Degradation of Plastocyanin in Copper-deficient Chlamydomonas reinhardtii

EVIDENCE FOR A PROTEASE-SUSCEPTIBLE CONFORMATION OF THE APOPROTEIN AND REGULATED PROTEOLYSIS*

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In the green alga Chlamydomonas reinhardtii, the copper-dependent accumulation of plastocyanin is affected via the altered stability of the cytochrome protein in copper-deficient versus copper-sufficient medium (t<sub>50</sub> < 20 min versus several hours). To understand the mechanism of plastocyanin degradation in vivo, the purified apoprotein was characterized relative to the holoprotein with respect to conformation and protease susceptibility. Circular dichroism spectroscopy revealed that the apoprotein in solution did not display the characteristic secondary structure displayed by the native or reconstituted holoprotein. The apoprotein was also susceptible to digestion in vitro by chymotrypsin whereas the holoprotein was resistant. High ionic conditions, which stabilize the folded structure of apoplastocyanin, also inhibit its degradation by chymotrypsin. These results suggest that one explanation for plastocyanin degradation in copper-deficient cells in vivo might be the increased susceptibility of the apo form to a luminal protease. Since apoplastocyanin is a normal biosynthetic intermediate for the formation of holoplastocyanin, the increased susceptibility of apoplastocyanin to proteolysis implies that degradative and biosynthetic activities would compete for the same substrate. However, characterization of an apoplastocyanin-accumulating mutant suggests that a plastocyanin-degrading protease is active only in copper-deficient cells. Thus, apoplastocyanin is rapidly degraded in copper-deficient cells, whereas its major fate in copper-supplemented cells is holoplastocyanin formation.

Plastocyanin is a small (97–104 amino acids), lumen-localized, copper-binding protein that functions in photosynthesis to catalyze electron transfer from cytochrome f of the membrane-associated cyt<sup>+</sup> b<sub>5<f>complex</f></sub> complex to P<sub>700</sub> in Photosystem I, and in respiration (in cyanobacteria) to catalyze electron transfer from the cytochrome b<sub>5</sub>f complex to the terminal oxidase (reviewed recently by Morand et al., 1994; Redinbo et al., 1994). It contains a single redox active copper (E<sub>0</sub> = -370 mV) and is referred to as a "blue" copper protein owing to the absorption properties of the oxidized form of the protein. The catalytic activity of plastocyanin is, of course, dependent on this copper center. Accordingly, copper-deficient plants or chelator-treated thylakoid membranes display loss of plastocyanin activity and impaired photosynthesis.

In the case of green algae and cyanobacteria, however, some species have the capacity to survive copper deficiency by inducing a soluble c-type cytochrome to serve as a functional backup for plastocyanin (Wood, 1978; Sandmann et al., 1983; reviewed by Merchant, 1995). In these organisms, if copper is available in amounts sufficient to satisfy the plastocyanin biosynthetic pathway, this copper protein accumulates and is used for photosynthetic electron transfer. Generally, cyt<sub>c</sub> is not detected in such cultures. However, under conditions of copper deficiency (which would limit or prevent the synthesis of a functional form of plastocyanin), green algae and cyanobacteria remain photosynthetically competent by inducing the synthesis of heme-containing cyt<sub>c</sub>. These organisms thus serve as excellent model systems for the study of the copper-dependent synthesis of plastocyanin.

Regulation of plastocyanin accumulation has been examined in Chlamydomonas reinhardtii, Scenedesmus obliquus, and Pediasastrum boryanum among the green algae, and Anabaena and Synechocystis spp. among the cyanobacteria (Merchant and Bogorad, 1986a, 1986b; van der Plas et al., 1989; Briggs et al., 1990; Bovy et al., 1992; Li and Merchant, 1992; Nakamura et al., 1992; Zhang et al., 1992; Ghassemian et al., 1994). Examination of mRNA and protein abundance as a function of copper concentration provides evidence for copper-responsive regulation at two stages in plastocyanin biosynthesis: 1) at the level of template accumulation (mRNA abundance) and 2) at the level of accumulation of mature protein. The relative contribution of processes regulating mRNA abundance versus protein abundance differs in various organisms and can depend upon the growth phase of the culture (reviewed by Merchant, 1996). In general, when both levels of control are displayed, lower concentrations of copper are required to induce the accumulation of plastocyanin-encoding mRNAs than are required for the accumulation of the protein. For the latter, the requirement for copper appears to be stoichiometric (Merchant et al., 1991). This suggests that the binding of copper to the polypeptide might be an important factor in determining the steady state abundance of the protein.

