Mechanism of KCN Inhibition of Photosystem I*

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Experiments with chloroplasts and purified spinach plastocyanin suggest a mechanism for KCN inhibition of Photosystem I. KCN inhibition can be bypassed by a detergent or reversed by replacement of the inactive plastocyanin. KCN bleaches and inactivates purified plastocyanin. KCN releases copper from chloroplast membranes and from purified plastocyanin. Cyanide does not bind to the apoprotein produced when plastocyanin is treated with KCN, and KCN-produced apoplastocyanin has a N-ethylmaleimide-reactive sulfhydryl group not found in holoplastocyanin. Apoplastocyanin is not active in restoring Photosystem I activity to plastocyanin-depleted membranes. Holoplastocyanin restores Photosystem I activities to plastocyanin-depleted membranes prepared from either control or KCN-treated chloroplasts to about the same extent. KCN-treated chloroplast membranes are found to have higher amounts of apoplastocyanin than do control chloroplast membranes. These results offer evidence that KCN removes the copper from plastocyanin in the chloroplast membrane, leaving the inactive apoplastocyanin which is unable to transfer electrons to Photosystem I.

Photosystem I is specifically inhibited by polycations (1, 2), mercury (3), and KCN (4). In each case, spectrophotometric evidence indicates that the site of inhibition is between cytochrome f and P700 (3, 5, 6). The site of action of plastocyanin is also between cytochrome f and P700 (7, 8). These spectrophotometric experiments suggest that plastocyanin is the site of inhibition, and there is some evidence to indicate that endogenous plastocyanin is inactivated by cyanide or mercury treatment (9), but to date, there is very little direct chemical evidence to define a mechanism by which KCN inhibits Photosystem I. We have investigated the effect of KCN on purified plastocyanin and on chloroplast-bound plastocyanin under similar conditions to those used for Photosystem I inhibition. Apoplastocyanin cannot catalyze electron transfer. The apoprotein contains a N-ethylmaleimide-reactive sulfhydryl group which is not accessible in the holoplastocyanin, suggesting that this sulfhydryl group is a ligand to the copper. We have investigated the plastocyanin in plastochroms which have been treated with KCN and demonstrated that the inactive plastocyanin described earlier (9) is actually apoplastocyanin.

**EXPERIMENTAL PROCEDURE**

Materials—Fresh spinach (Spinacia oleracea) was obtained from the local market. N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride and methyl viologen were purchased from Eastman Organic Chemicals and Schwarz/Mann, respectively. Radiochemicals, Aquasol and Protosol, were purchased from New England Nuclear. Insulin and a-chymotrypsinogen type II were purchased from Sigma. Horse heart myoglobin was purchased from Pentex Biochemicals.

Preparations—Chloroplasts were prepared in 0.4 M sucrose containing 0.05 M NaCl as described previously (1). Sonicated chloroplasts were obtained by diluting the chloroplast suspension to 0.5 mg of chlorophyll/ml with 0.4 M sucrose containing 0.05 M NaCl and sonicating for 2 min at maximum intensity with a Fisher 100-watt model SS 0 ultrasonic generator. After sonication, the chloroplast suspension was centrifuged at 104,000 x g for 1 hour, and the resulting pellet was resuspended in 0.4 M sucrose containing 0.05 M NaCl to give a concentration of 1 mg of chlorophyll/ml.

Chloroplasts were treated with KCN for 90 min by the method of Outrakul and Izawa (4). Chloroplast controls for the KCN inhibition treatment were done by substituting KOH for KCN. After treatment with KCN or KOH, the chloroplast suspension was centrifuged at 13,000 x g for 10 min and the resulting pellet was resuspended in 0.4 M sucrose containing 0.05 M NaCl to give a concentration of 1 mg of chlorophyll/ml.

TWEEN 20 chloroplasts were prepared by the methods of Barr et al. (10). Chloroplasts were centrifuged at 13,000 x g and the pellet was resuspended in 1% Tween 20 to 1 mg of chlorophyll/50 ml. This suspension was centrifuged at 48,000 x g for 10 min and the resulting pellet was resuspended in 0.4 M sucrose containing 0.05 M NaCl to 1 mg of chlorophyll/ml.

