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ISOLATION AND PROPERTIES OF PLASTOCYANIN FROM *ANABAENA VARIABILIS*

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SUMMARY

Plastocyanin is a copper protein found in photosynethetic tissue and it exhibits the properties of a physiological redox reagent. This protein has been purified from the blue-green alga *Anabaena variabilis*. Plastocyanin is required for a number of partial reactions of the photosynthetic electron transfer chain. These reactions include the transfer of electrons from reduced 2,3',6-trichlorophenolindophenol,*N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine and 2,3,5,6-tetramethyl-*p*-phenylenediamine to low potential oxidants. Reduced cytochrome *c* photooxidation does not appear to be dependent on plastocyanin. Cytochrome *f*, isolated from this alga, will partially replace plastocyanin in many of these reations. Inhibition of photosynthetic reactions by copper chelators appears to occur at some site other than the site of plastocyanin function.

INTRODUCTION

Plastocyanin was first isolated from higher plant leaves by KATOH *et al.*[1]. This protein is localized in the chloroplast and the experiments of KATOH AND TAKAMIYA provided direct evidence that plastocyanin is a participant in the photosynthetic electron transfer chain[2]. In their experiments, plastocyanin was removed from the chloroplast by sonication with a concomitant loss of $NADP^+$ Hill reaction activity. The activity could be partially restored on readdition of plastocyanin. Other lines of evidence for the role of plastocyanin in photosynthesis are the spectroscopic studies of URBACH AND FORK[3] on intact plants and the mutant studies of GORMAN AND LEVINE[4]. This paper describes the isolation of plastocyanin from the blue-green alga *Anabaena variabilis*. The purified protein was then used in reconstitution experiments with the photosynthetic particles isolated from this alga. Both plastocyanin and cytochrome *f* will restore the flow of electrons from several artificial donor systems to low potential Hill oxidants. The copper in plastocyanin does not seem to be the site of inhibition when Hill reaction activity is blocked by a number of compounds which act as copper chelators.

Abbreviations: TCIP,2,3',6-trichlorophenolindophenol; $TCIPH_2$, reduced form of TCIP; TMPD,*N*,*N*,*N'*,*N'*,-tetramethyl-*p*-phenylenediamine; DAD,2,3,5,6-tetramethyl-*p*-phenylenediamine; DCMU,3(3,4-dichlorophenyl)-1,1-dimethylurea; cupferon,*m*-nitrosophenyl-hydroxylamine.

MATERIALS AND METHODS

The procedures for growth of *A. variabilis* and for isolation of photosynthetic particles from this alga have been described earlier[5]. For the preparation of plastocyanin, 100 g fresh weight of packed cells of *A. variabilis* are resuspended and thoroughly sonicated in cold distilled water. The broken cell suspension is then brought to 60 % of saturation with $(NH_4)_2SO_4$ by addition of the solid salt. The suspension is centrifuged at 20000 × *g* for 15 min and the supernatant is filtered through glass-wool to remove the last traces of phycocyanin. The resulting solution is brought to saturation with $(NH_4)_2SO_4$ and allowed to stand for 48 h at 2°. A fluffy precipitate is collected by filtering through a thin pad of diatomaceous earth. The precipitated protein is resuspended in distilled water, dialyzed against 10^{-3} M phosphate buffer (pH 7.5) and passed through a DEAE-cellulose column to remove ferredoxin and other unwanted protein. The protein passing through the column is brought to 10^{-3} M with potassium ferricyanide so as to oxidize the plastocyanin. The preparation is dialyzed to remove excess ferricyanide and absorbed onto a CM-cellulose column previously equilibrated with 10^{-3} M phosphate buffer (pH 7.5). The column is washed briefly with 1 mM phosphate buffer (pH 7.5) and then the plastocyanin is eluted as a distinct blue band with 5 mM phosphate buffer (pH 7.5). The absorption spectrum of a typical preparation is presented in Fig. 1. The main visible absorption peak appears at 597 mμ and all the visible color is bleached on addition of a trace of sodium ascorbate. The ratio of the ultraviolet to visible absorption peak is 1.57.

