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The biosynthesis of membrane and soluble plastidic *c*-type cytochromes of *Chlamydomonas reinhardtii* is dependent on multiple common gene products

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Cytochrome *c6* functions in the thylakoid lumen to catalyze electron transfer from reduced cytochrome *f* of the cytochrome *b6f* complex to P700$^+$ of photosystem I. The biogenesis of mature cyt *c6* from cytosolically translated pre-apocytochrome *c6* involves numerous post-translational modifications including the proteolytic removal of a transit sequence and the covalent attachment of heme to two cysteinyl thiols on the apoprotein. Here, we report on the characterization of a previously unrecognized class of non-allelic mutants of *Chlamydomonas reinhardtii* that are blocked at the conversion of apocyt *c6* to holocyt *c6*. The mutants are acetate requiring since they are also deficient in cyt *f*, cyt *b* and the Rieske FeS protein. Pulse–chase studies indicate that heme attachment is not required for the two-step processing of pre-apocytochrome *c6* to apocyt *c6*, but is required for the stability of the mature protein. This is in contrast to the biosynthesis of mitochondrial cyt *c1* where heme attachment is required for the second processing step. We propose that the assembly of both holocytochrome *c6* and the cytochrome *b6f* complex are dependent on common gene products, possibly those involved in heme delivery or metabolism. This is the first suggestion that multiple loci are involved in the biosynthesis of both plastidic *c*-type cytochromes.

Key words: *Chlamydomonas reinhardtii*/chloroplast/cytochrome/heme

Introduction

Cytochrome *c6* (cyt *c6*) belongs to the family of soluble *c*-type cytochromes which function as electron carriers in the energy transducing electron transfer chains of mitochondria, respiring and photosynthetic bacteria, and some chloroplasts (see Mathews, 1985 for review). The metabolic function of these cytochromes is the catalysis of quinol oxidation by a membrane associated cyt *b*–*c* (*bc1*, *b6f*) complex that contains one *c*-heme, two *b*-hemes and an FeS center (see Figure 1 and Hauska *et al.*, 1983, for review). In most organisms that carry out oxygenic photosynthetic, specifically vascular plants, this reaction is catalyzed by the blue copper–protein plastocyanin, where the copper is the redox active cofactor that fulfils the role of the heme in cyt *c6* (Boulter *et al.*, 1977).

Although the electron transfer activities of cyt *c6* and plastocyanin depend on different redox centers, the biochemical equivalence, identical sub-organellar localization and similar physical (size, solubility, pI, redox potential) and catalytic properties of the two proteins are well documented (Gorman and Levine, 1966a; Wildner and Hauska, 1974; Kunert and Boger, 1975; Crofts and Wood, 1977; Wood, 1978; Bohme *et al.*, 1980; Bendall, 1982). In fact, some organisms, e.g. cyanobacteria and green algae, have genetic information for both plastocyanin and cyt *c6* and can use either protein in photosynthesis, the choice between them depending upon the availability of copper ions (Bohner and Boger, 1978; Wood, 1978; Merchant and Bogorad, 1986a). If copper is available, the organisms use plastocyanin to oxidize cyt *f* and reduce P700$^+$. If copper levels are insufficient to support plastocyanin synthesis at the stoichiometry required for photosynthesis, the organism uses cyt *c6* for this reaction.

In the unicellular green alga *Chlamydomonas reinhardtii*, the copper-dependent accumulation of plastocyanin is mediated by the altered stability of the holoprotein versus the apoprotein (Merchant and Bogorad, 1986b) whereas the copper-dependent expression of cyt *c6* is controlled at the transcriptional level (Merchant *et al.*, 1991). Under copper-deficient conditions the gene encoding cyt *c6* is actively transcribed; but when copper is available, transcription of this gene is greatly reduced. Both proteins are nuclear-encoded, cytosolically synthesized and post-translationally targeted to the lumen of the thylakoid membrane (Merchant and Bogorad, 1987a; Merchant *et al.*, 1990).

The structural and functional similarities between cyt *c6* and plastocyanin suggest that these proteins might share steps in the pathway by which the cytosolically translated pre-proteins are routed to the lumen of the thylakoid membrane. The deduced primary sequences of pre-apocyt *c6* (Merchant and Bogorad, 1987a) and pre-apoplastocyanin (Merchant *et al.*, 1990) reveal a typical 'two domain' transit sequence that is found in almost all nuclear-encoded pre-proteins that target the thylakoid lumen (Franzen *et al.*, 1989; von Heijne *et al.*, 1989; Merchant *et al.*, 1990). Based primarily on *in vitro* and *in organello* studies of the maturation and targeting of the precursors to two lumenal proteins (plastocyanin and a 33 kDa protein of the oxygen evolving complex), several groups have proposed that the translocation of the pre-proteins occurs in two steps (Weisbeek *et al.*, 1989; Bauerle *et al.*, 1991): transport across the envelope membranes to the stroma followed by transport across the thylakoid membrane to the lumen. A proteolytic processing step, catalyzed by a lumen-facing, thylakoid membrane-bound processing peptidase (Hageman *et al.*, 1986; Kirwin *et al.*, 1988), is proposed to occur either concomitant with or subsequent to the second translocation. Although this model proposes a stromal intermediate in the pathway, the *in vivo* existence of such an intermediate of authentic lumenal proteins has not been demonstrated. Further, it is unclear whether the hypothesized stromal intermediate is processed

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prior to the second translocation step (see Bauerle *et al.*, 1991, for discussion). Since cyt *c6* is also lumen-localized and the pre-protein contains the two-domain transit sequence, it is likely that its import utilizes the same pathway as does plastocyanin. The synthesis of the mature protein in each instance does however, require association with its cofactor—a step that is specific for and unique to each protein. In the case of plastocyanin, the cofactor (copper) is not required for import or processing of the precursor. *In vivo* and *in organello* studies indicate that the association of the targeted apoprotein with its cofactor occurs in the lumen of the thylakoid membrane (Merchant and Bogorad, 1986b; Li *et al.*, 1990). In the absence of association with copper, processed apoplastocyanin does not accumulate since it is rapidly degraded (Merchant and Bogorad, 1986b). In the case of the maturation of pre-apocyt *c6* to holocyt *c6*, a critical but uncharacterized step is the covalent attachment of heme to the apoprotein (catalyzed by a cyt *c6*/heme lyase) at some point in the pathway.

The biosynthesis of *c*-type cytochromes has been studied primarily in fungal experimental systems (Ohashi *et al.*, 1982; Nicholson *et al.*, 1987; Dumont *et al.*, 1988; Nargang *et al.*, 1988; Nicholson *et al.*, 1989) and more recently in bacteria (Kranz, 1989; Page and Ferguson, 1989, 1990; Brandner *et al.*, 1991; Ramseier *et al.*, 1991; Beckman *et al.*, 1992). Owing to its unique expression in green algae and cyanobacteria, the post-translational maturation of cyt *c6* has received much less attention. Nevertheless, it is an important problem since it addresses the general question of the synthesis of *c*-type cytochromes in plant systems, and is likely to be relevant to the biosynthesis of cytochrome *f* and perhaps other plastidic cytochromes as well. As a means of addressing the broad question of plastidic *c*-type cytochrome biogenesis, we are using a genetic approach to elucidate the post-translational events involved in cyt *c6* biosynthesis in *C.reinhardtii* with the specific goal of identifying the intermediates and their temporal sequence in the biogenesis of plastidic *c*-type cytochromes. In this report we define a general class of mutants in which a defect in the post-translational maturation of holocyt *c6* is associated with a defect in the assembly of the cyt *b6f* complex. Based on the biochemical characterization of these mutants we propose that (i) the mutants are blocked at a step(s) required for the attachment of heme to apocyt *c6*, (ii) the holocyt *c6* deficiency results from rapid turnover of the apocyt, and (iii) heme attachment is not required for import and processing of the pre-apocyt *c6*. We discuss also the possible biochemical basis for the connection between the cyt *b6f*-deficient and cyt *c6*-deficient phenotypes.

Results

Identification and characterization of a cyt c6-deficient mutant (F2D8)

In order to identify potential mutants with genetic blocks in the biosynthesis of cyt *c6*, UV-mutagenized, metronidazole (Flagyl)-enriched *C.reinhardtii* cells were screened for their inability to grow photoautotrophically. Metronidazole enrichment of mutagenized cultures has been used effectively by many laboratories to enrich for photosynthesis-deficient (i.e. acetate requiring) mutants. This compound, believed to accept electrons from reduced ferredoxin, spontaneously auto-oxidizes and generates toxic products (e.g. peroxides) in the process (Schmidt *et al.*, 1977). Since the toxicity of metronidazole depends on a functional photosynthetic electron transfer chain, survivors of metronidazole treatment are likely to be defective in one or more of the many functional components of the photosynthetic electron transfer chain and hence acetate requiring (see Figure 1). A cyt *c6*-deficient mutant could be distinguished from other non-photosynthetic cells since it would display an acetate requiring phenotype only under conditions of copper depletion (assuming normal plastocyanin function). However, we were unable to identify an acetate requiring, cyt $c6^-$ mutant by this simple differential screening strategy. We therefore screened for cyt $c6^-$ mutants by analyzing extracts from copper-deficient cultures of acetate requiring (both in the presence and absence of copper) clones by non-denaturing gel electrophoresis, followed by 'heme staining'. Cyt *c6*, as a small, acidic protein, is well separated from other proteins in soluble extracts of *C.reinhardtii* (e.g. Merchant *et al.*, 1991). The protein can be unambiguously identified in −Cu vs. +Cu extracts by its immunoreactivity or by heme-dependent peroxidase activity (e.g. Figure 2A and B, lanes marked 'wt'). Of 60 independently isolated acetate requiring clones that were screened, one clone, called F2D8, was identified that accumulated a much reduced level of holocyt *c6* (Figure 2A). By comparing the intensity of the heme-stained band in extracts of F2D8 with that of serial dilutions of extracts from wild-type cells, F2D8 was estimated to accumulate ~5% of the wild-type level of holocyt *c6*. Western blot analysis indicated that the decreased abundance of (heme-containing) holocyt *c6* is well correlated with a decreased abundance of the polypeptide. Immunoblot analysis of total cell extracts (including membranes) did not reveal any additional anti-cyt *c6* reactive polypeptide in F2D8. Since the antiserum is known to recognize both pre-apocyt *c6* and apocyt *c6* in an immunoprecipitation assay, these results indicate that the reduced level of holocyt *c6*

