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The assembly of cytochrome b_6/f complexes: an approach using genetic transformation of the green alga *Chlamydomonas reinhardtii*

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As an approach to the study of the biogenesis of the cytochrome b_6/f complex, we characterized the behaviour of its constitutive subunits in mutant strains of *Chlamydomonas reinhardtii* bearing well-defined mutations. To this end, we have constructed three deletion mutant strains, each lacking one of the major chloroplast *pet* genes: the Δ*petA*, Δ*petB* and Δ*petD* strains were unable to synthesize cyt *f*, cyt b_6 and subunit IV (suIV) respectively. Western blotting analysis, pulse-labelling and pulse–chase experiments allowed us to compare the cellular accumulation, the rates of synthesis and the turnover of the cyt b_6/f subunits remaining in the various strains. We show that the rates of synthesis of cyt b_6 and suIV are independent of the presence of the other subunits of the complex but that their stabilization in the thylakoid membranes is a concerted process, with a marked dependence of suIV stability on the presence of cyt b_6. In contrast, mature cyt *f* was stable in the absence of either suIV or cyt b_6 but its rate of synthesis was severely decreased in these conditions. We conclude that the stoichiometric accumulation of the chloroplast-encoded subunits of the cyt b_6/f complex results from two regulation processes: a post-translational regulation leading to the proteolytic disposal of unassembled cyt b_6 and suIV and a co-translational (or early post-translational) regulation which ensures the production of cyt *f* next to its site of assembly.

Key words: Chlamydomonas/cyt b_6/f/protein assembly

Introduction

A number of physiological functions borne by biological membranes depend on multiple-subunit protein complexes. Such multimeric membrane proteins have been implicated for instance in signal transduction, substrate import or energy transduction. Although the biochemical composition and overall membrane organization of these oligomeric proteins have been studied in some details (Shuman, 1987; Changeux, 1990; Cooper *et al.*, 1991; Cramer *et al.*, 1991), their mode of assembly in the membranes remains poorly understood. A key issue in this study is to understand how the cell keeps producing the various protein subunits to a level corresponding to the stoichiometric concentrations required for their assembly in a functional membrane complex. In prokaryotes, such a control mechanism may operate at the transcriptional level because, in most cases, the structural genes encoding the various subunits are clustered in operons. This is at variance with the case of eukaryotes where such gene clusters are rarely found. In the latter case, a major regulation step occurs at the post-translational level through a proteolytic disposal of the unassembled subunits. In most cases, this occurs at the level of the rough endoplasmic reticulum for membrane proteins routed to the plasma membrane: for instance the unassembled subunits of haemagglutinin or of the nicotinic acetylcholine receptor are retained in the endoplasmic reticulum and targeted for degradation (Gething *et al.*, 1986; Hurtley *et al.*, 1989; Blount and Merlie, 1990). For organellar membrane proteins, a post-translational degradation process has also been proposed to explain the pleiotropic deficiencies observed in mutant strains specifically blocked in the synthesis of one subunit only. Such is the case in several yeast respiration mutants (Dowhan *et al.*, 1985; Crivellone *et al.*, 1988) or in photosynthesis mutants of *Chlamydomonas reinhardtii* altered at the level of the photosystem I (PSI) complex (Girard-Bascou *et al.*, 1980), the PSII complex (Bennoun *et al.*, 1981; Erickson *et al.*, 1986; Jensen *et al.*, 1986; Kuchka *et al.*, 1988; de Vitry *et al.*, 1989), ATP synthase (Lemaire and Wollman, 1989) or the cyt b_6/f complex (Lemaire *et al.*, 1986; Chen *et al.*, 1993).