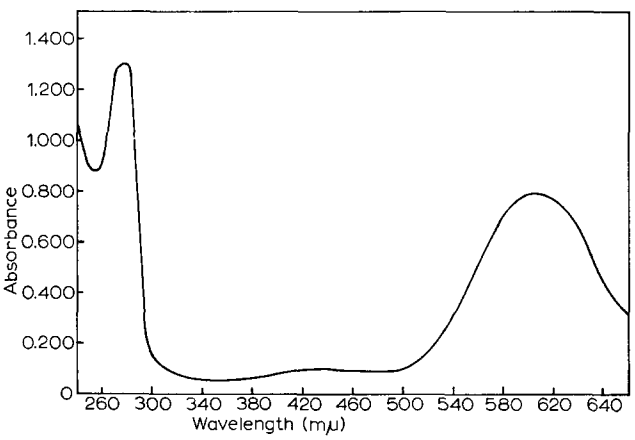

Fig. 1. Absorption spectrum of *A. variabilis* plastocyanin.

Cytochrome *f* can be isolated from the same preparation by eluting it from the CM-cellulose column with 0.02 M phosphate buffer (pH 7.5). Further purification of the cytochrome *f* of *A. variabilis* and its properties have been described[6]. The preparations of ferredoxin and NADPH–ferredoxin oxidoreductase (EC 1.6.99.4) from *A. variabilis* have both been described[6]. Procedures for measurement of photosynthetic electron transfer reactions have been previously reported in detail[7]. The contents of the reaction mixtures are recorded in the legends of the figures and tables.

All reagents used are commercially available with the exception of DAD which

was synthesized by the method of SMITH[8] in which durene (purchased from Matheson, Coleman and Bell Chemical Co.) was nitrated with HNO_3, then reduced with $SnCl_2$ in HCl to give the tin salt of DAD. The free amine was crystallized from a NaOH solution.

RESULTS

Since sonication at low ionic strength apparently releases plastocyanin from the photosynthetic structures, sonication of chlorophyll containing structures of *A. variabilis* was tested as a method for reversible extraction of this protein from an active electron transfer chain. The effect of increasing sonication time on a photoactive particle preparation from *A. variabilis* is shown in Fig. 2. Particles were diluted in 0.4 M sucrose–0.05 M NaCl to a chlorophyll concentration of 0.5 mg chlorophyll per

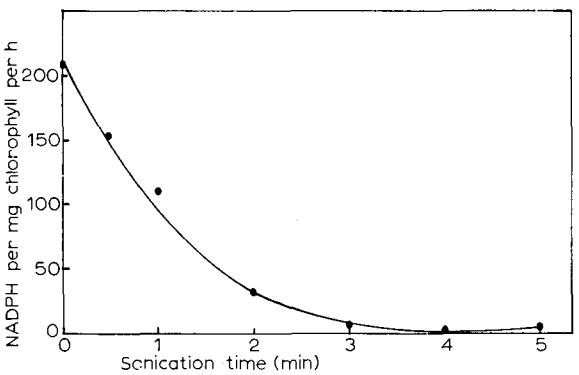

Fig. 2. The effect of sonication on the transfer of electrons from H_2O to $NADP^+$. Photosynthetic particles were sonicated and washed as described in the text. The reaction mixtures for measurement of $NADP^+$ reduction contained 5 μmoles phosphate buffer (pH 7.8), 5 μmoles Tris–HCl (pH 7.8), 10 μmoles $MgCl_2$, 0.4 μmole $NADP^+$, saturating amounts of ferredoxin and NADPH–ferredoxin oxidoreductase, and photosynthetic particles equivalent to 20 μg chlorophyll in a final volume of 1 ml. Illumination was for 2 min at 25° with an intensity of 15000 ft-candles white light.

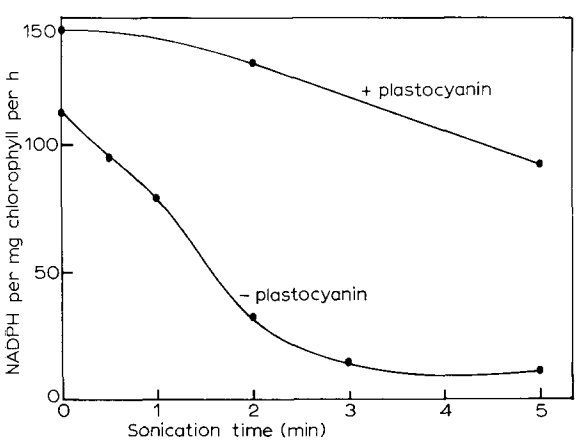

Fig. 3. The effect of sonication on the transfer of electrons from $TCIPH_2$ to $NADP^+$. Reaction conditions are the same as those described in the legend of Fig. 2 but for the addition of 10 μmoles sodium ascorbate and 0.1 μmole TCIP. Where indicated, 2.4 mμmoles plastocyanin were included.