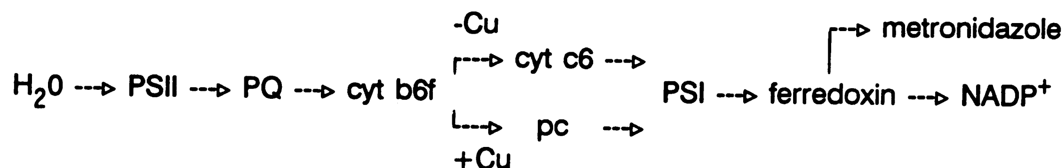

Fig. 1. Rationale for the analysis and characterization of cyt *c6*-deficient mutants. The diagram shows the sequence of electron transfer from plastoquinol to $NADP^+$ (or metronidazole) via either plastocyanin [if copper is available to the cell for holoplastocyanin formation (Merchant and Bogorad, 1986b)] or cyt *c6* [under conditions of copper deficiency (Merchant and Bogorad, 1987a; Merchant *et al.*, 1991)]. In wild-type cells, the use of plastocyanin versus cyt *c6* is mutually exclusive (Wood, 1978; Merchant *et al.*, 1991; K.Hill and S.Merchant, submitted). Mutations that affect only cyt *c6* (e.g. in the structural gene encoding the pre-apoprotein) should exhibit a conditional acetate requiring phenotype [i.e. the mutants should grow photoautotrophically in copper-supplemented medium (Merchant and Bogorad, 1987b)]. Mutations that affect the biosynthesis or function of multiple thylakoid membrane components (e.g. in the lumen targeting pathway or the heme biosynthetic pathway) would be expected to exhibit an acetate requiring phenotype independently of the use of plastocyanin or cyt *c6* for electron transfer.

in F2D8 cannot be explained by the accumulation of an aberrantly processed form of the protein in either a soluble or membrane associated form.

Cyt c6 deficiency in cyt b6f mutants

The lack of a conditional (with respect to copper availability) acetate requiring phenotype of F2D8 indicated that the cyt *c6* deficiency *per se* was not the cause of the acetate requiring phenotype since plastocyanin can functionally complement a cyt *c6* deficiency under conditions of copper availability (see Figure 1). For example, the plastocyanin-less mutant ac-208 exhibits an acetate requiring phenotype under conditions that repress cyt *c6* expression (copper availability), but is otherwise capable of photoautotrophic growth under conditions that permit cyt *c6* expression (copper deficiency) (Merchant and Bogorad, 1987b). One possible explanation for the copper-independent acetate requirement of F2D8 was that this mutant was deficient in plastocyanin expression as well as in cyt *c6* expression. A pleiotropic phenotype could arise, for example, from a single mutation that affected a pathway common to the biosynthesis of several proteins (see legend to Figure 1). We were able to discount the idea that the mutant was affected in the processing or targeting of lumenal proteins by showing that plastocyanin accumulated to normal levels in F2D8 when it was grown in copper-supplemented medium (Figure 2D). Thus, a more plausible explanation for the F2D8 phenotype was that this strain harbored a mutation that not only blocked the accumulation of cyt *c6*, but also blocked the expression of another component required for a different step of photosynthetic electron transfer. A possible candidate for this component was cytochrome *f*, the other plastidic *c*-type cytochrome. Indeed, it was found that membrane fractions of F2D8 do not accumulate cyt *f* (Figure 2C). Further analysis revealed that thylakoid membranes from F2D8 were deficient also in two other polypeptides of the chloroplast cyt *b6f* complex—holocyt *b6* and the Rieske iron–sulfur protein (not shown). It is likely that the cyt $c6^-$ and cyt $b6f^-$ phenotypes result from a single mutation since the deficiencies co-segregate in a back-cross ($P < 0.005$).

This result raised the possibility that the cyt *c6* deficiency noted in F2D8 might simply be a secondary consequence of a primary defect in the cyt *b6f* complex, since the latter is a substrate of cyt *c6*. In other words, it was possible that the cyt *b6f* complex was required for cyt *c6* accumulation. To test this possibility, five previously well-characterized cyt *b6f*-minus mutants were examined for their ability to synthesize holocyt *c6*. Three of these mutants [the chloroplast mutant B6 (Shochat *et al.*, 1990) and the non-allelic nuclear mutants F18 and ac-206 (Gorman and Levine, 1966b; Lemaire *et al.*, 1986; J.Girard-Bascou, personal communication)] were found to contain severely reduced levels of cyt *c6* (~1% of that found in wild-type) as estimated from either Western blots or heme-stained gels (Figure 2A and B). These three mutants were also phenotypically similar to F2D8 in that they accumulated normal levels of plastocyanin (Figure 2D) and mRNAs for cyt *f*, cyt *b6* and subunit IV, but did not accumulate immunoreactive cyt *f* (Figure 2C) or the Rieske FeS protein (not shown). We have also verified that thylakoid membranes from B6, ac-206 and F18 do not accumulate holocytochromes *b6* or *f* (Figure 3). The level of holocyt *c6* in extracts of mutant versus wild-type cells was further qauntitated by difference visible absorption spectroscopy. As shown in Figure 4 (and Table I), extracts from copper-deficient cultures of these cyt $b6f^-$ mutants do not exhibit an absorption peak corresponding to cyt *c6* (α

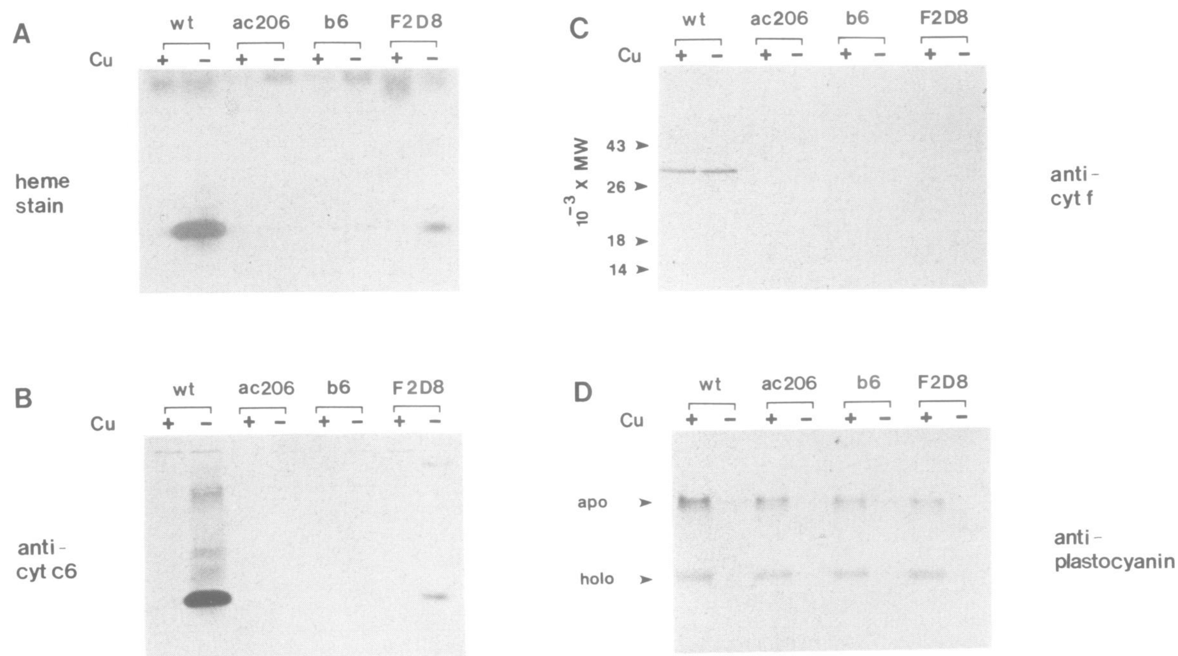

Fig. 2. Analysis of cytochrome *c6*, plastocyanin and cytochrome *f* content in wild-type and mutant strains. Extracts of soluble proteins (**A, B** and **D**) or total cellular protein (**C**) were prepared from the indicated strains after growth in either copper-supplemented (+) or copper-deficient (−) medium. Equivalent amounts of protein (30 μg) were separated on 16% non-denaturing (A, B and D) or 12% polyacrylamide denaturing (C) gels and then processed as follows: (A), TMBZ staining for heme-dependent peroxidase activity; (B), (C) and (D) immunoblots with anti-cyt *c6*, anti-cyt *f* and anti-plastocyanin, respectively.

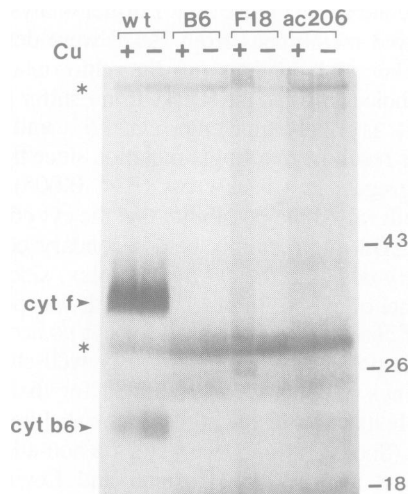

Fig. 3. Absence of holocyt *b6* and *f* in thylakoid membranes of cytochrome *b6f* mutants. Cellular fractions enriched in thylakoid membrane content were prepared from the indicated stains after growth in either copper-supplemented (+) or copper-deficient (−) medium. Thylakoid membranes (equivalent to 8 μg chlorophyll) were solubilized and separated on an SDS−denaturing polyacrylamide gradient (10−18%) gel. Heme-containing polypeptides (cytochromes *f* and *b6*) were visualized by staining with TMBZ. The bands indicated by the asterisks result from the presence of chlorophyll−protein complexes in the thylakoid membrane.

absorption maximum at 552 nm). Thus, *C.reinhardtii* strains B6, ac-206 and F18 appear to be similar to F2D8 in having a cyt *c6* deficiency that is associated with a cyt *b6f* deficiency.

