Absence of Plastocyanin in the Alga *Bumilleriopsis* and its Replacement by Cytochrome 553

Karl-Josef Kunert and Peter Böger

Fachbereich Biologie, Universität Konstanz

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Different preparative procedures for isolation and purification of plastocyanin were successful to obtain the pure protein from *Chlorella vulgaris*, *Scenedesmus acutus* and *Spinacia oleracea* but not from *Bumilleriopsis filiformis*. Lack of plastocyanin in this alga was further confirmed by the following in comparison with spinach chloroplast material:

a. Use of antibodies to block photosynthetic reactions generally dependent on plastocyanin;
b. *epr* spectral assessment;
c. treatment of chloroplasts with KCN and amphotericin B;
d. light-induced changes at 553 nm (=cytochrome f photooxidation).

The latter assays indicated that, in contrast to spinach, neither plastocyanin nor cytochrome f (=Cyt 553) is present in sonified or pyridine treated *Bumilleriopsis* chloroplasts. Photooxidation of (added) cytochrome 553 takes place not only without plastocyanin but is counteracted by the copper protein.

Introduction

After the discovery and characterization of plastocyanin by Katoh [1, 2] considerable effort has been undertaken to elucidate its role in the photosynthetic electron transport chain. Earlier reports about reconstitution experiments [3] or findings with mutants of *Chlamydomonas* [4] suggested a sequence Cyt f → Pc → P700. This was corroborated by several authors with detergent treated or sonified chloroplasts [5–7] and – along with the refinement of preparative techniques – also with reconstituted photosystem-I particles [8, 9]. Very recently, this sequence was evidenced with intact thylakoid membranes by inactivating plastocyanin directly at its site with specific agents such as $HgCl_2$ [10] or KCN [11, 12]. So, the sequence appears to be substantiated at least for higher plants.

However, up to now there has always been disagreement with this proposal. Particularly Fork and coworkers [13, 14] like to reverse the functional location of both redox proteins and are supported by others [15, 16].

A third proposal puts the redox carriers in series as first suggested by Kok *et al.* [17] and we, too, had some data in favour of it [18]. Some authors have extended this parallel scheme so far as not to ascribe cytochrome f but plastocyanin instead the main redox function in noncyclic electron transport [19].

Plastocyanin was isolated and (partly) purified from several algae: *Anabaena* [20], *Chlorella* [1, 21], *Chlamydomonas* [22], *Scenedesmus* (own findings, see also [23]). By comparative epr surveys [24] is was concluded that Pc is also present in the red alga *Porphyridium*. The occurence of cytochrome f was proven in all the species mentioned (see [25, 26] for references). It appears to occur in algae in a low molecular form throughout [25].

It was shown that algal cytochrome f is necessary for complete electron transport through both photosystems in *Euglena* [27, 52] and that it can be mutually exchanged with plastocyanin in photosystem I reactions of higher plant chloroplast (particles), although with different efficiency [13, 18, 28]. Little work has been done to elucidate the interaction of plastocyanin and cytochrome f in algae. Measurements of quantum efficiencies and kinetics of cytochrome-f oxidation and reduction in *Porphyridium* [29], for example, do indicate a crucial role for cytochrome f but not for plastocyanin (see also [30]). Ex-

Requests for reprints should be sent to P. Böger, Fachbereich Biologie, Universität Konstanz, *D-7750 Konstanz*, Giessberg.

Abbreviations: ΔA, difference of absorbance (optical density); Chl, chlorophyll; Cyt, cytochrome, a figure following indicates the absorbance maximum of the α-band; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Fd, ferredoxin (from *Bumilleriopsis*); MeViol, methylviologen (N,N′-dimethyl-4,4′-dipyridylium dichloride); Pc, plastocyanin; PS-I, PS-II, photosystem I or II, respectively; P700, reaction center chlorophyll at photosystem I; tricine-NaOH, N-tris(hydroxymethyl)-methylglycine buffer, adjusted with NaOH.

periments reported with cell-free systems of the procaryotic *Anabaena* [22, 31] showed that both algal Pc and cytochrome f catalyze redox reactions at photosystem I, however, these experiments were not aimed to answer the operational sequence. Experiments with eucaryotic algal chloroplast material are lacking since up to now only some species allow isolation of chloroplasts capable of noncyclic photosynthetic electron transport with rates comparable to those isolated from higher plants.

We have been pursuing investigations with a cell-free system of the coccoid heterokont alga *Bumilleriopsis filiformis* for several years. This species yields chloroplasts of high photosynthetic activity so badly needed *in vitro* [32]. In the following, data are given which indicate lack of plastocyanin and point to cytochrome f as the direct electron donor for photosystem I.

A preliminary report of these experiments has been presented [33].

Materials and Methods

1. Biological material

Bumilleriopsis filiformis. Vischer (from the stock collection of the latter author) and *Chlorella vulgaris* Beijerinck (= *C. prototheco*ides [34]; No. 211-11 h of the Algae Culture Collection, University of Göttingen) were grown in sterile liquid autotrophic mineral medium [35] in Fernbach flasks on a shaker as described previously [36]. The iron was complexed with EDTA. The cells were harvested after 7 – 8 days growth when the suspension had a density of about $4-5\times10^6$ cells with *Bumilleriopsis* and about $5-6\times10^7$ cells with *Chlorella. Scenedesmus acutus* (No. 276-3 a, Cult. Coll. Göttingen) was kindly provided by Dr. C. Soeder, Dortmund. There, the alga was grown in open flat containers in mineral medium [37] with trace elements [38] in a green-house without artificial light. Spinach was *Spinacia oleracea,* strain "ATLANTA", grown in the garden during April to June on thoroughly fertilized soil. However, in order to obtain chloroplasts with reproducible activity, it was grown in a controlled climate chamber at 20 °C on vermiculite with the nutrient solution according to Arnon [39], the $FeSO_4$ replaced by the Fe-EDTA complex. Fluorescent light with approx. 10^3 ft-c was given in an 8 hour light to 14 hour dark regime.

2. Preparative methods

a. Spinach plastocyanin

Approx. 2 kg of fresh spinach leaves, added to 400 ml of 0.02 M tricine-NaOH, pH 8,0, including 0.4 M sucrose were homogenized in a Waring Blendor (3 l capacity) and the homogenate extracted in a press (model Hafico).