The mechanisms underlying differential accumulation of the protein have been investigated further in Chlamydomonas by pulse-radiolabeling experiments. The results indicate that translation of the message as well as import and processing of the translation product occur normally and regardless of...
whether copper is present, which is consistent with a model in which association of copper with apoplastocyanin occurs after translocation of the protein into the thylakoid lumen (Merchant and Bogorad, 1986b; Li et al., 1990). However, in copper-deficient cells, mature plastocyanin is degraded ($t_\text{1/2} < 20$ min), whereas in copper-supplemented cells, the newly synthesized protein is extremely stable ($t_\text{1/2} >$ several hours). If the apoform of the protein were a better substrate for proteolysis than the holoform, the copper-dependent differential degradation of plastocyanin could be explained. In copper-deficient cells, the newly synthesized protein would remain in the protease-susceptible apoform, whereas in copper-supplemented cells, it would convert to the protease-resistant holoform. Crystalllographic studies reveal only minimal structural changes between apoplastocyanin and holoplastocyanin (Garrett et al., 1984); however, in solution, the apoprotein was noted to be much less stable (Koide et al., 1993). Thus, it is reasonable to suggest that it might indeed be more susceptible to one or more chloroplast proteases in vivo compared to the holoprotein, whose conformation is metal-stabilized (McLendon and Radany, 1978; Parsell and Sauer, 1989; Koide et al., 1993).

There are many well-documented examples of specific degradation of proteins in chloroplasts. In Chlamydomonas, the small subunit of Rubisco is selectively degraded if it cannot assemble with large subunits to form the multimeric holoenzyme (Schmidt and Mishkind, 1983). Likewise, in trypanosomes, the nuclear-encoded subunits of the chloroplast coupling factor are degraded in the absence of synthesis of the chloroplast-encoded subunits (Biekmann and Feierabend, 1985). In the case of chlorophyll-protein complexes, the apoproteins are degraded under conditions where chlorophyll synthesis is reduced (e.g. Bennett, 1981; Slovin and Tobin, 1982; Kim et al., 1994). And, for the electron transfer complexes, the pleiotropic effects of mutations affecting a single subunit of the photosynthetic complexes may have been shown, in many cases, to result from the specific degradation of the remaining subunits of the affected complex while other (unaffected) complexes accumulate normally (Erickson et al., 1988; Kuchka et al., 1989; Pakrasi et al., 1991; Takahashi et al., 1991; Kuras and Wollman, 1994). Distinct protease activities have been localized to chloroplasts, but their relationships to specific degradation processes have not yet been fully described (Liu and J. agendorn, 1984; Kuwabara and Hashimoto, 1990; Hooper and Hughes, 1992; Bushnell et al., 1993). Questions of interest with regard to proteolytic events in the chloroplast relate to location, regulation, substrate recognition, and substrate specificity. In the case of plastocyanin, the degradative activity is presumed to be lumens-localized, or at least thylakoid membrane-associated, since the fully processed form of the protein serves as the substrate for degradation.

We sought to compare the properties of purified apo- and holoplastocyanin with respect to protease susceptibility in vitro and in vivo with a view to increasing our understanding of 1) proteolytic processes in chloroplasts and 2) the mechanism of adaptation of the photosynthetic apparatus to copper-deficiency. The data presented in this work support a model in which apoplastocyanin is relatively unstable in solution. We also show that apoplastocyanin is highly susceptible in vitro to proteases. Nevertheless, the increased susceptibility of apoplastocyanin to proteolysis may not be sufficient to explain the regulation of its degradation in vivo. We propose that a specific mechanism exists for activating an apoplastocyanin-degrading protease in copper-deficient cells and that this additional factor is an important determinant of the rate of apoplastocyanin degradation in vivo.

**EXPERIMENTAL PROCEDURES**

Cell Growth and Culture—C. reinhardtii strain CC125 (wild-type) was grown at 22 °C under 125 μmol/m²/s illumination in either copper-deficient or copper-supplemented Tris acetate-phosphate (TAP) medium (Merchant and Bogorad, 1986a; Harris, 1988). A plastocyanin-deficient mutant, strain pC235, was maintained at room temperature in TAP medium under fluorescent house lights on the laboratory bench with occasional swirling. The mutant strain was transferred to an inoculum of 15–25 μmol/m²/s, 22 °C, with continuous agitation at 225 revolutions/min for 1 or 2 days before collecting cells for isolation of protein or other analysis.