Nevertheless, despite the identification of four independent mutants that have a cyt *c6* deficiency associated with a cyt *b6f* complex deficiency we can rule out the possibility that the cyt *c6* deficiency is a secondary consequence of a defect in the cyt *b6f* complex, since not all cyt *b6f*$^-$ mutants display the severe holocyt *c6* deficiency characterized by F2D8, B6, ac-206 and F18. Two cyt *b6f*$^-$ mutants, F1-9 (Garnier and Maroc, 1970; Maroc and Garnier, 1973) and FUD6 (Lemaire *et al.*, 1986), accumulate holocyt *c6* to ~20% (on a per mg protein basis) to 50−100% (on a per mg chlorophyll basis) of the level found in wild-type cells (Table I). This level of cyt *c6* is typical of that observed in other photosynthesis-minus mutants which often contain reduced levels of various chloroplast enzymes (on a per mg protein basis) owing to the pleiotropic effects that result from the absence of plastid function. For example, photosystem II (PSII) mutants contain holocyt *c6* at levels 50−60% (on a per mg protein basis) of that in wild-type cells while photosystem I (PSI) mutants contain somewhat less (20% of wild-type). Chloroplast genome deletion mutants (C2 and L210A) that result in the complete absence of both PSII and PSI and are hence devoid of photosynthetic electron transfer activity are also able to accumulate cyt *c6* to wild-type levels (Table I). A plastocyanin mutant (ac-208) and a Calvin cycle mutant (ac-214) also accumulate cyt *c6*. Thus, the severe

Table I. Cyt *c6* content in photosynthesis-minus mutants[a]

Strain	Defect	nmol cyt *c6*/mg chl	nmol cyt *c6*/mg protein
CC-124	Wild-type	1.5 ± 0.3[b]	1.6 ± 0.3[b]
F2D8	cyt *b6f* assembly	n.d.[c]	n.d.
B6	cyt *b6f* assembly	n.d.	n.d.
ac-206	cyt *b6f* assembly	n.d.	n.d.
F18	cyt *b6f* assembly	n.d.	n.d.
F1-9	cyt *b6f* assembly	1.3	0.4
FUD6	cyt *b6f* assembly	0.7	0.3
ac-21[d]	Rieske Fe/S	0.4	0.3
F34	PSII assembly	2.3	0.8
FUD7	*psb*A$^-$, PSII assembly	2.6	0.9
nac2-26	PSII assembly	2.5	0.8
L135F	*psa*A$^-$, PSI assembly	0.9	0.4
FUD26	*psa*B$^-$, PSI assembly	1.0	0.3
C3	*psa*B$^-$, PSI assembly	0.9	0.4
F14	PSI assembly	1.7	0.3
F1	PSI assembly	n.d.	n.d.
L210A	*tsc*A$^-$, PSI assembly *psb*A$^-$, PSII assembly	3.6	0.7
C2	*tsc*A$^-$, PSI assembly *psb*A$^-$, PSII assembly	2.3	1.6
ac-208	plastocyanin	3.3	2.6
ac-214	ribulose-5-phosphate kinase	n.m.	0.5

[a]The data in Table I are presented on the basis of chlorophyll content as well as protein content, since some photosynthesis-minus mutants have reduced levels of chloroplast pigments and proteins. Further, because cyt *c6* expression is highly dependent on cell density (see e.g. Merchant *et al.*, 1991), additional variability can be introduced from the fact that some mutants do not reach as high a cell density in liquid culture as do wild-type cells. Although every effort is made to harvest cells at comparable cell densities, this was not always practical.
[b]The values given for the wild-type strain are the mean and standard deviation of three independent experiments.
[c]n.d. not detectable; extracts from Cu-deficient cells of these strains contain so little holocyt *c6* (<0.1 nmol/mg protein) that it is not possible to distinguish it from the mitochondrial *c550* (see Figure 4).
[d]ac-21 (strain CC-2115 from the *Chlamydomonas* Stock Collection) is not completely acetate requiring.
n.m., not measured.

cyt *c6* deficiency (1–5% of wild-type) is noted only in a subset of *b6f*-deficient mutants (F2D8, B6, ac-206, F18) and is independent of a general defect in the photosynthetic apparatus (F1-9, FUD6, and others listed in Table I). This conclusion was supported by concomitantly analyzing cell extracts of each of the mutant strains (listed in Table I) for heme-dependent peroxidase activity (as in Figure 2A).

Biochemical basis for holocyt c6 deficiency

In order to determine the level at which the biosynthesis of holocyt *c6* is affected in these four mutants (F2D8, B6, ac-206 and F18), total RNA was isolated from copper-supplemented or copper-deficient wild-type and mutant cells and probed for the abundance of cyt *c6*-encoding messages (Figure 5A). The results indicate that mRNAs encoding cyt *c6* accumulate to normal levels in copper-deficient cultures of each of the four mutants (F2D8, B6, ac-206 and F18). Furthermore, the hybridizing messages are of the appropriate size (~700 nucleotides). As shown in Figure 5B, the messages are translatable *in vitro* and, in each case, yield a product that cross-reacts with anti-cyt *c6* and that corresponds in size to pre-apocyt *c6* [approximate mol. wt 14×10^3 (Merchant and Bogorad, 1986a)]. We conclude therefore that these four cyt $b6f^-$/cyt $c6^-$ mutants are unaffected in cyt *c6* mRNA accumulation. The absence of holocyt *c6* accumulation may therefore result from aberrant translation of the message *in vivo* or aberrant post-translational processing/maturation followed by rapid turnover.

To distinguish between these possibilities we examined the synthesis and stability of cyt *c6* in mutant and wild-type

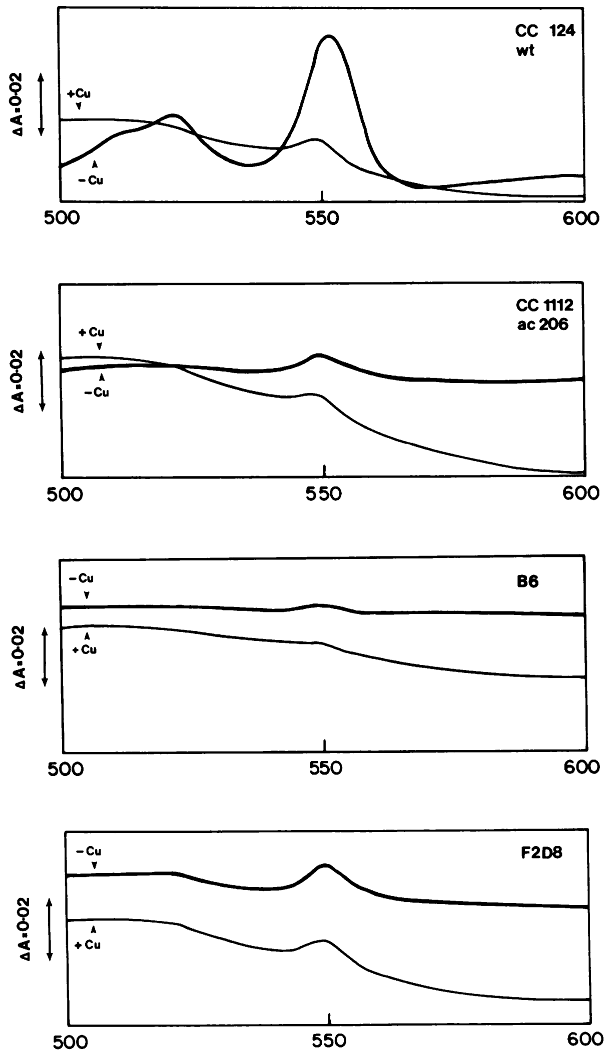

Fig. 4. Difference absorption spectroscopy of holocytochrome *c6* in wild-type versus mutant strains. Extracts containing total soluble protein were prepared from the indicated strains after growth in either copper-supplemented (+Cu, narrow trace) or copper-deficient (−Cu, heavy trace) medium. The difference absorption spectra shown were obtained after subtraction of the oxidized (potassium ferricyanide) spectrum from the reduced (sodium ascorbate) spectrum. The small peak at 549–549.5 nm was detected in extracts from all cyt $b6f^-$/$c6^-$ mutant strains grown in either the presence or absence of medium copper and most likely represents the mitochondrial cytochrome *c* (λ_{max} = 550 nm). The mitochondrial protein is not normally detected in extracts from copper-deficient cells since the cyt *c6* peak (λ_{max} = 552 nm) masks the cyt *c-550*. Owing to the constitutive presence of mitochondrial cyt *c-550*, the lower detection limit of this assay is ~10% of the level found in fully copper-deficient wild-type cells. The amount of cyt *c6* in the different mutant strains is shown in Table I and was calculated from these spectra as described in Materials and methods.

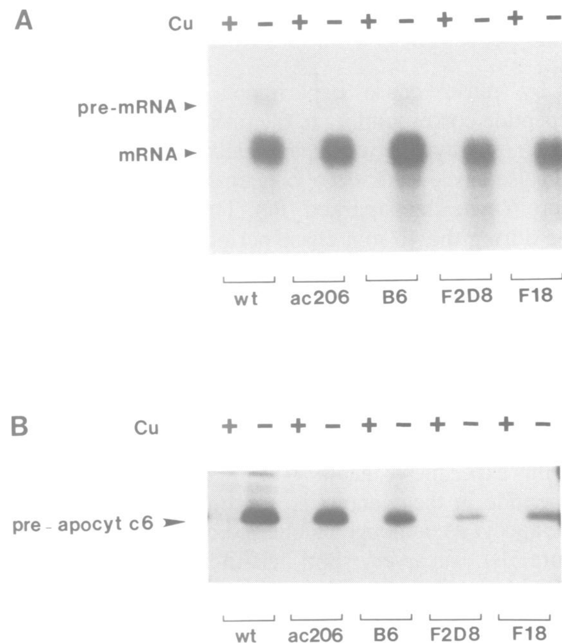

Fig. 5. Characterization of cyt *c6*-encoding messages in wild-type versus cyt $b6f^-$/cyt $c6^-$ mutants. (**A**) Total RNA was isolated from copper-supplemented (+) or copper-deficient (−) cultures of each of the strains. Equivalent amounts of total RNA (5 μg) were denatured with formaldehyde, separated by denaturing agarose gel electrophoresis and analyzed by Northern hybridization for cyt *c6*-encoding sequences. Since the abundance of mRNA in a given sample of total RNA changes over a 2-fold range as a function of the density at which the cells were collected for RNA isolation (K.Hill and S.Merchant, unpublished results), a duplicate blot was probed with a sequence encoding plastocyanin (not shown) as an internal control. (**B**) Equivalent amounts (9 μg) of total RNA were translated *in vitro* in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Polypeptides that were immunoreactive with anti-cyt *c6* serum were indirectly immunoprecipitated from equivalent amounts (by volume) of the translation products. The immunoprecipitates were visualized by fluorography after separation on an SDS-containing polyacrylamide gradient (12–18%) gel. Pre-apocyt *c6* has a predicted molecular weight of 15399 (Merchant and Bogorad, 1987a) but migrates on this gel system just above the 14 kDa molecular weight marker. Although each translation reaction was programmed with an equivalent amount of RNA the efficiency of translation varied between samples over a several-fold range.

