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THE PLASTOCYANIN CONTENT OF CHLOROPLASTS FROM SOME HIGHER PLANTS ESTIMATED BY A SENSITIVE ENZYMATIC ASSAY

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SUMMARY

1. A sensitive enzymatic assay of plastocyanin is described. The method is based on the stimulation of cytochrome *c* photo-oxidation in detergent-treated chloroplasts. Under appropriate conditions the rate of reaction is proportional to the amount of plastocyanin present and as little as 0.5 natom plastocyanin-copper may be determined in chloroplast samples.

2. The method is specific for plastocyanin when applied to sonic extracts of pea chloroplasts.

3. The plastocyanin content of chloroplasts from various higher plants varied from 2.0 to 9.0 natoms copper per mg chlorophyll whereas cytochrome *f* was present in quantities 2–5 times less (on a haem basis).

4. The total copper content of chloroplasts from most plants tested was in excess of the plastocyanin. In the case of barley chloroplasts the total copper was equal to the plastocyanin copper. We conclude that plastocyanin is likely to be the only copper compound involved in photosynthetic electron transport.

INTROCUCTION

The copper protein plastocyanin, since its discovery by KATOH[1] in the green alga *Chlorella ellipsoidea*, has been reported to be a component of several plants[2]. The occurrence of plastocyanin has always been connected with green, photosynthetic plant cells and said to be entirely absent from the chlorophyll-free tissues[3].

NIEMAN AND VENNESLAND[4], BISHOP *et al.*[5] and NIEMAN *et al.*[6] had already shown that photo-oxidation of reduced cytochrome *c* in detergent-treated chloroplasts requires a chlorophyll-containing particle as well as a soluble protein that was later identified by KATOH AND TAKAMIYA[3] with plastocyanin. Plastocyanin was found by the latter authors to be necessary for the photochemical reduction of $NADP^+$ by photosystem I particles[7]. The role of plastocyanin in cytochrome *c* photo-oxidation, according to KOK *et al.*[8] is to mediate electron transfer from cytochrome *c* to P700. KOK *et al.*[8] showed that the overall rate of reaction, with O_2 as final acceptor, was greatly stimulated by an auto-oxidizable dye such as methylviologen.

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The plastocyanin content of chloroplasts has been determined by spectrophotometric assay of a partially purified chloroplast extract[9]. This method requires rather large quantities of material. In the present paper a sensitive enzymatic assay of plastocyanin, based on the stimulation of cytochrome *c* photo-oxidation, is described. We have compared the amounts of plastocyanin, cytochrome *f* and copper in chloroplasts from several plants.

MATERIALS AND METHODS

Plant material

Pea and barley plants were grown in the greenhouse at a minimum temperature of 18°, with additional artificial light (14-h day) in winter and shading in summer. Etiolated barley plants were grown for 8 days in the dark, at 20°. Spinach leaves were obtained from Covent Garden Market. Orache (*Atriplex hortensis*) and parsley, garden-grown, were kindly provided by Dr. R. Hill. Tobacco seeds were obtained by kindness of Drs. H. Gaffron and R. Hill. Green tobacco is the cigar variety John William Broadleaf (*Nicotiana tabacum* L). Pale tobacco is its aurea mutant Su/su, which emerged from the same seed population[10].

Chloroplast preparation

Spinach and pea chloroplasts were prepared by a method similar to that of WALKER[11], by blending (4–7 sec) in a medium containing 0.33 M mannitol, 10 mM KH_2PO_4, 5 mM NaCl and 1 mM $MgCl_2$ (pH 7.5). The homogenate was squeezed through two layers of muslin, followed by gravity filtration through eight layers of muslin. Chloroplasts were sedimented by centrifuging for 1 min at 2000 × *g* (at r_{max}), washed and resuspended in the same medium. The chloroplast suspension contained 3–5 mg chlorophyll per ml.

To prepare broken plastids from barley leaves, 50 g freshly harvested leaves were ground with mortar and pestle, with 4 times their weight of the above medium containing 0.1 % bovine serum albumin. The homogenate was filtered as above and centrifuged for 30 sec at 2000 × *g*. Plastids were collected by centrifuging the supernatant for 15 min at the same speed, washed and resuspended. All operations were performed at 0–4°.