In a first method (compare [40, 22]) the sap was precipitated with a fourfold volume of acetone (– 15 °C), centrifuged (10 min, $10\,000\times g$), the pellet resuspended in 0.02 M tricine-NaOH, pH 8.0, dialyzed against the same buffer for at least 12 hours, centrifuged again as above, placed on a DEAE column (DE 52, Whatman) and treated further as described below for the algal plastocyanins. For oxidation of the plastocyanin during the preparation procedure the ferricyanide concentration should be approx. 5 times higher than indicated below.

The second, more elegant method, however, was to centrifuge the green sap at start for 2 min at $1000\times g$, + 4 °C, to discard the pellet and to spin the supernatant again for 5 min at $50\,000\times g$. Then the pellet was resuspended in tricine-NaOH, pH 8.0, adjusted to 2 – 3 mg of chlorophyll per ml and sonified in 40 ml portions for 2 min each with a Branson sonifier (mod. J-17 A, 300 Watts, with a macroprobe and the power setting at 6; maximum position at 12). The sample was cooled to + 4 °C with a continuous flow attachment connected to a refrigerated thermocirculator (Haake, Berlin, mod. KT 33). The combined samples were centrifuged for 30 min at $50\,000\times g$. Ammonium sulfate with 55% saturation was added to the supernatant (pH was somewhat below 6) which precipitates part of the flavoproteins and the rest of the green particles. The supernatant has a yellow-green color and contains the plastocyanin (besides yellow impurities). This supernatant turns dark-blue after addition of some K-ferricyanide (about 20 µl of a 2×10^{-2} M solution to 80 – 100 ml of supernatant). After dialysis for 12 hours against 0.02 M tricine-NaOH, pH 8.0, to remove the $(NH_4)_2SO_4$, the sample (which had lost its blue color) was oxidized again and placed on the first DEAE column (2×5 cm). The plastocyanin stuck at the top of the column, part of it in the blue form. It was washed with 300 ml of 0.06 M NaCl solution (if not mentioned otherwise, all sodium chloride solutions were buffered with 0.02 M tricine-NaOH, pH 8.0) until the eluate did not show any absorbance at 280 nm. The copper protein was then eluted with a linear gradient from 0.06 to 0.13 M NaCl. To the plastocyanin containing fractions (5 ml each) again ferricyanide was added (less than 5 µl of a 5×10^{-2} M solution per

fraction), those fractions combined, diluted 1:3 with 0.02 M tricine and placed on a second DEAE column (1 × 3 cm) and the protein eluted with 0.2 M NaCl; the volume of the eluate should be only 2 – 3 ml. It was further purified by passing through a Sephadex G-75 column (2 × 100 cm; elution with 0.05 M tricine-NaOH, pH 8.0), and concentrated on a small DEAE column as mentioned above.

A third procedure according to Siedow *et al.* [9] applies pyridine and sonification. All three methods yielded approx. the same amount of plastocyanin (about 1 mole protein per 400 moles of total chlorophyll). We used mostly the second one.

b. Algal plastocyanin (and cytochrome f)

A paste of about 400 g wet algal cells was suspended in tricine-NaOH, pH 8.0, adjusted to 2 – 3 mg Chl/ml suspension and sonified as described above. A fourfold volume of acetone (– 15 °C) was added, centrifuged, the pellet resuspended in tricine-NaOH and dialyzed as described for the acetone precipitation with spinach material. Then the green suspension was stirred (+ 4 °C) for 6 hours and centrifuged for 10 min at 15 000 × *g*. The supernatant has an intense yellow-green color in case of *Chlorella* and *Scenedesmus* and reddish-brown with *Bumilleriopsis*. It was placed on a first DEAE column (3 × 5 cm, Whatman DE 52) and the plastocyanin or the cytochrome 553 (only in the case of *Bumilleriopsis)* could be eluted with 0.2 M NaCl (NaCl solutions always buffered with 0.02 M tricine-NaOH, pH 8.0), the ferredoxin remained on top of the column. The plastocyanin in the eluate was oxidized with ferricyanide as described (for spinach Pc, 2nd procedure) and ammonium sulfate added up to 55% saturation (the eluate was buffered beforehand with 5 ml 1 M tricine-NaOH, pH 8.0, per 60 ml). After 12 hours of dialysis *vs* 0.02 M tricine-NaOH, pH 8.0, the sample was oxidized again with ferricyanide and placed on a second DEAE column (2 × 5 cm) and treated and eluted with a gradient from 0.06 to 0.18 NaCl. After a 1:3 dilution of the eluate this column step was repeated with a linear NaCl gradient of 0.06 to 0.13 M. The fractions were combined, oxidized completely with some ferricyanide and placed on the next (4th) DEAE column (1 × 3 cm) to be eluted thereafter with 0.2 M NaCl in a volume of 3 – 4 ml. This was passed through a Sephadex G-75 column (2 × 100 cm), eluted with 0.05 M of the buffer mentioned, and concentrated again on a small DEAE column (1 × 3 cm). – *Chlorella* yielded about 1 μmole Pc per 500 μmoles of Chl, with *Scenedesmus* the yield was somewhat less.

In all cases, after elution from the last DEAE column, all plastocyanin samples were dialyzed for only 1.5 to 2 hours *vs* 0.02 M tricine-NaOH, pH 8.0, and stored in 2 – 3 ml portions in the deep freeze. They are stable for months.

The plastocyanin preparations checked in the assays had a single band in the disc gel electrophoresis [25]. Their spectral and molecular characteristics will be described elsewhere. An extinction coefficient of 9.8 ($mM^{-1}\,cm^{-1}$) at 597 nm was used.

For preparation of algal cytochrome f (= Cyt 553 from *Bumilleriopsis*), no ferricyanide was added to the samples and one elution step with a gradient 0.06 to 0.13 M NaCl (at the second DEAE column, see above) proved to be sufficient to yield some fractions of highest purity. For a detailed preparative procedure of this cytochrome, its criteria and absorption spectra of both the reduced and oxidized forms see Lach *et al.* [25] (and also Fig. 7).

c. Preparation of chloroplasts and their treatment

Chloroplasts from *Bumilleriopsis* were prepared as described [36]; however, the homogenization medium was simplified: 0.4 M sucrose; 50 mM tricine-NaOH, pH 8.2; 10 mM NaCl; 5 mM $MgCl_2$; 2 mM Na-ascorbate was added just before use. This was mixed 1:1 (w/w) with a wet algal cell pellet and homogenized [36]. Addition of polyvinylpyrrolidone was not necessary for the experiments described herein. The "chloroplast suspension medium" consists of: 0.4 M sucrose, 50 mM tricine-NaOH, pH 8.2; 20 mM NaCl; 4 mM $MgCl_2$ and 10 mM K_2HPO_4. Chlorophyll concentration generally was about 3 mg/ml.