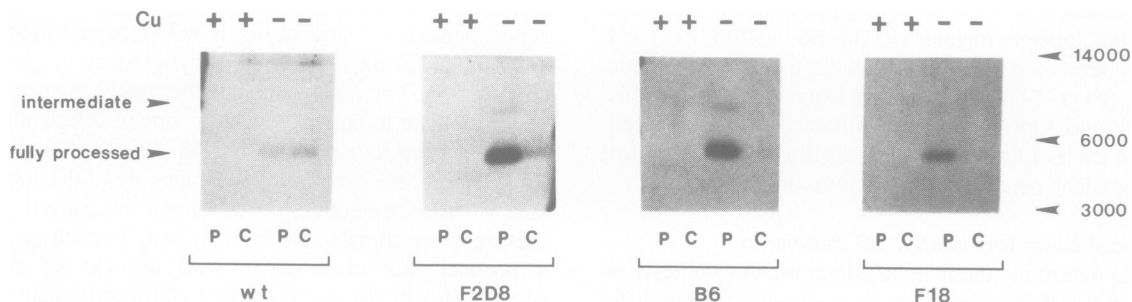

Fig. 6. *In vivo* synthesis and stability of cytochrome *c6* in wild-type and mutant strains. Cultures were grown to logarithmic phase ($2-4 \times 10^6$ cells/ml) in either copper-supplemented (+) or copper-deficient (−) medium and then labeled for 20 min in the presence of 1 mCi/ml $[^{35}S]Na_2SO_4$ (43 Ci/mg S, ICN Biomedicals, Inc. Irvine, CA) as described in Materials and methods. Labeling was terminated by diluting the isotope with the addition of a 20 mM Na_2SO_4 'chase'. Aliquots of the labeled cells were extracted into 100% acetone at 4°C either immediately after the addition of the chase ('P', pulse) or 40 min later ('C', chase). All samples were labeled to a specific activity of 0.04−0.13 TCA-precipitable c.p.m./cell. Anti-cyt *c6* reactive polypeptides were immunoprecipitated from equal volumes of solubilized acetone precipitate using either 10 μl (mutants) or 20 μl (wild-type) of anti-cyt *c6* serum. This amount of antiserum was sufficient to immunoprecipitate all of the cyt *c6* from mutant cell extracts. Cyt *c6* was not, however, quantitatively immunoprecipitated from extracts of wild-type cells since these cells, when grown in copper-deficient media, contain large pools of unlabeled, pre-existing cyt *c6*. Immunoprecipitates were separated on a SDS−denaturing polyacrylamide gradient (12−20%) gel and visualized by fluorography (75 day exposure to Kodak XAR-5 film). The specificity of the immunoprecipitation is evident in comparing the immunoprecipitates of extracts from copper-deficient cells (that express cyt *c6*) with those from copper-supplemented cells (that do not express cyt *c6*). The arrows and numbers (Daltons) to the right indicate the position to which molecular weight markers migrated in the gel. The band identified as 'intermediate' has been demonstrated to be a true precursor to the mature protein (unpublished).

cells by 'pulse−chase' experiments (Figure 6). A single polypeptide corresponding to the proteolytically processed, mature-sized cyt *c6* was specifically immunoprecipitated from pulse-labeled extracts of copper-deficient wild-type cells (Figure 6, wt, lanes marked 'P'). The labeled protein was stable during the 40 min chase period (Figure 6, wt, lanes marked 'C'). The immunoprecipitated polypeptide migrates on this electrophoretic system with an apparent molecular weight of just under 6×10^3 as does the purified holocyt *c6* (Merchant and Bogorad, 1986a). The mutants F2D8, B6 and F18 also synthesize a polypeptide with an electrophoretic mobility very similar to that of holocyt *c6* (Figure 6). But the newly synthesized protein is not stable. We estimate, from this experiment, that the half-life of the immunoreactive polypeptide in the mutants is <10 min.

Pre-apocyt *c6*, although recognized by the cyt *c6* antiserum (Figure 5B), has a very short half-life (<2 min) and is not detected in immunoprecipitates from pulse-labeled extracts of either wild-type or mutant cells in these experiments. However, the presence of another anti-cyt *c6* immunoprecipitable polypeptide was detected in each of the three cyt *b6f* mutants tested (Figure 6, arrow marked intermediate). This polypeptide is not detected in immunoprecipitates from extracts of wild-type cells since it is clearly present at a very low level compared with the major anti-cyt *c6* reactive polypeptide and immunoprecipitation from wild-type extracts was not quantitative in this experiment. Although the chemical nature of this polypeptide is not known, it could represent an *in vivo* intermediate in the holocyt *c6* maturation pathway based on the following criteria: (i) it is immunoprecipitated by the anti-cyt *c6* antiserum, (ii) it has apparent molecular weight of 8×10^3, intermediate between that of pre-apocyt *c6* (14×10^3) and holocyt *c6* (6×10^3), (iii) it is not synthesized in cells grown in copper-supplemented medium, and (iv) it is rapidly diluted during the chase period.

The electrophoretic analysis of the immunoprecipitates (Figure 6) does not resolve the small molecular weight difference between apo and holocyt *c6* (6×10^2 contributed by the prosthetic group). Nevertheless, it is unlikely that the protein synthesized (and degraded) in mutant cells is holocyt *c6* since 'mixing' experiments demonstrate that although the mutant protein migrates with a mobility close to that of purified holocyt *c6*, it does not comigrate with purified holocyt *c6* in all gel systems. Further, the instability of holocyt *c6* in these mutants would be difficult to rationalize since, ordinarily, holocyt *c6* is a very stable protein. For example, when transcription of the gene encoding cyt *c6* is repressed by the addition of cupric ions, the existing holocyt *c6* in *C.reinhardtii* cells decreases with $t_{1/2}$ of the order of hours largely as a result of dilution by cell division (Merchant and Boboard, 1986a). If a holocyt *c6* degrading mechanism were induced in the mutants, one would have to propose that it was very specific for holocyt *c6* since holoplastocyanin (the functional counterpart of holocyt *c6*) accumulates to normal levels in the mutants (Figure 2D). On the other hand, it is well-established that apoplastocyanin is rapidly degraded in *C.reinhardtii* (Merchant and Bogorad, 1986b). We therefore tested the possibility that the polypeptide synthesized and immunoprecipitated in the mutant cells is apocyt *c6* and that the cyt $b6f^-$/cyt $c6^-$ mutants are defective in the step of heme attachment.

To accomplish this it was necessary to distinguish holo from apocyt *c6* (Figure 7). Apocyt *c6* was prepared by chemical cleavage of the heme group from holocyt *c6* (as described in Materials and methods) and observed to migrate with an electrophoretic mobility slightly greater than that of holocyt *c6* on the SDS−PAGE system of Laemmli (Figure 8A). Nevertheless, this gel system could not resolve the two proteins when they were mixed prior to electrophoresis (not shown) and the tricine−SDS−PAGE system described by Schagger and von Jagow (1987) was used to separate apo from holocyt *c6* (Figure 7B). Purified holocyt *c6* and apocyt *c6* standards resolve on this gel system with apparent molecular weights of 6.9×10^3 and 6.5×10^3, respectively (Figure 7C).

The resolution of holo and apocyt *c6* standards by gel electrophoresis allowed us to test the hypothesis that the cyt *c6*-minus mutant strains are capable of synthesizing apocyt

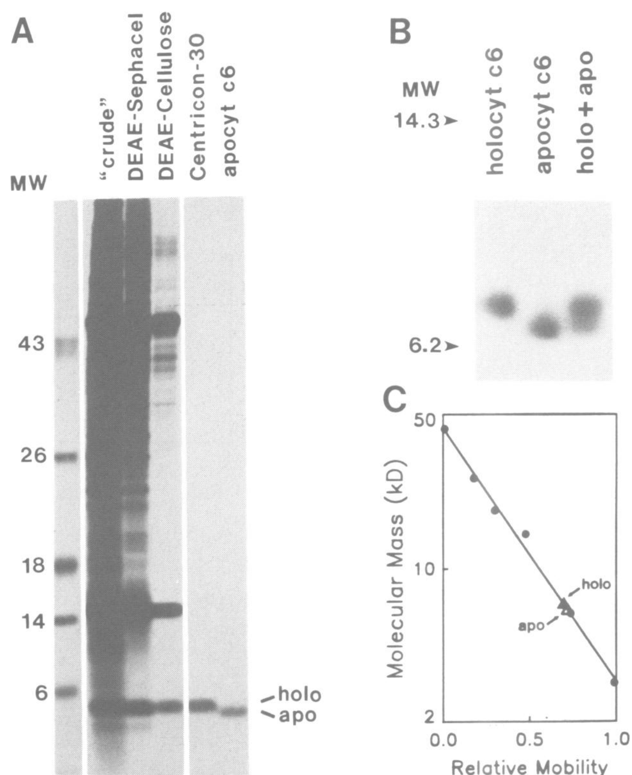

Fig. 7. Purification of ^{35}S-labeled holo and apocyt *c6*. Holocyt *c6* was purified from *in vivo* labeled cells (see Materials and methods). Apocyt *c6* was prepared by chemical removal of the heme group from holocyt *c6* as described in Materials and methods. (**A**) Aliquots (each containing an equivalent of 70 pmol cyt *c6*) from each step of the purification were analyzed on a SDS−polyacrylamide gradient (12−20%) gel and visualized by fluorography (3 day exposure to Kodak XRP-1 film). (**B**) Samples (500 c.p.m.) of purified holocyt *c6*, apocyt *c6* or a mixture of both ('holo + apo') were separated by tricine−SDS−PAGE (Schagger and von Jagow, 1987) and visualized by fluorography. The numbers (kDa) and arrows indicate the positions of molecular weight standards. (**C**) Estimation of the apparent molecular weight of purified holo and apo cyt *c6*. The mass of molecular weight standards (43.0, 25.7, 18.4, 14.3, 6.2 and 3.0 kDa: closed circles) was plotted as a function of their mobility in the gel shown in Figure 7B (least square fit, R = 0.996). The apparent molecular weights of holo (closed triangle) and apocyt *c6* (open triangle) were estimated by plotting their relative mobility on the curve (see text).

c6 but not holocyt *c6*. Wild-type and mutant cells grown in copper-deficient medium were characterized by the pulse−chase analyses and the immunoprecipitates were analyzed on the tricine-containing SDS−PAGE system. Both the wild-type strain and the cyt $b6f^-/c6^+$ mutant (FUD6) synthesize an immunoreactive polypeptide that comigrates with authentic holocyt *c6* (Figure 8; lanes 2, 9 and 16 compared with 8 and 15) and the protein is stable (Figure 8; lanes 3, 10 and 17 compared with 2, 9 and 16). The apparent molecular weight of this polypeptide was estimated to be 7.0×10^3 (Figure 8B). However, in the case of the four $b6f^-/c6^-$ mutants (B6, F2D8, ac206 and F18), each synthesizes an anti-cyt *c6* immunoreactive polypeptide that migrates with an increased electrophoretic mobility corresponding to an apparent molecular weight of 6.5×10^3 which is identical to the apparent molecular weight of the apocyt *c6* standard (Figure 8B), and this polypeptide is unstable *in vivo* (Figure 8; lanes 5, 7, 12 and 14 compared with lanes 4, 6, 11 and 13). We conclude that the mutants synthesize and process pre-apocyt *c6 in vivo* but are unable to convert the apocyt *c6* to holocyt *c6*. The apoprotein is degraded, resulting in the cyt *c6*-deficient phenotype. This conclusion is supported by (i) the molecular weight difference between the *de novo* synthesized protein in mutant versus wild-type cells (0.4×10^2, which is consistent with the apparent molecular weight difference between the authentic standards shown in Figure 7), and (ii) the short half-life of the protein synthesized in mutant cells (estimated from the data shown in Figure 8 to be < 10 min).