Analysis of Protein—Protein concentration was determined using the Coomassie Blue G-250 dye binding method as described by the manufacturer of the reagent (Pierce). Cell extracts containing soluble proteases were separated by non-denaturing gel electrophoresis (Li and Merchant, 1992), and proteases were visualized by staining with Coomassie Blue R-250 or silver staining (Oakley et al., 1980). Western blot analysis of plastocyanin levels was performed as described previously (Li and Merchant, 1992). Bound antibody was detected with an alkaline phosphatase-conjugated secondary antibody (Bio-Rad). To ensure that plastocyanin was quantitatively released into the supernatant fraction after freeze-thaw lysis of the cells, the pellet fractions were occasionally analyzed (after detergent solubilization) for immunoreactive plastocyanin species. In vivo radiolabeling and analysis of the radiolabeled products was carried out as described previously (Merchant and Bogorad, 1986).

Purification of Plastocyanin—Plastocyanin was purified as described by Redinbo et al. (1993) except that the final ultracentrifugation step on Centricon-30 was eliminated. No impurities were detected by silver staining of 1 μg of protein eluted from the G-50 column and analyzed after electrophoresis on SDS-containing polyacrylamide gels.

Preparation of Apoplastocyanin and Reconstitution of Holoplastocyanin—Apoplastocyanin was prepared by removal of Cu(I) from purified plastocyanin under a nitrogen atmosphere (see Tamilarasan and McMillin, 1986; Li and Merchant, 1992). In a typical reaction, 60 μm plastocyanin in 5 ml of 10 mM sodium phosphate (pH 7.0) was reconstituted with the addition of sodium ascorbate (5-fold molar excess) and dialyzed against 500 ml of a solution containing 25 mM Tris-Cl (pH 8.05) and 10 mM KCl for 2 h at 0 °C. Excess cyanide was removed by dialysis (also under a nitrogen atmosphere) against a solution containing 25 mM Tris-Cl (pH 8.05) for 3 h with one change. The yield of apoplastocyanin was about 84–90%. To prepare apoplastocyanin in a high salt solution, (NH₄)₂SO₄ was added to 0.5 M into the dialysis buffers. Reconstitution of holoplastocyanin was performed by direct addition of cupric ions (10-fold molar excess) in the form of cupric acetate into the above apoplastocyanin preparation (concentration ~60 μM in total volumes ranging from 1 to 5 ml). Excess copper was removed by dialysis against 500 ml of a solution containing 25 mM Tris-Cl (pH 8.05) with one change. Where indicated, the dialysis buffer included 0.5 M (NH₄)₂SO₄. All protein samples (apoplastocyanin and reconstituted holoplastocyanin) were stored at −20 °C for subsequent analysis including determination of protein concentration, difference spectroscopy, and protease susceptibility assays. About 50–85% of the apoprotein was reconstituted with copper by this method. Copper-containing plastocyanin was detected by a difference spectrum (ferri/cyanide-oxidized minus ascorbate-reduced), and the amount of holoplantoxy in was estimated using a difference extinction coefficient of 49 mM⁻¹ cm⁻¹ at 595 nm. The difference spectra representative of the samples of plastocyanin used for measurement of CD spectra and protease susceptibility are shown (Fig. 1). The visible and UV spectra (not shown) were recorded on a UV/scan 930 spectrophotometer. The estimation of holoplantoxy content was not affected by salt concentration.