ml and sonicated with a Bronwill Biosonic probe for varying lengths of time. Care was taken to keep the preparation at 5° or below. After sonication, the preparations were diluted to a concentration of 0.2 mg chlorophyll per ml and centrifuged at 105000 × *g* for 1 h. The supernatant, containing soluble protein, was removed and the chlorophyll-containing particles were resuspended in 0.4 M sucrose–0.05 M NaCl to a final chlorophyll concentration of 1 mg/ml. This procedure was used in all subsequent experiments to remove endogenous solubilized plastocyanin from the sonicated particles. As seen in Fig. 2, the Hill reaction activity to $NADP^+$ in the presence of saturating amounts of ferredoxin and NADPH–ferredoxin oxidoreductase drops rapidly in response to sonication. Addition of purified plastocyanin, purified cytochrome *f*, the two in combination or the crude solubilized protein from sonicated preparations would not restore this activity. Variation of the pH of sonication or of assay did not affect this result.

The shorter electron transfer sequence between TCIP and $NADP^+$ was tested next with better result. Fig. 3 shows that this activity, like the Hill reaction, is diminished by sonication and removal of the solubilized protein. However, readdition of purified plastocyanin largely restores the activity to the extracted particles. In this type of experiment, care must be taken to use purified ferredoxin and NADPH–ferredoxin oxidoreductase since crude preparations of these enzymes contain plastocyanin and cytochrome *f*.

TABLE I

PHOTOREDUCTION OF $NADP^+$ WITH $TCIPH_2$ AS ELECTRON DONOR

The reaction conditions are the same as those described for Fig. 4. Either 2.4 mμmoles plastocyanin or 3.0 mμmoles cytochrome *f* were added as indicated.

	NADPH per mg chlorophyll per h (μmoles)
Control	101
5 min sonication	30
5 min sonication + plastocyanin	132
5 min sonication + cytochrome *f*	77
5 min sonication + plastocyanin + cytochrome *f*	131

Cytochrome *f* is also able to restore electron transfer activity between $TCIPH_2$ and $NADP^+$. Table I illustrates this restoration. The maximum restoration with cytochrome *f* is not as great as that with plastocyanin, and the two in combination do not appear to act synergistically.

Fig. 4 shows saturation curves for the plastocyanin and cytochrome *f* responses of the $TCIPH_2$ to $NADP^+$ reaction using the extracted particles.

Several artificial electron donors were tested in place of TCIP. Fig. 5 shows the results of a comparison of reduced TCIP, TMPD, and DAD as electron donors for the photoreduction of a low potential Hill oxidant. In these experiments, methyl red was used as a terminal electron acceptor to avoid spurious differential effects of the various donor systems on the soluble enzymes required for $NADP^+$ reduction. In all cases an excess of the inhibitor DCMU was present to insure no flow of electrons from

water to $NADP^+$. The reaction from TMPD to $NADP^+$ is least affected by the extraction procedure, suggesting that this electron donor can partially by pass the carrier or carriers removed by the extraction. All three donor systems are completely restored by readdition of plastocyanin. A partial restoration of all reactions is seen on addition of cytochrome *f*. The combination of plastocyanin and cytochrome *f* is no better than plastocyanin alone.

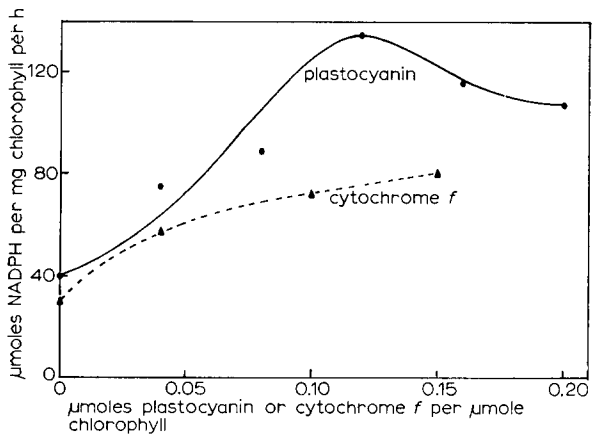

Fig. 4. Saturation of the TCIP to $NADP^+$ reaction with plastocyanin or cytochrome *f*. Reaction conditions are the same as those described for Fig. 3 but for the inclusion of $2 \cdot 10^{-5}$ M DCMU to block any flow of electrons from H_2O. The photosynthetic particles used in these assays were sonicated for 5 min and washed as described in the text. ●—●, plastocyanin; ▲---▲, cytochrome *f*.

