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Evidence for Cyanide and Mercury Inactivation of Endogenous Plastocyanin

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Abstract

Cyanide and mercury treatment of chloroplast membranes inactivates plastocyanin as shown by the inability of the extracted plastocyanin to restore electron transport in a bioassay on chloroplasts depleted of their endogenous plastocyanin by digitonin treatment. The extraction procedure did remove the enzyme from cyanide and mercury treated chloroplasts as shown by sodium dodecyl sulfate polyacrylamide electrophoresis of the extracts. This procedure normally shows a plastocyanin band at 11,000 dalton molecular weight and the band was present in extracts from control and cyanide or mercury treated membranes.

Introduction

Plastocyanin is generally accepted as an electron transport component in the redox pathway connecting Photosystems II and I [1], although its exact position is still disputed [2, 3]. Exogenously added plastocyanin restores electron flow in sonicated chloroplasts [4], mutants deficient of plastocyanin [5], histone treated chloroplasts [6], and in the agranal bundle sheath chloroplasts of maize [7]. Recently, Malkin and Bearden [8] provided ESR data showing that the plastocyanin copper atom

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undergoes light induced redox changes in intact chloroplasts consistent with the interpretation that plastocyanin is an obligate intermediate between the two photosystems.

Several electron transport inhibitors are believed to inactivate endogenous plastocyanin [9, 10, 11]. Katoh and Takamiya [12] have shown that KCN and $HgCl_2$ treatment of isolated plastocyanin removes the protein bound copper. Although Izawa *et al.* [11] and Kimimura and Katoh [13] have provided strong kinetic evidence that KCN inhibition of electron transport is due to plastocyanin destruction, they have not provided direct evidence for KCN inactivation of endogenous plastocyanin.

We have investigated the effect of KCN and $HgCl_2$ treatment on endogenous chloroplast plastocyanin by isolating and assaying the plastocyanin after such treatments. Assays included sodium dodecyl sulfate (SDS) gel electrophoresis of the plastocyanin fraction to characterize the protein and a plastocyanin bioassay similar to that of Plesničar and Bendall [14] to check for electron transfer activity of the plastocyanin extracted from KCN or $HgCl_2$ treated chloroplasts.

Methods

Chloroplasts were isolated from spinach in buffer containing 0.4 M sorbitol, 20 mM tris-HCl (pH 7.6), 10 mM KCl, 3 mM $MgCl_2$, 3 mM ascorbate, and 2 mg/ml BSA as described previously [15]. Unless otherwise stated, chloroplasts were resuspended in the same buffer without BSA and ascorbate. Ferricyanide reduction and cytochrome *c* (Cyt *c*) photooxidation were measured spectrophotometrically at 420 and 550 nm respectively in a Beckmann Acta III modified for side illumination. Red actinic light, approximately 5×10^5 ergs/cm^2/sec, was obtained from a 500 W quartz tungsten lamp after passing through 5 cm of a 1% $CuSO_4$ solution, an infra-red absorbing, and a red Corning filter No. 2030. The photomultiplier was shielded from actinic light by Corning filter No. 9782.

Reaction mixtures for cytochrome *c* photooxidation were essentially as described by Plesničar and Bendall [14] and contained in 3.0 ml, chloroplasts equivalent to 3-6 μg Chl/ml, 0.5% digitonin, 12 μM DCMU, 0.5 mM methylviologen (MV), 50 μM reduced cytochrome *c* (dithionite), 10 mM $KH_2PO_4 - KOH$ (pH 7.0), 10 mM NaCl, 1 mM $MgCl_2$, and sample containing plastocyanin. It was found that reaction mixtures pre-illuminated with red light for 30 sec prior to the addition of plastocyanin had faster and more reproducible rates of cytochrome *c* oxidation.

Ferricyanide reduction was measured in 3.0 ml reaction mixtures containing chloroplasts at 10-20 μg Chl/ml, 0.1 M sucrose, 40 mM

tricine – KOH (pH 8.2), 0.4 mM potassium ferricyanide, and 5 mM ammonium chloride.