The chlorophyll content of chloroplast preparations was determined spectrophotometrically by the method of ARNON[12].

Plastocyanin preparation

The method of plastocyanin preparation from parsley leaves (from which it is more readily obtained in solution than from spinach) has been developed in this laboratory by R. HILL and coworkers by the application of procedures developed by SAN PIETRO AND LANG[13] and KATOH[1]. The first part of the method separates plastocyanin, ferredoxin and ferredoxin–$NADP^+$ reductase in crude form; the second part is the purification of the crude plastocyanin by chromatography on DEAE-cellulose.

Washed leaves were homogenized in 1-kg batches in a 1-gallon Waring blendor in the presence of 900 g crushed ice, 100 ml 1 M Tris–0.8 M HCl buffer (pH 7.6), 1000 ml cold acetone (−15°). After homogenising for 1 min at medium speed the pulp was squeezed through four layers of muslin and centrifuged for 30 min at 1000 × *g* (r_{max})

in an MSE Major centrifuge at 0°. The supernatant after centrifuging was treated with 1.16 vol. of cold acetone with vigorous stirring. The suspension was allowed to stand in a cold room to allow the precipitate to settle, the supernatant was sucked off and the loose sediment centrifuged for 20 min at 1000 × *g*. The supernatant was decanted and the precipitate well mixed with a small volume of Tris–HCl buffer. Some protein dissolved but much insoluble material remained. This suspension was dialysed overnight against 0.06 M Tris–0.05 M HCl buffer (pH 7.6). Insoluble material was removed by centrifuging and the supernatant, containing plastocyanin in reduced form, ferredoxin, ferredoxin–$NADP^+$ reductase, *etc.*, was applied on a column of DEAE-cellulose (Whatman DE 23), 3 cm in diameter and 13 cm. in height, previously equilibrated with 0.06 M Tris–0.05 M HCl buffer (pH 7.6). The charged column was washed with 0.31 M Tris–0.25 M HCl solution. The flavoprotein enzyme was eluted slightly ahead of plastocyanin, which was identified in the eluate after the addition of potassium ferricyanide solution by the appearance of blue colour due to oxidized plastocyanin. Ferredoxin was eluted from the column with 0.5 M Tris–0.4 M HCl buffer (pH 7.6).

The crude plastocyanin preparation was applied to a DEAE column (Whatman DE 23, 2 cm in diameter and 10 cm in height) previously equilibrated with 0.02 M phosphate buffer (pH 6.9). Most of the flavoprotein enzyme was eluted with 0.05 M phosphate buffer. Elution of plastocyanin was started with 0.1 M phosphate buffer and continued with 0.2 M phosphate. The combined fractions containing reduced plastocyanin were diluted to a phosphate concentration of 0.05 M and chromatographed again on a similar column of Whatman DE 52 cellulose. Elution was performed with 0.1 M phosphate buffer.

In the final stage plastocyanin was purified in the oxidized form, after addition of a small excess of $K_3Fe(CN)_6$. The oxidized plastocyanin solution in 0.03 M phosphate was applied to a DEAE-cellulose column (2 cm × 20 cm Whatman DE 52) and eluted with 0.1 M phosphate. The absorbance of the fractions was determined at 278 and 597 nm[14], and fractions with $A_{278\,nm}/A_{597\,nm}$ less than 2.0 were pooled. The plastocyanin was concentrated by elution from a small column of DEAE-cellulose with 0.2 M phosphate. This yielded a solution containing 300 μM plastocyanin copper with $A_{278\,nm}/A_{597\,nm} = 1.6$. Absorbance at these two wavelenghts did not change significantly during storage at −15° for one year.

Estimation of plastocyanin in plastid preparations

Plastocyanin was determined by an enzymatic assay of the supernatant fraction after centrifuging sonically disrupted chloroplasts. For sonication, chloroplasts (approx. 0.5 mg chlorophyll) were resuspended in 5 ml 0.01 M phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$ and 10 mM NaCl. The conditions of sonication appear to depend on the type of sonicator, geometry of the system and density of suspension. With the Dawe "Soniprobe" type 1130A, probe 0.5 inch in diameter, we have found that 1 min sonication at 5 A is adequate for efficient breakage of membranes and reproducible extraction of plastocyanin. In most cases extraction of plastocyanin was complete in 15 sec. During sonication the temperature of the sample was kept below 10° by cooling the vessel in an alcohol–solid CO_2 bath. After sonication the sample was centrifuged 100 000 × *g* for 3 h. The clear and colourless supernatant was decanted and tested for cytochrome *c* photo-oxidase activity.

