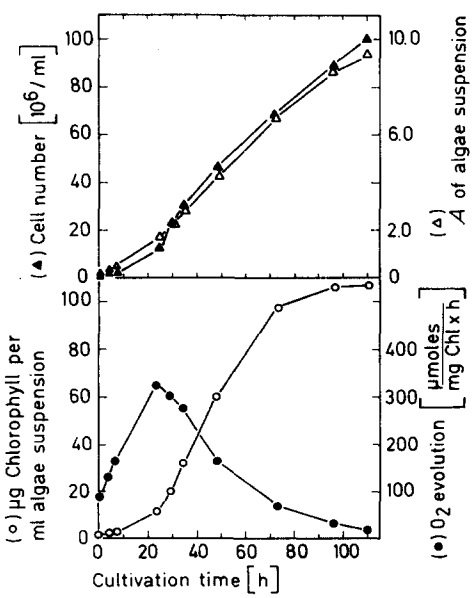

Fig. 2. Cultivation of *Scenedesmus acutus* and photosynthetic activity. Cell number was determined by direct counting. The absorbance of the culture was determined in a Zeiss spectrophotometer PM4 at 1000 nm after absorbance had been adjusted to 0.1–0.5. Chlorophyll of cells was extracted at 65 °C with methanol [19]. The data represent mean values of 3 experiments (deviation ±10 %). For oxygen uptake of the cell-free system see Fig. 3. Cellular activity was little influenced by adding bicarbonate or pregassing with N_2. Compare [17] for activity of heterotrophically grown cells.

Standardization of photochemical activity

Fig. 2 demonstrates the cell growth in the upper, cellular photosynthetic activity in the lower part. Although growth is quite linear from the second to the fourth culture day, the cellular activity is passing an optimum after the first 24 h of cultivation. Our cultivation time (48 h) should compromise between a necessary cell mass and a substantial photosynthetic activity assayed by the methylviologen mediated Mehler reaction ($H_2O \rightarrow MV$). With our cell material and homogenization conditions used (see Methods), rates of 25–30 μmol O_2 uptake per mg chlorophyll and hour were obtained in the methylviologen mediated Mehler reaction (see legend of Fig. 3). Lowering the ratio of algae to glass beads yields chloroplast material with decreased electron transport activity. It should be pointed out that with *Bumilleriopsis* the relation of algae to glass beads is less critical than with *Scenedesmus* [13].

Photoreactions

As demonstrated in Table I, O_2 uptake in the $H_2O \rightarrow MV$ system could not be stimulated by diphenylcarbazide [20], plastocyanin, and/or cytochrome 553, both from *Scenedesmus*. Addition of the first indicates that the diminished rate of electron transport is not due to inactivation of the water-splitting system. The overall reaction remained DCMU-sensitive. DAD/ascorbate doubled the oxygen uptake. Sonification reduced the rates by 50% of both the $H_2O \rightarrow MV$ and DAD/ascorbate $\rightarrow$ MV systems. Most interesting is the finding of the subsequent experiment dealing with interaction of the two redox proteins with Photosystem I in the presence of ascorbate, as shown in the right part of Table I. The rates increased by adding ascorbate and cytochrome 553 or ascorbate and plastocyanin. Inclusion of DAD did not change the stimulating effect of the redox proteins since both rates were additive. Such a stimulation by adding these redox proteins cannot be observed with chloroplasts from either *Bumilleriopsis* or spinach. In conclusion: these data indicate that in the

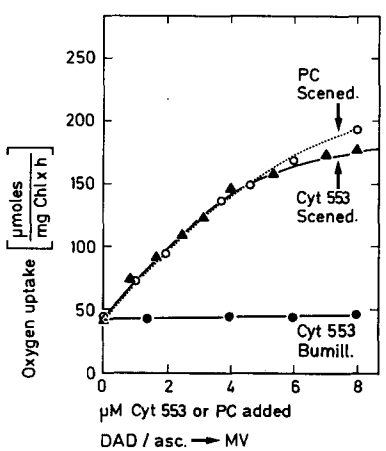

Fig. 3. Light-induced oxygen uptake by chloroplasts dependent on added plastocyanin and cytochrome 553 from *Scenedesmus*. System: DAD/ascorbate $\rightarrow$ MV. The 2 ml assay volume contained: 0.2 M Tricine, pH 8.0; 1 mM $MgCl_2$; 0.1 M sucrose; 0.15 mM MV; 0.15 mM sodium azide; 0.6 mM DAD; 4 mM sodium ascorbate; and chloroplast material equivalent to 60 μg of chlorophyll. For preparation of the latter see Methods, Section b. PC = plastocyanin.