Plastocyanin was isolated and purified by the methods of Anderson and McCarty (11). Apoplastocyanin was prepared from plastocyanin by the method of Katoh and Takamiya (12). Preparations of plastocyanin used in this publication were at least 97% pure as determined by sodium dodecyl sulfate gel electrophoresis.

Assays—Chlorophyll concentrations were estimated by the methods of Arnon (13).

The Photosystem I-dependent partial reaction of electron transport from ascorbate-reduced N,N,N',N'-tetramethyl-p-phenylenediamine to methyl viologen was performed as described previously (1), except that a reaction volume of 1.5 ml was used.

Photosystems I and II were measured as electron transport from H₂O to methyl viologen. Oxygen uptake was measured with a Yellow Springs Instrument Co. Clark-type oxygen electrode; changes in oxygen content were monitored continuously with a strip chart recorder. The reaction chamber was surrounded with a water jacket for temperature control at 25° and illuminated with a microscope lamp with a red filter (Baird Atomic, 680-nm narrow band pass). Light intensity in the chamber was approximately 6 x 10⁶ ergs/
cm$^{-2}$ s$^{-1}$. A typical reaction mixture contained chloroplasts equivalent to 85 nmol of chlorophyll, 400 nmol of methyl viologen, 2 nmol of gramicidin, 250 nmol of Tris-HCl, pH 6, and water to 1.5 ml final volume.

Plastocyanin was assayed spectrophotometrically at 597 nm by the method of Katoh (14). Purity was estimated by comparing the visible extinction coefficient of plastocyanin with the protein concentration estimated by ultraviolet light absorption (15).

Copper concentrations were determined on a Perkin-Elmer 403 atomic absorption spectrophotometer calibrated with appropriate standard copper solutions.

Carbon 14 containing samples were counted in a Beckman LS-100 liquid scintillation counter using 10 ml of Aquasol and a 100-μl sample volume.

Sodium dodecyl sulfate gels were sliced in 1-mm sections, digested with 300 μl of 85% Protosol for at least 24 hours at ambient temperature, and diluted with 10 ml of Aquasol before liquid scintillation counting.

Sodium dodecyl sulfate analytical disc gel electrophoresis was carried out by a modification of the method of Swank and Munkres (16). Gels (5 × 50 mm) were 12% acrylamide, 0.5% N,N-methylenebisacrylamide, 0.1 M H$_3$PO$_4$ (adjusted to pH 8.4 with dry Tris base), 0.1% sodium dodecyl sulfate, 0.5 M urea, and 0.075% N,N,N',N'-tetramethyl-N,N'-tetramethylethylenediamine. A 25-μl aliquot of 10% ammonium persulfate solution was added to initiate gel formation. Electrophoresis reservoir buffer was of the same composition as the gel except it had no acrylamide, N,N-methylenebisacrylamide, or N,N,N',N'-tetramethylethylenediamine. Protein samples were prepared by mixing equal volumes of the protein solution and a solution containing 4% sodium dodecyl sulfate, 0.02% EDTA; 0.2 M Tris-acetate, pH 8.4; and 1 M urea; followed by a 5-min incubation at 100°. Pre-electrophoresis was carried out at 8 mA/tube for 1.5 hours. Protein samples were stacked on the gel by electrophoresis at 1 mA/tube for 1 hour followed by electrophoresis at 4 mA/tube until bromphenol blue tracking dye reached the end of the gel. Gels were fixed and stained in a solution containing 1.25 g of Coomassie blue R250, 227 ml of methanol, 46 ml of glacial acetic acid, and water to 500 ml, for 3 to 5 hours.

The gels were diffusion destained in a solution containing 200 ml of isopropyl alcohol, 200 ml of glacial acetic acid, and water to 500 ml. The gels were scanned at 590 nm with a Gilford 240 spectrophotometer equipped with a Gilford linear transport attachment.

Molecular weights were determined on Sephadex G-50 (fine) using the standards insulin, 6,000; myoglobin, 17,000; and α-chymotrypsinogen A, 25,000. The Sephadex gel was equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, unless otherwise stated. Fractions were collected from the 1 x 60 cm column at a flow rate of about 5 ml/hour.

