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Regulation by Copper of the Expression of Plastocyanin and Cytochrome c_{552} in *Chlamydomonas reinhardi*

SABEEHA MERCHANT AND LAWRENCE BOGORAD*

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

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Plastocyanin and cytochrome c_{552} are interchangeable electron carriers in the photosynthetic electron transfer chains of some cyanobacteria and green algae (P. M. Wood, Eur. J. Biochem. 87:9–19, 1978; G. Sandmann et al., Arch. Microbiol. 134:23–27, 1983). *Chlamydomonas reinhardi* cells respond to the availability of copper in the medium and accordingly accumulate either plastocyanin (if copper is available) or cytochrome c_{552} (if copper is not available). The response occurs in both heterotrophically and phototrophically grown cells. We have studied the molecular level at which this response occurs. No immunoreactive polypeptide is detectable under conditions where the mature protein is not spectroscopically detectable. Both plastocyanin and cytochrome c_{552} appear to be translated (in vitro) from polyadenylated mRNA as precursors of higher molecular weight. RNA was isolated from cells grown either under conditions favorable for the accumulation of plastocyanin (medium with Cu^{2+}) or for the accumulation of cytochrome c_{552} (without Cu^{2+} added to the medium). Translatable mRNA for preapoplastocyanin was detected in both RNA preparations, although mature plastocyanin was detected in *C. reinhardi* cells only when copper was added to the culture. Translatable mRNA for preapocytochrome, on the other hand, was detected only in cells grown under conditions where cytochrome c_{552} accumulates (i.e., in the absence of copper). We conclude that copper-mediated regulation of plastocyanin and cytochrome c_{552} accumulation is effected at different levels, the former at the level of stable protein and the latter at the level of stable mRNA.

Plastocyanin, a type I copper protein found in plant and algal chloroplasts, functions in photosynthesis as a mobile electron carrier between the membrane-bound cytochrome b_6-f complex and P-700, the reaction center of photosystem I (see reference 11 for review). In some green algae, plastocyanin may be functionally replaced by a soluble *c*-type (c_{552} or c_{553}) cytochrome (42, 49), which is analogous to the mobile cytochromes c_{555} and c_2 in anoxygenic green and purple photosynthetic bacteria (34, 37). This *c*-type cytochrome is distinct from the membrane-bound cytochrome *f* (48). Its properties are similar to those of plastocyanin with respect to solubility, size (approximately 11,000 daltons), and redox potential (around 370 mV) (4, 6, 15, 20). When both proteins are isolated from the same organism, the isoelectric points are in the same range—although among species the proteins range from acidic (pI 3.8) to basic (pI 9.5) (26). Since the charge on the carrier is believed to be important for its ability to catalyze P-700 reduction (12, 16), it was suggested that the paired variation in pI was the result of parallel evolution of both proteins in response to a change in the same reaction partner (26). Both proteins can catalyze the reduction of P-700 in vitro in reconstituted systems (30).

In algae which are capable of expressing both plastocyanin and the soluble *c*-type cytochrome, the choice between the accumulation of one or the other appears to depend principally upon the availability of copper in the medium (40, 49). If the amount of available copper is not sufficient to support plastocyanin accumulation at the stoichiometry required for photosynthesis (22), the cell uses the *c*-type cytochrome as an alternate and additional carrier.

In the green alga *Chlamydomonas reinhardi*, the plastocyanin-to-chlorophyll ratio in the cell can be changed from 1:150 to 1:10,000 by manipulating the concentration of Cu^{2+} in the medium. This change is accompanied by a concomitant, converse change in the cytochrome c_{552}-to-chlorophyll ratio from 1:100,000 to 1:600 (49). In *Scenedesmus acutus* in a full medium with 20 μM added Cu^{2+}, plastocyanin is found at a ratio of 1 per 150 chlorophyll molecules (9); whereas when Cu^{2+} is not available for plastocyanin synthesis *S. acutus* cells accumulate an immunoreactive polypeptide precursor of plastocyanin of higher apparent molecular weight (approximately 14,000) and perhaps an apoprotein (7, 8). At the same time the level of soluble plastidic cytochrome c_{553} increases to about 1 per 100 chlorophyll molecules (9, 40). The experiments with *Chlamydomonas* sp. cells (49) were not designed to detect precursors or apoproteins of plastocyanin. However, based on the inability to reconstitute spectroscopically detectable plastocyanin by the addition of Cu^{2+} to extracts from Cu^{2+}-depleted cells, Wood (49) suggested that the apoprotein does not accumulate under conditions where the mature protein is absent.

Our work is aimed at defining the signal which regulates the accumulation of plastocyanin and cytochrome c_{552} in *C. reinhardi* and the mechanism of signal transduction. We have therefore employed immunochemical methods to detect apoproteins, precursors, and translatable mRNAs for these proteins to extend the above observations. We describe a preliminary model for the regulation of the accumulation of plastocyanin and cytochrome c_{552} in *C. reinhardi*.