system used the soluble redox proteins added back to the membrane are able to accept exogenous electrons (from ascorbate) and donate them to photosystem I, DAD being unnecessary. Accordingly, methylviologen mediated oxygen uptake of sonified particles was not impaired provided either plastocyanin or cytochrome 553 were added (Fig. 5, curve 1; see legend for rates). Fig. 3 demonstrates in more detail the similar redox activity of these proteins (comp. 21, 22 for higher plant chloroplast particles). It should be noted that cytochrome 553 from the alga *Bumilleriopsis* was without effect.

Cytochrome 553 photooxidation

The bound (endogenous) cytochrome 553 of the isolated untreated chloroplast material was photooxidized to 35 %. Photooxidation could not be increased by adding plastocyanin (Fig. 4, tracings 1 and 2). Also added soluble cytochrome 553 was photooxidized to this extent only (tracings 3, 4). Additional plastocyanin did not increase the absorbance change at 553 nm (not shown). It should be noted that omission of ascorbate increased the extent of photooxidation by 5–10 % only. Although less than 5 % of cytochrome 553 was solubilized by sonification treatment, its photooxidation was substantially decreased (tracing 5 of Fig. 4). In this case, however, added plastocyanin brought back the deflection almost to the value of the non-sonified chloroplast material (comp. tracings 7 and 1) indicating that plastocyanin had been the limiting component.

Added soluble cytochrome 553 was also oxidized to 35 % without added plastocyanin (tracing 7). In the presence of the latter, the photooxidation was somewhat higher (tracing 8), and seems to represent the addition of both the deflections of tracing 6 (endogenous) and 8 (added cytochrome 553), both in the presence of plastocyanin.

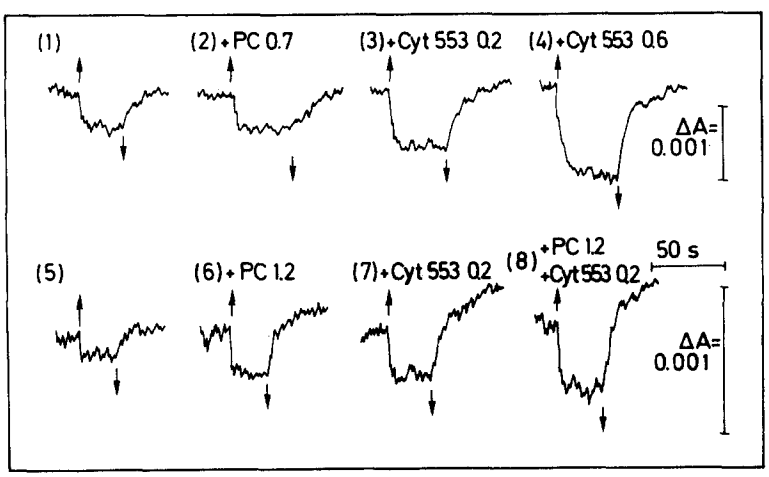

Fig. 4. Cytochrome 553 photooxidation of *Scenedesmus* chloroplast material. Chlorophyll was 0.15 mg, *P*-700 content about $5 \cdot 10^{-2}$ nmol, and cytochrome 553 bound to the thylakoids $2.4 \cdot 10^{-2}$ nmol per 3 ml assay volume; the reaction mixture included 0.2 M Tricine, pH 8.1; 0.1 M $MgCl_2$; 0.1 M sucrose; 0.05 mM MV and 2 mM sodium ascorbate. For further details see Methods. Tracings 5–8 demonstrate the findings with sonified chloroplast material. Figures behind the redox proteins are nmoles. See reference [4] for determination of *P*-700. PC = plastocyanin.