KCN treatment was performed as described by Izawa *et al.* [11] in a volume of about 40 ml containing chloroplasts at about 0.5 mg Chl/ml, 0.1 M sucrose, 1 mM $MgCl_2$, 0.1 M tricine (pH 7.8), 50 μM potassium ferricyanide, and 30 mM KCN. $HgCl_2$ treatment was performed in 40 ml of buffer containing chloroplasts at 0.5 mg Chl/ml, 0.1 M sucrose, 1 mM $MgCl_2$, 0.1 M tricine – KOH (pH 7.8) and 2.0 mM $HgCl_2$. Flasks were sealed and stored in the dark at 0-4° C for 60 min. Control chloroplasts were treated identically except that KCN and $HgCl_2$ were omitted from the incubation.

Plastocyanin was routinely isolated from chloroplasts by the following procedure. After KCN or $HgCl_2$ treatment, washed or unwashed chloroplasts, equivalent to 10-20 mg Chl in 2-40 ml buffer (see legends for details), were slowly added to pre-chilled (–15° C) acetone to a final volume of 90% acetone (v/v). The resulting flocculent was allowed to settle, most of the acetone decanted, and the precipitate collected by centrifugation. Soluble protein was extracted into 10 ml of buffer containing 20 mM tricine – KOH (pH 7.6) and 10 mM NaCl. The extract was adsorbed onto a DEAE-cellulose column (5 mm x 7 cm) equilibrated with the same buffer. The column was washed extensively with tricine buffer and tricine buffer plus 0.1 M NaCl. Plastocyanin was then eluted from the column in tricine buffer plus 0.3 M NaCl and 0.5 ml fractions were collected and assayed for plastocyanin activity.

Purified plastocyanin was a generous gift from Drs. R. Barr and G. A. Peters. The concentration was calculated using the extinction coefficient of 9.8 $mM^{-1} cm^{-1}$ [20].

Protein concentration was determined by the method of Lowry *et al.* [16]. SDS polyacrylamide gel electrophoresis was performed according to the method described by Hoober [17]. Molecular weights were determined as described by Weber and Osborn [18] using ovalbumin (M.W. 45,000), chymotrypsinogen A (M.W. 25,000) and ribonuclease A (M.W. 13,700) as protein standards.

Results

Plastocyanin isolation and assay

The DEAE elution profile for plastocyanin isolated from untreated washed chloroplasts is shown in Fig. 1. Typically, approximately 80% of the total plastocyanin recovered from the DEAE column is eluted in a final volume of 1.5 ml of tricine buffer. Because the total amount of plastocyanin isolated is generally too small to determine spectrophoto-