MATERIALS AND METHODS

Cell growth and culture. *C. reinhardi* 2137 (from Laurens Mets, University of Chicago) was grown either photoheterotropically in Tris-acetate-phosphate medium or

* Corresponding author.

phototrophically in minimal medium (18, 45). Copper-free medium was prepared by using a trace element solution (27) from which copper sulfate was omitted. All glassware used in the preparation of media and maintenance of the culture was washed with 6 N HCl. The cells were maintained for several generations in copper-free medium to ensure that they were indeed free of added copper.

Purification of plastocyanin. Plastocyanin was purified from *C. reinhardi* by modifications of procedures described previously (2, 19, 49). *C. reinhardi* cells were grown photoheterotrophically in TAP medium containing 8 μM chelated copper. Cells (10 liters) were collected by centrifugation at 5,000 × *g* for 5 min. Pelleted cells were washed once in 10 mM sodium phosphate (pH 7.0). The cells were frozen slowly at −70°C in 10 mM sodium phosphate (pH 7.0) at a concentration equivalent to 1 to 2 mg of chlorophyll per ml and thawed at room temperature. Broken cell debris was pelleted from the thawed materials, and the plastocyanin-containing supernatant was saved. The pellet was suspended in 10 mM sodium phosphate (pH 7.0), and the freeze-thaw-centrifugation step was repeated. Ammonium sulfate (38 g) was dissolved in 100 ml of supernatant from the previous step. The insoluble material was removed by centrifugation (30,000 × *g*, 10 min), the plastocyanin in the supernatant was oxidized by the addition of potassium ferricyanide, and the supernatant (approximately 150 ml) was loaded on to a 50-ml Sepharose 4B column equilibrated with 1.9 M ammonium sulfate in Tris-KCl buffer (50 mM Tris chloride [pH 7.5], 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA). Plastocyanin retained on the column was eluted isocratically. The fractions containing plastocyanin (approximately 30 ml) were pooled, dialyzed overnight (with one change) against 1 liter of Tris-KCl buffer, and loaded onto a DEAE-cellulose column (30 ml) equilibrated in Tris-KCl. The column was washed with 1 volume of column buffer, after which the plastocyanin was eluted by a KCl gradient (10 to 300 mM) in the same buffer. Plastocyanin was detected in the column fractions by its absorbance at 597 nm after the addition of potassium ferricyanide (to 1 mM). The plastocyanin-containing fractions were pooled, concentrated on an Amicon YM 2 membrane, and further purified on a Sephadex G-50 fine column (40 by 1.6 cm). The purity was estimated from a silver-stained gel (Fig. 1) and from amino-terminal sequencing as being greater than 99%.

Purification of cytochrome c_{552}. Cytochrome c_{552} was purified essentially as described by Gorman and Levine (19, 20) and Wood (49) with minor modifications as listed below. The protein was concentrated by ultrafiltration on Amicon YM 2 membranes instead of ion-exchange chromatography, DEAE-Sephacel was used instead of DEAE-cellulose, and Sephadex G-50 fine was used rather than Sephadex G-75.

Antibodies. Antibodies were prepared by popliteal lymph node injection of purified antigen (100 to 200 μg) into rabbits, followed by multiple intradermal boost injections (44).

Western blots. Proteins were separated on polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) (31) and transferred to a nitrocellulose membrane (pore size, 0.1 μm) electrophoretically (50 V, 2 to 3 h) in 25 mM Tris–192 mM glycine–20% methanol (46). Antigen was detected by using monospecific antibodies against cytochrome c_{552} or plastocyanin at 1:100 dilution and the Immun-Blot detection kit from Bio-Rad Laboratories. Calf serum (10%) was used instead of the gelatin (1%) recommended in the Bio-Rad protocol to decrease nonspecific reactions. Detergent was not used in the washes.