Discussion

We have identified a class of cyt $b6f^-$ mutants (F2D8, B6, ac-206 and F18) that are also deficient (<5% of wild-type) in the accumulation of the soluble, plastidic holocyt *c6* (Table II). The abundance of cyt *c6* mRNA (hybridizable and translatable) in these mutants demonstrates that the mutation(s) responsible for the cyt *c6* deficiency act(s) at either a translational or post-translational level. We cannot definitively exclude the possibility that a decrease in the translation of pre-apocyt *c6 in vivo* contributes to some extent to the phenotype of the mutants. However, it is unlikely that translation of the cyt *c6* message is primarily responsible for the phenotype in this class of mutants, since the polypeptide is synthesized to significant extents in all the mutants of this class. However, in each case the newly synthesized polypeptide is rapidly degraded in the mutants but not in the wild-type cells (Figure 8). The instability of cyt *c6* in mutant cells is probably a consequence of the inefficient attachment of heme to the apoprotein, since the newly synthesized protein comigrates with an apocyt *c6* standard rather than with holocyt *c6*. The half-life of the protein in mutant cells is estimated to be < 10 min and thus accounts for the low level of cyt *c6* in mutant cells.

The proposal that the apoprotein of cyt *c6* is unstable *in vivo* is well precedented from studies of the role of cyt *c*/heme lyase in the biosynthesis of (mitochondrial) cyt *c*, where it was noted that apocyt *c* in *Neurospora crassa* and apoiso-1-cyt *c* of *Saccharomyces cerevisiae* are rapidly degraded ($t_{1/2}$ of the order of minutes) in cyt *c*/heme lyase-deficient strains (Nargang *et al.*, 1988; Dumont *et al.*, 1990). An alternative possibility is that the cyt *c6* synthesized in the four $b6f^-$ mutants is improperly processed at its N-terminus and therefore subject to degradation. However, preliminary characterization of the protein synthesized in one of the mutants (ac-206) suggests that it has the same N-terminus as the wild-type holoprotein. Thus, it is more likely that this class of mutants is defective in the formation of holocyt *c6* from apocyt *c6*.

In determining the molecular basis for the cyt $c6^-$ phenotype, the correlation between the cyt $c6^-$ and the cyt $b6f^-$ phenotype should be accounted for. As indicated in Table I, the cyt $c6^-$ phenotype is specifically associated with a subset of $b6f^-$ mutants. Each of the four representatives of this subset (F2D8, B6, ac-206, F18) contain normal levels of the chloroplast-encoded messages for cyt *f*, cyt *b6* and subunit IV (Figure 9). Pulse-labeling experiments have shown that the mutants synthesize cyt *f* (not shown) and are therefore likely to be affected at a post-translational step in the assembly of the complex. Mutations that affect other photosynthetic functions (PSII, PSI, plastocyanin, Calvin cycle) do not, in general, affect cyt *c6*

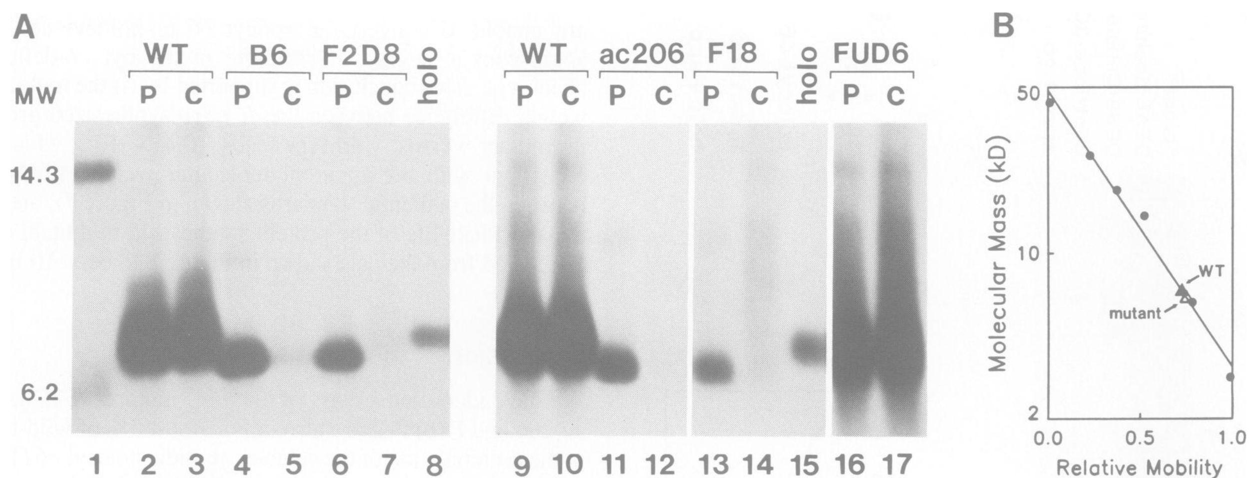

Fig. 8. Resolution of apocytochrome versus holocytochrome *c6* in wild-type and mutant strain. (**A**) Cells of the indicated strain were grown to logarithmic phase ($2-5 \times 10^6$ cells/ml) in copper-deficient medium and then labeled for 20 min in the presence of 1.5 mCi/ml [^{35}S]Na_2SO_4 (1060−1600 Ci/mmol, carrier-free, NEN Research Products) as described in Materials and methods. Labeling was terminated by diluting the isotope with a Na_2SO_4 'chase' added to a final concentration of 20 mM. Aliquots of labeled cells were extracted into 100% acetone at 4°C either immediately after the addition of the chase ('P', pulse) or 40 min later ('C', chase). All samples were labeled to a specific activity between 7.4 and 13.9 TCA-precipitable c.p.m./cell. Cyt *c6* was immunoprecipitated from equal volumes of solubilized acetone precipitate from each strain using either 10 μl (B6, F2D8, ac-206 and F18) or 40 μl (wild-type and FUD6) of anti-cyt serum (see also legend to Figure 6). Immunoprecipitates were separated on a high resolution tricine−SDS−PAGE system (Schagger and von Jagow, 1987) as described in Materials and methods and visualized by fluorography using Kodak XAR-5 film (exposure times: lanes 1−8, 5 days; lanes 9−12 and 16−17, 1 day; lanes 13−15, 6 days. The left (lanes 1−8) and right panels (lanes 9−17) represent two independent experiments. Lanes 8 and 15 show the mobility of a holocyt *c6* standard. The numbers to the left (kDa) indicate the positions of molecular weight of cyt *c6* immunoprecipitated from wild-type and mutant strains. The mass of molecular weight standards in lane 1. (**B**) Estimation of the apparent molecular weight of *c6* immunoprecipitated from wild-type and mutant strains. The mass of molecular weight standards (43.0, 25.7, 18.4, 14.3, 6.12 and 3.0 kDa; closed circles) was plotted as a function of their mobility in the gel shown in Figure 8A, left panel (least squares fit, $R = 0.993$). The apparent molecular weight of cyt *c6* synthesized by wild-type (closed triangles) and mutant (open triangles) strains was estimated by plotting their relative mobility on the curve (see text).

levels significantly. One might argue that the cyt *c6*$^-$ phenotype in the four *b6f*$^-$ mutants is a secondary consequence of the cyt *b6f* deficiency. For example, the *b6f* complex may play a role *in vivo* in maintaining the availability of the reduced substrate (ferroheme) for the putative cyt *c6*/heme lyase. It is known that the cyt *c* and *c1*/heme lyases of *N.crassa* require a source of reductant (NADH and flavin nucleotides) in *in vitro* catalyzed reactions (Nicholson and Neupert, 1989; Nicholson *et al.*, 1989). Or, for example, the cyt *c6*/heme lyase or a processing peptidase may be structurally associated with the *b6f* complex. However, the properties of F1-9 and FUD6 (Table II), which are deficient in cytochromes *b6* and *f* but have normal levels of cyt *c6*, indicate that a cyt *c6* deficiency is not an obligatory consequence of a cyt *b6f*$^-$ deficiency. The converse, viz, that cyt *c6* is required for the maintenance of cyt *b6f*, is not the case since cyt *c6* is not normally synthesized by wild-type cells in copper-supplemented medium whereas the polypeptides of the cyt *b6*f complex are (Figures 2A and C, 3).