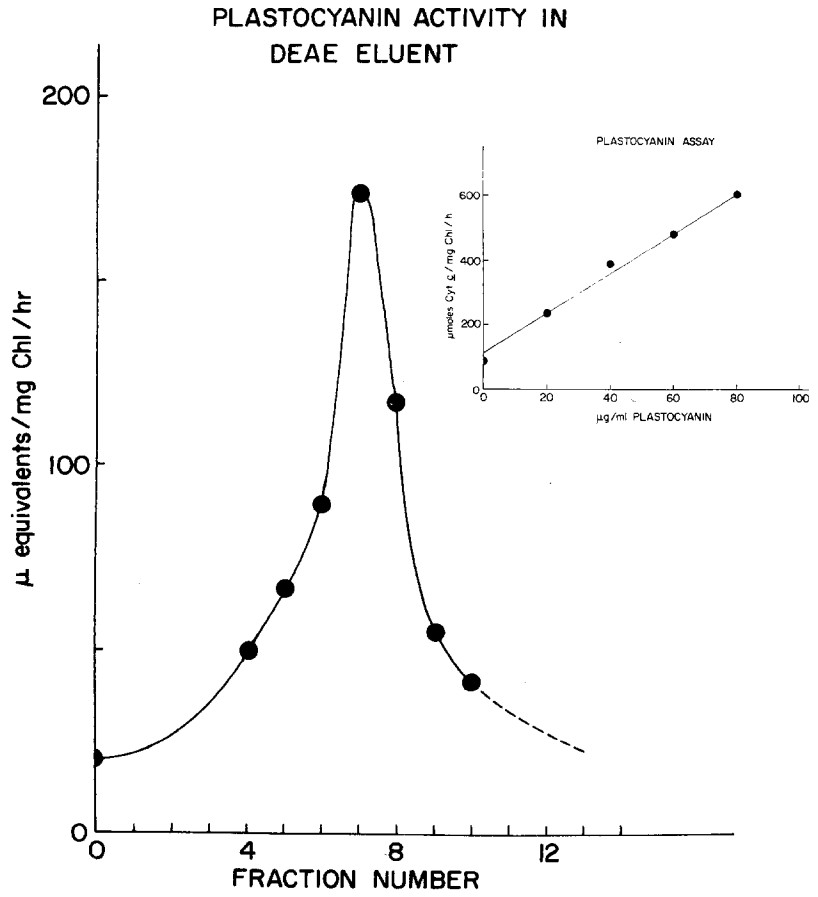


Figure 1. Plastocyanin elution profile from a DEAE column. 4.0 ml of spinach chloroplasts, 5.0 mg Chl/ml, were treated with 90% acetone (v/v) and the soluble protein extracted as described in Methods. Fractions from the DEAE column were assayed for plastocyanin activity. Insert: Cytochrome *c* photooxidation versus plastocyanin concentration. The rate of cytochrome *c* photooxidation was measured, as described in Methods, as a function of the amount of purified plastocyanin added to the reaction mixture.

metrically (less than 50 nmoles), the plastocyanin-dependent, Photosystem I catalyzed oxidation of reduced cytochrome *c* assay described by Plesničar and Bendall [14] was used to detect and quantitate the amount of plastocyanin recovered. The insert to Fig. 1 shows that this assay is directly proportional to the plastocyanin content of the sample. The rate of cytochrome *c* oxidation is linear to at least 2 μM plastocyanin (final concentration) whereas the most concentrated sample

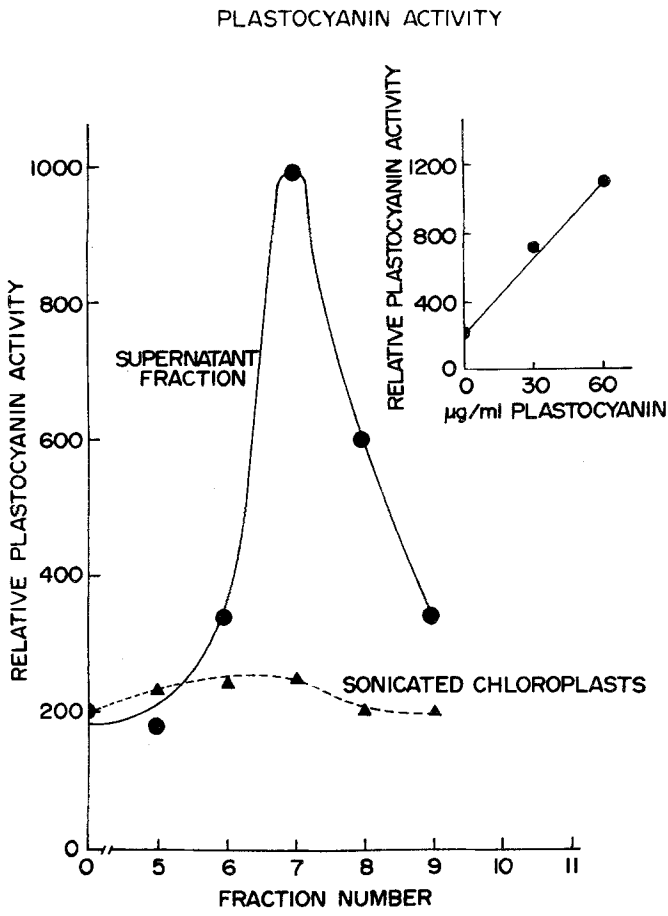


Figure 2. Plastocyanin content in sonicated chloroplasts. 30 ml of spinach chloroplasts, 1 mg Chl/ml, were sonicated for 2 x 30 sec. in a Bronson Sonicator (power setting 5). The suspension was centrifuged at 144,000 x G for 2 hrs and the pellet resuspended in 5.0 ml 20 mM tricine – KOH (pH 7.6) buffer containing 10 mM NaCl. Both the supernatant and lamellae fractions were fractionated with 90% acetone and soluble protein extracted as detailed in Methods. The insert is identical to Figure 1.

assayed contained about 1 µM plastocyanin (final concentration in the reaction mixture).