Preparation of RNA. RNA was isolated and fractionated either by the procedure of Schmidt et al. (43) or as described below. Cells from 1 liter of middle- to late-log-phase culture were collected by centrifugation (5 min at 5,000 × *g*) and washed in 300 ml of cold medium. The pellet was suspended in approximately 10 ml of 50 mM Tris chloride (pH 7.5)–40 mM EDTA–10 mM 2-mercaptoethanol, transferred to a mortar containing liquid nitrogen, and ground to a fine powder under liquid nitrogen. Five milliliters of Tris-equilibrated phenol (33) was added to the powder. The cells were thawed in the mortar with occasional grinding, and the paste was transferred to a 30-ml centrifuge tube. Five milliliters of chloroform-isoamyl alcohol (24:1) was added to the paste, and the suspension was shaken vigorously. The aqueous phase was separated from the phenol-chloroform-isoamyl alcohol by centrifugation at 6,000 × *g* for 5 min. The aqueous phase was reextracted with phenol and chloroform-isoamyl alcohol until the interface was clear. The aqueous phase was then extracted twice with an equal volume of chloroform-isoamyl alcohol (24:1). The nucleic acids were precipitated at −20°C by the addition of 2.5 volumes of cold ethanol. The pellet was washed once with 70% ethanol, dried under vacuum, and suspended in 2 ml of distilled water. RNA was then precipitated from solution by the addition of an equal volume of 4 M LiCl (0°C, 1 h). The pellet was suspended in water, precipitated by the addition of 2.5 volumes of ethanol, washed two times with 70% ethanol, dried under vacuum, and suspended in 1 ml of distilled water. The yield of RNA from 1 liter of cells was about 10 mg. Total RNA was separated into polyadenylated [poly(A)$^+$] and poly(A)$^-$ fractions on a 2- to 3-ml poly(U)-Sepharose column as described by the manufacturer (P-L Biochemicals, Inc., Milwaukee, Wis.). The RNA sample (1 ml) was diluted fivefold with starting buffer and passed through the column. The eluate was reapplied to the column. Unbound RNA [poly(A)$^-$] was precipitated overnight at −20°C by the addition of 2.5 volumes of ethanol. The column was washed with starting buffer until the absorbance at 260 nm was stable (about 5 column volumes). Poly(A)$^+$ RNA was eluted in 90% formamide–10 mM Tris chloride (pH 7.5)–10 mM EDTA–0.2% Sarkosyl and precipitated in ethanol, as described above, after the addition of sodium chloride to 0.7 M. The precipitates were washed two times with 70% ethanol, dried under vacuum, and suspended in 300 μl of distilled water. The RNA was analyzed by gel electrophoresis on 1.2% agarose gels containing formaldehyde (33) and by translation in vitro as described below. The purity of the poly(A)$^+$ and poly(A)$^-$ fractions was checked by hybridization of the RNA separated on formaldehyde gels to either a poly(A)$^+$-specific probe (pcf 8-31), a cDNA clone for the nuclear-encoded *C. reinhardi* β-tubulin (50), or a plastid RNA-specific probe (pZmc 427), the cloned gene for the 32-kilodalton quinone-binding polypeptide associated with photosystem II (3, 5, 17). RNA was blotted onto GeneScreen and hybridized to nick-translated probes according to protocols supplied by the manufacturer (New England Nuclear Corp., Boston, Mass.).

In vitro translation and immunoprecipitation. Total RNA (5 μg) or fractionated RNA (0.1 to 1.0 μg) was translated in vitro with rabbit reticulocyte lysate as described by the manufacturer (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) in 30-μl reaction mixtures. Proteins were labeled with [^{35}S]methionine. Translation reactions were stopped after 90 min by the addition of 1 μl of pancreatic RNase (10 mg/ml), followed by incubation for 10 min at 30°C. The samples were heated at 90°C for 5 min after the addition of an equal volume of solution containing 4% SDS–5

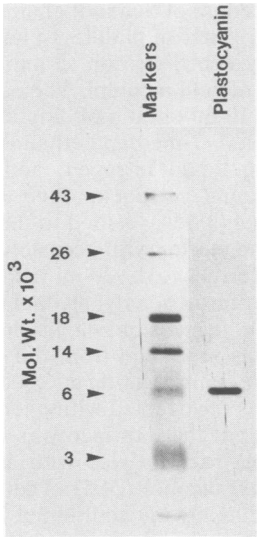

FIG. 1. SDS-15% polyacrylamide gel of purified *C. reinhardi* plastocyanin. Left lane contained unlabeled molecular weight markers from Bethesda Research Laboratories, 1 μg per band; right lane contained 5 μg of plastocyanin, purified as described in Materials and Methods. The gels were run, fixed, and stained as described in Materials and Methods. The stain is sensitive to 5 ng of protein.

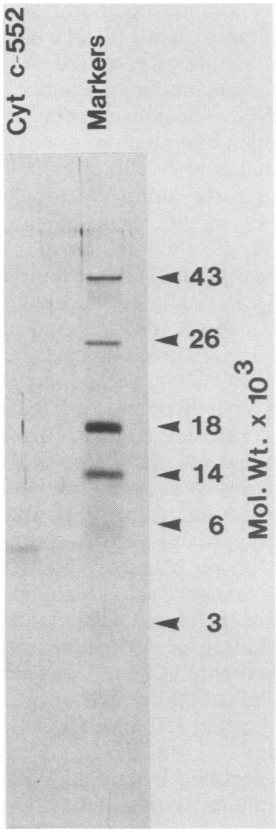

FIG. 2. SDS-15% polyacrylamide gel of *C. reinhardi* cytochrome c_{552}. Left lane contained 0.2 μg of cytochrome c_{552}, purified as described in Materials and Methods. The band can also be stained for heme-dependent peroxidase activity on 3,3'-diaminobenzidine (35). Right lane contained unlabeled molecular weight markers as in Fig. 1. The gel was treated as described in Materials and Methods.

mM EDTA–40 mM Tris chloride (pH 7.5). A volume equivalent to 1/15 of the reaction was removed for analysis by SDS-polyacrylamide gel electrophoresis. The remaining sample was diluted with 4 volumes of immunoprecipitation buffer containing 40 mM Tris chloride (pH 7.5)–150 mM NaCl–2% Nonidet P-40–2 mM EDTA. Aprotinin was added to a final concentration of 1.2 trypsin inhibitor units per ml (43). The samples were treated with preimmune serum at room temperature to remove nonspecifically reacting proteins. The immunoglobulin G (IgG) was removed by incubation with IgGSORB (25 μl of a 10% suspension) for 90 min. Each sample was then incubated with the appropriate antibody for 2 h at room temperature or overnight at 4°C, followed by incubation with IgGSORB for 90 min at room temperature with continuous agitation. IgGSORB was centrifuged through a solution containing 30% sucrose (28) to separate bound antigen-antibody complexes from the translation mixture. The pellets were washed four times with immunoprecipitation buffer, transferred to a new tube, washed once in 0.9% NaCl (14), and suspended in sample buffer for gel electrophoresis. Antigen was released from the IgGSORB by heating the sample for 5 min at 90°C. IgGSORB was removed by centrifugation (5 min at 10,000 × *g*), and the supernatants were analyzed by gel electrophoresis.

