Molecular Genetic Identification of a Pathway for Heme Binding to Cytochrome $b_6^*$

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Heme binding to cytochrome $b_6$ is resistant, in part, to denaturing conditions that typically destroy the noncovalent interactions between the $b$ hemes and their apoproteins, suggesting that one of two $b$ hemes of holocytochrome $b_6$ is tightly bound to the polypeptide. We exploited this property to define a pathway for the conversion of apo- to holocytochrome $b_6$, and to identify mutants that are blocked at one step of this pathway. *Chlamydomonas reinhardtii* strains carrying substitutions in either one of the four histidines that coordinate the $b_h$ or $b_l$ hemes to the apoprotein were created. These mutations resulted in the appearance of distinct immunoreactive species of cytochrome $b_6$, which allowed us to specifically identify cytochrome $b_6$ with altered $b_h$ or $b_l$ ligation. In gabaculine-treated (i.e. heme-depleted) wild type and site-directed mutant strains, we established that (i) the single immunoreactive band, observed in strains carrying the $b_h$ site-directed mutations, corresponds to apocytochrome $b_6$ and (ii) the additional band present in strains carrying $b_l$ site-directed mutations corresponds to a $b_h$-heme-dependent intermediate in the formation of holocytochrome $b_6$. Five nuclear mutants ($ccb$ strains) that are defective in holocytochrome $b_6$ formation display a phenotype that is indistinguishable from that of strains carrying site-directed $b_l$ ligand mutants. The defect is specific for cytochrome $b_6$ assembly, because the $ccb$ strains can synthesize other $b$ cytochromes and all $c$-type cytochromes. The $ccb$ strains, which define four nuclear loci ($CCB1$, $CCB2$, $CCB3$, and $CCB4$), provide the first evidence that a $b$-type cytochrome requires trans-acting factors for its heme association.

Quinol oxidizing complexes, the cytochrome $b_{1}$ complex of mitochondria and bacteria, and its chloroplast counterpart, the cytochrome $b_{6}/f$ complex, couple translocation of protons across the membrane to oxidation of lipophilic electron carriers (quinols) and reduction of small hydrophilic proteins (reviewed in Refs. 1 and 2).

In the green alga *Chlamydomonas reinhardtii*, the cytochrome $b_{6}/f$ complex comprises seven subunits. The four large subunits are in a 1:1:1:1 ratio (3, 4). Three of them encode by chloroplasitc genes (5), $petA$ (encoding cytochrome $f$, a $c$-type cytochrome), $petB$ (encoding cytochrome $b_{6}$), and $petD$ (encoding subunit IV); whereas the Fe$_{5}$S$_{2}$ Rieske protein is encoded by a nuclear gene, $PetC$ (6). In addition, the cytochrome $b_{6}/f$ complex contains three transmembrane subunits of low molecular mass (~4 kDa), the products of the chloroplast genes $petG$ (7–9) and $petL$ (10) and the nuclear gene $PetM$ (11, 12).

The cytochrome $b_{6}/f$ complex binds five cofactors: two $b$ hemes (high potential $b_h$ and low potential $b_l$), one $c$ heme, a Fe$_{5}$S$_{2}$ cluster, and a chlorophyll a (13–16). In $c$-type cytochromes, the heme is covalently attached to the protein by thioether linkages between the sulfhydryl groups of (usually) two cysteine residues and the vinyl groups of the tetrapyrrole ring. In $b$-type cytochromes, the heme is believed to be noncovalently bound to the protein through pairs of histidines, which serve as axial ligands for the iron atom.

Cytochrome $b_{6}$ and subunit IV are homologous, respectively, to the first four $a$-helices and next three $a$-helices of cytochrome $b$ of the cytochrome $b_{c}$ complex (17). $b_h$ and $b_l$ hemes are associated with cytochrome $b_{6}/f$ heme on the luminal side of the thylakoid membrane (close to the quinol oxidizing site) and $b_l$ heme on the stromal side (close to the quinone reducing site).

The crystallographic structure of the $b_{c}$ complex from bovine heart mitochondria is being resolved (18, 19). The two hemes are bis-histidine-coordinated (20) and span the membrane bilayer approximately perpendicularly to the membrane plane (21). The two pairs of histidines that coordinate the central iron atoms are conserved in all cytochrome $b$ (22) and located on helices B and D as follows in *C. reinhardtii*: (B)His$_{100}$-$b_h$-His$_{202}$C and (B)His$_{96}$-$b_l$-His$_{187}$D (5).

Numerous mutations of cytochrome $b$ have been obtained in photosynthetic bacteria (*Rhodobacter* species) and mitochondria (reviewed in Ref. 23). By contrast, only a few mutations in $petB$ that alter cytochrome $b_{6}$ are known (24–26). These mutations were generated in *C. reinhardtii*, which is a unique system for mutational studies of the cytochrome $b_{6}/f$ complex owing to the availability of chloroplast gene replacement methodology coupled with the fact that the cytochrome $b_{6}/f$ complex is dispensable for growth in *C. reinhardtii*.