KCN treatment

As shown in Fig. 5, preincubation of *Scenedesmus* chloroplast material for 60 min with KCN invariably leads to inhibition of O_2 uptake of 50–60 % in the DAD/ascorbate → MV (curve 3) and H_2O → MV system (curve 4) as well. With DAD/ascorbate as electron donor, however, activity could be restored to the control value after various intervals of KCN treatment by adding back either cytochrome 553 or plastocyanin (curve 2 in Fig. 5). Loss of activity was almost as small as observed within the 60 min chloroplast ageing (see inserted column).

After sonification (Methods, part c) and subsequent removal of solubilized plastocyanin by centrifugation, photosynthetic oxygen uptake, although exhibiting a decreased rate, was no longer susceptible to KCN inhibition (Fig. 5, curve 1).

In a further assay, *Scenedesmus* and spinach chloroplast material (300 μg chlorophyll/ml) was incubated with KCN for 60 min. Together with a non-treated control the chloroplasts were sonified (see Methods) and centrifuged for 1 h at 250 000 $\times g$. The supernatants were then assayed by light-induced ascorbate oxidation with digitonin Photosystem I particles from spinach [28]. As shown in Table II, the

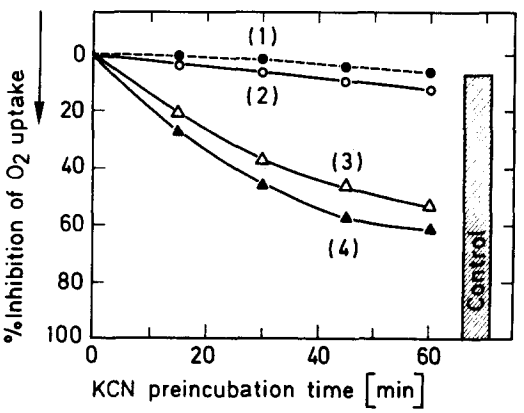

Fig. 5. Light-induced oxygen uptake in the DAD/ascorbate → MV system after chloroplast pretreatment by KCN. 0.1 ml *Scenedesmus* chloroplast material with 300 μg of chlorophyll was mixed with 0.9 ml of an ice-cold medium consisting of: 30 mM KCN; 0.2 M Tricine, pH 8.0; 1 mM $MgCl_2$; 60 μM potassium ferricyanide and 0.1 M sucrose. At the intervals indicated 0.2 ml of this KCN incubation medium was added to 1.8 ml of the assay mixture described in the legend of Fig. 3. Thereby the cyanide was diluted to 2.7 mM preventing inactivation of the redox proteins which were added afterwards as noted (plastocyanin 4 nmol, cytochrome 553 2 nmol). Chloroplast superoxide dismutase was still inhibited by this cyanide concentration, the oxygen uptake being enhanced by 25–50 % vs. a cyanide-free control. Curve (1): KCN pretreatment with sonified chloroplast fragments. (Without added redox proteins the rate was 25 μmol $O_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$.) When plastocyanin or cytochrome 553 were added, rates attained 200 and 142 μmol $O_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$, respectively. With or without the redox proteins added no KCN inhibition was detected. Curves (2) to (4) represent data with non-sonified chloroplast material. Curve (2): with added plastocyanin or cytochrome 553; controls without cyanide treatment were 110 μmol $O_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$, with plastocyanin added, and 130 μmol $O_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$ with added soluble cytochrome 553. Curve (3): no plastocyanin or cytochrome 553 added; control rate: 50 μmol $O_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$. Curve (4): O_2 uptake with water as donor, no DAD/ascorbate present; control rate for chloroplasts without cyanide treatment was 24 μmol $O_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$. The column represents the rate stability over a 60 min ageing of chloroplast material without KCN incubation.

TABLE II

ASCORBATE PHOTOOXIDATION BY DIGITONIN PHOTOSYSTEM I PARTICLES FROM SPINACH

Assay according to ref. 28; chlorophyll: 5 μg/ml. A rate of 22 μmol $O_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$ was obtained without pure plastocyanin (=PC) or supernatants and subtracted from the data shown.