Gel electrophoresis. Samples were separated on 12 or 15% polyacrylamide gels containing 0.1% SDS (31). The gels were then stained (38) or fixed and fluorographed (10) as appropriate for the experiment.

Materials. Materials were from the following sources: Ammonium sulfate (Schwarz-Mann, Cambridge, Mass.); Sephadex G-50 fine, poly(U)-Sepharose, and Sepharose 4B (P-L Biochemicals); DE-52 (Whatman Ltd., Maidstone, England); IgGSORB (The Enzyme Center, Malden, Mass.); [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.); calf serum (Flow Laboratories, McLean, Va.); pancreatic RNase and aprotinin (Sigma Chemical Co., St. Louis, Mo.); rabbit reticulocyte lysate in vitro translation system and unlabeled, ^{14}C-labeled, and prestained molecular weight markers (Bethesda Research Laboratories, Gaithersburg, Md.); nitrocellulose membrane (Schleicher & Schuell Co., Keene, N.H.); Immun-Blot (GAR-HRP) assay kit (Bio-Rad Laboratories, Richmond, Calif.); Sarkosyl (International Biotechnologies, Inc., New Haven, Conn.).

RESULTS

Purification of plastocyanin and cytochrome c_{552}. The observation that pea plastocyanin will bind to unsubstituted Sepharose beads (2) was exploited to purify plastocyanin from *C. reinhardi*. The protein was greater than 50% pure after this step, so that one additional column (DEAE-cellulose) was sufficient to purify the protein. The advantage of this procedure over that employed by Gorman and Levine (19) is the significant improvement in yield resulting from fewer chromatographic steps. The absorption spectrum of the oxidized protein after the removal of potassium ferricyanide by gel filtration on Sephadex G-50 fine is indistinguishable from the published spectrum (19). The protein is >99% pure as evidenced by the single polypeptide on a silver-stained SDS-polyacrylamide gel (Fig. 1) and the unique amino-terminus sequence (data not shown). Although the protein migrates with a relative mobility of 6,000 daltons on a 15% polyacrylamide gel containing SDS, the minimum molecular weight as estimated from its amino acid composition is approximately 10,500.

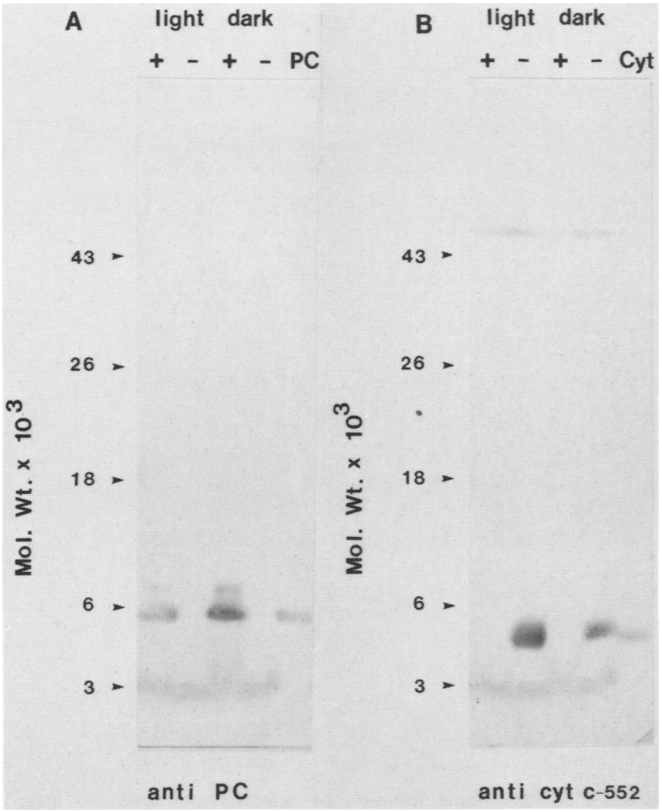

FIG. 3. Western blot of cell extracts from *C. reinhardi*. Cells (at a concentration equivalent to 1 to 2 mg of chlorophyll per ml) were frozen and thawed in 10 mM sodium phosphate (pH 7.0). Cell material equivalent to 20 μg of chlorophyll was denatured by heating to 90°C for 2 min in sample buffer for gel electrophoresis (31) at a concentration equivalent to 0.5 mg of chlorophyll per ml. Insoluble material was removed by centrifugation, and the proteins in the supernatant were separated on a 12% polyacrylamide gel containing 0.1% SDS by electrophoresis at 50 V for 16 h. Prestained molecular weight markers were used as standards on every gel. Transfer to nitrocellulose and immunodetection of plastocyanin and cytochrome c_{552} are described in Materials and Methods. Lanes: +, cells grown in a full medium containing 8 μm $CuSO_4$; −, cells grown in a medium free of added Cu^{2+}, estimated concentration of Cu^{2+} about 100 nM (49) PC, purified plastocyanin; Cyt, purified cytochrome c_{552}; light, cells grown photoheterotrophically; dark, cells grown heterotrophically. Anti-*C. reinhardi* plastocyanin was used as the primary antibody on the blot shown in A, and anti-*C. reinhardi* cytochrome c_{552} was used on the blot shown in B. The dark smudge with an apparent mobility of 3,000 is chlorophyll.