Spinach chloroplasts were prepared from preilluminated leaves. Freshly harvested leaves were put into a 500 ml beaker with some ice-water and illuminated with gentle stirring with a 100-W bulb for approx. 30 min; then 15 g were homogenized for 2 sec in a Sorvall Omnimixer in 40 ml of the cold homogenization medium used for *Bumilleriopsis*. The homogenate was filtered through 4 layers of cheesecloth and 1 layer of nylon tissue (40 μm mesh) and centrifuged for 1 min at 4000 × *g*, the pellet then suspended in the "suspension medium" noted above.

For preparation of photosystem I particles from *Bumilleriopsis* the pH of the chloroplast suspension was increased with NH_4OH to 8.9 and 0.5 ml of pyridine added per 100 mg of Chl [9]. After 30 min of stirring (at + 4 °C) the suspension was sonified for 5 min with a Branson sonifier (power setting at 6; with a cooled continuous flow cell; see under chapter 2/a). Then, Tween 20 was added in a final concentration of 1% (w/w). After 20 min of stir-

ring, then centrifuging for 1 hour at 50 000 × *g*, the pellet was discarded and the supernatant centrifuged again for 30 min at 50 000 × *g*. The supernatant contained the PS-I particles which were spun down for 1 hour at 144 000 × *g*. The pellet was resuspended in 0.05 M tricine-NaOH, pH 8.0, and passed through a Sephadex G-75 column (3 × 60 cm, elution with the same buffer). The main chlorophyll containing fractions were combined, concentrated about 4:1 in a cold glycerol-water mixture (1:1 v/v), and stored at −20 °C without loss of activity for about 6 months (= pyridine-Tween 20-chloroplast particles, photosystem-I particles).

The photosystem-I particles from spinach were prepared according to the method of Siedow *et al.* [9] and eventually concentrated and stored like the *Bumilleriopsis* particles.

For sonification, the *(Bumilleriopsis)* chloroplast suspension was adjusted to 0.2 mg Chl/ml and treated successively in 10 ml portions for 4 min with the microprobe of a Branson sonifier (see above, power setting at 3). The sample was cooled in an ice-NaCl mixture; the temperature should not rise higher than 10 °C. 2 × 10 ml of the sonified sample were mixed with 120 ml of ice-cold 2.5% NaCl solution and centrifuged for 15 min at 250 000 × *g* (at the bottom of the vials; Beckman L2-65B). The pellet was evenly suspended in the chloroplast suspension mixture (see above) and adjusted to 2 mg Chl/ml.

d. Preparation of antibodies

2 mg (= 0.5 ml) of *Scenedesmus* plastocyanin or *Bumilleriopsis* cytochrome 553, respectively, were mixed with 1 ml of borate buffered saline (NaOH 0.9 g; H_3BO_3 10.3 g and NaCl 7.8 g per liter) and 1.5 ml complete Freund's adjuvants. This was injected partly into the muscles of the back, the hind leg and under the skin of the neck of a rabbit (bought in the local market). After 3 days the procedure was repeated, after 3 – 4 weeks a booster shot with 3 mg of protein and incomplete adjuvans was given in the same manner. After a further week the titer was checked by the Ouchterlony technique, and repeated a week later. By then, generally, the titer had reached its maximum and the animals were sacrificed, and as much blood as possible was taken from the neck vein. After standing for about 4 hours at room temperature, the blood was centrifuged for 10 min at 4000 × *g* and the supernatant mixed with an aliquot of 0.9% NaCl solution buffered with 0.02 M phosphate, pH 7.0. After cooling to 0 °C, 20 ml of saturated ammonium sulfate was added dropwise with stirring to 20 ml of the antiserum and the mixture stirred for one further hour in an ice bath. Then, after centrifugation for 20 min at 10 000 × *g*, the precipitated antiserum was resuspended in less than 10 ml of 0.9% NaCl (including 0.02 M phosphate buffer, pH 7.0) and stored at −20 °C after a 3 – 4 hours dialysis against the same medium.

e. KCN treatment of the chloroplast was carried out according to a slightly modified procedure of Izawa *et al.* [12]: 0.1 ml of chloroplasts (with 0.3 mg of Chl) were mixed with 0.9 ml of an ice-cold medium consisting of 30 mM KCN; 0.2 M tricine-NaOH, pH 7.9; 1 mM $MgCl_2$; 60 μM K-ferricyanide and 0.1 M sucrose. The control samples contained 0.2 M tricine-NaOH, pH 8.2. After standing for the time indicated at 0 °C, aliquots of this preincubation mixture were added to the reaction medium indicated in the legend of Fig. 5.

For amphotericin preincubation, 0.1 ml of the chloroplast suspension (see part 2 c) was mixed with 0.9 ml of 0.2 M tricine-NaOH, pH 8.2, including 1 mM $MgCl_2$ and 0.1 M sucrose. A 0.5 ml fraction there of was mixed with 10 μl of 20 mM amphotericin B (dissolved in dimethylformamide-HCl) and incubated at 20 °C [41].

3. Instruments

Cytochrome f photooxidation was measured with an Aminco-Chance dual wavelength spectrophotometer (model DW-2 of American Instruments, Silver Spring, Md.). A cuvette with a 3 ml volume had a magnetic stirring device and could be illuminated by an actinic light source. This was a 40-W Unitron lamp (Unitron Instruments Company, Newton Highlands, Ma.) with light defined by a red filter (Schott RG 630, 2 mm) and additional interference filters (Balzers Filtraflex) giving monochromatic light of 652 and 707 nm, respectively; see also legend of Fig. 7. The end-on photomultiplier was protected against scattered light by a Filtraflex DT green filter and a Corning blue-green filter No. 9788. For further instrumental details see legends.