In contrast to knowledge accumulated on the biosynthetic pathway of the tetrapyrrole cofactors and on the process of covalent attachment of $c$ hemes to their apoproteins (reviewed in Refs. 27–30), conversion of $b$-type apocytochromes to their holo-form has received little attention, possibly due to the difficulty in distinguishing heme association defects from defects in other aspects of cytochrome $b$ assembly. Since hemin can bind, without catalysis, to synthetic peptides that mimic helices B and D of cytochrome $b$ (31), one view is that heme binding to cytochrome $b$ should also be uncatalyzed *in vivo*. This view is uncontested by virtue of the fact that protein factors involved in the catalysis of heme binding to cytochrome...
b or b6 have not been identified. The hemes of mitochondrial and bacterial cytochrome b are not detected after SDS-PAGE1 of membranes or purified complexes (32, 33), and this undoubtedly accounts for the dearth of information on cytochrome b assembly. By contrast, heme(s) of cytochrome b6 can be visualized after electrophoresis (3, 34–36). Here, we demonstrate that tight binding of heme is a unique aspect of the chloroplast cytochrome b6 (which may reflect a different mode of association, perhaps covalent, of at least one of the two hemes), and we exploit this property to (i) establish a temporal order of heme binding to apocytochrome b6 in a multistep pathway in vivo and (ii) identify mutants that are blocked specifically at the step of heme insertion into the b6 site. This has been accomplished by monitoring the synthesis of cytochrome b6 in strains carrying site-directed alterations of b6 or b6 ligands and in nuclear mutants with similar phenotypes. These nuclear mutants define four loci.

MATERIALS AND METHODS

Strains—Wild type (WT) strain CC125 mt+ was used for UV mutagenesis, and 137c was used for chloroplast transformation and genetic analysis. Transforms were grown in Tris acetate phosphate (TAP) medium, pH 7.2, at 25 °C under dim light (5–6 μE).

Isolation of Nuclear Mutants Deficient in Cytochrome b6 Complexes—WT cells grown to a density of 3–5 × 106 cells/ml were transferred to Petri dishes exposed to 260-nm UV irradiation for 1–5 min with constant agitation. The cells’ viability was approximately 20–30%. Irradiated cells were transferred to the dark for 2 h to minimize photoreversion. The cells were then plated on thin agar slabs (2% TAP agar poured over Miracloth circles) over TAP agar plates. Plates were returned to the dark for 24–48 h. Agar slabs were transferred to TAP agar plates containing 20 μM metronidazole and placed in high light (50–60 μE). After 24–48 h, agar slabs were transferred to fresh TAP agar plates and maintained under dim light for 2–3 weeks until colonies were apparent (50–150 colonies/plate). Surviving colonies were tested for fluorescence induction kinetics (3, 38); mutants showing a decreased cytochrome b6 fluorescence and a fluorescence of cytochrome b6-deficient mutants (26) were chosen.

Mutagenesis and Plasmids—Site-directed mutagenesis was performed in Escherichia coli as by Kunkel (39) on plasmid pWB (24), which encompasses the whole petB coding sequence and its flanking regions. Mutated products were verified by sequencing. Plasmid pWF6 was constructed by introducing the 1.9-kilobase pair EcoRV-Smal fragment of plasmid pUC-tp-A-XaA (40), bearing the aadA cassette, at the unique EcoRV site of plasmid pWF (24). In the resulting pWF6 plasmid, the aadA cassette is located 309 base pairs downstream from the end of the petA coding region and is transcribed from the same strand as the petA gene.

Chloroplast Transformation Experiments—C. reinhardtii cells were transformed by particle bombardment as by Boynton et al. (41). We attempted to complement the SpetB strain, deleted for the petB gene (24), with the mutated petB genes (Table I); after bombardment, transformant cells were plated on minimum medium (MM) under high light. Since no phototrophic transformants were recovered by the above procedure, we used the WT strain in co-transformation experiments with plasmid pWF6, which confers resistance to spectinomycin. Cells were grown for approximately six generations in the presence of 0.5 mM 5-fluoroexoridine before transformation (42) and plated after transformation on TAP-spectinomycin (100 μg/ml)1 under dim light to select for spectinomycin-resistant clones. Resistant clones were then screened by fluorescence to choose those that were defective in cytochrome b6 activity. The transformants were confirmed to be homoplasmic for the petB mutation by restriction fragment length polymorphism analysis and DNA filter hybridization with specific probes (data not shown). The introduced mutations were further confirmed by direct sequencing of the mutated petB genes in these transformants (data not shown).

Genetic Analysis—Crosses were done according to Harris (43), and complementation analysis between nuclear mutants was done according to Goldschmidt-Clermont et al. (44). For reversion tests, mutant strains were grown in TAP to a density of 2 × 106 cells/ml−1; the cells were collected by centrifugation and resuspended in MM to a density of 2 × 106 cells/ml−1. One-half ml was spread onto MM agar plates (10 plates) and maintained under high light for 2–3 weeks, at which time colonies were counted. Recombination tests were performed to detect tight linkage; at the same time, at least 30 zygotes isolated from crosses between different mutant strains were transferred separately to either TAP or MM agar plates. They were kept under dim light on TAP plates during 2 weeks or high light on MM plates for 3–4 weeks; the number of zygotes giving rise to colonies was estimated on each plate; the number of tetratype and nonparental ditype tetrad tears was estimated as a6/a2 (where a6 is the number of zygotes that give rise to colonies on minimal medium, a2 is the number of zygotes transferred to minimal medium, b6 is the number of zygotes that give rise to colonies on TAP, and b2 is the number of zygotes transferred to TAP. Protein Isolation, Separation, and Analysis—Biochemical analyses were carried out on cells grown to a density of 2 × 106 cells/ml−1. For analysis of polypeptide contents, samples were resuspended in 100 mM DTT, 100 mM Na2CO3, and solubilized in the presence of 2% SDS at 100 °C for 50 s. When indicated, nonheated samples were solubilized in the presence of 2% SDS at room temperature. Polypeptides were separated in the Laemmli system (45) using 12–18% acrylamide gels in the presence of 8 μM urea or using 15% acrylamide gels containing 0.1% SDS. Heme staining was detected by peroxidase activity of heme binding sulfhydryl probes. Hemes were grown in high light (50–60 μE) after 24–48 h, agar slabs were transferred to fresh TAP agar plates and maintained under dim light for 2–3 weeks until colonies were apparent (50–150 colonies/plate). Surviving colonies were tested for fluorescence induction kinetics (3, 38); mutants showing a decreased cytochrome b6 fluorescence by immunoblotting and a fluorescence of cytochrome b6-deficient mutants (26) were chosen.