Using this assay, Fig. 2 shows the results of an experiment in which chloroplasts were extensively sonicated and the plastocyanin isolated from both the supernatant and the lamellae fractions. Over 90% of the total plastocyanin has been released into the supernatant by the

sonication treatment. Using the calibration curve (insert to Fig. 2) obtained with purified plastocyanin of known concentration and measuring the activity of the plastocyanin released into fractions 6-9, we calculate that we have recovered about 7.5 $\times 10^{-8}$ moles of plastocyanin from 30 mg of chlorophyll, or a chlorophyll to plastocyanin ratio of 400-450:1. This is in good agreement with other reported values [8, 14, 19] and indicates a 75-85% recovery of the total plastocyanin from the chloroplasts. Other experiments have shown that sonication prior to the 90% acetone fractionation is not necessary to recover 80% of the total plastocyanin.

Gel Electrophoresis of Plastocyanin

The molecular weight of native spinach plastocyanin is about 21,000 and the protein contains two atoms of copper per molecule [20]. Milne and Wells [21] have evidence that spinach plastocyanin is a dimer of identical subunits with a minimum molecular weight of 10,626. Figures 3A and 3B show SDS gel electrophoresis protein profiles of purified

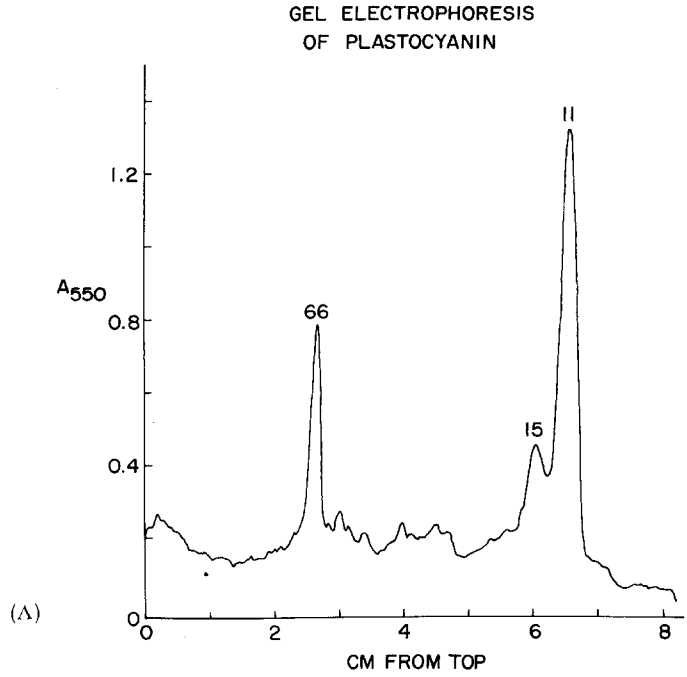
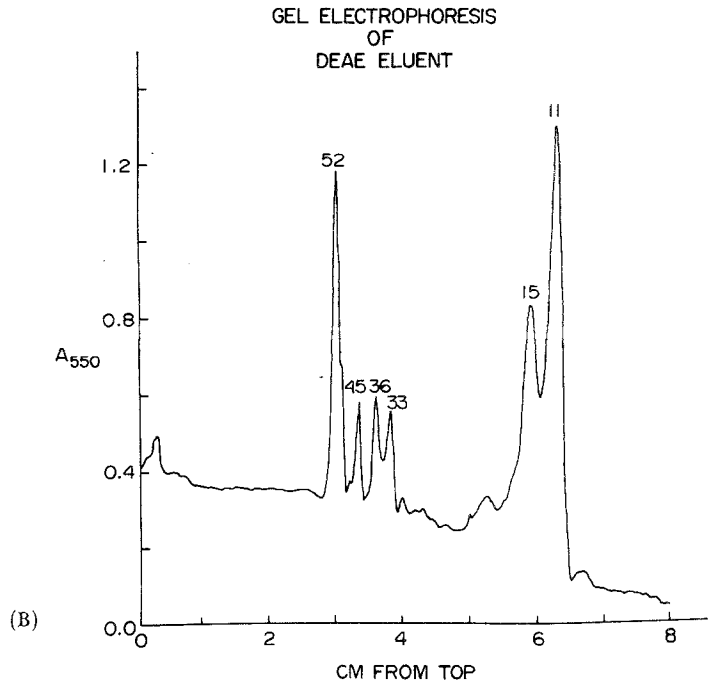