Measurement of Circular Dichroic Spectra—The CD spectra were measured in a Jasco 600 spectropolarimeter (J. panop SPS) in a 0.1-mm cylindrical quartz cuvette with samples containing 1) purified (apoplastocyanin), 2) purified holoplastocyanin, or 3) reconstituted holoplastocyanin (all at 0.56 mg/ml) in a solution buffered with Tris-Cl (pH 8.05). Measurements at 2-nm intervals (at 2 cm⁻²/cm thickness, averaged 1024 scans, measured over 4 measuring cycles) were made at room temperature. The spectrum of the solvent was subtracted from the spectra of proteins. The CD spectra of plastocyanin after modification of the Cystein Side Chain in Apoplastocyanin—To apo- or holoplastocyanin (12 μg in 50 μl of a solution containing 25 mM Tris-Cl (pH 8.05), 1 μl of 4 M maleimidylphenyl methyl or cyanide (Molecular Probes Inc., Eugene, OR) in dimethyl formamide was added. After 60 min at room temperature, the sample was diluted 20-fold with 10 mM sodium phosphate (pH 7.0), and 2 μl of dithiothreitol was added.
from a 100 mM stock solution to a final concentration representing a 50-fold molar excess with respect to the maleimidyphenyl methylcoumarin concentration. Under these conditions, the dissociation of copper from holoplasticyanin is avoided. The protein in the diluted sample was subsequently concentrated in a Centricon-3 unit (Amicon Corp., Beverly, MA) to remove excess dye and dithiothreitol. One-third of the concentrated sample was analyzed by electrophoretic separation in polyacrylamide gels under non-denaturing conditions to resolve the various forms of plasticyanin (Li and Merchant, 1992). Each of the samples was also analyzed on an SDS-containing gel after reduction of disulfide linkages to confirm that each form indeed comigrated after denaturation.

Protease Susceptibility Assay—10 μg of protein (holoplasticyanin or apoplasticyanin) was incubated at 25 °C with 0.02 units of chymotrypsin (diluted from a 1 mg/ml stock) in a solution buffered with 100 mM sodium Tricine (pH 8.0) with or without the addition of 0.5 M (NH₄)₂SO₄, as indicated, in a total volume of 30 μl. The reaction was timed from the addition of protease. At each time interval, a 5-μl aliquot was removed and transferred to another tube containing phenylmethylsulfonyl fluoride (1 μl of a 1 mg/ml stock solution in 100% isopropyl alcohol) to stop the reaction. The product was diluted with H₂O so that the final salt concentration was <50 mM. Each sample was analyzed by non-denaturing electrophoresis in a polyacrylamide gel as described (Li and Merchant, 1992). The protein was visualized by staining with a silver reagent (Oakley et al., 1980). The specific activity of chymotrypsin at different salt concentrations, assessed according to the manufacturer’s instructions using BTEE (N-benzoyl-l-tyrosine ethyl ester) (Sigma) as a substrate, was found to be independent of salt concentration within the range tested (0–0.5 M (NH₄)₂SO₄).

Strain pc235—The plasticyanin-deficient strain pc235 was chosen from a collection of non-photosynthetic mutants enriched by metronidazole treatment after UV mutagenesis (Harris, 1989). Survivors of the metronidazole enrichment step were tested on minimal and TAP media to confirm their acetate-requiring phenotype. Western blot analysis of cell extracts prepared from non-photosynthetic mutants allowed us to identify strains displaying a plasticyanin-minus (or plasticyanin-deficient) phenotype. Five such mutants (referred to as pc strains) were identified. The defect in these strains appeared to be localized to plasticyanin biosynthesis, since the pc mutants displayed a conditional acetate-requiring phenotype. Specifically, pc strains were acetate requiring in copper-containing medium but not in copper-deficient medium (when plastocyanin function is taken over by cytochrome c₅₅₅). One of these copper-deficient strains, strain pc235, which exhibited a leaky non-photosynthetic phenotype, was found to accumulate higher levels of apoplasticyanin, but lower levels of holoplasticyanin, relative to wild-type strains, despite full copper supplementation of the medium. Northern blot analysis of the mRNA isolated from pc235 indicated that it contains normal amounts of plasticyanin encoding messages. The mRNA encoded a preproprotein that could be precipitated with antibodies raised against plasticyanin and that co-migrated with the wild-type pre-protein. In vivo radiolabeling experiments indicated that plasticyanin synthesis and maturation in this strain was normal (see Fig. 6) and comparable to a wild-type strain. Further, sequence analysis of the plasticyanin-encoding genomic DNA in pc235 indicated a wild-type sequence for the entire transcribed region, including the intron. The phenotype therefore was concluded to result from a mutation lying outside the structural gene for plasticyanin. The defect in pc235 is thus attributed to a trans-acting factor that determines either the formation of holoplasticyanin from apoplasticyanin or that stabilizes the holoprotein in vivo after it is assembled. In either case, most of the plasticyanin in strain pc235 must be thermodynamically less stable (see “Results”).