### RESULTS

#### Molecular Weight of Plastocyanin

In order to establish the stoichiometry of copper, plastocyanin, and chlorophyll, we were prompted to re-examine the molecular weight of spinach plastocyanin. We have found that purified plastocyanin is a monomer of 10,600 daltons when measured by Sephadex G-50 fine gel chromatography in 0.1 M sodium phosphate buffer with suitable standards. The same results were obtained with 0.01 M, 0.001 M phosphate buffer and with distilled water. However, when the plastocyanin was passed through a column equilibrated with distilled water, about one-third of the total protein precipitated and passed through the column in the void volume. All calculations in this paper are based on the molecular weight of plastocyanin being 10,500.

**Effect of Triton X-100 on KCN-Inhibited Chloroplasts—**

Triton X-100 reversed KCN inhibition. Table I shows that following KCN treatment as described under "Experimental Procedure," chloroplasts were 70% inhibited in their ability to catalyze the Photosystem I-driven electron transfer from ascorbate-reduced N,N,N',N'-tetramethyl-p-phenylenediamine to methyl viologen. Both the control chloroplasts and the KCN-inhibited chloroplasts showed strong stimulation of rate upon addition of Triton X-100. Although the absolute rate was not as high as that of the control, the KCN chloroplasts are stimulated 3 times more than the control chloroplasts, and the degree of inhibition for the Triton X-100-treated KCN chloroplasts is reduced from 70% to 15%. The high rates observed in the presence of Triton X-100 were not dependent on the presence of N,N,N',N'-tetramethyl-p-phenylenediamine.

**Effect of Plastocyanin on Sonicated KCN-Inhibited Chloroplasts—**

Mild sonication removes plastocyanin (17, 18) from chloroplasts. Upon readdition of purified plastocyanin, high rates of Photosystem I activity can be measured as the transfer of electrons from ascorbate-reduced N,N,N',N'-tetramethyl-p-phenylenediamine to methyl viologen. Table II shows that both control chloroplasts and KCN-treated chloroplasts have low levels of Photosystem I activity after sonication. Upon addition of purified plastocyanin, the activity of both preparations shows a strong stimulation of about 11-fold.

**Effect of KCN on Purified Plastocyanin—**

The addition of small increments of KCN bleaches the blue color of oxidized plastocyanin. On further addition of KCN, the protein denatures and precipitates. Fig. 1 shows that as a sample of plastocyanin was added to KCN, the absorbance at 600 nm decreased, and the absorbance at 597 nm increased.

### TABLE I

<table>
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<tr>
<th>Treatment</th>
<th>μmol of O2/mg of chlorophyll/hr</th>
<th>Fold stimulation</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control chloroplasts</td>
<td>820</td>
<td></td>
<td></td>
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<tr>
<td>KCN-treated chloroplasts</td>
<td>240</td>
<td></td>
<td>71%</td>
</tr>
<tr>
<td>Control chloroplasts + 0.02 ml of 1 % Triton</td>
<td>10,000</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>KCN-treated chloroplasts + 0.02 ml of 1% Triton</td>
<td>8,500</td>
<td>35</td>
<td>15%</td>
</tr>
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</table>

### TABLE II

**Effect of plastocyanin on sonicated KCN-inhibited chloroplasts**

Chloroplasts were prepared as described under "Experimental Procedure." KCN treatment was performed as described under "Experimental Procedure." Photosystem I was measured as electron transfer from ascorbate-reduced N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride to methyl viologen as described under "Experimental Procedure." The indicated amounts of 1% Triton X-100 were added prior to turning on the light.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>μmol of O2/mg of chlorophyll/hr</th>
<th>Fold stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonicated chloroplasts</td>
<td>180</td>
<td>11</td>
</tr>
<tr>
<td>Sonicated chloroplasts + 4 nmol of plastocyanin</td>
<td>2000</td>
<td>11</td>
</tr>
<tr>
<td>Sonicated KCN chloroplasts</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Sonicated KCN chloroplasts + 4 nmol of plastocyanin</td>
<td>1600</td>
<td>11</td>
</tr>
</tbody>
</table>
purified, oxidized plastocyanin is titrated with KCN, the loss of biological activity and the absorbance of 597 nm decline in the same way. At a KCN concentration of 0.35 mg/ml and a plastocyanin concentration of about 0.4 mg/ml, the plastocyanin is no longer able to stimulate the rate of oxygen uptake in the sonicated chloroplast preparation. At this point, KCN had not caused any turbidity in the plastocyanin solution.