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MM, minimum medium; TAP, Tris acetate phosphate medium; TMBZ, 3,3’,5,5’-tetramethylbenzidine; WT, wild type; E, einstein(a).
continued to stain for heme by the TMBZ assay even after these treatments (Fig. 1A). To confirm that this property is unique to cytochrome \(b_6\), we used a membrane preparation from bovine heart mitochondria to analyze the heme-cytochrome \(b\) interaction after electrophoresis on a 15% acrylamide gel containing 0.1% SDS (Fig. 1B). This gel system resolves the closely migrating mitochondrial cytochrome \(b\) and \(c_1\) and a high mobility band, typical of cytochrome \(c\), with no evidence for cytochrome \(b\) staining. By contrast, cytochrome \(b_6\) was heavily stained with TMBZ under these conditions (Fig. 1B). These experiments point to unusually tight binding of heme(s) to cytochrome \(b_6\), i.e., resistant to denaturation. Since cytochrome \(b_6\) did not stain with TMBZ more heavily than cytochrome \(f\), which binds only one heme, we suggest that only part of the \(b\) heme complement of cytochrome \(b_6\) is tightly associated with the polypeptide.

**Mutation of the Heme-binding Histidines Revealed Distinct Forms of Cytochrome \(b_6\) That Are Resolved by Gel Electrophoresis and Correspond to \(b\) Site and \(b_6\) Site Mutants**—To determine whether we could distinguish which of the two hemes bound tightly to apocytochrome \(b_6\) and whether both heme binding sites were required for cytochrome \(b_6\) assembly, we used site-directed mutagenesis to construct \(petB\) genes in which one of the four heme-liganding histidines, either His\(_{100}\) and His\(_{202}\) to \(b\), or His\(_{86}\) and His\(_{187}\) to \(b\), were substituted individually (Table I). At least three independent transformants for each modification were characterized for their biochemical properties. None of the transformants displayed a heme-stainable cytochrome \(b_6\) after SDS-PAGE of solubilized membranes, and the level of heme-stained cytochrome \(f\) was reduced to that observed in a WT (data not shown). Immunodetection indicated that in each transformant cytochrome \(f\) accumulated to 10% of the WT level, and cytochrome \(b_6\) and subunit IV were present in trace amounts (Fig. 2A).

Analysis of the immunoreactive species in the transformants versus the WT strain indicated a striking change in the migration pattern of cytochrome \(b_6\) (Fig. 2A). Holocytochrome \(b_6\) migrated as a broad diffuse band in the WT (Fig. 2A, lane WT, and dilution series in bottom part), as does in \(\Delta petA\) and in \(\Delta petD\) mutants (bottom part of Fig. 2A), which synthesize holocytochrome \(b_6\), while it migrated as discrete sharp bands in the \(petB\) site-directed heme-binding mutants. (The diffuse pattern in the WT strain does not result from overloading, because the same pattern is noted in dilute samples or in the \(\Delta petA\) and \(\Delta petD\) strains, which accumulate much less holocytochrome \(b_6\).) The \(\Delta petB\) lane serves as a control to indicate that the diffuse band in WT indeed corresponds to cytochrome \(b_6\) and that a band, noted with an asterisk, is an unrelated cross-reacting signal. The contaminating polypeptide appears in the region of cytochrome \(b_6\) only when electrophoresis is carried out in the presence of 8 M urea (Fig. 2A, second part). This cross-reacting band is not visible in the absence of urea (Fig. 2A, third part). In the four transformants bearing substitutions of the \(b_6\) heme ligands, cytochrome \(b_6\) migrated as a single band below the contaminating \(\ast\) band (Fig. 2A, second part, \(b_6\) transformants). By contrast, in the four transformants bearing substitutions of the \(b_6\) heme ligands, cytochrome \(b_6\) migrated