Miscellaneous Materials—“Gold-label” chemicals for preparation of copper-free medium were purchased from Aldrich. All other chemicals were purchased from either Fisher Scientific or Sigma, unless otherwise specified.

RESULTS

Preparation of Apoplasticyanin and Its Reconstitution with Copper—In previous work (Merchant and Bogorad, 1986b), one of us had noted that newly synthesized plasticyanin was degraded in copper-deficient cells but was stable in copper-supplemented cells. A simple explanation for differential degradation was that copper-deficient cells contained a form of plasticyanin that was more susceptible to degradation. Since copper-deficient cells were found to synthesize and process plasticyanin precursors at approximately the same rate as copper-supplemented cells, it was likely that mature apoplasticyanin was the protease-susceptible species in vivo. To test the properties of apoplasticyanin with respect to its structure and susceptibility to proteases, it was necessary to prepare purified apoplasticyanin, and this was accomplished by chelation of copper with cyanide (Fig. 1). Visible spectroscopy confirmed that the preparation of apoplasticyanin displayed very little absorbance characteristic of the blue copper center. The yield of apoplasticyanin in various preparations ranged from 84 to 90%. The ability of apoplasticyanin to reconstitute with copper indicated that the preparation was not irreversibly denatured (Fig. 1C).

The structure of the native protein in the crystal form revealed that it is an eight-stranded, anti-parallel β-sandwich (Colman et al., 1978; Guss and Freeman, 1983). Removal of the copper from the crystal did not affect the structure very significantly, and it was concluded that copper was not required for the folding of the polypeptide into its native structure (Garrett et al., 1984). Nevertheless, in vitro folding studies revealed that although apoplasticyanin was capable of folding in the absence of copper, the folded structure was not stable in solution in the absence of high concentrations of salt (Koide et al., 1993). Our preparation of apoplasticyanin was therefore characterized by CD spectroscopy for the diagnostic β-strand spectrum (Draheim et al., 1986) (Fig. 2). The spectra of the native holo-
protein and the reconstituted holoprotein were very similar. In fact, the estimated secondary structure of the reconstituted holoprotein was not significantly different from the secondary structure of the native protein (Table I). However, the spectra of the reconstituted protein, the data were input into the Secondary Structure Estimation program (j apan Spectroscopic Co., Ltd., J apan), and the amount of each type of secondary structure was calculated against the reference spectra consisting of a set of spectra of purified holoplantocyanin at a series of different concentrations (ranging from 0.1 to 2.3 mg/ml). The secondary structure content of purified holoplantocyanin was calculated from the distribution of species (Li et al., 1992). To demonstrate more con- cates that our preparation was indeed capable of adopting the native structure and was not irreversibly denatured.

Analysis of apoplastocyanin preparations by electrophoresis under non-denaturing conditions generally revealed three species. In earlier work, we had identified the three species as apoplastocyanin, a disulfide-linked dimer of apoplastocyanin, and residual holoplantocyanin (Li and Merchant, 1992). Similar analysis of spinach apoplastocyanin revealed the same population of species (Li et al., 1990). Bacterial amicyanin also readily dimerizes once the copper is removed from the holoprotein (Kumar and Davidson, 1992). To demonstrate more convincingly that the species identified as apoplastocyanin indeed contained a reactive accessible thiol group (as opposed to holoplantocyanin where the single cysteine provides a ligand to the copper, or the dimer where the cysteine participates in the disulfide bond), the preparation was treated with a fluorescent maleimide (Fig. 3). As expected, only the band corresponding to apoplastocyanin was found to react with the thiol reagent.

Protease Susceptibility of Apoplastocyanin in Vitro—The CD spectra, electrophoretic mobility (Figs. 2 and 3), and increased immunoreactivity (Li and Merchant, 1992) of preparations of apoplastocyanin are all supportive of a different conformation of apoplastocyanin relative to holoplantocyanin. To test whether the apoprotein is indeed more susceptible to proteases compared to holoplantocyanin, equal amounts of apo- and holoplantocyanin were separately incubated with proteases, and the residual protein was analyzed by silver staining after electrophoretic separation on a non-denaturing gel (Fig. 4). Apoplastocyanin was found to be highly susceptible to digestion by either trypsin (not shown) or chymotrypsin (Fig. 4A) whereas holoplantocyanin was resistant for an extended period of time during an identical incubation. Conditions (high salt) which stabilize the folded conformation of apoplastocyanin (Koide et al., 1993) also inhibit degradation (Fig. 4B). The protease assays were highly reproducible; identical results were obtained when the experiment was repeated under the same conditions. These data suggest that the unfolded conformation is indeed more susceptible to proteolysis. Since the same results are obtained with trypsin, chymotrypsin, or (as yet uncharacterized) proteases found in extracts of chloroplasts (data not shown), it is unlikely that there is a single particularly suscep- tible site on the polypeptide.