The cyt b_6/f complex is a central component of the photosynthetic electron transport chain of higher plants, green algae and cyanobacteria (Cramer *et al.*, 1991; Anderson, 1992). It catalyses the oxidation of quinols and the reduction of plastocyanin and participates in the establishment of the proton motive force used in the synthesis of ATP. This protein complex comprises four major chloroplast-encoded subunits, the *petA* gene product corresponding to a *c*-type cytochrome (cyt *f*), the *petB* gene product corresponding to a *b*-type cytochrome with two haems (cyt b_6), the *petD* gene product (subunit IV, or suIV), and the *petG* gene product, corresponding to a small subunit of ~4 kDa. Of these four chloroplast-encoded subunits, only cyt *f* possesses a presequence which is cleaved during synthesis. At least two nuclear gene products assemble with the rest of the complex in *C.reinhardtii*: the Rieske protein which contains an iron sulfur centre and subunit V, the function of which remains unknown (Lemaire *et al.*, 1986).

The ability to generate photosynthesis mutants from *C.reinhardtii* by conventional mutagenesis and to grow them in heterotrophic conditions with acetate as a carbon source (Harris, 1989), has allowed the isolation of many mutants defective in cyt b_6/f activity. In a previous biochemical study (Lemaire *et al.*, 1986) we have shown that the lack of synthesis of some specific chloroplast-encoded subunit, cyt *f* or suIV, prevented the accumulation of the other subunits of the complex. In contrast the chloroplast-encoded subunits of the cyt b_6/f complex accumulated to a significant extent in the thylakoid membranes in the absence of detectable amounts of the Rieske protein. This subunit has recently been demonstrated to be a peripheral membrane protein in *C.reinhardtii* (Breyton *et al.*, 1993; de Vitry,

1993). A similar accumulation of the cytochrome moiety of the complex in the absence of the Rieske protein has been reported for the *bc* complexes from photosynthetic bacteria (Davidson *et al.*, 1992) or yeast mitochondria (Crivellone *et al.*, 1988). However a *Lemna* mutant, whose primary defect was reported to be in the expression of the Rieske protein, displayed no accumulation of the rest of the cyt b_6/f complex (Bruce and Malkin, 1991).

A more detailed characterization of the assembly process of cyt b_6/f complexes required the use of mutants displaying gene lesions defined at the molecular level. An efficient procedure for chloroplast gene transformation by homologous recombination, has recently been described in *C.reinhardtii* (Boynton *et al.*, 1988). Moreover, several selective non-photosynthetic markers have been developed (Boynton *et al.*, 1990; Goldschmidt-Clermont, 1991). One of them, the *aadA* expression cassette (Goldschmidt-Clermont, 1991) is a useful tool for disrupting any chloroplast gene involved in photosynthesis. In the present study, we have constructed deletion mutants, specifically deleted in either the *petA*, *petB* or *petD* genes. We have analysed the behaviour of the various subunits of the cyt b_6/f complex remaining in these transformed strains. We report that cyt b_6 and suIV are independently translated but that stabilization of suIV is tightly dependent on the presence of cyt b_6. Most strikingly, we show that high rates of cyt *f* synthesis require the presence of suIV and cyt b_6 in the thylakoid membranes. We suggest that cyt *f* synthesis is driven by a cyt b_6/suIV precomplex.

Results

Inactivation of the petA, petB and petD genes

We have previously cloned the *Hin*dIII restriction fragments of the *petA*, *petB* and *petD* genes (Büschlen *et al.*, 1991). This led to disruption of the open reading frame (ORF) of the *petA* and *petB* genes because of the presence of an intragenic *Hin*dIII site. Continuous *petA* and *petB* genes and a *petD* fragment of suitable size for transformation were reconstituted from the cloned fragments after the *Hin*dIII sites not involved in the fusion process had been destroyed by exonuclease treatment (see Materials and methods).