| Nucleotide and amino acid sequences | New restriction sites | Plasmid (Strain) |
|-----------------------------------|----------------------|-----------------|
| ...ATT CAC CGT TGG...             |                      | WT              |
| I\(^{105}\) H\(^{106}\) D\(^{107}\) W\(^{108}\) |                      | WT              |
| ...GGG CGG...                     | BsuHI pB86A          | (TB86A)         |
| Ala                               |                      |                 |
| ...TCG CGA...                     | NruII pB86S          | (TB86S)         |
| Ser                               |                      |                 |
| ...GTT TTA CAC GTT TTC...         |                      | WT              |
| V\(^{289}\) L\(^{290}\) H\(^{291}\) V\(^{292}\) F\(^{293}\) |                      |                 |
| ...TTA GAC GTC...                 | AatII pB100D         | (TB100D)        |
| Asp                               |                      |                 |
| ...TTA CTA GCT...                 | SpeI pB100L          | (TB100L)        |
| Leu                               |                      |                 |
| ...AGT CAC ACT TTC...             |                      | WT              |
| S\(^{186}\) H\(^{187}\) T\(^{188}\) F\(^{189}\) |                      |                 |
| ...GGT ACC...                     | KpnI pB187G          | (TB187G)        |
| Gly                               |                      |                 |
| ...AGT ACT...                     | ScaI pB187S          | (TB187S)        |
| Ser                               |                      |                 |
| ...CAC TTT TTA ATG ATT CGT AAA... |                      | WT              |
| H\(^{202}\) F\(^{203}\) L\(^{204}\) M\(^{205}\) I\(^{206}\) R\(^{207}\) K\(^{208}\) |                      |                 |
| ...GAC TTC TTA ATG ATC CGG AAA... | BspEI pB202D         | (TB202D)        |
| Asp                               |                      |                 |
| ...CAG TTC CTG ATG ATT CGT AAA...  | AluNI pB202Q         | (TB202Q)        |
| Glu                               |                      |                 |
accumulation (Fig. 2A) or synthesis (Fig. 2B).

Synthesis of Cytochrome b₆ in the Presence of Gabaculine, an Inhibitor of the Tetrapyrrole Biosynthetic Pathway, Reveals a b₆—Heme-dependent Intermediate in the Biogenesis of Cytochrome b₆—To order the electrophoretic species identified in the strains carrying b₆ versus b₆ ligand mutations in the context of holocytochrome b₆ maturation, synthesis of cytochrome b₆ was monitored in cells depleted of heme. This was accomplished by treating cells with gabaculine, which prevents tetrapyrrole synthesis, for 6 h prior to and during a pulse-labeling experiment. Radiolabeled cells were solubilized and analyzed by urea/SDS-PAGE. In gabaculine-treated WT cells, neosynthesized cytochrome b₆ migrated as a discrete band at the front of the diffuse band observed in untreated cells (Fig. 3A) in a pattern reminiscent of that of radiolabeled b₆ site mutants (see Fig. 2B). By contrast, the pool of cytochrome b₆ that had accumulated in the membranes and was preexisting was still visualized as a broad, diffuse band whether or not the cells were treated with gabaculine (Fig. 3B). Again, the absence of a signal in the ∆petB strain authenticates the identity of the bands in the WT and TB202Q strains. Thus, heme depletion by gabaculine treatment prevents heme assembly into neosynthesized apocytochrome b₆ but has no effect on preexisting cytochrome b₆. In gabaculine-treated b₆ transformants (e.g., strain TB187G, Fig. 3B) we observed no change in the electrophoretic position of the cytochrome b₆ band. In contrast, in gabaculine-treated b₆ transformants (e.g., strain TB202Q), most of the newly synthesized cytochrome b₆ doublet is converted to a discrete single band migrating in the position of the lower band.
of the doublet (Fig. 3A). Since the pool size of cytochrome \(b_6\) accumulated in the mutant is small, reflecting the shorter half-life of the mutant protein, the effect of gabaculine treatment on the upper band is apparent even when unlabeled extracts are examined in immunoblotting experiments (Fig. 3B); specifically, the proportion of the upper band of the doublet is highly decreased relative to that of the lower band. These results confirmed that (i) the lower band of the doublet occurring in \(b_6\) strain mutants corresponds to apocytochrome \(b_6\) and (ii) the upper band, whose synthesis is strongly reduced upon gabaculine treatment, is a heme-dependent intermediate in the formation of holocytochrome \(b_6\). We suggest a temporal order of heme association with apocytochrome \(b_6\) such that the \(b_6\) heme is assembled prior to the \(b_6\) heme.

A Set of Nuclear Mutants of \(C.\) reinhardtii Presents the Same Phenotype as the \(b_6\) Transformants—To characterize the nuclear factors involved in the biogenesis of cytochrome \(b_{6f}\) complexes, we have isolated a number of nuclear mutants deficient in cytochrome \(b_{6f}\) activity (3, 51), all of which, including the \(ccb\) strains described below, displayed fluorescence characteristics typical of mutants with impaired cytochrome \(b_{6f}\) activity. In contrast to the WT strains, their fluorescence yield (26) rose continuously to an \(F_{\text{max}}\) level (Fig. 4). They were not altered in photosystem II, since this defect would cause a loss in variable fluorescence and a block at the \(F_{\text{max}}\) level (see the flat fluorescence trace that is observed in mutants lacking photosystem II reaction centers in Fig. 4). The collection of mutants was screened by TMBZ staining, for the loss of cytochrome \(b_6\) and reduced accumulation of cytochrome \(f\), and by immunoblotting.

The set of \(ccb\) nuclear mutants accumulated low amounts of distinct forms of cytochrome \(b_6\), which are visualized after electrophoretic separation of solubilized membrane proteins (Fig. 5). These forms of cytochrome \(b_6\) were revealed either by immunodetection (Fig. 5A) or by autoradiography (Fig. 5B), correspond in all \(ccb\) mutants to the doublet, which is similar to that observed in transformants with altered \(b_6\) axial ligands. This suggests that the \(ccb\) strains are blocked at the same step at which the engineered \(b_6\) site mutants are blocked, viz. at the step of conversion of the \(b_6\) heme-dependent intermediate to the holocytochrome (see Fig. 9). The nuclear \(Ccb\) genes would then be proposed to encode factors required for the proper interaction of the \(b\)-type hemes with apocytochrome \(b_6\) to produce holocytochrome \(b_6\). To support this model, representative \(ccb\) mutants were treated with gabaculine to test whether the \(ccb\) strains would behave exactly like the \(b_6\) site mutants (representative example in Fig. 6). Indeed, gabaculine treatment inhibited the formation of the \(b_6\) heme-dependent intermediate (upper band of doublet), and this is apparent both in “pulse” radiolabeling experiments, where de novo synthesis of cytochrome \(b_6\) is monitored (Fig. 6A, compare plus lane to minus lane for \(ccb4–2\)), and also when cytochrome \(b_6\) accumulation is assessed by immunoblotting (Fig. 6B, note the depletion of the upper band corresponding to the \(b_6\) heme-dependent intermediate).