It is not surprising that high salt (which stabilizes the native conformation) only delays rather than prevents degradation. If the kinetic barrier to unfolding is small and the two species (i.e. protease-resistant and protease-susceptible) are in equilibrium (see Koide et al., 1993), the degradation of one form would eventually lead to the degradation of the entire population of molecules. Thus, the fact that the apoprotein can adopt a native structure in the absence of the cofactor does not preclude its recognition as a non-native structure by a protease. The identity of the protease in vivo is not known, but the location of the substrate demands a proteolytic mechanism for plastocyanin degradation in the thylakoid lumen.

Protease Susceptibility of Plastocyanin in Vivo—A model for plastocyanin biosynthesis has been developed from experimental work in numerous laboratories (Grossman et al., 1982; Hageman et al., 1986; Smeekens et al., 1986; Kirwin et al., 1988; Hageman et al., 1990; Li et al., 1990; Bassham et al., 1991; Howe and Merchant, 1993). Pre-apoplastocyanin, synthesized in the cytosol, is translocated across the envelope...

**TABLE I**

Comparison of reconstituted holoplantocyanin secondary structure to native holoplantocyanin.

| Type of secondary structure (%) | Native holoplantocyanin | Reconstituted holoplantocyanin |
|--------------------------------|-------------------------|--------------------------------|
| α-Helix                       | 5.0 ± 0.1               | 5.1 ± 0.1                       |
| β-Strand                      | 43.7 ± 0.7              | 43.4 ± 0.9                      |
| β-Turn                        | 24.4 ± 0.3              | 24.5 ± 0.1                      |

**Fig. 2.** Far UV CD spectra of apo-, holoprotein, and reconstituted plastocyanin. The plastocyanin content of each sample was the same (0.56 mg/ml). Each sample was in a solution containing 25 mM Tris-Cl (pH 8.05) in a cuvette with a pathlength of 0.1 mm. All measurements were made at room temperature. Thin solid line, apoplastocyanin; thick solid line, holoplantocyanin; dashed line, reconstituted plastocyanin.

**Fig. 3.** Accessibility of the cysteinyl thiol in apo- versus holoplantocyanin to reaction with maleimidylphenyl methylcoumarin. Apo- and holoplantocyanin were treated with a thiol-reactive reagent, maleimidylphenyl methylcoumarin, for 60 min. Equivalent amounts of apo- and holoplantocyanin were analyzed as follows after separation by electrophoresis through a 15% polyacrylamide gel under either non-denaturing (A) or denaturing (B) conditions: under UV-illumination to detect the fluorescent reagent (lanes 3, 4, 7, and 8) or by staining with Coomassie Blue R-250 to detect protein (lanes 1, 2, 5, and 6). The arrows on the left point to apoplastocyanin (a), disulfide-linked dimer of apoplastocyanin (d), holoplantocyanin (h). The arrows and the numbers on the right indicate the size of the molecular weight markers separated on the SDS-containing denaturing gel.
membranes of the chloroplast and processed by a stromal peptidase into intermediate apoplastocyanin. The intermediate is further transported across the thylakoid membrane into the lumen and processed by a lumen-facing protease. The mature apoplastocyanin, which is formed after the second translocation step, is a substrate for two alternative pathways. Under copper-supplemented conditions, apoplastocyanin is a substrate for the formation of holoplastocyanin (Li et al., 1990). Under copper-deficient conditions, apoplastocyanin is a substrate for degradation (Merchant and Bogorad, 1986b; this work). The inferred existence of a proteolytic mechanism in the lumen and the susceptibility of apoplastocyanin to such a protease suggests that degradation of apoplastocyanin could be in competition for the biosynthetic reaction of holoplastocyanin formation, unless holoprotein formation in copper-supplemented cells occurs much faster than proteolysis. Alternatively, there is the possibility that the proteolytic activity could be regulated by copper. We therefore sought to determine whether the relative thermodynamic instability of apoplastocyanin and its increased protease susceptibility in vitro was sufficient to account for its degradation in vivo in copper-deficient cells, or whether the plastocyanin-degrading proteolytic activity might actually be regulated in vivo.