Effect of KCN on the Copper of Plastocyanin—Since the blue color of oxidized plastocyanin is due to a bound copper (19), and since KCN bleaches this blue color (Fig. 1), it was of interest to investigate the relationship between the KCN-induced bleaching and the copper binding to the plastocyanin. About 250 nmol of plastocyanin were treated with enough K14CN to completely decolorize and inactivate the plastocyanin. The plastocyanin was placed on a Sephadex G-50 column after the KCN treatment. The resulting fractionation can be observed in Fig. 2. In the lower panel of this figure, the copper is seen to be completely removed from the plastocyanin by treatment with the K14CN, and the copper co-chromatographs exactly with the K14CN. When pure plastocyanin is chromatographed without KCN treatment, the copper peak always corresponds exactly to the 280-nm peak of the plastocyanin. Calculations show that the amount of copper removed from the plastocyanin corresponds to the amount of copper expected in plastocyanin.

KCN-induced Copper Removal from Chloroplasts—Copper ions are released from chloroplasts incubated under the conditions used for KCN inhibition (Fig. 3). In this experiment, the concentrations of chloroplasts and buffer components were 10-fold higher than in the normal incubation, so that enough copper would be released to the supernatant solutions to permit detection by atomic absorption spectrophotometry. It is apparent that at 90 min of incubation (the time used for inhibition of Photosystem I reactions), KCN had caused the release of 0.6 µg of copper/ml compared to only 0.1 µg/ml in control samples. The extra copper released due to KCN comes to 2.5 µg/12.5 mg of chlorophyll (a plus b) used in the experiment. We can calculate a theoretical amount of 3.6 µg of plastocyanin copper in the original 12.5 mg of chlorophyll, assuming 1 plastocyanin/250 chlorophylls (a plus b) in a ratio of 2.8 to 1 (2) with average molecular weight of 897. Thus, the copper released amounted to 69% of that theoretically available in the plastocyanin present, which correlates well with the 71% inhibition of chloroplasts subjected to the same treatment (Table I).

Effect of Plastocyanin and Apoplastocyanin on Plastocyanin-Depleted Membrane—Barre et al. have reported that washing chloroplasts in dilute Tween 20 removes all plastocyanin from the membrane and inhibits the H2O to methyl viologen Hill reaction. Readdition of purified plastocyanin restores this activity (10). In Table III, Photosystems I and II are measured as electron flow from water to methyl viologen. The chloroplasts are seen to have high rates of electron flow only when
the unlabeler gramicidin is present. The oxygen uptake is completely sensitive to diuron. The Tween 20 chloroplasts have no oxygen uptake activity unless plastocyanin is present. Exogenous plastocyanin supports oxygen uptake from water to methyl viologen similar to that observed with the control chloroplasts, and the plastocyanin-stimulated rate is found to be completely diuron sensitive. When apoplastocyanin is substituted for plastocyanin, there is no oxygen uptake.

**N-Ethyl[14C]Maleimide Labeling of Apoplastocyanin**

Others have reported a sulphydryl of variable reactivity in plastocyanin (12, 20). Fig. 4 shows the elution profile of N-ethylmaleimide-treated plastocyanin and apoplastocyanin. Purified plastocyanin and apoplastocyanin prepared from purified plastocyanin were separately mixed with N-ethyl[14C]maleimide and incubated 1.5 hours at 25°C. After incubation, the samples were placed on Sephadex G-25 (fine) columns and fractions were collected. The elution profile for the holoplastocyanin shows that it is not labeled by the N-ethyl[14C]maleimide. The apoplastocyanin, however, is strongly labeled by the same treatment. Ferricyanide has some ultraviolet absorption and a peak in Fig. 4B is present. Small amounts of ferricyanide were added to oxidize any holoplastocyanin present and to allow the calculation of the per cent of apoplastocyanin as described under "Results." The addition of 1 ml of water was made to each fraction before assaying for the absorbance at 590 nm or counting a 20-μl aliquot.