4. Chemicals

General chemicals and buffers were from Merck AG, Darmstadt; "pro analysi" quality was used throughout, including those for algae cultures. Cytochrome c and methylviologen were from Serva, Heidesberg; amphotericin B from Calbiochem, Los Angeles, USA. The detergents were from Sigma, St. Louis, Mo., USA.

Results

Fig. 1 demonstrates the principle for comparative isolation of plastocyanin and algal cytochrome f.

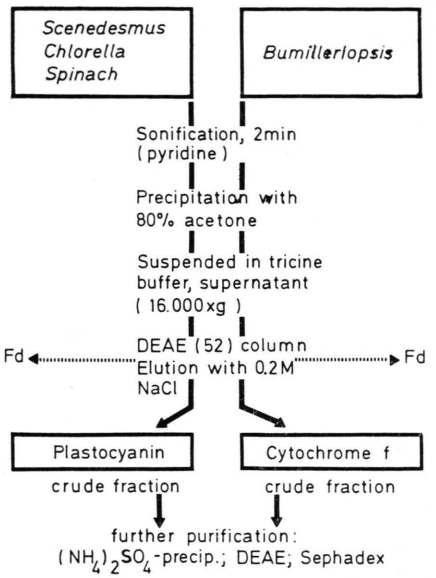

Fig. 1. Scheme for comparative isolation of plastocyanin and cytochrome f from different algae and spinach. For explanations and certain deviations from this scheme see "Materials and Methods".

The first two preparative steps in Fig. 1 are to be considered as possible alternatives. With the three species (noted in the upper left) plastocyanin isolation was successful regardless of certain modifications of the procedure as described in detail in the previous section.

The addition of pyridine and sonification of the chloroplast material proved to be successful to extract Pc completely, although with pyridine the yield of the pure protein was only approx. 10% higher than treating the chloroplasts with either 80% acetone or by sonification alone. This was found with *Spinacia*, *Chlorella* and *Scenedesmus*. Therefore, spinach photosystem I particles prepared according to the "pyridine method"[9] did not exhibit a light-induced 554 nm-change without plastocyanin added (see Fig. 8), as was already reported[9]. Also prolonged sonification of spinach chloroplasts (up to 4 min) did not increase the yield of plastocyanin, nor did addition of pyridine plus detergents (*e.g.* 1% Tween 20) *and* sonification combined.

Spinach, *Chlorella* and *Scenedesmus* (treated in detail as described under "Methods") yielded plastocyanin but practically no soluble cytochrome f could be isolated in addition. Also Gorman and Levine[22] reported a very small yield of the latter protein from *Chlamydomonas* (using the acetone treatment of the cells). We obtained completely different results with *Bumilleriopsis*. Treated in the same manner, Pc could never be isolated but rather the soluble cytochrome 553 (= cytochrome f from *Bumilleriopsis*, see Lach *et al.*[25, 42]) was found thereby, in a molar ratio to Chl a of approx. 1:300, substantiating the figure reported[25]. This lack of Pc was also repeatedly confirmed with considerable preparative modifications as outlined above.

Seemingly, the chloroplasts of this alga have no plastocyanin under the culture conditions mentioned. In Fig. 2 the lack of plastocyanin is further evidenced. Photosystem-I particles supplemented with aliquots of the "crude fraction" (of Fig. 1) cannot catalyze the photooxidation of reduced mammalian cytochrome c when the cytochrome 553 antibody is added (tracings I and II) whereas a Pc antibody had no effect (III). Care was taken to be sure that the

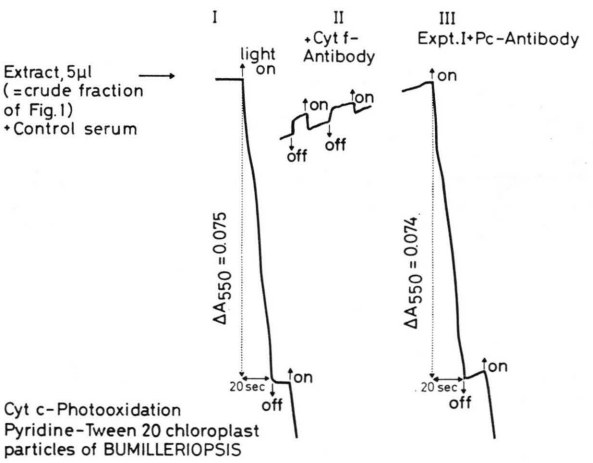

Fig. 2. Influence of antibodies on the chloroplast extract of *Bumilleriopsis* (= "crude fraction", see Fig. 1) measured by photooxidation of Cyt c. Antibodies were from *Bumilleriopsis* cytochrome 553 and *Scenedesmus* plastocyanin. The reaction mixture contained in a final volume of 1 ml: 10 µmoles of phosphate buffer pH 7.0; 0.03 µmole of reduced mammalian cytochrome c; 0.05 µmole of DCMU; 0.02 µmole of methylviologen, and subchloroplast particles from *Bumilleriopsis* equivalent to 5 µg of chlorophyll. The reaction was performed in open cuvettes placed in a Hitachi/Perkin-Elmer spectrophotometer (model 124) equipped with a 40-W Unitron lamp, its light focused as actinic light source (side illumination; wavelength >610 nm defined by a 2 mm Schott cut-off filter including a 2 mm KG1 heat filter. Saturating light intensity at the cuvette was $5-8 \times 10^6$ ergs/cm^2·sec). Decrease of optical density was followed for 3×20 sec on a Hitachi recorder, model 56M, with an absorbance diplay down to 0—0.1; temperature 20—22 °C. The cytochrome c was reduced beforehand with some solid sodium dithionite, and the excess removed by 1 min of aeration (Plesničar and Bendall[44]).

reaction was completely uninhibited also with a manifold excess of antibody against plastocyanin. The result was obtained using either an antibody against the plastocyanin from *Scenedesmus* (as shown) or spinach. It is generally observed (*e. g.* with antibodies against the Fd-NADP reductase from different plant and algal species; unpublished results) that an antibody never has such a marked specificity as not to inactivate its antigen somewhat regardless of its plant origin. So, if there were Pc present in the "crude fraction" of *Bumilleriopsis*, the photooxidation should at least have been *partially* inhibited. – The amount of antibody necessary for complete inhibition was determined beforehand as demonstrated in Fig. 3 (with limiting concentration of Pc or Cyt 553, respectively).