Purified *C. reinhardi* cytochrome c_{552} also migrates with an abnormally low apparent molecular weight (Fig. 2). However, its amino acid composition is similar to that of other cytochromes c_{552} (26) and indicates a minimum molecular weight of around 11,000.

Detection of polypeptides reactive to antibodies against *C. reinhardi* plastocyanin and cytochrome c_{552}. Monospecific antibodies against plastocyanin and cytochrome c_{552} were used to identify precursors, if any, of plastocyanin and cytochrome c_{552} in cells growing in the absence and presence of added copper (Fig. 3). Under conditions where

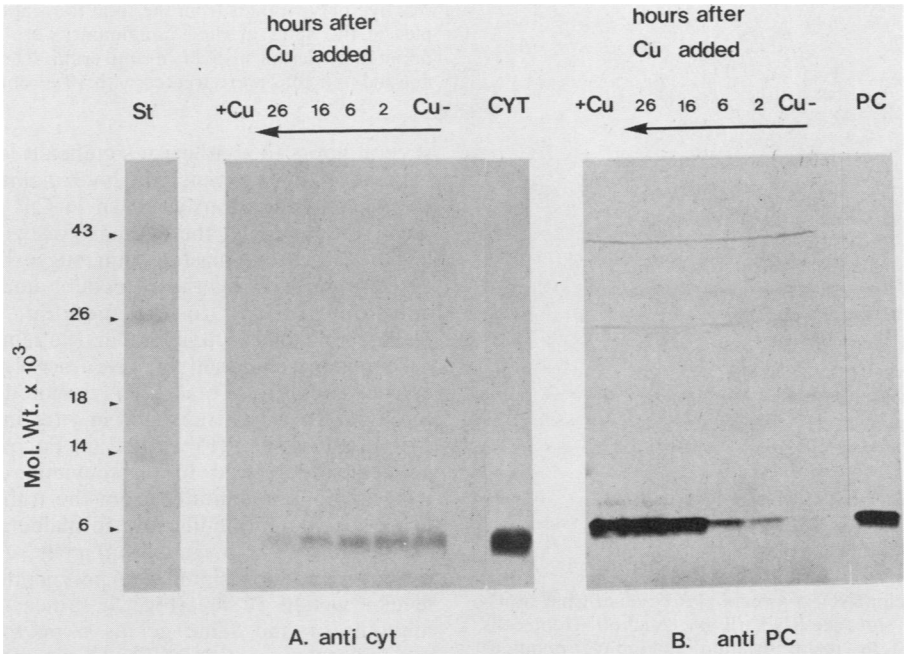

FIG. 4. Time course for induction of plastocyanin and loss of cytochrome c_{552} upon addition of Cu^{2+} (8 μM) to cultures maintained in the absence of added Cu^{2+}. A, Western blot of cell extracts from *C. reinhardi*. The blot was treated with anti-cytochrome c_{552}. B, Western blot of cell extracts from *C. reinhardi*. The blot was treated with anti-plastocyanin. Samples were prepared as described in legend to Fig. 3, except that the proteins were separated on a 15% polyacrylamide gel containing 0.1% SDS. St, prestained molecular weight markers, +Cu, cells maintained on 8 μM added Cu^{2+}; Cu−, cells maintained for several generations in the absence of added Cu^{2+}; 2, 6, 16, and 26, Cu^{2+} (8 μM Cu EDTA) was added to copper-depleted cultures during a period of exponential growth, and the cells were sampled 2, 6, 16, 26 h later, respectively; CYT, purified cytochrome c_{552}; PC, purified plastocyanin. The doubling time of the culture was about 10 h.

plastocyanin is spectroscopically detectable ($+Cu^{2+}$), a strong immunoreactive polypeptide which comigrates with purified plastocyanin is visible (Fig. 3A). When the cells are copper depleted so that plastocyanin does not accumulate, no immunoreactive material is detected. The results are independent of the growth conditions of the culture. The cells show the same response under conditions of phototrophic, photoheterotrophic, or heterotrophic growth. In some experiments a band migrating slightly slower than plastocyanin was detected in cells containing plastocyanin. It is not known whether this is a precursor form or an isoform of plastocyanin. That it is not a contaminating polypeptide is clear, since it is never seen in cells that do not accumulate plastocyanin.