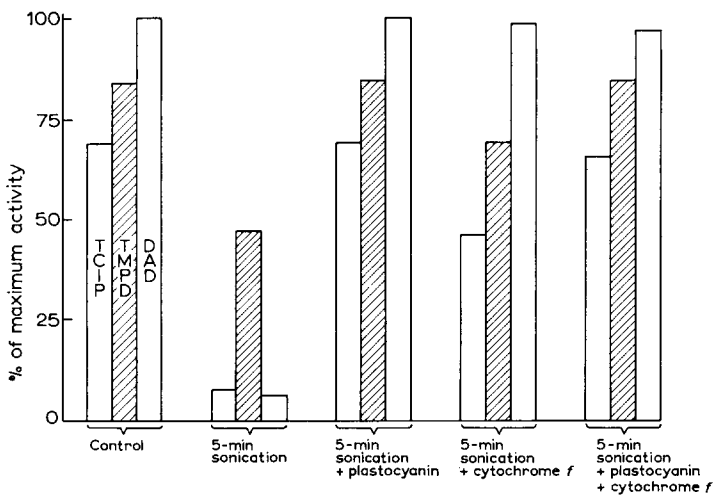

Fig. 5. Comparison of artificial electron donors in the photoreduction of methyl red. These reactions were run under anaerobic conditions achieved by several cycles of evacuation and flushing with N_2 gas. All reaction mixtures contained 20 μmoles sodium ascorbate, 0.05 μmole methyl red, and photosynthetic particles equivalent to 30 μg chlorophyll in a final volume of 1.5 ml. In all experiments, $1.3 \cdot 10^{-5}$ M DCMU was included to prevent the utilization of H_2O as an electron donor. Where indicated, 4 mμmoles plastocyanin, 5 mμmoles cytochrome *f*, 0.1 μmole TCIP, 0.2 μmole TMPD, and 1.6 μmoles DAD were used. Methyl red reduction was measured by the change in absorbance at 430 mμ and the absolute rates with this oxidant were comparable to that observed with $NADP^+$.

The photo-oxidation of reduced mammalian cytochrome *c* gives a different response to sonication and removal of solubilized protein from the photosynthetic apparatus. As can be seen in Fig. 6, prolonged sonication, sufficient to completely abolish the $TCIPH_2$ to $NADP^+$ reaction, has no affect on the photo-oxidation of reduced cytochrome *c*. The addition of cytochrome *f* stimulates this reaction and greater stimulation is achieved with plastocyanin. A combination of plastocyanin and cytochrome *f* gives an even greater stimulation.

Similar experiments were performed in the presence of benzyl viologen to avoid rate limitations in the movement of electrons on the low potential end of the chain[9].

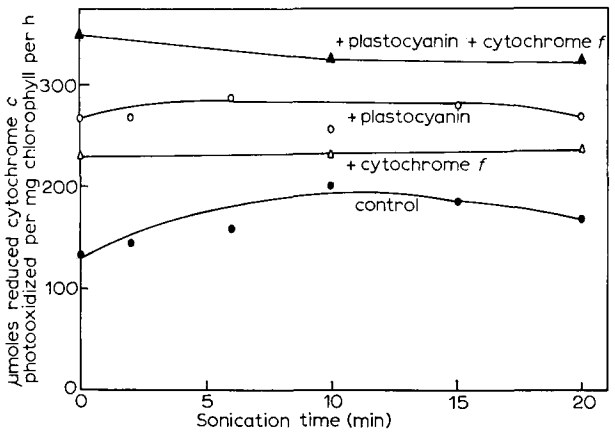

Fig. 6. Photo-oxidation of reduced cytochrome *c*. These reaction mixtures contained the detergent Tween 20 at a final concentration of 0.5%, 20 μmoles Tris–HCl (pH 7.8), 30 μmoles $MgCl_2$, 1 mg reduced mammalian cytochrome *c*, and photosynthetic particles containing 20 μg chlorophyll in a final volume of 1.0 ml. Either 2.4 mμmoles plastocyanin or 3 mμmoles cytochrome *f* were added as indicated.