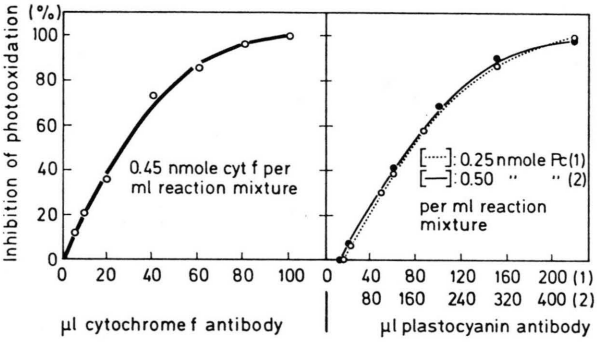

Fig. 3. Titration curves of cytochrome 553 and plastocyanin with their respective antibodies. The degree of antigen inactivation was measured by cytochrome c photooxidation as described in the legend of Fig. 2. Rate of the (uninhibited) control was approx. 30 μmoles of cytochrome c oxidized per mg Chl and hr (see Fig. 6). The reaction with Pc was checked with two concentrations of antigen and antibody, respectively.

Furthermore, it is important to note that the rate observed in Fig. 2 with the 5 μl aliquot of the "crude fraction" corresponds to only 0.002 μmole of cytochrome 553. This protein concentration (of 2 μM) in the assay is far from the saturating level giving a rate of only 142 μmoles cyt c oxidized/mg Chl x hour, which was also obtained with the same concentration of highly purified cytochrome 553 as is shown in Fig. 6. As pointed out there, a cyt c photooxidation rate due to Pc simply adds up to a rate catalyzed by Cyt 553. So, in the experiment of Fig. 1, the activity measured was due *only* to the heme protein present in the "crude fraction" checked.

The epr spectra given in Fig. 4 corroborate this conclusion: Lack of plastocyanin in the supernatant

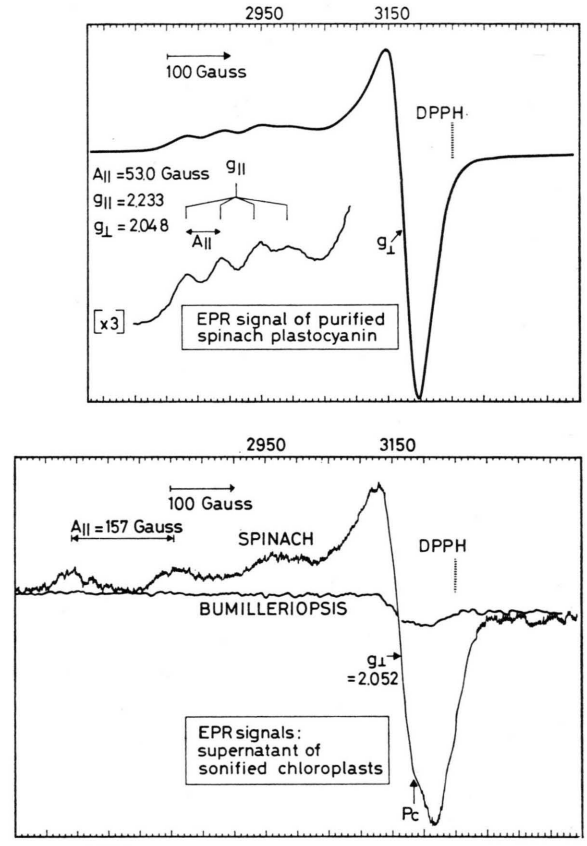

Fig. 4. EPR signal of purified spinach plastocyanin and of the supernatant of sonified chloroplasts (see Fig. 1 and preparative methods) from spinach an *Bumilleriopsis*. The epr tube contained in a 0.4 ml volume either 46 nmoles of spinach plastocyanin or the supernatant of a chloroplast suspension equivalent to 1.5 mg of chlorophyll. 0.25 mM ferricyanide was included in all samples. The instrument was a VARIAN E-3 with a temperature control unit V-6040; temperature 109 °K; field span 3000 ± 500 Gauss; microwave frequency approx. 9.12 GHz; microwave power 20 mW; modulation frequency 100 KHz and amplitude 10 Gauss. Time constant 1 sec; scan rate 125 Gauss/min; receiver gain 5×10^{-4} (times 3, see inset). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used as a resonance marker.

of sonified *Bumilleriopsis* chloroplasts is opposed to the observation of the typical signal for Pc in the corresponding extract from spinach [43] (this was also the case when detergents or pyridine were present additionally). The Pc signal of this crude fraction, however, is not completely identical with that of the pure spinach Pc. The typical narrow hyperfine splitting constant for the pure protein has disapparead, giving way to the broad one generally observed with complexes containing the copper in an ionogenic form. So, it appears that besides native Pc there was additional copper in the

sample [44, 45], which was also indicated by the signal heterogeneity in the signal minimum (see arrow). The amount of Pc calculated was about 1 mole per 200 moles of Chl, which is close to the figure reported [44] but about twice as high as obtained by our outlined preparation procedure. Most authors, however, noted a ratio of Chl: Pc of about 500:1 (see *e. g.* l. c. [14]).

From preliminary epr experiments with chloroplast *particles* the same results can be concluded as from the findings just mentioned above. Detailed studies (using lower temperatures) are under way in this laboratory.

As reported long ago [46], and verified recently by the most elegant studies of the Good group [11, 12], KCN reacts quite readily and specifically with plastocyanin. Therefore, the experiment represented by Fig. 5 was undertaken with a result expected