A polypeptide reactive with antibody against cytochrome c_{552} can be detected only in cultures which are depleted of copper (Fig. 3B). This polypeptide comigrates with purified cytochrome c_{552} and can be detected in photoautotrophic, photoheterotrophic, and heterotrophic, copper-depleted cultures of *C. reinhardi*. Once again, precursors of cytochrome c_{552} or an apoprotein cannot be detected in cultures which do not contain spectroscopically detectable cytochrome c_{552}.

When Cu^{2+} (8 μM, Cu-EDTA) is added to a copper-depleted culture, the plastocyanin does not immediately reach the level maintained in cells grown with copper (Fig. 4), demonstrating that a polypeptide precursor to plastocyanin is not accumulating in copper-depleted cells. Although plastocyanin is detectable in 2 h, the cell requires several hours to synthesize significant levels and about two generation times to reach the level maintained in cells which have been continuously grown in Cu^{2+}-supplemented medium. Interestingly, the decrease of cytochrome c_{552} parallels the increase in plastocyanin, suggesting that cessation of cytochrome c_{552} synthesis occurs immediately upon the addition of Cu^{2+} to the medium, rather than after plastocyanin has accumulated to the required stoichiometry.

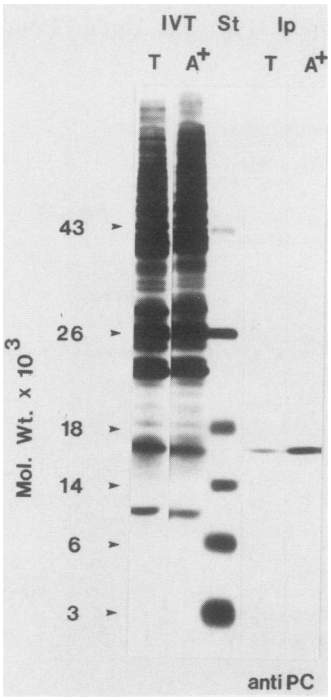

FIG. 5. Immunoprecipitation of preapoplastocyanin from translation products of *C. reinhardi* RNA. RNA isolation (from cells grown with added Cu^{2+}), in vitro translation, and indirect immunoprecipitation are described in Materials and Methods. Lanes: T, 5 μg of total RNA translated in a 30-μl reaction mixture; A^+, 0.1 μg of RNA selected on poly(U)-Sepharose; St, ^{14}C-labeled high-molecular-weight markers. Total products of in vitro translation (IVT) are shown on the left of the molecular weight markers. Immunoprecipitates (Ip, with 10 μl of anti-plastocyanin) from the in vitro translation mixtures are shown on the right.

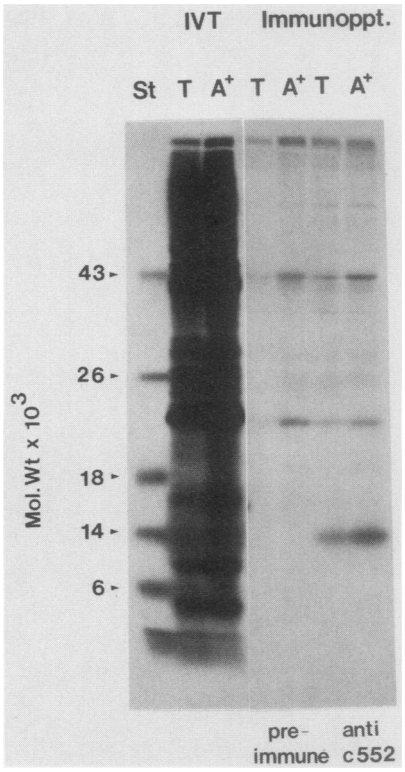

FIG. 6. Immunoprecipitation of preapocytochrome c_{552} from translation products of *C. reinhardi* RNA isolated from cells grown in the absence of added Cu^{2+}. Lanes: ST, ^{14}C-labeled molecular weight markers; T, 5 μg of total RNA translated in a 30-μl reaction mixture; A^+, 0.1 μg of RNA selected on poly(U)-Sepharose; IVT, total products of translation; Immunoppt., immunoprecipitation of reactive polypeptides from the total translation products. The samples in the lanes marked preimmune were treated with 10 μl of preimmune serum instead of antiserum. The samples in the lanes marked anti c552 were treated with 10 μl of antiserum.

Immunoprecipitation of precursors to plastocyanin and cytochrome c_{552}. Total RNA isolated from cells of *C. reinhardi* 2137 was translated in vitro in the rabbit reticulocyte lysate system (Fig. 5 and 6). Polypeptides which were antigenically related to plastocyanin or cytochrome c_{552} were immunoprecipitated from the translation mixtures by using specific antibodies and formaldehyde-fixed *Staphylococcus aureus* (Cowan strain) cells. Antibody to plastocyanin immunoprecipitated a polypeptide of apparent molecular weight 17,000 (Fig. 5). Since the purified protein migrates on the same gel as a polypeptide of apparent molecular weight, 6,000 (Fig. 1), we conclude that, like the barley and wheat plastocyanins (39), *C. reinhardi* plastocyanin is synthesized in precursor form. The primary translation product from mRNA for spinach plastocyanin appears to be larger than the one from the mRNA for *C. reinhardi* plastocyanin and has an apparent molecular weight of 25,000 (23). However, the purified spinach and *C. reinhardi*