Additions	μmol O_2 uptake $\cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$	Treatment of chloroplast supernatants with KCN
Controls:		
(1) +1.1 nmol PC, *Scenedesmus*	220	25
(2) +2.2 nmol PC, *Scenedesmus* + Supernatants of sonified chloroplasts (=SN)	416	26
(3) +SN, spinach	464	25
(4) +SN, spinach+KCN	25	24
(5) +SN, *Scenedesmus*	81	33
(6) +SN, *Scenedesmus*+KCN	44	33

supernatant of KCN-treated chloroplast material from both spinach (No. 4) or *Scenedesmus* (No. 6) had poor stimulating activity in contrast to the supernatants obtained from the non-treated control (Nos. 3, 5). When the added plastocyanin and the supernatants were subsequently treated again with 15 mM KCN for 60 min, the data of the right column of Table II were obtained. In all samples the rate was down to an unspecific basal value. It has been shown that KCN treatment produces the enzymatically inactive apoprotein of *Scenedesmus* plastocyanin [18].

A light minus dark difference spectrum of (untreated) chloroplasts measured during photooxidation clearly shows a trough at 553–554 nm (Fig. 6A) occasionally with a distinct shoulder at 559 nm, indicating that primarily the *f*-type cytochrome is oxidized under our conditions. The preparation shown in Fig. 6B has little cytochrome *b*-559. Sometimes, more is present giving rise to a substantially increased

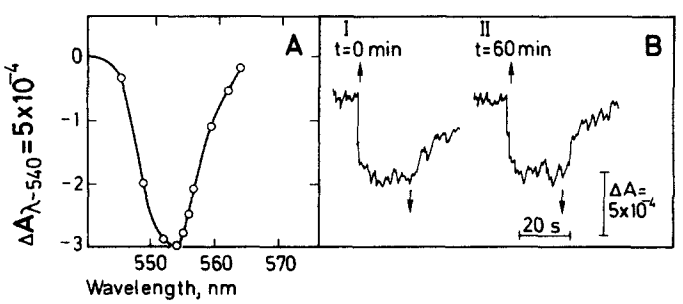

Fig. 6. A: Light minus dark difference spectrum of *Scenedesmus* chloroplast material. Reaction conditions and medium as noted in B, except for the portion of the KCN mixture; chlorophyll was 150 μg/ml. B: Cytochrome 553 photooxidation with *Scenedesmus* chloroplasts pretreated by KCN for 60 min. (I) control; (II) after KCN incubation. The assay consisted of 0.6 ml KCN incubation medium, including chloroplasts equivalent to 0.18 mg chlorophyll and 2.4 ml of the reaction mixture according to legend of Fig. 4. Absorbance changes were determined at 553 nm with 540 nm as reference (see Methods).

559 nm absorbance change after KCN treatment overlapping the contribution of cytochrome 553 absorbance changes. However, estimates of cytochrome 553 according to equations of [29] indicate that cytochrome 553 photooxidation was not impaired by KCN treatment of *Scenedesmus* chloroplasts.

DISCUSSION

Since algal chloroplasts possess cytochrome *f* (e.g. cytochrome 553 in *Scenedesmus* or *Bumilleriopsis* and cytochrome 552 in *Euglena*) which can be isolated in a soluble form, it should be possible to remove it, leaving most of the plastocyanin in the membrane. Our cell homogenization procedure alone partially removes cytochrome 553, but half of it at least remains bound to the thylakoid membrane. Although it is evident that loss of electron transport activity has some bearing on the amount of cytochrome 553 left in the membrane, the relationship is not directly correlated. It appears that dependent on cultivation time, part of the cytochrome *f* remains intrinsically bound, with a variable molar relationship to plastocyanin. Currently, we speculate on the possibility that cytochrome 553 might substitute for plastocyanin in *Scenedesmus* under certain physiological conditions. Further work with *Scenedesmus* of different cultivation ages and cytochrome 553 content is needed to clarify this point. Also for higher plants data were reported that part of the cytochrome *f* may reside inaccessibly to polar oxidants within the membrane, and part of it may be close to the aqueous surface [23].