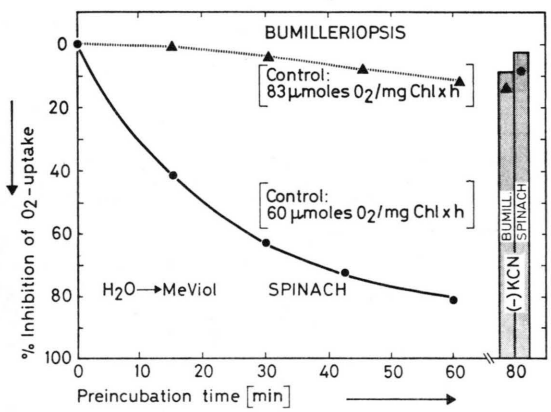

Fig. 5. Light-induced O_2-uptake by KCN treated chloroplasts, mediated by methylviologen with water as electron donor. At the intervals indicated 0.2 ml of the preincubation mixture was added to 2.8 ml of reaction mixture consisting of 0.2 M tricine-NaOH buffer, pH 8.2, 1 mM $MgCl_2$, 0.1 M sucrose, 0.15 mM methylviologen and 0.15 mM sodium azide. Final chlorophyll concentration was always 60 µg/3 ml. Reaction was followed with a YSI-oxygen electrode device equipped with a Rikadenki B-161 recorder. Light source was a 100 W iodine lamp (from Spindler & Hoyer, Göttingen) with filters as mentioned in the legend of Fig. 2; light intensity was about 4.8×10^5 ergs/cm²·sec. Total reaction time did not exceed 2 min.

from the foregoing data. The electron transport in spinach chloroplasts was markedly affected by preincubation with approx. 30 mM KCN, whereas with *Bumilleriopsis* chloroplasts decrease of activity was little less than the control and due to some inhibition of PS-II activity.

The corresponding experiment with amphotericin B also gave analogous results (Table I): No substantial effect on the electron transport with *B.*-chloroplasts but severe inhibition of spinach plastids. That is: No plastocyanin is present in *Bumilleriopsis*.

Table I. Light-induced O_2-uptake with methylviologen and water as electron donor by chloroplasts preincubated for 60 min with amphotericin B. The assays contained 2% dimethylformamide. For the preincubation see "Materials and Methods", for reaction mixture and experimental details legends of Fig. 5.

Additions	(1) µmoles O_2-uptake/ mg Chl × h * Preincubation time 0 min	(2) 60 min	(3) Inhibition in % of control (=1)
Spinach			
(−) Amphotericin	60	49	18
(+) Amphotericin	60	27	55
Bumilleriopsis			
(−) Amphotericin	83	68	18
(+) Amphotericin	83	68	18

* $H_2O \rightarrow$ MeViol.

Plastocyanin, although absent, nevertheless is a potent redox carrier when tested with *Bumilleriopsis* subchloroplast particles. In Fig. 6, it is demonstrated how effective the cytochrome c photooxidation could proceed with either cytochrome 553 or plastocyanin (from *Scenedesmus*). Half the maximum velocity was attained when the cytochrome was substituted by the copper protein. It should be pointed out that Cyt c photooxidation with sonified *Bumilleriopsis* chloroplasts or particles is strictly dependent on the addition of either one of the two redox proteins (comp. Fig. 2).

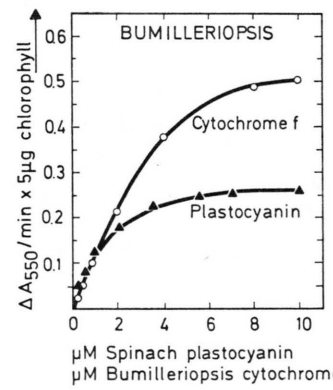

Fig. 6. Dependence of cytochrome c photooxidation by photosystem-I particles of *Bumilleriopsis* on purified cytochrome 553 (cytochrome f) and plastocyanin. The assay was performed as described in the legend of Fig. 2. $\varepsilon_{\text{cyt c, 550}}$ (reduced minus oxidized) = 19.0 (mM^{-1} cm^{-1}).