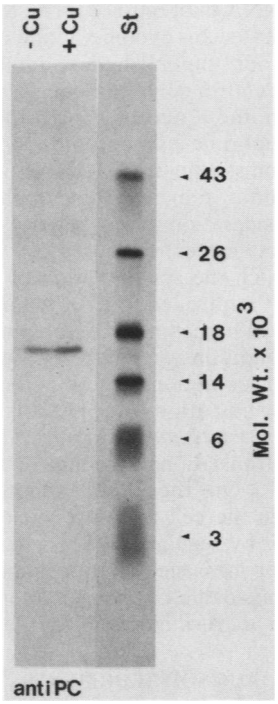

FIG. 7. Immunoprecipitation of preapoplastocyanin from translation proucts of RNA isolated from cells grown with or without added Cu^{2+}. Lanes: −Cu, immunoprecipitate from translation products of 5 μg of total RNA isolated from cells grown without added Cu^{2+}; +Cu, immunoprecipitates from translation products of 5 μg of total RNA from cells grown with 8 μM added Cu^{2+}; St, ^{14}C-labeled molecular weight markers.

plastocyanins also show a rather large difference in size on 15% SDS–polyacrylamide gels (14,000 and 6,000, respectively). Since both proteins have very similar molecular weights (approximately 10,500) on the basis of their amino acid sequence and composition (11; our unpublished results), it is not possible to guess at the true size of the precursors based on the mobilities of the proteins on SDS-polyacrylamide gels. The same is true for cytochrome c_{552}, which is also translated from *C. reinhardi* RNA as a precursor. A polypeptide of apparent molecular weight 14,000 can be specifically immunoprecipitated from the translation reaction mixtures with antiserum against cytochrome c_{552} (Fig. 6). The messages for preapoplastocyanin (17,000) and preapopcytochrome c_{552} (14,000) are both selectable on poly(U)-Sepharose (Fig. 5 and 6), indicating that the mRNAs are poly(A)$^+$. The translation products from poly(A)$^-$ RNA did not contain any proteins which were antigenically related to plastocyanin or cytochrome c_{552}, suggesting that plastocyanin and cytochrome c_{552} are nuclear encoded in *C. reinhardi*. The purity of the poly(A)$^+$ and poly(A)$^-$ RNA was judged by Northern analysis as described in Materials and Methods. The poly(A)$^-$ RNA fraction did not appear to contain any message for a nuclear gene-specific probe (β-tubulin) and was 100-fold enriched [over the poly(A)$^+$ fraction] for RNA which hybridized to a probe specific for a chloroplast gene. The products of poly(A)$^-$ RNA translated by rabbit reticulocyte lysate are minor compared to those of an equal amount of total RNA. If plastocyanin and cytochrome c_{552} are nuclear-encoded, cytoplasmically synthesized proteins destined for the chloroplast, the precursors probably contain an NH_2-terminus "transit" sequence (13), which is required for their posttranslational uptake into the organelle (23, 36). Part of this transit sequence may be designed to aid transport across the thylakoid membrane, since both proteins are probably located in the luminal space.

Level of regulation by Cu^{2+}. mRNA which directs the synthesis of preapoplastocyanin by rabbit reticulocyte lysate is present in cells grown with added Cu^{2+} as well as in cells grown in the absence of Cu^{2+} (Fig. 7). Since plastocyanin does not accumulate in cells grown without Cu^{2+} (Fig. 3A), it may be concluded that either the level of plastocyanin in these cells is regulated by translation or the (pre)apoprotein is unstable in the absence of the metal ligand and is degraded. mRNA for preapocytochrome c_{552}, on the other hand, is present only in cells which are grown in the absence of added Cu^{2+} (Fig. 8), indicating that Cu^{2+} may directly or indirectly inhibit transcription of the gene for cytochrome c_{552} or that Cu^{2+} may decrease the stability of the message.

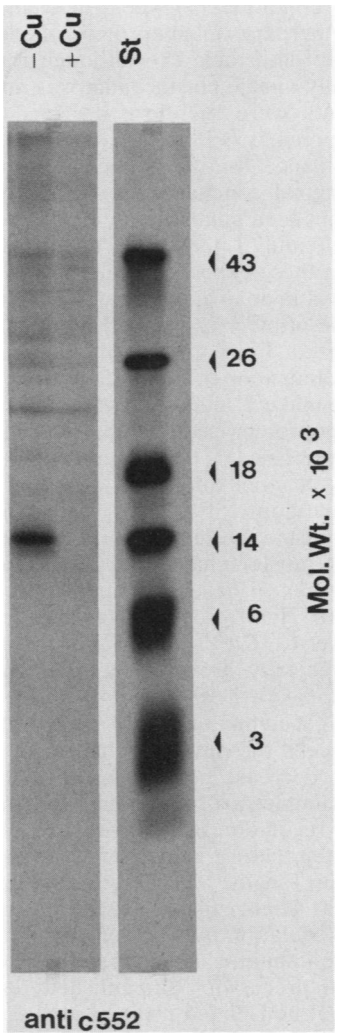

FIG. 8. Immunoprecipitation of preapocytochrome c_{552} from translation products of RNA isolated from cells grown with or without added copper. Lanes: −Cu, immunoprecipitate from translation products of 5 μg of total RNA isolated from cells grown without added Cu^{2+}; +Cu, immunoprecipitate from translation products of 5 μg of total RNA isolated from cells grown with Cu^{2+} added to 8 μM; St, ^{14}C-labeled molecular weight markers.