Figure 3. SDS gel electrophoresis protein profiles of purified and crude plastocyanin. (A) Purified plastocyanin, (B) Sample number 7 from Figure 1. The numbers refer to molecular weights ($\times 10^3$ daltons) determined by co-electrophoreses with protein standards.



spinach plastocyanin and the plastocyanin containing eluent from the DEAE columns. The major band in 3A occurs at 11,000 daltons and is in good agreement with the molecular weight of plastocyanin as determined by Milne and Wells [21]. As shown in 3B, a major band from the DEAE eluent containing plastocyanin occurs at 11,000 daltons and it is reasonable to identify this polypeptide as plastocyanin. Other bands, however, are also present in the DEAE eluent indicating that this fraction cannot be considered a pure preparation.

KCN and $HgCl_2$ Treated Chloroplasts

To determine whether KCN and $HgCl_2$ treatments of intact (Class II) chloroplasts inactivate plastocyanin as suggested by Izawa *et al.* [11], and Kimimura and Katoh [13], chloroplasts were treated and the plastocyanin isolated as described above. Table I shows typical results. As previously reported [11, 13] both treatments markedly inhibit ferricyanide reduction. Our isolation procedure showed a recovery of approximately 14% and 0% of the total plastocyanin activity from KCN and $HgCl_2$ treated chloroplasts respectively.

TABLE I. Activity of Cyanide and Mercury Treated Chloroplasts

	$H_2O \rightarrow$ Ferricyanide (μequ/mg Chl/h)	Plastocyanin activity recovered
A		
Control chloroplasts	280	3223[a]
KCN Treated Chloroplasts	43	467
% Control	15	14
B		
Control Chloroplasts	432	2800
$HgCl_2$ Treated Chloroplasts	146	0
% Control	34	0

[a]One unit of plastocyanin activity is defined as 0.001 A_{550}/min/7 μg Chl.

Different chloroplast preparations were treated with KCN and $HgCl_2$ and assayed as described in Methods. Plastocyanin was isolated from 40 ml suspensions containing chloroplasts at 0.5 mg Chl/ml. The recovered plastocyanin activity was calculated after pooling those fractions eluted from a DEAE column containing at least 10% activity above background.

The plastocyanin activity is based on the bioassay of digitonin treated chloroplasts as described in Methods.

Figure 4 shows the SDS polyacrylamide gel electrophoresis protein profiles from the DEAE eluent of control and KCN treated chloroplasts. The gel patterns from both samples are identical and both contain a substantial amount of the plastocyanin polypeptide (occurring at 11,000 daltons). Similar results were found for the protein profiles of the DEAE eluent from $HgCl_2$ treated chloroplasts. Apparently neither KCN nor $HgCl_2$ treatment hinders the isolation of (inactive) plastocyanin.

Discussion

Using the sensitive assay for plastocyanin described by Plesničar and Bendall [14] we routinely recovered about 80% of the total plastocyanin from untreated chloroplasts. SDS polyacrylamide gel electrophoresis of our DEAE protein fractions shows a well defined band at 11,000 daltons which we have identified as plastocyanin by co-electrophoresis against highly purified plastocyanin. Our determined molecular weight is in good agreement with the value of 10,626 daltons obtained by Milne and Wells [19] using amino acid analysis and sephadex gel chromatography.

KCN and $HgCl_2$ treatment of intact chloroplasts has been reported by Izawa and co-workers [11] and Kimimura and Katoh [13] to inhibit the reduction of hydrophilic electron acceptors (e.g. ferricyanide) but not hydrophobic electron acceptors (e.g. oxidized *p*-phenylenediamine).