The characteristics of a weakly non-photosynthetic mutant strain of C. reinhardtii, pc235 (see “Experimental Procedures”), permitted us to distinguish between these possibilities. This strain provides plastocyanin with a wild-type primary sequence but has reduced plastocyanin function owing to decreased thermodynamic stability relative to the holoprotein. However, other algae has been assumed to result from its decreased thermodynamic stability relative to the holoprotein. Nevertheless, Koide et al. (1993) noted that the fact that the mutant is capable of synthesizing plastocyanin under these conditions (Fig. 6). Thus, we conclude that a protease-sensitive conformation may be a prerequisite for apoplastocyanin degradation, but it is not sufficient in vivo.

**DISCUSSION**

Degradation of Apoplastocyanin—The degradation of apoplastocyanin in copper-deficient cells of C. reinhardtii and other algae has been assumed to result from its decreased thermodynamic stability relative to the holoprotein. However, the mechanism by which the apoprotein might be recognized as a suitable substrate for proteolysis over the holoprotein was not well understood. Unlike the light-harvesting chlorophyll a/b-binding protein, which requires its cofactor for folding (Paulsen et al., 1993), copper is not required for the folding of this polypeptide into its native structure. In fact, x-ray crystallography and solution NMR studies indicate that the structures of the apo and holoprotein are virtually identical (Garrett et al., 1984; Kaeber et al., 1993). Nevertheless, Koide et al. (1993) noted that the folded conformation of the apoprotein was only slightly more stable than the unfolded conformation, and further, that the refolding of the unfolded apoprotein was slow. The differential degradation of plastocyanin in copper-deficient versus

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**FIG. 4.** Pro tease susceptibility of apo- versus holoplastocyanin. 10 μg each of apo- or holoplastocyanin was incubated with 0.02 units of chymotrypsin in 100 mM Tris (pH 8.0) in a total volume of 30 μl for the indicated amounts of time. The reaction was terminated by the addition of phenylmethylsulfonyl fluoride to >100 μM. Portions of the products (about 200 ng each lane) were loaded onto alternate lanes of a 12% polyacrylamide gel for electrophoresis under non-denaturing conditions (Davis, 1964), which separates apo- and holoplastocyanin (Li and Merchant, 1992). The residual protein was visualized by staining with a silver reagent (Oakley et al., 1980). In panel B, 0.5 mM (NH₄)₂SO₄ was added to the samples before the incubation with protease. The arrows point to the positions of migration of apo- and holoplastocyanin. The products of digestion are probably too small (<15 amino acids residues) to be retained on the gel. In the absence of added protease, apoplastocyanin is not degraded.

**FIG. 5.** Differential accumulation of apoplastocyanin under copper-supplemented versus copper-deficient conditions. C. reinhardtii strain CC-125 (WT) and plastocyanin-deficient strain (pc235) were cultured in copper-supplemented (+) or copper-deficient (-) conditions as described under “Experimental Procedures.” Cells (usually 100-ml cultures) were collected at stationary phase (about 1 × 10⁷ cells/ml for the wild-type strain and 5 × 10⁶ cells/ml for strain pc235) and resuspended in a minimal volume (~200 μl) of a solution containing 10 mM sodium phosphate (pH 7.0). The concentrated cells (equivalent to ~1–2 mg chlorophyll/ml) were lysed by two freeze-thaw cycles (freeze at ~80 °C and thaw at room temperature). The supernatant, collected after centrifugation (15,850 × g) at 4 °C, was identified as the soluble cell extract and separated by electrophoresis through a 15% polyacrylamide gel under non-denaturing condition (Li and Merchant, 1992). Total soluble protein equivalent to 1.0 OD₅₆₅ in a 1-ml protein assay was loaded onto each lane. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane which was subsequently decorated with plastocyanin-specific antibodies. The arrows indicate apoplastocyanin (a), dimer of apoplastocyanin (d), and holoplastocyanin (h). Differences in the abundance of various species of plastocyanin do not result from differential release of the protein from the mutant versus the wild-type cells, or from copper versus copper cells, during preparation of cell extracts.
copper-supplemented cells can thus be easily explained by the differential protease susceptibility of an unfolded protein compared to a folded one (reviewed by Dice, 1987; Gottesman and Maurizi, 1992). In this work, we have demonstrated that, at low ionic strength, apoplasticocyanin does not exhibit a native structure (Fig. 2), and it is indeed highly protease susceptible (Fig. 4). It is not irreversibly denatured under these conditions as evidenced by its ability to reconstitute with copper (Figs. 1 and 2); hence, the in vitro preparation may be a fair representation of the polypeptide in vivo. However, the finding that apoplasticocyanin in mutant strain pc235 is degraded in copper-deficient cells but not in copper-supplemented cells (Fig. 5) indicates that the increased susceptibility of apoplasticocyanin is only one component of the system. The other is the protease, which our work suggests might be regulated by copper.