**Labeling Soluble Proteins from Chloroplasts after KCN Treatment**—The ability to label the sulphydryl group of apoplastocyanin but not the holoplastocyanin suggested the experiment shown in Fig. 5. If the KCN removes the copper from plastocyanin and leaves the apoplastocyanin in the membrane, then it should be possible to label and to identify the apoplastocyanin from KCN-treated chloroplasts but not the holoplastocyanin from control chloroplasts.

Fig. 5 shows that the KCN-treated chloroplasts release proteins which are similar in electrophoretic profile to those released from control chloroplasts. Both extracts had been incubated with N-ethyl[14C]maleimide. As seen in the upper panel of Fig. 5, the KCN treatment causes the appearance of a large peak in N-ethyl[14C]maleimide counts per minute in a protein released from chloroplasts, and this peak corresponds to the Rf = 0.64 plastocyanin peak. The control preparation releases some labeled protein which appears as a shoulder at Rf = 0.64 but the extent of labeling is only one-third of that seen in the KCN-treated preparation.

The detailed procedure for the experiment shown in Fig. 5 is important because it offers the basis for our belief that the apoplastocyanin remains in the membrane following copper removal by KCN. Chloroplasts were prepared and inhibited with KCN as described under "Experimental Procedure." The chloroplasts were centrifuged to remove the KCN, the copper, and any proteins which were not membrane-bound. The chloroplasts were resuspended and assayed to ensure that inhibition had occurred. Following the assays, the chloroplasts were precipitated in -10°C acetone, resuspended in buffer, centrifuged, and the soluble protein was collected on a DEAE-column. The DEAE-column was eluted with 0.3 M NaCl, and fractions containing protein were pooled and treated with N-ethyl[14C]maleimide. Aliquots of the N-ethylmaleimide-labeled protein were subjected to sodium dodecyl sulfate gel electrophoresis, and Fig. 5 shows the 590-nm scan of the gels. After the gels were scanned, they were sliced into 1-mm slices and counted. Just prior to sodium dodecyl sulfate gel electrophoresis, 60 μg of purified plastocyanin were added to both the control and the KCN-treated samples, so that the plastocyanin peaks could be identified accurately.

**TABLE III**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>μmol of O2/mg of chlorophyll/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplasts</td>
<td>222</td>
</tr>
<tr>
<td>Chloroplasts, no gramicidin</td>
<td>100</td>
</tr>
<tr>
<td>Chloroplasts + diuron</td>
<td>0</td>
</tr>
<tr>
<td>Tween 20 chloroplasts</td>
<td>0</td>
</tr>
<tr>
<td>Tween 20 chloroplasts + 1.4 nmol of plastocyanin</td>
<td>255</td>
</tr>
<tr>
<td>Tween 20 chloroplasts + 5.7 nmol of apoplastocyanin</td>
<td>0</td>
</tr>
<tr>
<td>Tween 20 chloroplasts + 1.4 nmol of plastocyanin + diuron</td>
<td>0</td>
</tr>
</tbody>
</table>

**Fig. 4. A, N-Ethyl[14C]maleimide labeling of holoplastocyanin.** Addition of 50 μl of N-ethyl[14C]maleimide (1 mg of N-ethylmaleimide/ml at 2 μCi/μmol) to 0.33 ml of holoplastocyanin (1.5 mg/ml) was followed by incubation at 25°C for 1.5 hour. After incubation, the mixture was loaded on a 1 x 30 cm Sephadex G-25 fine column equilibrated with 0.1 M potassium phosphate buffer at pH 8.0 at 25°C. Fractions of 20 drops were collected at 30 s/drop. Fractions were assayed for absorbance at 280 nm, and 20-μl aliquots of each fraction were counted as described under "Experimental Procedure." A, K*CN cpm, O--O. B, the same protocol as in A except apoplastocyanin was used which had some K3Fe(CN)6 present to oxidize any holoplastocyanin present and to allow the calculation of the per cent of apoplastocyanin as described under "Results." The addition of 1 ml of water was made to each fraction before assaying for the absorbance at 590 nm or counting a 20-μl aliquot.