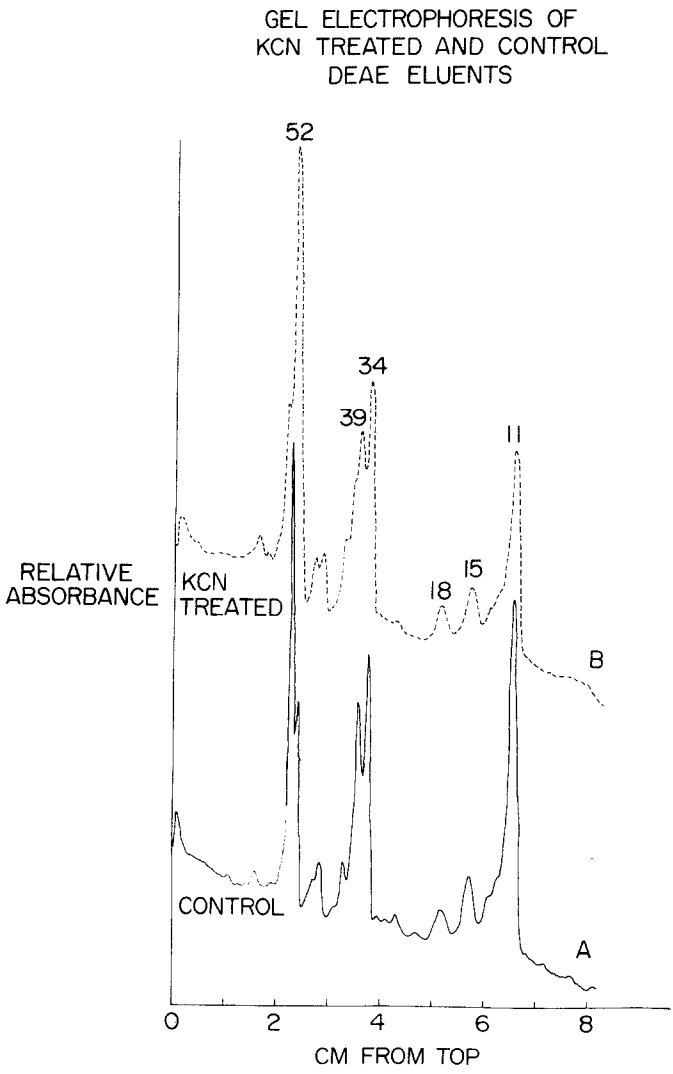


Figure 4. Comparison of the protein profiles of the plastocyanin containing DEAE eluents from control and KCN treated chloroplasts. Samples electrophoresed were the DEAE eluents from (A) control and (B) KCN treated chloroplasts isolated as in Table IA.

From kinetic analysis and the use of various inhibitors, they concluded that a probable target for inhibition is plastocyanin. Both KCN and $HgCl_2$ react quite rapidly with isolated plastocyanin to remove or replace the bound copper from the apoprotein [12, 22]. The question still remained as to whether *in situ* plastocyanin was, in fact, the site of KCN or $HgCl_2$ treatment.

We isolated the plastocyanin from KCN and $HgCl_2$ treated chloroplasts and were able to recover only a small fraction of the original activity (Table I), as defined by the plastocyanin bioassay. The lack of photochemical activity in the bioassay of treated chloroplast extracts is probably not due to carry over of the inhibitors for even when the chloroplasts are extensively washed prior to the isolation of the plastocyanin to remove the inhibitors, the plastocyanin isolated is inactive. In addition, purified plastocyanin added to a reaction mixture containing inactive plastocyanin restores the photochemical oxidation of cytochrome *c*. SDS gel electrophoresis protein profiles (Fig. 4) of the DEAE fraction containing the plastocyanin shows that the lack of activity is not due to the absence of plastocyanin in the crude protein extract. Thus, our results support the contention of Izawa *et al.* [11], Ouitrakul and Izawa [22] and Kimimura and Katoh [13] that the site of KCN and $HgCl_2$ inhibition is indeed plastocyanin.

Acknowledgements

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