Scenedesmus chloroplasts prepared by simply breaking the cell with glass beads are different from those of *Bumilleriopsis* prepared in the same manner. Only the *Scenedesmus* particles react with added (ascorbate-)reduced plastocyanin or cytochrome 553 exhibiting a light-induced methylviologen mediated oxygen uptake. This is not observed with spinach or *Bumilleriopsis* chloroplasts either. For these the treatment and often in addition the presence of detergents in the reaction mixture or sonification are required to demonstrate plastocyanin-dependent Photosystem I reactions. Furthermore, with *Bumilleriopsis* chloroplasts, these treatments solubilize 80–90 % of all cytochrome 553, as they do with *Euglena*. Our *Scenedesmus* chloroplast material also demonstrates that its plastocyanin (concentration calculated on the basis of the blue copper chromophore) is about as effective as *Scenedesmus* cytochrome 553. In subchloroplast particles of higher plants the copper protein is by far more active than the cytochrome [21, 22, 27], probably due to specificity problems, since algal cytochromes were tested (comp. Fig. 3). The inverse effect is found with *Bumilleriopsis* chloroplasts [4]. The data of Fig. 3 demonstrate that reconstitution experiments are successful only when redox proteins and corresponding thylakoids from the same organism are used, which has been done with the systems from higher plants in one case [6]. Those authors found photooxidation of added spinach cytochrome *f* to correspondingly depleted particles to be dependent on the presence of plastocyanin. It should be noted that only 10 % of the spinach cytochrome *f* added was photooxidized [6], and it was not ruled out that possibly some endogenous (bound) cytochrome *f* remained in the particle and was activated by the plastocyanin added. Our findings with *Scenedesmus* particle indicate a substantial photooxidation of added cytochrome 553, regardless of the copper protein being present or not. Photooxidation of soluble cytochrome 553 or 552 without plastocyanin added was also

reported for *Bumilleriopsis* chloroplasts [4] and *Euglena* [9], respectively, both organisms being apparently devoid of plastocyanin (own findings [9]).

This is the first report, to our knowledge, of a direct reaction of cytochrome 553 with Photosystem I of plastocyanin-containing algal thylakoids. It is achieved without detergents, thereby minimizing possible artificial redox arrangements in a disturbed thylakoid fragment. Simple breakage of the cell may, nevertheless, cause artefacts with which we have to work at present.

KCN treatment leads to a 50 % inhibition of electron transport activity only when no additional plastocyanin or cytochrome is supplied (Fig. 5). This is in contrast to spinach chloroplasts where an 80–90 % inhibition is observed [15, 4, 24]. Furthermore, photooxidation of membrane-bound cytochrome 553 is not inhibited by KCN treatment (Fig. 6B), two findings indicative of a plastocyanin-independent pathway of cytochrome 553 photooxidation. Sonified *Scenedesmus* chloroplast particles are comparable to higher plant subchloroplast fragments: plastocyanin is released and the released copper protein can be inactivated by KCN treatment (see Table II). These sonified chloroplasts are no longer susceptible to KCN inhibition. With both water and DAD/ascorbate as electron donors they exhibit electron transport activity, although at decreased rates (Table I; Fig. 5). Addition of plastocyanin to these chloroplast particles stimulates photooxidation of bound cytochrome 553. This cannot be explained at present but may indicate that sonification not only releases plastocyanin but also (in contrast to KCN-treated chloroplasts) prevents an alternative direct interaction of bound cytochrome 553 with the reaction center of Photosystem I.

These findings indicate that in vivo a sequence cytochrome 553 → plastocyanin → Photosystem I as found for higher plant plastids appears to be operating possibly besides a second one, in which cytochrome 553 is directly oxidized by the Photosystem I reaction center (cf. [26]). Our data show that there is enough *P*-700 to account for such a separate pathway. For this reaction, it is unclear whether the electrons are donated from Photosystem II or other sources, possibly directly from cytochrome b_6, as discussed for *Euglena* [9]. The latter proposal is in contrast with the findings of higher plants, where cytochrome b_6 oxidation requires plastocyanin [24].

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