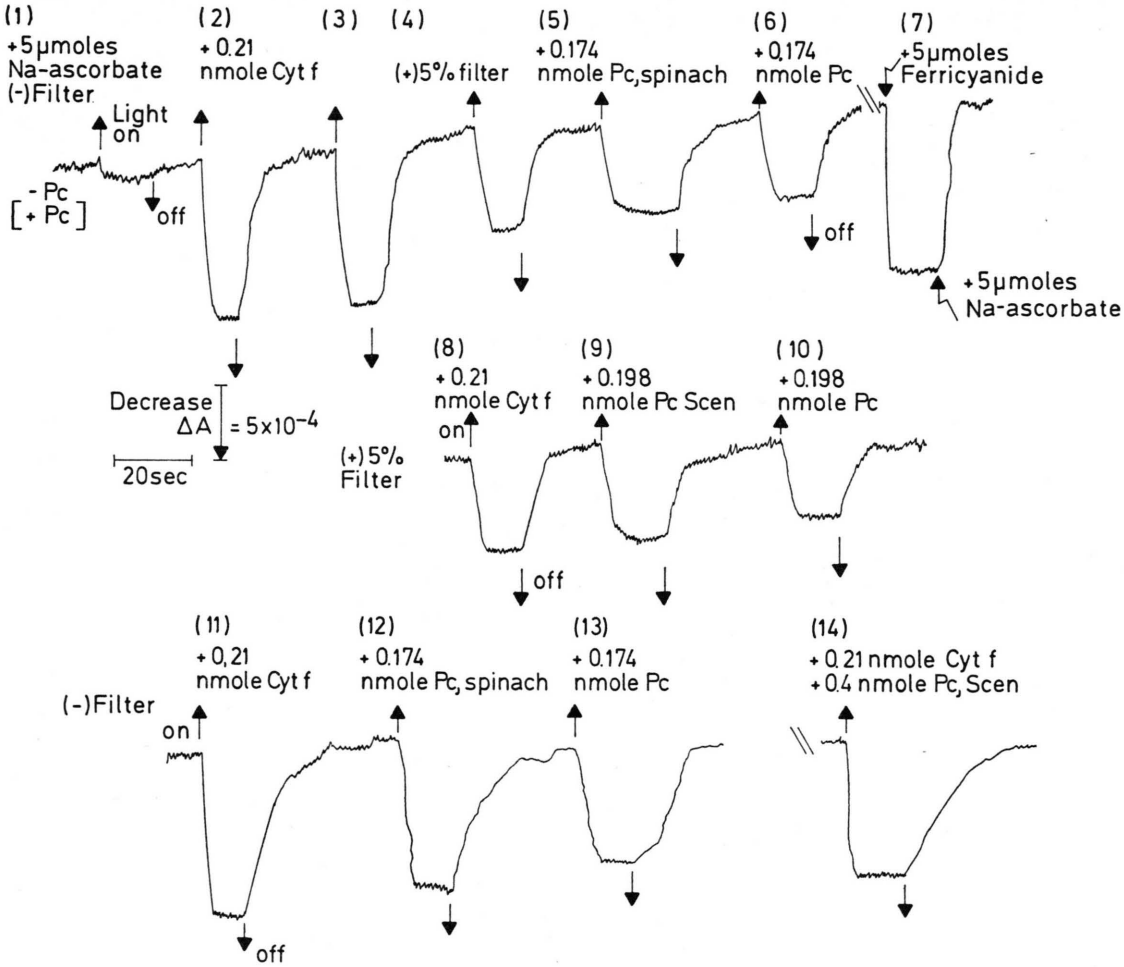

Fig. 7. Absorbance changes of cytochrome 553 (= Cyt f) with sonified *Bumilleriopsis* chloroplasts. The basal reaction mixture had a final volume of 3 ml containing: 0.2 ml sonified chloroplasts equivalent to 90 μg of chlorophyll and 2.8 ml of 0.2 M tricine-NaOH, pH 8.2, including: 0.1 M sucrose; 0.1 M $MgCl_2$; 0.05 mM methylviologen. Subsequent additions are indicated in the figure. Absorbance changes were determined at 553 nm with 541 nm as reference with the AC spectrophotometer. Ambient temperature was 25 °C. The sample was illuminated with 707 or 652 nm light both of 0.8 to 1×10^5 ergs/cm^2·sec when no (intensity) filter was inserted (the 5% filter diminished the intensity by 95%). Speed: 1 inch/sec. The ΔA span indicated corresponded to a tenth of the full paper width of the recorder. Tracing No. (1) was identical with either plastocyanin present or not. The sonified chloroplasts had a molar ratio of P700: Chl = 1:230; *i.e.* 0.39 nmole of P700 was present in the assay. For determination of P700 the extinction difference at 702 nm was measured between a ferricyanide and Na-ascorbate treated sample according to Wild *et al.* [50] using a differential extinction coefficient of 64 mM^{-1}·cm^{-1} (Hiyama and Ke [51]); instrument: Hitachi/Perkin-Elmer spectrophotometer, model 124, with a recorder display of 0 to 0.1 absorbance. The differential millimolar extinction coefficient, reduced minus oxidized, for *Bumilleriopsis* cytochrome f at 553 and 541 nm was determined to be 15.0 (mM^{-1}·cm^{-1}) from tracing No. (7); complete oxidation and reduction of the cytochrome was verified by separate spectral analysis.

Figs 7 and 8 demonstrate data describing cytochrome f photooxidation. In contrast to spinach, sonified *Bumilleriopsis* chloroplasts did not exhibit any light-induced change at 553 nm with either Pc added or not (Fig. 7, tracing No. 1). These particles contained no traces of cytochrome f (= Cyt 553), as was proven by spectral assessment. In tracings Nos. 2 and 3 cytochrome 553 was added in approx. the same amount as P700 present. The rapid photooxidation was complete (as seen by the identical extent of the deflections Nos. 2, 3, and 7, respectively; the latter demonstrating the redox reactions done by chemical means). The reaction was completely reversible in the dark; 707 nm light could be substituted by 650 nm light without changing the deflections. It is clear, therefore, that no transport