The \(b_6\) Heme-dependent Intermediate Is Resistant to Denaturing Treatments—In an attempt to assess the nature of the modification that gives rise to the \(b_6\) heme-dependent intermediate (upper band), crude membrane preparations from WT, \(\Delta petB\), \(WT\), \(\Delta petB\), \(\Delta petB\), \(\Delta petB\), and \(\Delta petB\), and \(\Delta petB\) strains were treated with phosphate/acetone, which should release noncovalently bound hemes. The migration pattern of cytochrome \(b_6\) species remained unchanged in all strains after phosphate/acetone treatment (Fig. 7). Therefore, neither the diffuse aspect of cytochrome \(b_6\) in the WT strain nor the upper band of the doublet in \(b_6\) site-directed mutants and nuclear \(ccb\) mutants can be accounted for by noncovalent association of heme with the polypeptide. To test whether the \(b_6\) heme-dependent intermediate (upper band) was associated with heme, heme staining assays were conducted after separation of membrane proteins under less denaturing gel conditions, such as solubilization on ice with 1% SDS or 0.88% octyl-glucoside, 0.22% SDS and electrophoresis at 4 °C in the absence of urea. Nevertheless, we could not detect a heme-staining band in the region of cytochrome \(b_6\) in any of the \(b_6\) transformants or the \(ccb\) mutants after SDS-PAGE, despite attempts to use more sensitive heme detection methods sensitive to 1% of the WT content in cytochrome \(b_6\) (data not shown).

\[\text{Genetic Analysis Shows That Four Nuclear Loci Are Required for the Conversion of the} \ b_6\ \text{Heme-dependent Intermediate to Mature Holocytochrome} \ b_6\ —\text{The number of loci represented by the} \ ccb\ \text{strains was determined by genetic analyses. Two nuclear mutants with similar phenotypes,} \ M\#30 \text{and} \ M\#35, \text{obtained by random integration of transforming DNA (37), were also included in this analysis, although they displayed a much lower fertility. In recombination tests, it is assumed that mutations in the same gene should be closely}\]
linked, thereby preventing a high frequency of recombination events. The frequencies of tetratype and nonparental ditype tetrads were estimated from crosses between the various mutants (Table II, upper part). Three mutant strains gave rise to WT progeny when crossed with all of the other mutants, therefore each defining a locus, respectively CCB1, CCB2, CCB3 (C for cofactor binding, C for cytochrome \( b_6 \) complex, and B for subunit PetB). Another three strains (ccb4–1, 4–2, 4-M\( ^{35} \)) failed to recombine with each other but were able to recombine with the three mutants above. (The apparently lower recombination frequency for M\( ^{35} \) is probably due to its lower fertili-

**Fig. 5.** Accumulation and synthesis of cytochrome (cyt.) \( b_6 \) subunits in nuclear mutants. A, content of cytochrome \( b_6 \) subunits analyzed by immunoblotting experiments as in Fig. 2A. The strains are identified by the alleles they carry at the nuclear CCB loci (see Table II). B, autoradiogram of a urea/SDS-PAGE gel showing synthesis of chloroplast-encoded proteins during a 5-min labeling in the presence of \( ^{14} \)-acetate and an inhibitor of cytoplasmic translation as in Fig. 2B.

**FIG. 6.** Gabaculine treatment prevents the formation of slowly migrating products of the petB gene in ccb mutants. Cytochrome (cyt.) \( b_6 \) was untreated (−) or treated (+) with gabaculine. Analysis by urea/SDS-PAGE is shown.\( ^{a} \) Chloroplast-encoded protein synthesis in cells of WT and nuclear mutant ccb4–2. The cells were pulse-labeled in the presence of cycloheximide (8 \( \mu \)g/ml) and gabaculine (1 mM). Immunodetection of cytochrome \( b_6 \) accumulation in simplified membrane preparations with an antibody specific for cytochrome \( b_6 \), as in Fig. 3.

**Fig. 7.** Migration pattern of petB gene products in WT and mutant strains treated with phosphate/acetone. Migration pattern of cytochrome (cyt.) \( b_6 \) species in membrane preparations derived from WT and mutant strains, untreated (−), or treated (+) with phosphate/acetone. Polypeptides were revealed with an antibody specific for cytochrome \( b_6 \) after urea/SDS-PAGE.

**TABLE II**

Complementation and recombination analysis of nuclear mutants

| Gene Alleles | ccb1–1 | ccb2–1 | ccb3–1 | ccb4–1 | ccb4–2 | ccb4-M\( ^{35} \) |
|-------------|--------|--------|--------|--------|--------|----------------|
| ccb1–1      | ND     | ND     | ND     | ND     | ND     | ND             |
| ccb2–1      | ND     | ND     | ND     | ND     | ND     | ND             |
| ccb3–1      | ND     | ND     | ND     | ND     | ND     | ND             |
| ccb4–1      | ND     | ND     | ND     | ND     | ND     | ND             |
| ccb4–2      | ND     | ND     | ND     | ND     | ND     | ND             |
| ccb4-M\( ^{35} \) | ND     | ND     | ND     | ND     | ND     | ND             |

\(^{a}\) Mutant strain ccb4-M\( ^{35} \) has lower frequencies of tetratype and nonparental ditype tetrads because of a strong mortality.