Cytochrome *c* photo-oxidation has been followed spectrophotometrically at

550 nm. The following reagents were added to a 1-cm spectrophotometer cuvette: 0.08 μmole reduced cytochrome *c*, 15 mg digitonin, the sample for assay or standard plastocyanin (0.3–2.0 nmoles) and 10 mM phosphate buffer (pH 7.0) to a final volume of 3 ml; these were followed by 5 μl 0.6 M KCN, 10 μl 40 μM 3(3,4-dichlorophenyl)-1,1-dimethylurea, 5 μl methylviologen chloride (1 mg/ml) and 10 μl pea chloroplasts containing 4.5 μg chlorophyll. The phosphate buffer contained 1 mM $MgCl_2$ and 10 mM NaCl. Cytochrome *c* dissolved in this buffer was reduced with a small excess of $Na_2S_2O_4$ which was then removed by aeration. Digitonin (Analar, British Drug Houses, Ltd.) was dissolved by warming to give a 1 % solution in the same buffer.

The sample in a spectrophotometric cuvette was illuminated intermittently through a red glass filter and a water filter 15 cm thick by means of a 500-W tungsten lamp projector. The total time of illumination (15–60 sec at each exposure) was dependent on the rate of cytochrome *c* photo-oxidation. The absorbance changes caused by illumination were measured with a Unicam SP 500 spectrophotometer.

Cytochrome f

Cytochrome *f* was determined by direct spectrophotometry of the chloroplast preparation with a sensitive split-beam spectrophotometer made in the Johnson Research Foundation, University of Pennsylvania. Optical separation of cytochome *f* from other cytochrome components was achieved by plotting a difference spectrum for samples treated with hydroquinone and ferricyanide in the presence of the non-ionic detergent Triton X-100. The method is based on the measured oxidation–reduction potentials of chloroplast cytochromes[15] and the observation that treatment of chloroplasts with Triton X-100 converts the cytochrome *b*-559 of high oxidation potential ($E_0' = +0.37$, pH 7) into a compound of lower potential that is no longer reducible by hydroquinone. Triton X-100 has no significant effect on the spectrum either of purified cytochrome *f* (H. E. DAVENPORT, personal communication) or of cytochrome *f* in chloroplasts. The chloroplast preparation was diluted in a medium containing 0.33 M mannitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 50 mM potassium phosphate buffer (pH 6.5) and 1 % (w/v) Triton X-100 to give a concentration of about 650 μg chlorophyll in 4 ml. The preparation was divided equally between two 1-cm cuvettes and the base-line was drawn with the spectrophotometer. 5 μl 0.5 M hydroquinone was added to one cuvette, 5 μl 0.5 M $K_3Fe(CN)_6$ to the other and the difference spectrum determined between 580 and 520 nm. The concentration of cytochrome *f* was calculated from the height of the α-peak at 554 nm above a line drawn through the isosbestic points at 543.5 and 560 nm (D. S. BENDALL, unpublished results) for which a millimolar extinction coefficient of 19.7 $cm^2 \cdot mmole^{-1}$ (ref. 16) was used.

Copper content

The copper content of chloroplasts was determined by the colorimetric method of VAN DE BOGART AND BEINERT[17] by measurement of the absorbance of the bathocuproin complex in *n*-hexanol.

RESULTS

Comments on the method for plastocyanin determination

The extraction of plastocyanin by sonication of chloroplasts under the conditions given under MATERIALS AND METHODS is at least 90 % complete, as judged by the

activity of the supernatant and pellet after centrifuging. Extraction is less complete if the chlorophyll concentration is too high during sonication, even when more prolonged sonication gives no further improvement.

The time-course of photo-oxidation of reduced cytochrome *c* by plastocyanin in the presence of digitonin-treated chloroplasts is illustrated in Fig. 1. The reaction is linear with time at least for the initial period of 2 or 3 min. The rate of dark cytochrome *c* oxidation is practically zero and with low chlorophyll and plastocyanin concentrations the initial rate of reaction is proportional to the amount of plastocyanin present. The choice of chlorophyll concentration is limited by the sensitivity of the method on the one hand and the rate of spontaneous cytochrome *c* photo-oxidation on the other. In the presence of 4.5 μg chloroplast chlorophyll the initial rate of cytochrome *c* photo-oxidation is proportional to the plastocyanin concentration up to 2 nmoles of plastocyanin (Fig. 2).