DISCUSSION

The results presented above demonstrate that no polypeptide that is a precursor to either plastocyanin or cytochrome c_{552} is accumulated under conditions where the mature protein is absent. Although Wood (49) had established that plastocyanin was present only in *Chlamydomonas* sp. cells which were grown in a medium containing added Cu^{2+} and was replaced by cytochrome c_{552} if the medium did not support the accumulation of plastocyanin, he was not able to directly detect apoproteins since his observations were based on spectroscopic measurements. He attempted to look for the apoprotein by trying to reconstitute plastocyanin from extracts of Cu^{2+}-depleted cells plus added Cu^{2+} and was led to conclude that the apoprotein did not accumulate under conditions where plastocyanin was not detectable. However, the inability to reconstitute plastocyanin from cell extracts need not necessarily imply that the apoprotein is absent; it may merely reflect the difficulty in achieving reconstitution of plastocyanin from the apoprotein. In *S. acutus*, under conditions of Cu^{2+} deficiency, a putative precursor and perhaps an apoprotein of plastocyanin are immunologically detectable (8), although reconstitution of spectroscopically visible plastocyanin was not achieved (9). Our experiments were therefore designed to detect immunoreactive precursors in *C. reinhardi*, if present. In the absence of evidence for the existence of precursors, we support the original conclusion of Wood (49) and suggest that plastocyanin accumulation may be differently regulated in *C. reinhardi* and *S. acutus*. The possibility that the antisera used in these experiments do not recognize precursors of plastocyanin or an apoprotein is discounted in light of the fact that the primary translation product synthesized in vitro is recognized. Furthermore, the kinetics of accumulation of plastocyanin upon the addition of Cu^{2+} to the growing culture (Fig. 4) make it unlikely that a pool of accumulated precursor or apoprotein exists within the cell.

There are several signals that the cell might use to regulate cytochrome c_{552} accumulation. The possibilities include the direct measurement of Cu^{2+}, the measurement of plastocyanin, or the requirement for a functional photosynthetic electron transfer chain. The direct measurement of Cu^{2+} by *C. reinhardi* cells is intimated by the following observations. (i) Heterotrophically grown cells show the same response to Cu^{2+} as do phototrophically and photoheterotrophically grown cells, implying that dependency on photosynthesis is not a prerequisite for cytochrome c_{552} accumulation. (ii) Upon addition of Cu^{2+} to Cu^{2+}-depleted cells the ratio of cytochrome c_{552} to chlorophyll begins to decrease before plastocyanin has accumulated to a stoichiometrically significant extent (Fig. 4), implying that the regulation of cytochrome c_{552} accumulation occurs at a step before stoichiometric accumulation of plastocyanin. (iii) Finally, a plastocyanin-deficient mutant of *C. reinhardi* has been isolated (32). This mutant (ac-208) shows normal regulation by Cu^{2+} of cytochrome c_{552} accumulation and no immunochemically detectable plastocyanin or accumulated precursors (unpublished results). Such a mutant could not be isolated if the absence of plastocyanin were able to induce cytochrome c_{552} accumulation, i.e., if plastocyanin itself were a regulatory agent. In *S. acutus*, some of the effects of Cu^{2+} on plastocyanin and cytochrome c_{553} accumulation can be mimicked by Ag^{+}, suggesting that regulation of plastocyanin and cytochrome expression is mediated via a metal-sensing system (41).

Both plastocyanin and cytochrome c_{552} are translated from poly(A)$^+$ mRNA in precursor form. Since their synthesis in vivo is inhibited by cycloheximide and not by chloramphenicol (41; our unpublished results on plastocyanin synthesis in *C. reinhardi* cells), it appears that both proteins are nuclear genome encoded. All other chloroplast cytochromes (membrane cytochromes b_6, b_{559}, and *f*) are plastid encoded and synthesized (1, 24, 25, 47). The potential nuclear localization of the gene for cytochrome c_{552} should be taken into consideration in any general model for assembly of plastid cytochromes.

The level at which the accumulation of plastocyanin and cytochrome c_{552} is controlled in *C. reinhardi* is different for the two proteins. Whereas mRNA for plastocyanin is present under all conditions, mRNA for cytochrome c_{552} is present only under conditions where cytochrome c_{552} accumulates. The accumulation of plastocyanin therefore is either affected by translational control (by Cu^{2+}) or by instability of the translational product or apoprotein in the absence of Cu^{2+}. On the other hand, the amount of cytochrome c_{552} in the cell is controlled either at the transcriptional level or by regulation of processing and turnover of the transcript or message. The mechanism by which the cell measures the available Cu^{2+} poses an intriguing problem which we hope to address biochemically and genetically.

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