Each *pet* gene was then deleted by exchanging a suitable restriction fragment containing most or all of the corresponding *pet* ORF for the *aadA* cassette which confers spectinomycin/streptomycin resistance in the chloroplast of *C.reinhardtii*. This cassette is a chimeric gene of 1.9 kb, resulting from the fusion of the 5′ untranslated region of *atpA* with the *aadA* gene from *Escherichia coli* and the 3′ untranslated region from the *rbcL* gene (Goldschmidt-Clermont, 1991). To delete *petA*, we inserted the *aadA* cassette in place of a 1024 bp *Bgl*II–*Acc*I fragment, carrying most of the *petA* ORF. To delete *petD*, a *Hin*dIII restriction site was introduced by site-directed mutagenesis, 6 bp downstream of the *petD* stop codon. The 530 bp *Hin*dIII fragment carrying the entire *petD* ORF was then replaced with the *aadA* expression cassette. To delete *petB*, we introduced one restriction site on each side of the *petB* ORF. An *Eco*RI site was introduced 39 bp upstream of the *petB* start codon and a *Bam*HI site was introduced 31 bp downstream of the stop codon. The 718 bp *Eco*RI–*Bam*HI fragment was then replaced with the *aadA* expression cassette.

Wild type (WT) cells were bombarded with these constructs carrying the *aadA* cassette in the direct orientation. Transformants were selected on TAP plates containing spectinomycin and screened for their fluorescence induction kinetics after dark adaptation. As expected for mutants lacking cyt b_6/f activity, the transformants displayed fluorescence induction kinetics with a continuous rise phase up to the F_{max} level (not shown).

DNA analysis of the transformants

The transformants were subcloned for several rounds until they became homoplasmic for the mutated genome. Shown on Figure 1 is a comparative Southern analysis of total DNA from strains with deletions in the *petB* gene (Figure 1A), in the *petA* gene (Figure 1B) and in the *petD* gene (Figure 1C). In each case, three similar homoplasmic transformants obtained independently (lanes 2, 3 and 4) are compared with the WT (lanes 1). The DNA of the Δ*petB* transformants was digested with *Acc*I. Hybridization with a 1.4 kb *Dde*I–*Nsi*I probe (probe B) gave two bands of 1.2 and 2.4 kb with the WT DNA and one 4.8 kb band with the DNA of the Δ*petB* transformants (Figure 1A). The 4.8 kb band was also detected in the transformants using the aad probe. This size corresponded to the insertion of the 1.9 kb *aadA* gene in place of the 0.7 kb deletion in the *petB* gene which contained an *Acc*I site. The DNA from Δ*petA* transformants was digested with *Eco*RV and *Bam*HI. Hybridization of these digests with a 553 bp *Acc*I–*Eco*RV probe (probe A) showed the absence of the 4.4 kb *Bam*HI–*Eco*RV band in all the Δ*petA* transformants (Figure 1B). Instead, we observed a 5.3 kb band consistent with the the 1.9 kb *aadA* gene insertion in this region. The same band was observed in the transformants when hybridized with a 1.0 kb *Xho*I–*Pst*I probe corresponding to the *aadA* gene (probe aad). The DNA of the Δ*petD* transformants was digested with *Eco*RV and *Pst*I. A 0.6 kb KpnI–*Hin*dIII fragment and a 0.7 kb *Pst*I–*Hin*dIII fragment were used as probes (probes D). As shown in Figure 1C, two bands of 2.7 and 3.0 kb were observed with the WT DNA, corresponding respectively to the upstream and downstream parts of the *petD* ORF. Δ*petD* transformants yielded two other bands of 3.8 and 3.3 kb. The size of the first one was consistent with that of a fusion of the 1.5 kb upstream region of the *aadA* cassette with the upstream region of the *petD* ORF (2.7 kb) bearing a 0.4 kb deletion. The size of the second one was consistent with that expected from the fusion of the 0.5 kb downstream region of the *aadA* cassette with the downstream part of the *petD* ORF (3.0 kb) bearing a 0.2 kb deletion. As expected, only the 3.8 kb band was detected in the transformants by the aad probe.

Consistent with the above DNA analysis, each type of transformant displayed a selective absence in one transcript, the 0.8 kb *petB* transcript in the Δ*petB* transformant, the 1.4 kb *petA* transcript in the Δ*petA* transformant and the 0.9 kb *petD* transcript in the Δ*petD* transformant (results not shown).