\(^{b}\) Mutant strain ccb1–1 has a tendency to revert.

\(^{c}\) ND, not determined.

Therefore, the three strains define alleles at a single locus, CCB4. We were unable to characterize further the locus of mutant M\( ^{35} \) because to its very low fertility. The conclusions from the recombination analysis were confirmed by complementation analysis (44). Only the two closely linked mutations in strains ccb4–1 and ccb4–2 failed to complement each other (Table II, lower part). The complementation tests confirmed that the two strains carried mutated alleles of the same gene. Thus, the genetic analysis of these six nuclear mutants define four different nuclear genes. Since the five mutations complement with at least three other mutations, they are all recessive mutations.

**Analysis of Double Mutants Confirms That the ccb Strains Are Affected at the Same Step as Are the bh Site Mutants, Which Follows the Step Affected in the btb Site Mutants**—To confirm the temporal order of cytochrome \( b_6 \) assembly suggested above (see Fig. 9 also), we generated double mutants by crossing the ccb strains with the chloroplast transformants lacking either a \( b_6 \) or a \( b_7 \) liganding histidine. The \( b_6 \) TB202Q \( m^ {t} \) transformant was crossed with each of five nuclear ccb mutant strains (\( ccb1–1 \), \( ccb4–1 \) \( m^ {t} \)), while the \( b_7 \) TB187G \( m^ {t} \) transformant was crossed with the nuclear mutants \( ccb1–1 \) and \( ccb4–2 \) \( m^ {t} \). The resulting tetrads were dissected to recover the four progeny of the zygotes. All progeny had inherited the chloroplast \( H^ {202} \) → Gln or His\( ^ {187} \) → Gly mutations, transmitted uniparentally by the \( m^ {t} \) parent, while only two members of the tetrad inherited the nuclear mutant allele transmitted by the \( m^ {t} \) parent, the two other members having a WT nuclear genome. We analyzed the cytochrome \( b_6 \) content in the progeny from these crosses by immunoblotting. In all crosses, the four members of the tetrad presented the same phenotype, which was identical to the
phenotype of the chloroplast parent. Upon electrophoresis, cytochrome $b_6$ migrated as a doublet for the progeny of TB182Q X ccb4–1 crosses, but it migrated as a single sharp band for the progeny of TB187G X ccb4–2 crosses (Fig. 8). This confirms that the $b_6$ site mutation affects a step before that affected in the ccb strains and that the phenotype conferred by the $b_6$ site mutation is the same as that conferred by the mutations in the trans-acting CCB loci.

**DISCUSSION**

The unique denaturation-resistant association of heme with cytochrome $b_6$ suggested an unusual mode of heme binding, and it also permitted us to dissect the heme assembly pathway in *C. reinhardtii* by exploiting genetic approaches in C. *reinhardtii*. Chloroplast mutants, obtained by site-directed mutagenesis of either one of the two histidines in each pair that coordinate $b_6$ and $b_6^*$, were used as templates to define a distinctive phenotype for mutations affecting heme association with cytochrome $b_6$. Upon urea/SDS-PAGE, cytochrome $b_6$ migrates as a broad and diffuse band in the WT strain, whereas it is replaced by a sharp band corresponding to apocytochrome $b_6$ in $b_6^*$ transformants and a distinct doublet in $b_6$ transformants. The additional species observed in $b_6^*$ transformants was identified as a $b_6$ heme-dependent intermediate in the assembly of holocytochrome $b_6$. These characteristic features were used to identify a set of nuclear mutants that displayed the same phenotype as $b_6$ site mutants. The mutants represent four nuclear loci, which we propose encode factors required specifically for the heme assembly into apocytochrome $b_6$.

**Conversion of Apocytochrome $b_6$ to Holocytochrome $b_6$**

We propose that the single, gabaculine-resistant sharp band, detected in strains where the His ligands to $b_6$ and $b_6^*$, we conclude that the diffuse aspect of WT cytochrome $b_6$ results from its interaction with the two hemes. Proper folding of the polypeptide in the environment of the $b_6$ site is required for formation of the diffuse migrating species. For instance, substitution of Leu204 by a proline residue in the vicinity of His250 ($b_6$ ligand) yields an electrophoretic doublet, presumably due to misfolding of the $b_6$ attachment site (26).

The ccb mutants and the $b_6/b_6^*$ chloroplast transformants are nonphototrophic strains and are deficient in the various subunits of the cytochrome $b_6/f$ complex. Since the abundance of *petB* transcripts and the rate of synthesis of cytochrome $b_6$ polypeptides were not decreased in these strains, their low content of cytochrome $b_6$ polypeptides must result from their increased proteolytic susceptibility because of impaired heme binding.