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<td>0</td>
</tr>
</tbody>
</table>

**Fig. 5 shows that the KCN-treated chloroplasts release**

1The abbreviations used are: pCMP, p-chloromercuribenzoate; diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, N-tris(hydroxymethyl)methylglycine; Mes, 2N-morpholinoethane sulfonic acid.
KCN treatment and that the apoprotein remained bound to the control. This indicates that copper was removed by the KCN-treated sample shows J-fold greater labeling than does control. For one minute, it is observed that the plastocyanin peak in the electrophoretic mobility of apo- and holoplastocyanin were the same, with N-ethylmaleimide. After normalization of the counts under these conditions. The regions of the gel which are under the peak at R, = 0.35 became significant labeled by the KCN treatment. Of the two peaks, only the protein under the peak at R, = 0.35 becomes significantly labeled with N-ethyl[14C]maleimide. After normalization of the counts per minute, it is observed that the plastocyanin peak in the KCN-treated sample shows 3-fold greater labeling than does the control. This indicates that copper was removed by the KCN treatment and that the apoprotein remained bound to the membrane.

**DISCUSSION**

Katoh reported that plastocyanin from spinach existed as a dimer of 21,000 daltons based on data obtained from the analytical ultracentrifuge (14). Each monomer contains one tightly bound copper atom which is responsible for the blue color of the protein in the oxidized state (19). The 21,000 dalton value has been widely cited in the literature (9, 12, 19, 21, 22). However, plastocyanins from many other plant species are known to be monomers of 10,500 daltons (21).

We have found that Sephadex gel chromatography of spinach plastocyanin with suitable low molecular weight standards shows that the spinach protein behaves as a monomer of about 10,500 daltons. In further support of the 10,500 dalton value, we have reported elsewhere NMR experiments on spinach and *Anaabaena variabilis* plastocyanin which are consistent only with monomeric structures (23).

It was reported earlier that Triton X-100 allows direct ascorbate reduction of P700 in control and polycation-inhibited chloroplasts (24). Polycations, like KCN, have been postulated to inhibit Photosystem I at the plastocyanin site (1, 2, 4). Triton X-100 reverses the KCN inhibition, apparently by allowing the direct reduction of P700 by ascorbate as in the reversal of polycation inhibition. This result indicates that the P700 is not inhibited or damaged by the KCN treatment, and confirms the optical and EPR measurements of Izawa *et al.* who found that KCN treatment had no effect on P700 oxidation by Photosystem I (6). Since KCN inhibits cytochrome f photooxidation by P700 or its direct reduction by ascorbate, we feel that it is likely that plastocyanin is the site of inhibition.

If KCN is to inhibit Photosystem I at the level of plastocyanin, one would expect KCN to affect the purified protein in some way. Fig. 1 shows that the loss of absorbance at 597 nm is paralleled by the loss of the ability to respond in a Plesnicar-type plastocyanin assay (25). Since the blue color is caused by the bound copper (19), it seemed possible that the KCN was binding to or removing the copper from the plastocyanin. Fig. 2 shows the effects of KCN on purified plastocyanin. The copper is completely removed and separated from the newly formed apoplastocyanin in this experiment. When holoplastocyanin without KCN treatment is subjected to Sephadex gel filtration, the absorbance at 280 nm, the absorbance at 597 nm, and the copper as measured in the atomic absorption spectrophotometer co-chromatograph.

KCN might cause the release of the copper from the plastocyanin which remains in the membrane as the apoprotein. Table II shows that sonications, a procedure known to remove all plastocyanin from chloroplast membranes, allows both control and KCN-treated membranes to be stimulated to the same extent on addition of purified plastocyanin. We interpret this to mean that the KCN treatment causes no damage to the photosynthetic electron transport apparatus other than the formation of apoplastocyanin.