At the present time, the identity of this protease is not known. Various types of proteases have been identified in chloroplasts including metalloproteases and ATP-dependent proteases (Liu and J agendorf, 1986; Gray et al., 1990; Hooper and Hughes, 1992; Bushnell et al., 1993). Although we have noted an ATP-dependent protease activity in chloroplast extracts that recognizes apoplasticocyanin preferentially over holoplasticocyanin as a substrate (data not shown), the absence of ATP in the thylakoid lumen (see Cline et al., 1992) makes it unlikely that this activity is the putative luminal protease responsible for apoplasticocyanin degradation. Our identification of the substrate for this protease (apoplasticocyanin) and our ability to prepare reasonable amounts of radiolabeled substrate now opens the door for the assay and identification of a luminal protease and the copper-responsive system that might regulate it. Toward this end, we have also characterized preparations of apoplasticocyanin by electrophoretic separation under non-denaturing conditions. Independently (e.g. Li et al., 1990), a similar analytical method had indicated that preparations of apoplasticocyanin contained multiple species that differed with respect to electrophoretic mobility. In this work, we have identified the species corresponding to apoplasticocyanin by the basis of the chemical reactivity of the cysteinyl thiol (Fig. 3), while in previous work we identified the species corresponding to cysteine-linked apoplasticocyanin dimers (Li and Merchant, 1992).

Protein Degradation in Chloroplasts—There are many examples of specific degradation of proteins in chloroplasts (Mishkind et al., 1985; Vierstra, 1993). The substrates include the unassembled subunits of multimeric proteins, multimeric complexes lacking or containing a single mutated polypeptide and the apoplypptide of unassembled holoproteins (exemplified by the work of Apel and Kloppstech, 1980; Bennett, 1981; Merchant and Bogorad, 1986b; Howe and Merchant, 1992; Kimata and Thell, 1994). It is likely that in each case the substrates are thermodynamically destabilized relative to the native structures. However, in some cases their degradation appears to be a species-specific phenomenon. For instance, in C. reinhardtii, apocyt f is rapidly degraded whereas in etiolated wheat seedlings, apocyt f accumulates (Anderson and Gray, 1991; Howe et al., 1994). The difference can probably be attributed to the lack of a suitable protease in one system versus the other, since the substrate (apocyt f) is probably equally destabilized in both systems. Likewise, in cyanobacteria, a mutation in the psaC gene results in the accumulation of a non-functional PSI complex in the photosynthetic membrane (Mannan et al., 1991) whereas in Chlamydomonas, the same mutation results in the degradation of the entire complex (Takahashi et al., 1991). Once again, the conservation of PSI structure between cyanobacteria and eukaryotic chloroplasts suggests that the complex is perhaps equally destabilized (thermodynamically) in either system, but might be degraded in one and not the other owing to the occurrence of a suitable protease in one system but not the other. Our results (Fig. 5) suggest that the same might be true for plastocyanin, i.e. its degradation in vivo depends upon the regulated activity of a luminal protease.

Summary—Two models were proposed to account for differential degradation of plastocyanin in copper-deficient cells. First, that copper-deficient cells contained a form of plastocyanin that was more susceptible to proteolysis (i.e. apoplasticocyanin) and second, that copper-deficient cells induced a plastocyanin-specific protease activity in order to ensure that copper might be available for other, perhaps indispensable, copper enzymes (e.g. cyt oxidase). In this work, we suggest that both models are correct. We have demonstrated that apoplasticocyanin is the preferred substrate for proteolysis, but copper-deficient growth conditions are additionally required for the degradation of apoplasticocyanin (in mutant strain pc235).

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