The rate of cytochrome *c* photo-oxidation by purified plastocyanin depends on the quality and age of the chloroplast preparation. Therefore each experiment has its own calibration curve.

We have found it necessary, at least in the case of barley, to prepare chloroplasts for extraction of plastocyanin from fresh leaves. When there are many samples to assay, the sonic extract of chloroplasts may be stored at $-15°$ for at least 24 h without loss of plastocyanin.

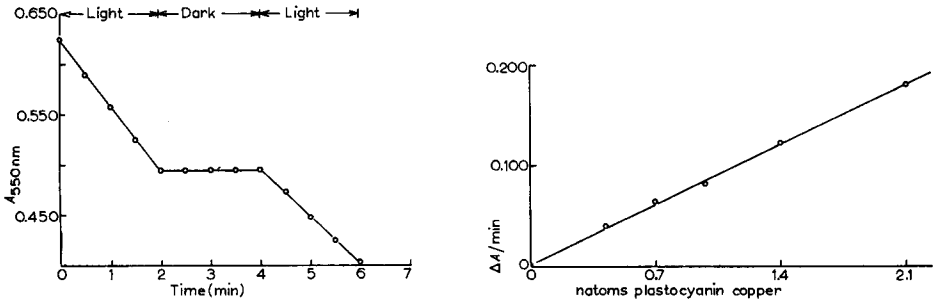

Fig. 1. The time-course of photo-oxidation of reduced cytochrome *c* by plastocyanin in the presence of digitonin-treated chloroplasts. The conditions of the experiment are described in the text.

Fig. 2. Calibration curve for the enzymatic assay of plastocyanin. An absorbance change 0.100 corresponds to the oxidation of 5.2 nmoles cytochrome *c*.

Comparison of the enzymatic and direct spectrophotometric methods

In order to check the validity of the enzymatic method comparative experiments have been performed with pea chloroplasts. Chloroplasts containing approx. 53 mg chlorophyll were suspended in 100 ml of 0.01 M phosphate buffer (pH 7.0) and sonicated at 5 A for 1 min, in 5-ml portions. After centrifugation at 100000 x *g* the supernatant was dialysed overnight against 1 mM phosphate (pH 7.5), freeze dried and resuspended in 5 ml of water. The solution was clarified by centrifuging and the pale green supernatant was assayed for plastocyanin by the enzymatic method and by direct spectrophotometry. The enzymatic method gave a concentration of 18.9 nmoles/ml, which was not significantly different from the value of 17.5 nmoles/ml obtained by direct spectrophotometry. The extinction coefficient of $4.9 \cdot 10^6$ cm^2·atom Cu^{-1} at 597 nm was used[14].

TABLE I

COMPOSITION OF CHLOROPLASTS FROM VARIOUS PLANTS

Each figure represents the mean value of the number of experiments shown in parentheses.

Plant	*Plastocyanin (natoms copper/mg chlorophyll)*	*Cytochrome f (natoms haem/mg chlorophyll)*	*Total copper (natoms/mg chlorophyll)*
Pea	6.2 (7)	1.1 (3)	13.2 (3)
Spinach	5.7 (7)	1.2 (3)	-
Barley	5.2 (7)	1.5 (4)	5.2 (3)
Orache	8.9 (3)	3.2 (3)	27.0 (3)
Green tobacco	2.0 (3)	1.0 (3)	3.8 (3)
Pale tobacco	5.7 (3)	2.4 (3)	20.9 (3)

Content of plastocyanin, copper and cytochrome f in chloroplasts of various plants

The plastocyanin contents of chloroplasts from various plants determined by the enzymatic method are summarized in Table I, together with the corresponding figures for cytochrome *f* and total copper. Although plastocyanin and cytochrome *f* exhibit somewhat similar properties and are closely associated in the photosynthetic electron transport chain, there is considerable variability both in the absolute amount of each component and in the ratio of one to the other. A substantial amount of plastocyanin has also been found in etiolated leaves from 8-day-old barley seedlings. In the case of green barley leaves the total plastocyanin content was approx. 2.5 nmoles/g fresh weight leaf. The leaves from etiolated plants contained essentially the same amount.