Accumulation of the cyt b_6/f subunits

SDS–PAGE of purified thylakoid membranes, followed by silver or haem-staining, revealed that the strains with deletions in either *petA*, *petB* or *petD* genes displayed a pleiotropic deficiency in all cyt b_6/f subunits (results not shown). The use of specific antibodies allowed us to detect

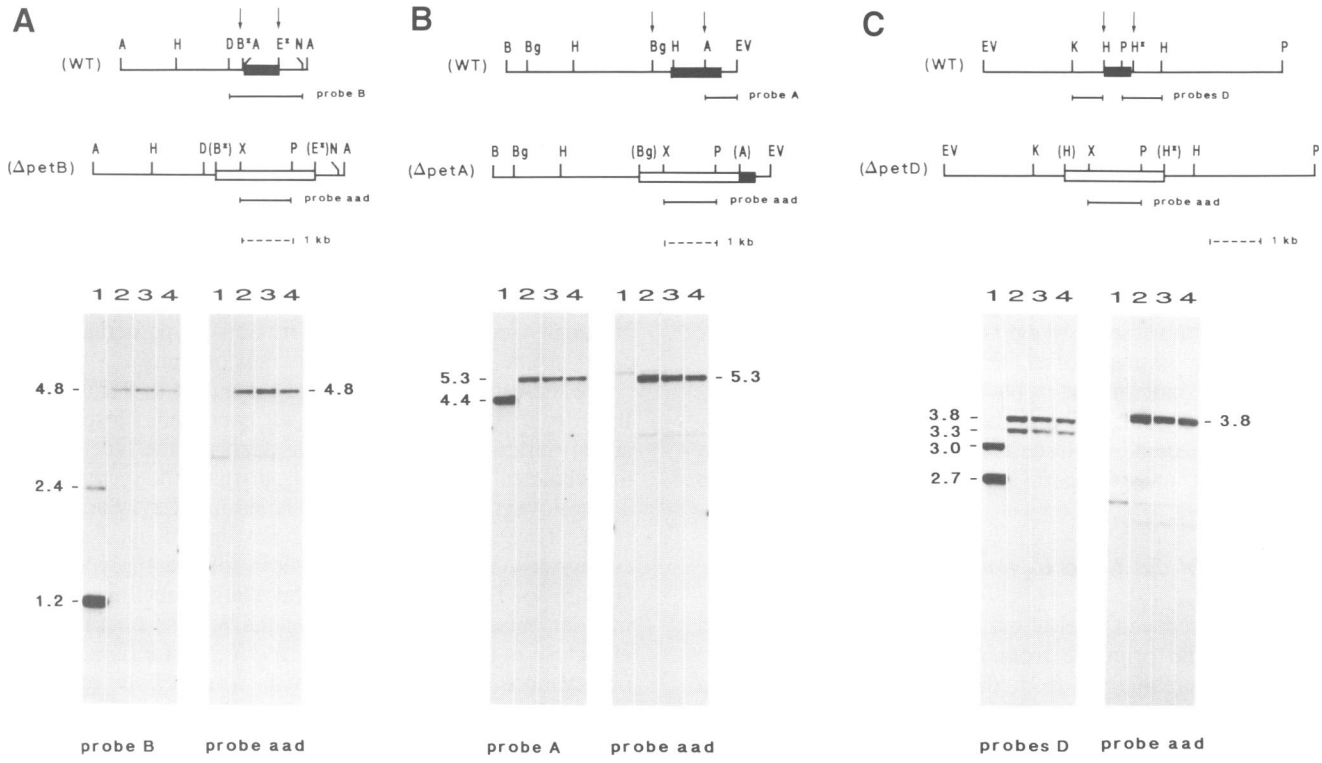

Fig. 1. DNA filter hybridization analysis of WT and transformants. Total DNA of the WT and of three transformants of each kind was digested, electrophoresed on 0.8% agarose gels, transferred onto Hybond-N membranes and hybridized. Schematic restriction maps of the deleted regions are shown at the top of each panel for the WT (upper part) and for the transformants (lower part). Black bars represent the coding region of the *pet* genes. Opened bars represent the *aadA* cassette. Fragments used as probes are underlined. Restriction sites are: A, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; D, *Dde*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; N, *Nsi*I; P, *Pst*I; X, *Xho*I. Restriction sites introduced by mutagenesis are pointed out with asterisks. Arrows show restriction sites used to introduce the *aadA* cassette. Restriction sites deleted during