If copper removal is in fact the mechanism of KCN inhibition of chloroplast Photosystem I, it should be possible to observe release of copper from the chloroplasts. Fig. 3 shows that the difference between the amounts of copper released from KCN and control chloroplasts after 90 min of incubation with KCN was about 2.5 μg of copper for chloroplasts equivalent to 12.5 mg of chlorophyll. The theoretical calculations under "Results" were based on the report by Arntzen *et al.* which gives a ratio of one plastocyanin molecule of molecular weight 21,000/500 chlorophyll molecules (26). The ratio should be modified to one plastocyanin molecule/250 chlorophyll molecules based on plastocyanin's molecular weight of 10,500.
This ratio agrees nicely with the data of Plesnicar et al. which show one plastocyanin copper molecule/200 chlorophyll molecules (25). Therefore, the calculations indicate that about 3.5 μg of plastocyanin copper should be present in chloroplasts equivalent to 12.5 mg of chlorophyll. If the copper released from the chloroplasts was in fact from plastocyanin, a net release of about 70% of the total plastocyanin copper was achieved by KCN treatment. Table I indicates that a typical KCN inhibition of chloroplasts amounts to about 70%. The strong correlation between these numbers and the results achieved with the KCN treatment of purified plastocyanin support the conclusion that the copper observed in the supernatant fluid of KCN-treated chloroplasts is largely copper removed from plastocyanin.

To obtain the data reported in Table III, we used a method for making plastocyanin-free chloroplast membrane as reported by Barr et al. (10). This method produces membranes which can be restored to good Hill activity and which show good coupling of phosphorylation to electron transport. Sonicated membranes do not show these activities. We feel that this plastocyanin-depleted membrane preparation warrants further study since it allows much better retention of chloroplast activities.

Others have reported a pCMB-reactive sulfhydryl group in plastocyanin (12, 21). This pCMB-reactive sulfhydryl group is found in both the holo- and apoplastic forms, although the reactivity of the apoplastic form is much more rapid. Fig. 4 shows a N-ethylmaleimide-reactive sulfhydryl group in the apoplastic form which is not present in the holoplastic form of plastocyanin. Plastocyanin has only a single sulfhydryl per monomer in the apoplastocyanin which is not present in the holoplastocyanin. The reaction with the apoplastocyanin is much more rapid. It is found in both the holo- and apoplastocyanin, although the data seem to support Katoh’s conclusion that the sulphydryl is a ligand of the copper and, therefore, unreactive to N-ethylmaleimide in the holoprotein. It is also possible that the copper merely blocks the accessibility of the N-ethylmaleimide to the sulfhydryl group. We have no evidence which would favor one possibility over the other, but the data seem to support Katoh’s conclusion that the single sulfhydryl group of plastocyanin is in fact a ligand to the copper atom. In either case, the conclusion that the sulfhydryl group’s environment is affected by the copper, seems warranted. A similar conclusion was reached with parsley plastocyanin. Graziani et al. have reported that the sulfhydryl group is in the vicinity of the copper based on EPR studies (27).

To prove that KCN removes the copper but not the protein from the membrane, KCN-treated chloroplasts were washed free of soluble protein by centrifugation prior to extraction and N-ethylmaleimide labeling of the apoplastic form. The presence of the labeled apoplastic form as seen in Fig. 5 indicates that the apoplastic form must have remained bound to the chloroplast membrane after copper removal by KCN.

Selman showed that following KCN or mercury treatment, the plastocyanin could be identified using sodium dodecyl sulfate gel electrophoresis as an 11,000 dalton peak. It was also shown that the plastocyanin from these inhibited preparations was inactive in a Plesnicar plastocyanin assay (9). We have used techniques similar to Selman and have shown that the inactivated plastocyanin is apoplastic in the case of KCN treatment. The mercury inactivation might be a slightly different case. Katoh speculated, due to the high affinity of mercuric ions for sulfhydryl groups, and due to the one to one stoichiometry between copper removed and mercury present, that the mercury-inactivated endogenous plastocyanin might not be the apoprotein but might be the mercury adduct of plastocyanin. Further experiments will be required to substantiate this possibility.

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