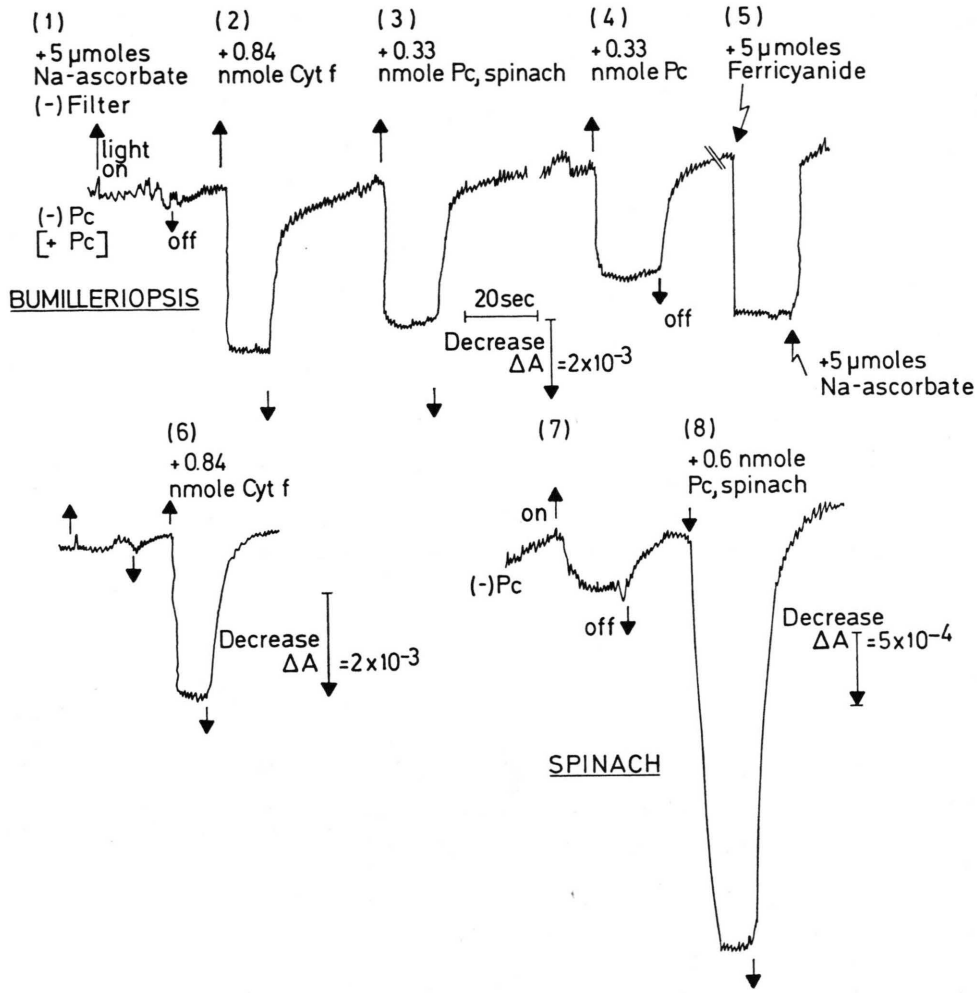

Fig. 8. Absorbance changes of cytochrome f with photosystem-I particles of *Bumilleriopsis* and spinach. Algal subchloroplast (pyridine-Tween 20) particles were prepared as noted in "Materials and Methods", those from spinach according to Siedow *et al.* [9]. The reaction mixture contained in a final volume of 3 ml: 0.05 M tricine-NaOH, pH 8.0; 0.05 mM methylviologen and particles equivalent to 130 µg of chlorophyll in the tracings Nos. (1) to (5) and 110 µg of Chl with spinach chloroplast particles (Nos. 7, 8); the assay with the latter also contained 0.125% digitonin. Subsequent additions are noted in the figure. For measurements see legend of Fig. 7; spinach particles were checked at 554 nm (with 540 nm light as reference. For the differential extinction coefficient see Nelson and Neumann [49]). *Bumilleriopsis* PS-I particles had a molar ratio of P700: Chl = 1: 65, *i. e.* 2 nmoles of P700 were present in the assay of Nos. (1) to (5) and 0.84 nmole in No. (6). Methylviologen could be omitted without altering the deflections observed, as was the case with plastocyanin in tracing (1).

of electrons from photosystem II to cytochrome 553 was operating, otherwise the extent of the photooxidation would have been smaller. A decreased deflection was observed when the actinic light was diminished by a 5% filter (tracing No. 4 – 6). – Surprisingly, the tracings Nos. 5 and 6 indicated a substantial inhibition of cytochrome 553 photooxidation (about 13 and 18%) due to spinach Pc added. This was observed also with *Scenedesmus* plastocyanin although the effect was smaller (Nos. 8 – 10). With high intensity actinic light the inhibition was even higher (Nos. 11 – 14), approx. 30% with 0.35 nmole Pc added. This is completely different from the finding with spinach chloroplast (particles). With those, Pc is an essential component to achieve the light-induced 554 change of cytochrome f (Fig. 8, Nos. 7, 8); in the case of higher plant chloroplasts the latter is still present in the subchloroplast particles, thereby confirming data of previous reports [7–9]. The other tracings in Fig. 8 show that photosystem-I particles from *Bumilleriopsis* (prepared with Tween 20 and pyridine)

yielded principally the same data as was explained in Fig. 7 with sonified algal chloroplasts. Almost a 100% cytochrome photooxidation was observed with P700 present either 2.5 times in excess of the heme protein (tracing No. 2) or in the same concentration (No. 6).

So, in conclusion, the experiments reported above with chloroplasts and subchloroplast particles from *Bumilleriopsis* reveal a fundamental deviation from the corresponding ones for spinach. Plastocyanin is not only not a necessary redox component but interferes with cytochrome 553.

Discussion

Data showing the lack of a component within the thylakoid membrane are, of course, more susceptible to critical objections than positive indications. So, for example, we cannot exclude the possibility that the amount of Pc present in this alga is so small that it escaped detection, being nevertheless of obligatory functional importance. This is highly improbable, however, since these are data accumulating indicative for the occurrence of functionally linked redox carriers in a molar ratio of 1:1 (or higher) based *e. g.* on the Cyt f content. This was found in *B.*-chloroplasts for the Fd-NADP reductase, Fd and Cyt b 559 ([48]; Böger, unpubl.) and also by Wild and coworkers in chloroplasts of Sinapis [47] (comp. [44, 49]). Furthermore, if there were traces of functionally active Pc in our sonified chloroplasts or particles, one should not expect a 100% photooxidation of added cytochrome 553 in the presence of ascorbate. This would imply an improbable rapid turnover activity of this hypothetical small amount of the copper protein.

As found with cytochrome 553, however, the method of extraction is quite critical. Deviations from the procedures described generally left some Cyt f (10%) in the membrane, which could always be detected *e. g.* by the Cyt c photooxidation assay. These traces were found when the sonification time and power were decreased or the pyridine omitted. These procedures had no deleterious effect on Pc, and we are inclined to conclude that it should have been detected by our assays if it were present, since we have amply demonstrated (Figs 3 and 6) that Pc is quite active in redox reactions associated with PS-I.

Spinach subchloroplast particles contain Cyt f which (when adding Pc) undergoes photooxidation only to 12 – 14% [8] or 50 – 60% in our case, as was also reported by Siedow *et al.* [9]. This incomplete reaction may be due to some denaturation of the heme protein (or the PS-I reaction center). *Bumilleriopsis* particles and sonified chloroplasts gave no deflection with Pc added but instead they had to be supplemented with Cyt 553, before being subsequently photooxidized to 100%. This indicates that the reaction site of PS-I is neither impaired nor occupied by some (inactivated) inhibiting redox component. (The chloroplast particles, however, contain b-cytochromes, which will be reported elsewhere.) – Furthermore, the inhibition of Cyt 553 photooxidation by small amounts of Pc gives strong evidence that there is no Pc in the chloroplast material tested. This plastocyanin inhibition is of a competitive type *vs* cytochrome. Both redox proteins seemingly introduce their electrons at the same site of the PS-I system. Therefore, Cyt c photooxidation is stimulated in an *additive* manner when both redox carriers are given together; apparently both are also easily reduced by the excess of Cyt c employed.

It should further be noted that gently sonified *B.*-chloroplasts do not need the addition of any detergent to let the Cyt-553 photooxidation go. So, with *Bumilleriopsis* the objections are not valid against possible artefacts caused by the action of detergents [14]. Particles from higher plant chloroplasts need the use of detergents either in the reaction mixture [7–9, 44] or during preparation of the particles (own findings). Furthermore, in contrast to spinach [8, 9], *Bumilleriopsis* subchloroplast particles are enriched in P700 (see legend of Fig. 8).

With respect to PS-I photoreactions dealt with herein *Bumilleriopsis* chloroplasts possess some rather unique properties:

1. They are devoid of Pc, and gentle sonification yields particles which have no traces of cytochrome f either. This is hardly attainable with spinach chloroplasts [8].
2. Cytochrome f (Cyt 553) is photooxidized to approx. 100% when added in a molar ratio of $\geqq 1$ to P700.
3. No detergent is needed for this photooxidation.
4. Subchloroplast particles are enriched in P700 *vs* chlorophyll.

Furthermore, our findings from comparative functional assays demonstrate that data from higher plant chloroplasts should be transferred with some caution into general schemes of photosynthetic electron transport.

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