We conclude therefore that the class of mutants described here are blocked in the conversion of apocytochrome *c6* to holocytochrome *c6* and suggest that the block results from the same defect that causes *b6f*$^-$ phenotype. This defect may lie in a plastidic heme lyase that (unlike the mitochondrial equivalents) functions *in vivo* to convert both apocytochromes *f* and *c6* to their mature forms, or in a lumenal chaperonin or disulfide reductase that maintains the apoprotein or the cysteinyl thiols in a conformation appropriate for reaction with the lyase. Another possibility is that heme delivery to the lumen (the probable suborganellar site of heme attachment to apocyt *c6* and *f*) or heme delivery to the lyase is affected (see below and Figure 10). A decrease in any one of these functions would

Table II. Summary of the characterization of cyt *b6f*-deficient mutants

Polypeptide[b]	Mutants[a]					
	F2D8	B6	ac-206	F18	F1-9	FUD6
cyt *c6*	−	−	−	−	+	+
cyt *f*	−	−	−	−	−	−
cyt *b6*	−	−	−	−	−	−
Rieske FeS	−	−	−	−	−	−
cyt *b559*	n.d.[c]	+	+	+	+	n.d.
cyt *c550*	+	+	+	+	+	+

[a]F2D8, ac-206, F18 and F1-9 are nuclear mutants. The mutations in B6 and FUD6 map to the chloroplast genome.
[b]The levels of holocyt *c6* accumulated in each of the mutants was measured in this work as described in Materials and methods. The levels of cyt *f*, cyt *b6* and the Rieske FeS protein were tested either in this work by Western blot analysis of electrophoretically separated thylakoid membrane proteins or were previously determined in other work by Western blot analysis, spectral analysis or by heme staining [B6 (Shochat *et al.*, 1990), ac-206 (Gorman and Levine, 1966b), F18 (Bendall *et al.*, 1986; Lemaire *et al.*, 1986) F1-9 (Garnier and Maroc, 1970; Marco and Garnier, 1973), FUD6 (Lemaire *et al.*, 1986)]. Cyt *b559* was identified spectrally in ac-206, F18 and F1-9 (Bendall *et al.*, 1986; Garnier and Maroc, 1970; Maroc and Garnier, 1981). Its presence in B6 is inferred since B6 has normal PSII function and cyt *b559* is required for PSII accumulation (Pakrasi *et al.*, 1991; Shochat *et al.*, 1990). Cyt *c550* was identified by its absorption spectrum and by its heme-dependent peroxidase activity in thylakoid membrane fractions (Atteia *et al.*, 1992).
[c]Not determined.

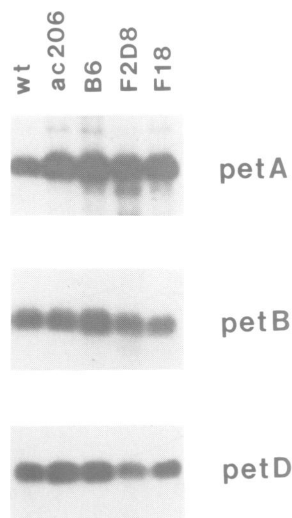

Fig. 9. Accumulation of chloroplast messages encoding polypeptides of the cytochrome *b6f* complex in wild-type versus cyt *b6f*⁻/cyt *c6*⁻ mutants. Total RNA was isolated from copper-deficient cultures of each of the strains. Equivalent amounts of total RNA (3 μg) were separated by denaturing agarose gel electrophoresis and analyzed by Northern hybridization for the presence of pet A (encoding cyt *f*), pet B (encoding cyt *b6*) and pet D (encoding Subunit IV) messages. Cloned DNA probes were generously provided by Dr Francis-Andre Wollman (pet A), Dr Deb Berthold (pet B) and Dr Robert Spreitzer (pet D).

reduce or prevent heme attachment to apocyts *c6* and *f*. The *b6f* deficiency may then result from the instability of the other polypeptides of the complex (cyt *b6*, Rieske iron–sulfur protein, subunit IV) in the absence of cyt *f* (e.g. Lemaire *et al.*, 1986; Cirvellone *et al.*, 1988; Konishi *et al.*, 1991). The cyt *b6f*⁻ cyt *c6*⁻ phenotype could also result from a deficiency in plastidic heme biosynthesis. If heme biosynthesis is affected, the likely target would be ferrochelatase since this is the only step that is unique to plastidic heme synthesis (Castelfranco and Beale, 1983). We do not favor this third possibility since F18 and ac-206 have been demonstrated to contain cyt *b559* (Table II; Bendall *et al.*, 1986), a cytochrome that is essential for PSII structure and function (Pakrasi *et al.*, 1991). Furthermore, we have noted that all four mutants, F2D8, B6, ac-206 and F18, contain wild-type levels of mitochondrial cyt *c* [an 11 kDa protein referred to previously (Lemaire *et al.*, 1986) as h_2 (Table II)]. Thus all four mutants are capable of synthesizing other heme proteins and we conclude that heme synthesis is unlikely to be significantly affected in these four mutants. Since at least three of the mutants are non-allelic (ac-206 maps to linkage group XIV (Harris, 1988), an F18/ac-206 diploid exhibits wild-type fluorescence [J.Girard-Bascou, unpublished results) and B6 results from a plastid genome mutation (Schochat *et al.*, 1990)], each of the mutant phenotypes may well have a different biochemical basis. The F2D8 phenotype is probably caused by a mutation that is distinct from that in F18 and ac-206 since it can be distinguished (albeit subtly) from the others. For example, (i) its acetate requiring phenotype is slightly 'leaky' and (ii) a small proportion of apocyt *c6* can be converted into holocyt *c6* at a slow rate resulting in detectable levels of accumulated holocyt *c6* (e.g. Figure 2A and B). The existence of multiple, non-allelic loci involved in a single step (heme attachment) is consistent with the notion that several biochemical functions might be required for normal catalysis of this reaction. These functions might include (i) factors required to deliver substrates to the heme lyase, (ii) factors required to maintain the substrates in a conformation appropriate for binding to the heme lyase or (iii) perhaps the heme lyase itself. Nevertheless, we cannot at present rule out the possibility that only a single gene product is required for heme attachment, and that the other mutants result from defects in the expression of that single gene. Further genetic, molecular and biochemical characterization of these mutants should allow us to determine the biochemical bases for these defects.

If heme attachment is indeed blocked in this class of mutants, our results indicate that heme attachment is not required for proteolytic procesing of the pre-protein. It is possible then that the fully processed protein is the substrate for heme attachment. Since the active site of the thylakoid processing peptidase faces the lumen (Kirwin *et al.*, 1988) this suggests that heme attachment to apocyt *c6* likely occurs in the lumen (Figure 10). It is also likely that holocyt *f* synthesis occurs in the lumen since the N-terminal domain containing the prosthetic group is lumen-localized and is suggested to be co-translationally transported across the thylakoid membrane on the basis of its synthesis on thylakoid-bound ribosomes (Alscher *et al.*, 1978; Gray *et al.*, 1984; Willey *et al.*, 1984). This is the first instance where it has been suggested that the biosynthesis of the membrane and soluble *c*-type cytochromes in eukaryotes depend on common gene products [as is the case in bacteria (Kranz, 1989; Ramseier *et al.*, 1991; Beckman *et al.*, 1992)]. The post-translational pathway for maturation of cyt *c6* (including heme attachment) is fully active in copper-supplemented cells when the cyt *c6*-encoding gene is completely silent (J.Quinn and S.Merchant, unpublished results). This finding is consistent with the above suggestion. We also suggest for the first time that heme transport or delivery to the lumen might be an important step in the pathway of chloroplast *c*-type cyt maturation. The identification of an anti-cyt *c6* reactive polypeptide with a size intermediate between pre-apocyt *c6* and cyt *c6* (Figure 7) is another provocative observation, since the existence of a partially processed intermediate in the targeting pathway for 'naturally occurring' lumenal proteins (Figure 10) remains a controversial issue (Bauerle and Keegstra, 1991; Bauerle *et al.*, 1991). Ongoing work (unpublished) indicates that the species identified here is indeed, like the partially processed mitochondrial cyt *c1* (Ohashi *et al.*, 1982; Nicholson *et al.*, 1989) a kinetically competent intermediate in the pathway. With the recent proposal that heme delivery to the periplasm is required for bacterial cyt *c2* synthesis, and the observation that genes involved in the bacterial process share sequence similarity with organellar ORFs (Beckman *et al.*, 1992), the analogy between organellar and bacterial cyt *c* synthesis (Hartl and Neupert, 1990) is further strengthened. The work of Ramseier *et al.* (1991) and Beckman *et al.* (1992) suggests that six to nine genes may be required for bacterial cyt *c* synthesis. As many (or perhaps more) might be required in the highly compartmented eukaryotic cells. The description of the dual cyt *c6*⁻/cyt *b6f*⁻ phenotype simplifies the identification of additional mutants (and loci) involved in plastidic *c*-type cyt maturation since mutations affecting transcription or translation of the