**Conversion of Apocytochrome $b_6$ to Holocytochrome $b_6$ Is a Specifically Assisted Process: Mutations in Four Different Nuclear Genes Result in the Same Phenotype as $b_6$ Transformants**—The five ccb mutants that display a phenotype identical to that of $b_6$ transformants are altered specifically in the biosynthesis of cytochrome $b_6$ and not in the general pathway of $b_6$-type heme biosynthesis. The argument for this conclusion is as follows: (i) the ccb mutants are not deficient in cytochrome $b_6\text{apo}$, since they exhibit fluorescence induction curves characteristic of cytochrome $b_6/f$ mutants, whereas cytochrome $b_6\text{apo}$ mutants, because they do not accumulate photosystem II (54), should have no variable fluorescence; (ii) holocytochrome $f$ formation is normal (i.e., it stains for heme) in ccb mutants although its abundance is reduced due to disruption of assembly of the cytochrome $b_6/f$ complex; (iii) the synthesis and accumulation of cytochrome $c_6$ occurs normally in these strains (data not shown); and (iv) the mitochondrial cytochromes are present as evidenced by normal TMBZ staining of the *c* type cytochromes and normal growth by dark respiration on TAP (data not shown). We therefore conclude that the ccb mutants are affected in nuclear factors specifically required for the proper binding of the $b_6$-type hemes to apocytochrome $b_6$ or for the folding of the apoprotein in a conformation suitable for heme association.

Genetic analysis indicates that the mutations belong to four nuclear loci, CCB1, CCB2, CCB3, and CCB4. Since three of the four complementation groups are represented by only one mutation, it is likely that the number of loci involved in the process may be higher. The number of nuclear genes involved in this single assembly process may seem rather high; the biochemis-
try of this process is not understood in any system, and it is therefore not possible to speculate on the function of these loci. In Saccharomyces cerevisiae, at least two loci (CBP3 and CBP4) have been identified as encoding candidate cytochrome b assembly factors, but the inability to distinguish heme association mutants from those affected at an early step in bc₃ complex assembly has precluded a definitive functional assignment to these loci (55, 56).

One Heme May Be Covalently Attached to Cytochrome b₆—Surprisingly, denaturing conditions, such as acid/acetone treatment used to prepare globin from hemoglobin (50), which are generally considered to dissociate noncovalently bound hemes from proteins, did not affect the shape of the electrophoretic bands for cytochrome b₆; neither the WT diffuse band nor the doublet in b₆ transformants or in ccb mutants was converted to a sharp single band. We could show, by TMBZ staining after urea/SDS-PAGE, that WT cytochrome b₆ retained a b heme after phosphate/acetone or acetone/acid extraction. Heme binding to cytochrome b₆ also resisted boiling SDS. It should be noted, however, that it did not heme-stain more than cytochrome f, which binds only one heme. This observation suggests that only part of the b heme complement of cytochrome b₆ shows high binding affinity for the apoprotein.

The stability of a hemoprotein form of cytochrome b₆ is in sharp contrast with the various reports that b hemes are lost after SDS-PAGE of bacterial cytochrome b (32), mitochondrial cytochrome b (Ref. 33 and Fig. 1B), or the b-type cytochrome from Chlorobium limicola, which shares intermediate properties between cytochrome b and cytochrome b₆ (57). The unusual stability of b heme binding to cytochrome b₆ strongly suggests some linkage that cannot be formed within cytochrome b from bc complexes. Some residues close to the His ligands of the b₆ hemes, conserved in sequences of cytochrome b₆, are absent from cytochrome b, including the one from C. limicola, could be involved in interactions with the tetrapyrrole ring. Given the similar absorption spectra of cytochrome b₆ and cytochrome b, any covalent linkage of cytochrome b₆ hemes ought to occur through atoms that are not conjugated with the macrocycle. Site-directed mutations of residues that are candidates for providing covalent ligands to the hemes in the vicinity of b₁ or b₆ should allow us to settle this point.

Pathway from Apocytochrome b₆ to Holocytochrome b₆—From the various electrophoretic patterns of cytochrome b₆ forms, it seems reasonable to suggest that the products of the CCB loci catalyze the association of the b₆ heme to the b₁-binding intermediate form of cytochrome b₆. A schematic view of the apo- to holo-conversion for cytochrome b₆ is presented in Fig. 9 with the following steps: membrane integration of apo-cytochrome b (A), formation of a b₁ heme-dependent intermediate (B), b₆ heme binding to b₁-dependent intermediate requiring Ccb-encoded trans-acting factors, perhaps responsible for the tight heme-binding. It may sound paradoxical that the CCB loci-assisted step is at the level of b₆ binding to the b₁ intermediate form. The latter is stable in denaturing conditions, thereby supporting a tight b₁ heme-binding of b₁ rather than b₆ to cytochrome b₆. This raises the question of whether the form of cytochrome b₆ revealed in the b₁ and ccb mutants is a genuine assembly pathway intermediate in cytochrome b₆ biogenesis or perhaps a dead end process that occurs only in the mutants. In this view, proper covalent binding of b₁ to the apocytochrome may require the concerted presence of the b₁ substrate and the Ccb gene products. In the absence of either of these factors, spontaneous covalent binding of b₁ in an inappropriate conformation may occur at low yield generating the b₁-dependent intermediate. Regardless, the occurrence of this form has permitted us to resolve the two steps in holocytochrome b₆ formation.