The copper content of chloroplast preparations from various plants ranged from 3.8 to 24.0 natoms/mg chlorophyll (Table I). The most interesting case was that of barley chloroplasts, for which the total copper content was equal to the plastocyanin copper. In other experimental plants the copper content was 2–3 times higher than the corresponding plastocyanin content. The very wide range of values for the copper content in chloroplasts isolated from different plants indicates that in general there is no close relation between the copper and plastocyanin contents of plants.

DISCUSSION

Method for plastocyanin determination

The photo-oxidation of cytochrome *c* and its dependence on various factors has been studied in detail by NIEMAN AND VENNESLAND[4], BISHOP *et al.*[5], NIEMAN *et al.*[6], KATOH AND TAKAMIYA[18] and KOK *et al.*[8]. They showed that light saturation is rather difficult to achieve and that light becomes increasingly limiting as higher amounts of chloroplast chlorophyll are used. The reaction is insensitive to various poisons, but appears to be stimulated by high concentrations of $(NH_4)_2SO_4$(refs. 5, 6). BISHOP *et al.*[5] have found a pronounced effect of phosphate on cytochrome *c* photo-oxidation.

We have used throughout the same light intensity for the samples as for the

standards. The calibration curve showed good reproducibility when fresh chloroplast preparations (obtained by the same procedure from the same type of material) were compared. The rate of loss of activity tended to vary from one preparation to another. $(NH_4)_2SO_4$ (1 M) exhibited an inhibitory effect on the rate of cytochrome *c* oxidation. In our experimental conditions 50 % variation of phosphate concentration does not significantly influence the rate of photo-oxidation. With a high concentration of phosphate (0.1 M) in the sonicating medium a longer time of sonication is needed.

The agreement between the results of the two methods, spectrophotometric and enzymatic, indicates that the enzymatic method is specific for plastocyanin when applied to extracts of chloroplasts from higher plants even though Euglena cytochrome *f* is known to catalyse photo-oxidation of cytochrome *c* by spinach chloroplasts. The enzymatic method requires much smaller quantities of material and employs a relatively simple procedure.

Content of plastocyanin, copper and cytochrome f in chloroplasts of various plants

The plastocyanin contents of pea, spinach and green barley chloroplasts are very similar. The same results expressed as a number of chlorophyll molecules per atom of plastocyanin copper give a figure close to 200. KATOH *et al.*[2], using an acetone extraction with subsequent purification and spectrophotometric determination, have found for spinach chloroplasts the ratio of chlorophyll to plastocyanin to be 300. They stressed though that this figure represents a maximal value. The actual molar ratio of chlorophyll to plastocyanin copper should be lower due to losses in the extraction procedure.

Orache and tobacco chloroplasts show significantly different contents of plastocyanin and also of cytochrome *f*. These difference in tissues that, as far as we know, perform the same type of photosynthesis indicate that the number of chlorophyll molecules per molecule of electron transport component is not a rigid number and could have a wide range of values in different plants.

The relative concentration of copper in spinach chloroplasts expressed as moles of chlorophyll per atom copper amounts to 130–160, according to KATOH *et al.*[2]. Close agreement between the amount of total copper and plastocyanin copper in green barley chloroplasts indicate that the main function of copper in the chloroplast is that of a constituent element of plastocyanin. An excess amount of copper in orache, tobacco and pea chloroplasts does not seem to be necessarily involved in photosynthetic reactions of chloroplasts.

By studying green and pale mutants of tobacco plants, SCHMID[10] and SCHMID AND GAFFRON[19] have presented the concept of variation in the size of the photosynthetic unit. According to them, it is the pigment content that changes, and the enzymes which limit the dark reaction of photosynthesis have not suffered in proportion. Similar conclusions can be drawn from our results with green and pale tobacco plants. However, according to our results, stoicheiometry of the two electron carriers studied, plastocyanin and cytochrome *f*, also appears to vary in different plants. Although these carriers seem to be performing closely related functions in photosynthetic electron transport their variable amounts in chloroplasts of different plants indicate a more complex situation which needs further investigation.

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