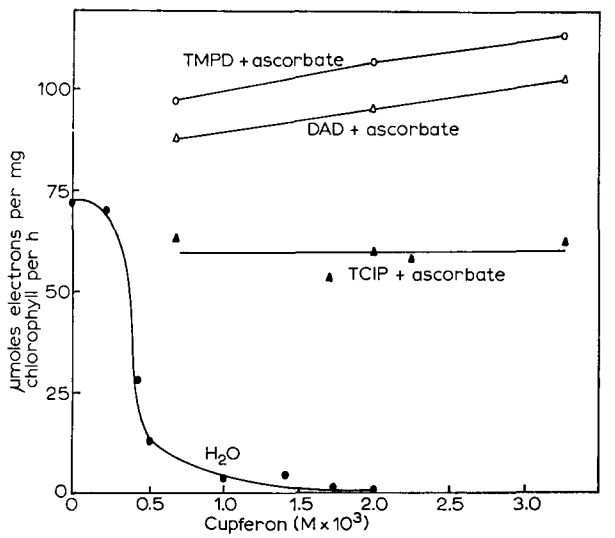

Fig. 7. Inhibition of methyl red reduction by cupferon. The reaction conditions are described in the legend of Fig. 5.

The inclusion of this auto-oxidizable terminal electron acceptor uniformly increased the rates of reduced cytochrome *c* photo-oxidation about 4-fold for each of the conditions described in Fig. 5.

A number of copper chelating reagents have been reported as inhibitors of the photosynthetic electron transfer sequence[10,11]. Fig. 7 shows the results of comparison of the effects of varying concentrations of the chelator cupferon on the Hill reaction activity and the partial reactions from reduced TCIP, DAD, and TMPD. It is evident that the reduction of methyl red with electrons from H_2O is completely blocked by cupferon while no inihibitory effect and perhaps a slight stimulation is seen for the transfer of electrons from the artificial donor systems. Similar results were obtained with the copper chelators salicylaldoxime and ethyl xanthylic acid. The cupferon inhibition could not be reversed by washing the photosynthetic particles or by washing in the presence of excess metal ions to remove the chelator.

DISCUSSION

The isolation of plastocyanin in *A. variabilis* extends the distribution of this electron carrier to a procaryote. It will be interesting to see if plastocyanin is also found in the electron transfer chain of photosynthetic bacteria. The procedure used in our isolation of plastocyanin differs from that used with higher plant preparations in that the *A. variabilis* protein is absorbed on CM-cellulose and passed through DEAE-cellulose. The higher plant plastocyanin is absorbed on DEAE-cellulose[1]. A systematic comparison of these proteins from the two sources may reveal other differences. FUJITA *et al.* have recently reported the further purification of a higher plant plastocyanin with an $A_{597\,m\mu}/A_{278\,m\mu}$ ratio equal to unity[12]. It may prove possible to further purify the *A. variabilis* protein to achieve a similar ratio.

Activity measurements suggest that the photosynthetic particles of *A. variabilis* are more sensitive to irreversible damage by sonication than are spinach chloroplasts. KATOH AND SAN PIETRO were able to show partial restoration of electron flow from H_2O to $NADP^+$ on addition of plastocyanin to extracted chloroplasts. This was not possible with the *A. variabilis* preparations. The good restoration of the $TCIPH_2$ to $NADP^+$ sequence by plastocyanin seems to indicate that sonication not only removes plastocyanin but causes irreversible damage closer to the oxygen evolving step. The operational interchangeability of plastocyanin and cytochrome *f* permits no inference as to the relative positions of these two carriers in the sequence. Perhaps they operate in parallel to one another as suggested by KOK[13]. The comparison of various electron donors to the partial electron transfer sequence would indicate that $TCIPH_2$ and reduced DAD are both oxidized by a plastocyanin-dependent sequence. The plastocyanin involvement in the DAD to $NADP^+$ reaction of spinach chloroplasts has been shown in the work of TREBST *et al.*[14]. TMPD may partially react at a non-plastocyanin-dependent site since the TMPD to methyl red reaction is only partially lost on plastocyanin removal. Similar results with spinach chloroplasts have been reported by VERNON, SHAW AND LIMBACH[15]. The oxidation of reduced cytochrome *c* is not affected by very long sonication and washing suggesting that the basal rate oxidation does not involve plastocyanin. As reported by Kok, RURIANSKI AND HARMON[9], both plastocyanin and cytochrome *f* will increase the flow of electrons into the chain, but they are not obligatory intermediate carriers.

Copper has long been postulated as a carrier in the photosynthetic electron transfer sequence and the discovery of plastocyanin has verified this postulate. However, plastocyanin accounts for only half the copper found in the higher plant chloroplast[16]. The experiments reported here show that copper chelators block the Hill reaction but do not affect the partial reactions which do depend on plastocyanin. Thus plastocyanin is not inhibited by these chelators. Rather some site closer to the oxygen evolving step is sensitive to inhibition by copper chelators. This sensitive site may contain copper although it does not necessarily follow that the chelators are inhibitory only by virtue of their ability to bind copper.

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