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REFERENCES
1. Cramer, W. A., Martinez, S. E., Huang, D., Tae, G.-S., Everly, R. M., Heynann, J. B., Cheng, R. H., Baker, T. S., and Smith, J. L. (1994) J. Bioenerg. Biomembr. 26, 31–47
2. Cramer, W. A., Soriano, G. M., Panomarev, M., Huang, D., Zhong, H., Martinez, S. E., and Smith, J.-L. (1996) Annu. Rev. Plant Mol. Biol. 47, 477–508
3. Lemaire, C., Girard-Bascou, J., Wollman, F.-A., and Bennoun, P. (1986) Biochim. Biophys. Acta 851, 229–238
4. Pierre, Y., Breyton, C., Kramer, D., and Popot, J.-L. (1995) J. Biol. Chem. 270, 29342–29349
5. Bieschlen, S., Choquet, Y., Kuras, R., and Wollman, F.-A. (1991) FEBS Lett. 284, 257–262
6. de Vitry, C. (1994) J. Biol. Chem. 269, 7603–7609
7. Pierre, Y., and Popot, J.-L. (1993) C. R. Acad. Sci. Ser. III, 1404–1409
8. Breyton, C., de Vitry, C., and Popot, J.-L. (1994) J. Biol. Chem. 269, 7597–7602
9. Berthold, D. A., Schmidt, C. L., and Malkin, R. (1995) J. Biol. Chem. 270, 29290–29298
10. Takahashi, Y., Rahire, M., Breyton, C., Popot, J.-L., Joliot, P., and Rochaix, J.-D. (1996) EMBO J. 15, 3498–3506
11. de Vitry, C., Breyton, C., Pierre, Y., and Popot, J.-L. (1996) J. Biol. Chem. 271, 10667–10671
12. Ketchner, S. L., and Malkin, R. (1996) Biochim. Biophys. Acta 1273, 195–197
13. Hurt, E. C., and Hauksa, G. (1983) FEBS Lett. 153, 413–415
14. Bald, D., Krup, J., Boekema, E. J., and Rogner, M. (1992) in Research in Photosynthesis (Murata, N., ed) Vol. I, pp. 629–632, Kluwer Academic Publishers, Dordrecht, The Netherlands
15. Huang, D., Everly, R. M., Cheng, R. H., Heynann, J. B., Schagger, H., Sled, V., Ohnishi, T., Baker, T. S., and Cramer, W. A. (1994) Biochemistry 33, 4401–4409
16. Pierre, Y., Breyton, C., Lemoine, Y., Robert, B., Vernotte, C., and Popot, J.-L. (1997) J. Biol. Chem. 272, 21901–21908

FIG. 9. Schematic pathway of the conversion of apocytochrome to holocytochrome b₆ and patterns after urea/SDS-PAGE. A, membrane integration occurs even in absence of heme association. B, there is formation of a b₁ heme-dependent intermediate, which can be prevented by heme depletion (gabaculine treatment). C, Ccb1–Ccb4 encoded nuclear factors are necessary for the production of the holo-form showing both b₆ heme binding and b₁ binding. D, holocytochrome b₆ accumulates in a protease-resistant form, upon association with the other b₆f subunits.
17. Widger, W. R., Cramer, W. A., Hermann, R. G., and Trebst, A. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 674–678
18. Yu, C.-A., Xia, J.-Z., Kachurin, A. M., Yu, L., Xia, D., Kim, H., and Deisenhofer, J. (1996) Biochim. Biophys. Acta 1275, 47–53
19. Xia, D., Yu, C.-A., Kim, H., Kachurin, A. M., Zhang, L., Yu, L., and Deisenhofer, J. (1997) Science 277, 60–66
20. Simpkin, D., Palmer, G., Delvin, F. J., Mckenna, M. C., Jensen, G. M., and Stephens, P. J. (1989) Biochemistry 33, 176–185
21. Degli Esposti, M., De Vries, S., Crimi, M., Ghelli, A., Patarnello, T., and Meyer, A. (1993) Biochim. Biophys. Acta 1143, 243–271
22. Brasseur, G., Saribas, A. S., and Daldal, F. (1996) Biochim. Biophys. Acta 1275, 61–69
23. Kuras, R., and Wollman, F.-A. (1994) EMBO J. 13, 1019–1027
24. Simpkin, D., Palmer, G., Delvin, F. J., Mckenna, M. C., Jensen, G. M., and Stephens, P. J. (1989) Biochemistry 33, 176–185
25. Kuras, R., and Wollman, F.-A. (1994) EMBO J. 13, 1019–1027
26. Finazzi, G., Büschlen, S., de Vitry, C., Rappaport, F., Joliot, P., and Wollman, F.-A. (1997) Biochemistry 36, 2867–2874
27. Zito, F., Kuras, R., Choquet, Y., Koos, H., and Wollman, F.-A. (1997) Plant Mol. Biol. 33, 79–86
28. Berry, E. A., Huang, L.-S., and DeRose, V. J. (1991) J. Biol. Chem. 266, 9064–9077
29. Hurt, E. C., and Hauska, G. (1981) Eur. J. Biochem. 117, 591–599
30. Phillips, A. L., and Gray, J. C. (1983) Eur. J. Biochem. 137, 553–560
31. Wu, M., and Tzagoloff, A. (1989) J. Biol. Chem. 264, 1122–1130
32. Crivellone, M. D. (1994) J. Biol. Chem. 269, 21284–21292
33. Hurt, E. C., and Hauska, G. (1984) FEBS Lett. 168, 149–154
34. Bannoun, P., and Deleglise, P. (1982) in Methods in Chloroplast Molecular Biology (Edelman, M., Hallock, R. B., and Chua, N.-H., eds) pp. 25–38, Elsevier Biomedical Press, Amsterdam
35. Robertson, D. E., Farld, R. S., Moser, C. C., Urbauer, J. L., Mulholland, S. E., and Tzagoloff, A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1721–1725
36. Ascoli, F., Rossi Ferri, M. R., and Antonini, E. (1981) Methods Enzymol. 76, 72–87