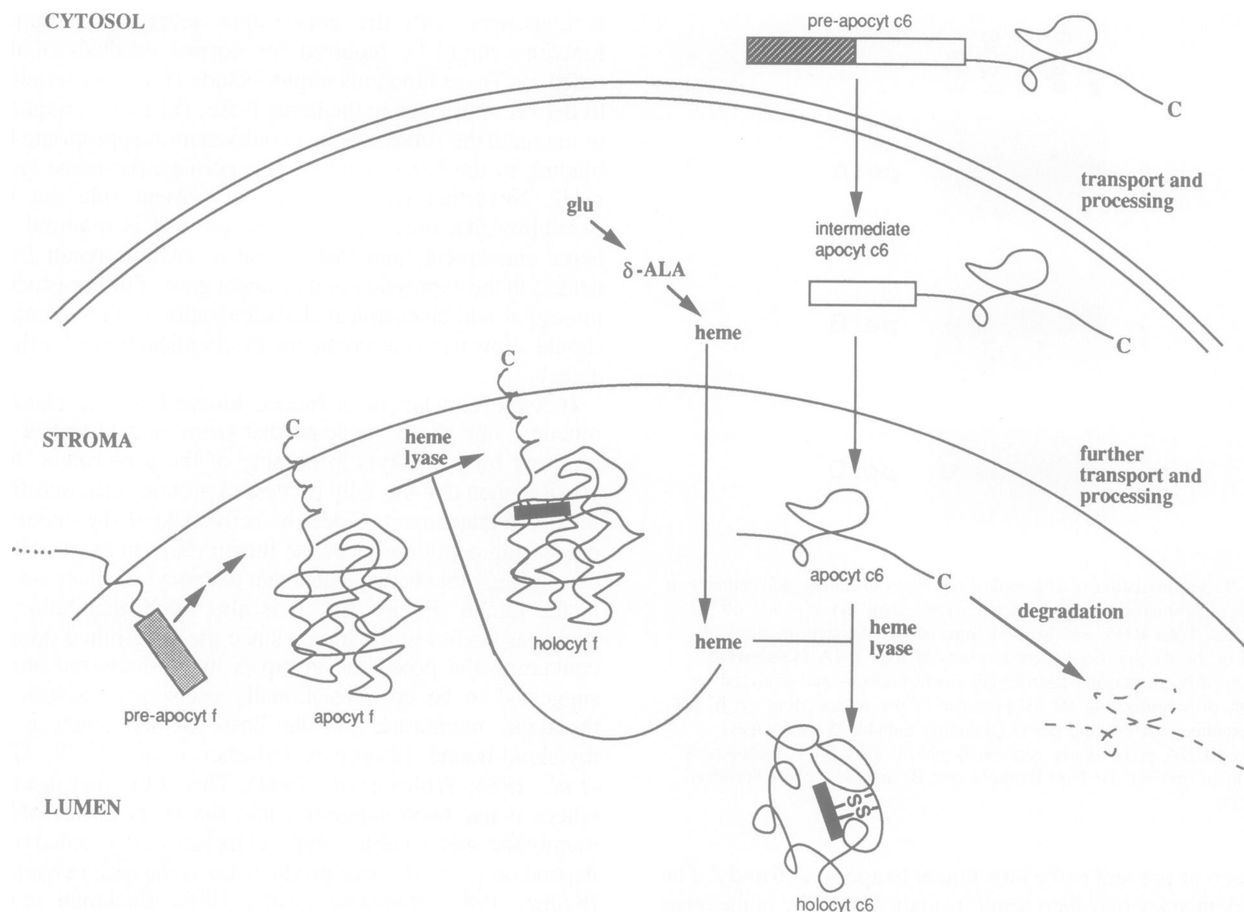

Fig. 10. A proposed biosynthetic pathway for plastidic *c*-type cytochromes. Pre-apocyt *c6* is synthesized on cytosolic ribosomes with a two-domain transit sequence (von Heijne *et al.*, 1989). The C-terminus is indicated. The striped box represents the N-terminal domain that signals envelope translocation and the unfilled box the one that signals thylakoid translocation. Translocation across the envelope membrane, followed by proteolytic removal of the first domain by a stromal processing protease generates an intermediate that is a substrate for translocation across the thylakoid membrane (Weisbeek *et al.*, 1989). The existence of a kinetically competent intermediate remains a controversial issue (Bauerle and Keegstra, 1991; Bauerle *et al.*, 1991). However, the identification of a putative intermediate *in vivo* in this work (Figure 6) supports the two-step processing pathway. Translocation of the intermediate across the thylakoid membrane and removal of the second domain by a thylakoid peptidase (Kirwin *et al.*, 1988) generates apocyt *c6* in the lumen. This processing step is independent of heme attachment since the mutants analyzed in this work do not appear to be blocked at processing. The fully processed apocyt *c6* is proposed to be a substrate for heme attachment, catalyzed by a cyt *c6*/heme lyase. The solid, thick line represents a heme group (edge view) in the holocytochome. If heme attachment is inefficient, the apoprotein is rapidly degraded as is apoplastocyanin (Merchant and Bogorad, 1986b). If heme attachment to apocyt *f* is catalyzed by the same enzyme that catalyzes holocyt *c6* formation, or if holocyt *f* formation depends on gene products that are also required for holocyt *c6* formation (e.g. heme binding proteins, heme transport systems, lumenal chaperonins or disulfide reductases), mutations that affect these functions would be expected to result in the cyt $c6^-$/cyt f^- dual phenotype. The absence of the entire *b6f* complex could simply be a secondary consequence of the cyt *f* deficiency since cyt *f* is membrane-anchored by a C-terminal transmembrane segment, indicated here as a helical line (see e.g. Lemaire *et al.*, 1986; Gerhus *et al.*, 1990; Konishi *et al.*, 1991). At present, the steps in the maturation of cyt *f* are not known. That is, whether processing or heme attachment occur co-translationally or post-translationally. Delta-aminolevulinic acid is abbreviated δ-ALA. The envelope membranes are indicated as a pair of curved lines and the thylakoid membrane as a single line. Since the existence of lumenal heme binding proteins, chaperonins or disulfide reductases is speculative, they are not included in the figure.

genetic information for the apoproteins are not expected to exhibit the dual phenotype.

Materials and methods

Strains and cell culture

Chlamydomonas reinhardtii wild-type strains (CC-124 and CC-125) and all photosynthesis-minus mutant strains (acetate requiring) were obtained either from the *Chlamydomonas* culture Collection, Duke University, or as follows: B6 and C2 from Dr Laurens Mets, University of Chicago; L210A and L135F from Dr Michel Goldschmidt-Clermont, University of Geneva; FUD26 and C3 from Dr Jacqueline Girard-Bascou, Institut de Biologie Physico-chimique; F18 and FUD6 from Dr Francis-Andre Wollman, Institut de Biologie Physico-Chimique; nac2-26 from Dr Michael Kuchka, Lehigh University; and FUD7 from Dr Jeanne Erickson, UCLA. Cells were grown at 22°C under dim light (25 $\mu E/m^2/s$) in Tris-acetate-phosphate (TAP) medium in which the copper concentration was reduced to <3 nM (Merchant and Bogorad, 1986a; Harris, 1988). A reduced sulfate TAP medium, prepared by using $MgCl_2$ in place of $MgSO_4$, was used for *in vivo* labeling experiments.

Isolation of F2D8

Cultures of the wild-type strain CC-124 were grown to a cell density of 5×10^6 cells/ml in TAP medium. A 25 ml volume of stirred cells was irradiated in a sterile Petri dish (15 × 100 mm) with a germicidal UV light for 6 min at a distance of 68 cm (4.7 $\mu W/cm^2$ at the surface of the cells). The survival ratio of the irradiated cells was determined to be ~25%. Typically, three 25 ml aliquots of irradiated cells were pooled, diluted 2-fold with TAP medium, and then divided into several smaller (10 ml) cultures. These cells were transferred to an incubator (22°C, 250 r.p.m.) and allowed to grow for 3 days in complete darkness followed by 5 days of growth in dim light (25 $\mu E/m^2/s$). At this point metronidazole (Flagyl, Sigma Chemical Company, St Louis, MO) was added to the mutagenized cultures, to a final concentration of 20 mM, and the cells were transferred to bright light (125 $\mu E/m^2/s$) for 1.5 days in order to enrich for clones that were

defective in photosynthetic electron transfer (Schmidt *et al.*, 1977). Acetate requiring clones were identified among the metronidazole-enriched survivors by their ability to grow heterotropically (on TAP medium) but not photoautotrophically (on minimal medium) under illumination of 25 $\mu E/m^2/s$. The holocyt *c6* content of single isolates of these acetate requiring clones was determined by analyzing soluble proteins extracted from individual clones grown in copper-deficient medium by non-denaturing gel electrophoresis and subsequent staining of the gels for heme-dependent peroxidase activity. The acetate requiring phenotype of the cyt $c6^-$ mutant described in this work, F2D8, was inherited as a Mendelian mutation in a backcross to a wild-type strain (CC-125). Progeny from the cross were characterized by Western blot analysis for the cyt $c6^-$ and cyt f^- phenotypes. The two phenotypes were determined to co-segregate ($P < 0.005$).

Preparation of cell extracts and thylakoid membrane fractions

Cultures were grown to late-logarithmic phase ($0.5-1.0 \times 10^7$ cells/ml), collected by centrifugation (7500 *g* for 5 min at 4°C), washed once in ice-cold phosphate buffer (10 mM phosphate-Na, pH 7.0) and resuspended in the same buffer at a concentration equivalent to 0.5−1 mg chlorophyll/ml. The cells were broken by subjecitng the concentrated cell suspensions to two slow freeze−thaw cycles (−80°C to room temperature over a period of several hours). The released, soluble proteins were collected as supernatants after centrifugation (12 000 *g* for 15 min) at 4°C. As judged by immunoblot analysis, cyt *c6* is quantitatively recovered in these supernatants. The insoluble pellet does not contain significant amounts of cyt *c6*. The protein and chlorophyll contents of the same strain grown in either copper-supplemented or copper-deficient medium were not significantly different.

To obtain extracts containing 'total' cellular protein, one volume of concentrated cell suspension was diluted with an equal volume of denaturation buffer (3% SDS, 62.5 mM Tris−Cl, pH 6.8, 10% glycerol, 10% β-mercaptoethanol, 0.1% bromophenol blue) and the mixture was heated in a boiling water bath for 5 min. Following centrifugation (12 000 *g* for 10 min), the supernatants were collected for electrophoretic separation and further analysis.

Cell extracts enriched in thylakoid membrane content were prepared as follows. Cultures at late-logarithmic phase (0.5×10^7 cells/ml) were harvested by centrifugation, washed once in an ice cold solution containing 50 mM Na-tricine, pH 8.0, and adjusted to a chlorophyll concentration of 0.5 mg/ml with the same buffer. Three ml aliquots of the cell suspension were sonicated in 15 ml Corex tubes (Corning Glass Works, Corning, NY) in a bath type sonciator (Laboratory Supplies, Hicksville, NY) for 25 s. The entire procedure was performed on ice. Unbroken cells were removed from the sonicated cell suspension by centrifugation at 3000 *g* for 45 s. Thylakoid membranes were collected from the resulting supernatants by centrifugation at 48 000 *g* for 10 min. The pelleted membranes were resuspended in 0.15 mM NaCl, 10 mM Na-tricine, pH 8.0 to a chlorophyll concentration of 0.5−2 mg/ml and then solubilized in the following mixture: 1% SDS, 0.05 M Na_2CO_3, 0.05 M dithiothreitol, 12% sucrose and 0.05% bromophenol blue.

Gel electrophoresis and Western blotting

Protein samples were separated either on non-denaturing polyacrylamide gels (Merchant *et al.*, 1991), on the SDS-denaturing polyacrylamide gel system described by Laemmli (1970), or the tricine−SDS−polyacrylamide gel system described by Schagger and von Jagow (1987). The latter type of gel included a 4% total acrylamide (3.88% acrylamide, 0.12% bisacrylamide) stacking gel (3 cm), a 10% total acrylamide (9.7% acrylamide, 0.3% bisacrylamide) spacer gel (2 cm) and a 16.5% total acrylamide (15.5% acrylamide, 1% bisacrylamide) separating gel (17 cm). These gels were typically run at 125 V for 36 h. Heme-containing proteins were visualized in gels by tetramethylbenzidine (TMBZ) staining (Thomas *et al.*, 1976). ^{35}S-labeled proteins were visualized by autoradiography of gels that were fixed and infiltrated with fluor according to Bonner and Laskey (1974).

For Western blot analysis, separated proteins were electrophoretically transferred (50 V, 2−3 h at 4°C) from non-denaturing or denaturing gels to Immobilon-P transfer membranes (Millipore Corporation, Bedford, MA) in a solution containing 25 mM Tris, 192 mM glycine, 20% methanol (Towbin *et al.*, 1979). The immunoreactive polypeptide of interest was probed with polyclonal antibodies against *C.reinhardtii* cytochrome *c6*, *C.reinhardtii* plastocyanin, spinach cytochrome *f* (from Dr Richard Malkin, University of California−Berkeley), or maize cytochrome *b6* and Rieske iron−sulfur protein (from Dr Alice Barkan, University of Oregon). Antigen−antibody complexes were detected by the use of a horseradish peroxidase-conjugated second antibody as described previously (Merchant and Bogorad, 1986a).

RNA analysis by Northern blots and by in vitro translation

Total RNA was isolated from late-log cultures and characterized by Northern hybridization analysis as described previously (Merchant *et al.*, 1991). For analysis by *in vitro* translation, total RNA (9 μg in H_2O) was translated by a rabbit reticulocyte lysate in the presence of [^{35}S]methionine (1150 Ci/mmol, Amersham Corporation, Arlington Hts, IL) according to instructions provided by the supplier (Promega Corporation, Madison, WI). Pre-apocyt *c6* was immunoprecipitated from the translation reaction essentially as described previously (Merchant and Bogorad, 1986a) with the modification that the SDS concentration in the denatured translate (2% w/v) was diluted with immunoprecipitation buffer to a final concentration of 0.2% instead of 0.4%.

Quantitation of holocyt c6

Soluble extracts were prepared as described above from 500 ml cultures grown to late log phase ($0.5-1.0 \times 10^7$ cells/ml) in either copper-supplemented (10 μM) (+Cu) or copper-deficient (−Cu) TAP medium. The extracts were adjusted to a volume of 1.8 ml with 10 mM phosphate-Na^+ buffer, pH 7.0 and the protein concentration was determined against a standard solution of bovine serum albumin as described below. The extracts were divided into two equal portions (0.9 ml), one of which was reduced with sodium ascorbate and the other oxidized with potassium ferricyanide. The absorption spectra of the reduced and oxidized extracts were recorded separately against a blank of phosphate buffer on a Uvicon 930 spectrophotometer. A difference spectrum was obtained by subtraction of the oxidized spectrum from the reduced spectrum. A $\Delta\epsilon_{552}$ (reduced−oxidized) for holocyt *c6* of 21 $mM^{-1} cm^{-1}$ was determined using purified *C.reinhardtii* holocyt *c6*. The amount of cyt *c6* in extracts prepared from cells grown in copper-deficient medium was calculated from the height of the α band maximum (552 nm), measured above a baseline drawn so as to intersect the difference spectrum at the isosbestic points 542 and 561 nm. The constitutive presence (with respect to medium copper supplementation) of a small peak at 549.0−549.5 nm probably reflects the mitochondrial cytochrome *c550* (see Figure 4). The contribution that this cytochrome makes toward the cyt *c6* Δ-absorbance at 552 nm (ΔA_{552}) was corrected for by subtracting the ΔA_{552} value obtained with +Cu extracts from the ΔA_{552} value obtained with −Cu extracts. Owing to the constitutive presence of cyt *c550*, the limit of detection of this method for quantitation of cyt *c6* in mutant strains is ~10% of the levels found in the wild-type strain.

Analysis of de novo synthesized proteins by in vivo labeling

Cells were labeled by modifications of the procedure described by Schmidt and Mishkind (1983). Cells were grown to a density of $2-5 \times 10^6$ cells/ml in reduced-sulfate TAP media (see above) either supplemented with copper (10 μM $CuCl_2$) or not, harvested by centrifugation (7500 *g* for 5 min at 22°C), and gently resuspended to a cell density of 1×10^8 cells/ml in sulfate-free TAP medium. Sulfate-free TAP medium was prepared by excluding Hutner's trace elements (Harris, 1988) from reduced-sulfate TAP medium. The concentrated cell suspension was incubated in dim light (25 $\mu E/m^2/s$) for 45 min prior to the addition of the radioisotopic label. 'Pulse−chase' labelling was performed as described in the figure legends. Protein synthesis and degradation were stopped by removing 0.5 ml aliquots of labeled cells into 4.5 ml of ice-cold acetone (100%). The effectiveness of the labeling and chase periods in these experiments was established by monitoring the incorporation of ^{35}S into protein during the course of the experiment as follows. Aliquots (10 μl) of cells were removed from the labeling reaction into 1 ml ice-cold acetone at various times during the experiment. Acetone precipitates were solubilized as described below. Incorporation of ^{35}S into protein in the solubilized acetone pellets was determined by trichloroacetic acid precipitation as described by Pratt (1984) with the exception that Na_2SO_4, cysteine and methionine were added to all trichloroacetic acid solutions to final concentrations of 10 mM, 0.1% (w/v) and 0.1% (w/v), respectively.

The acetone precipitated proteins were collected by centrifugation and dissolved in 0.3 ml sample buffer (2% SDS, 60 mM Tris−Cl, pH 8.6, 60 mM dithiothreitol, 1 mM EDTA, 5 mM aminocaproic acid, 1 mM benzamidine, 1 mM phenylmethylsulfonylfluoride). The solution was heated in a boiling water bath for 5 min, centrifuged (12 000 *g* for 2 min) to remove undissolved material, and then either used immediately in immunoprecipitation reactions or stored at −70°C. Prior to immunoprecipitation of anti-cyt *c6* reactive polypeptides, equal volumes of the dissolved acetone precipitate from each strain were diluted 10-fold with immunoprecipitation buffer (40 mM Tris−Cl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2% Nonidet-40) and then heated for 5 min in a boiling bath. This solution was treated with a suspension (10% in 40 mM phosphate, pH 7.2, 150 mM NaCl, 0.05% sodium azide) of formalin-fixed and heat-killed *Staphylococcus aureus* cells (IgGSORB, The Enzyme Center, Malden, MA) (100 μl per

10 μl of antiserum that was to be used for immunoprecipitation) in order to pre-absorb proteins that bound non-specifically to the IgGSORB. After a 30 min incubation at room temperature on a platform shaker, the IgGSORB was removed by centrifugation (12 000 *g*, 5 min). To precipitate anti-cyt *c6* reactive polypeptides, 10−40 μl of antiserum (see appropriate figure legend) was added to each supernatant and the mixture was incubated at 4°C for 6−12 h. The antibody−antigen complex was collected after treatment with IgGSORB. The IgGSORB precipitate was washed three times with 5 ml of immunoprecipitation buffer and once with 0.9% NaCl. The final pellet was denatured in 30 μl of sample buffer (3% SDS, 10% glycerol, 62.5 mM Tris−HCl, pH 6.8, 0.1% bromphenol blue) at 100°C for 5 min. The undissolved material was removed by centrifugation (12 000 *g*, 2 min) and the supernatant was analyzed by gel electrophoresis.

Purification of ^{35}S-labeled apocyt c6

The plastocyanin-less mutant ac-208 was grown in 1.2 l of copper-deficient, reduced-sulfate TAP medium. At a cell density of 1.5×10^6 cells/ml, $[^{35}S]SO_4$ (43 Ci/mg S, ICN Biomedicals Inc., Irvine, CA) was added to a final concentration of 5 μCi/ml. Upon reaching late-log phase (66 h after addition of the radiolabel), the culture was harvested by centrifugation and washed once with ice-cold buffer (10 mM sodium phosphate, pH 7.0) containing 5 mM Na_2SO_4. Holocyt *c6* was purified from the cell paste essentially as described previously (Merchant and Bogorad, 1986a) except that a Centricon-30 filter system (Amicon Corporation, Danvers, MA) was used at the last stage of purification instead of a gel filtration column. Apocyt *c6* was prepared by a modification of a previously described procedure (Fisher *et al.*, 1973). Specifically, heme was cleaved from the holocyt *c6* by adding 8 μl of glacial acetic acid and 1.3 mg Ag_2SO_4 to 7.7 nmol of ^{35}S-labeled holocyt *c6* (170 c.p.m./pmol) in 90 μl of H_2O. The mixture was incubated at 40°C for 12 h in the dark and then centrifuged at 12 000 *g* for 10 min to remove precipitated heme aggregates. Greater than 90% of the radiolabel was retained in the pellet, indicating that the cyt *c6* polypeptide was also precipitated by this treatment. In order to cleave the silver mercaptide bond, the brown-colored precipitate was washed once with acetone/0.1 N acetic acid, collected by centrifugation, dried in air and solubilized in 0.1 ml of 6 M guanidine HCl, 1 M dithiothreitol, 0.05 M ammonium acetate, pH 5.0. Although some residual insoluble material remained, >50% of the label was recovered in the supernatant following centrifugation of the solubilized mixture at 12 000 *g* for 10 min. This supernatant was applied to a G-25 gel filtration column (1 × 24 cm) equilibrated with 0.05 M ammonium acetate, pH 5.0. The ^{35}S eluting in the void volume was dialyzed against 10 mM sodium phosphate, pH 7.0, for 2 h and then water for 1 h. The dialyzed material was concentrated in a vacuum centrifuge (Speedvac). The final recovery of ^{35}S by this procedure was ~25%. The apocyt *c6* generated by this procedure was devoid of the heme-dependent absorption bands at 410 and 552 nm. Furthermore, the electrophoretic mobility of the major labeled polypeptide in the apoprotein preparation was slightly greater than that of holocyt *c6* (Figure 8). Longer exposures of these autoradiographs revealed a polypeptide migrating with an apparent molecular weight of 18−20 kDa, possibly a dimeric form of apocyt *c6*. The apocyt *c6* preparation was subjected to 30 cycles of repetitive Edman degradation (UCLA Microsequencing Facility) and the radioactivity released at each cycle was measured by scintillation counting. Two major peaks of ^{35}S were released at cycles 19 and 26, which correspond to Met19 and Met26 of the mature polypeptide (Merchant and Bogorad, 1987a). This confirmed that the chemically prepared apocyt *c6* possesses the correct N-terminus. The fractions corresponding to the cysteine residues at positions 14 and 17 did not contain a significant amount of radiolabel.

Miscellaneous procedures

Chlorophyll concentrations were determined from the absorbance at 652 nm of whole cells extracted with acetone:methanol (80:20), using the extinction coefficients of Arnon (1949). Protein concentrations were determined using the Coomassie Blue dye binding reagent and instructions provided by Pierce Chemical Co. (Rockford, IL).

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