cloning are indicated in brackets; (**A**) *Acc*I digests of the DNA from the WT (lane 1) and from three Δ*petB* transformants (lanes 2, 3 and 4) probed for the *petB* gene (probe B) and for the *aadA* cassette (probe aad). (**B**) *Bam*HI/*Eco*RV digests of the DNA from the WT (lane 1) and from three Δ*petA* transformants (lanes 2, 3 and 4) probed for the *petA* gene (probe A) and for the *aadA* cassette (probe aad). (**C**) *Eco*RV/*Pst*I digests of the DNA from the WT (lane 1) and from three Δ*petD* transformants (lanes 2, 3 and 4) probed for the *petD* gene (probe D) and for the *aadA* cassette (probe aad).

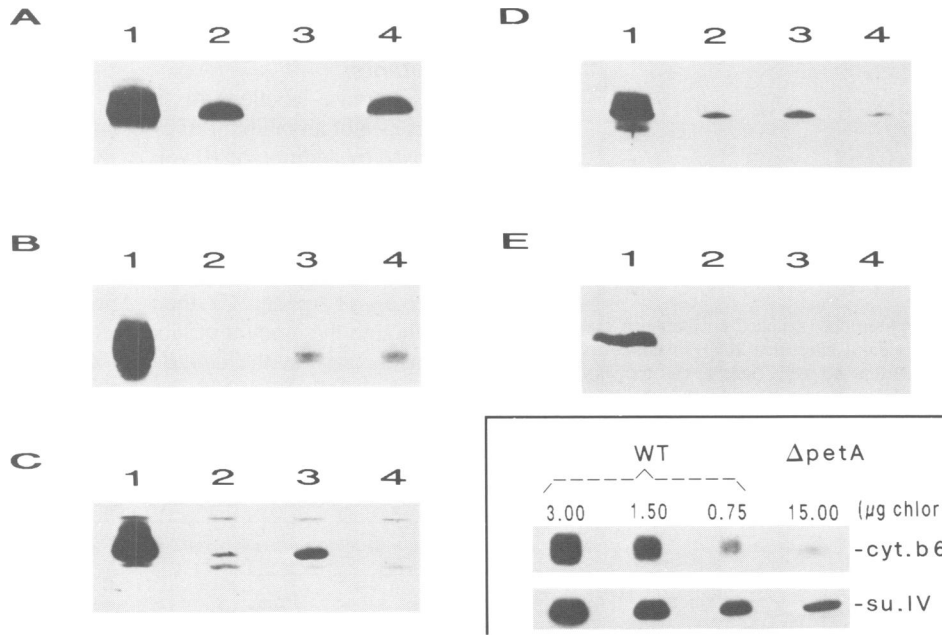

Fig. 2. Immunoblots of thylakoid membrane polypeptides from the WT (lane 1) and transformants Δ*petB* (lane 2), Δ*petA* (lane 3) and Δ*petD* (lane 4). Thylakoid membranes were subjected to urea/SDS−PAGE, electroblotted onto nitrocellulose membranes and reacted with antisera against cyt *f* (**A**), the C-terminus of cyt b_6 (**B**), the N-terminus of suIV (**C**) or the Rieske protein (**D**). (**E**) After electophoresis, thylakoid membranes were electrotransferred onto PVDF membrane and reacted with an antiserum against the *petG* product. **Inset**: immunodetection of cyt b_6 and suIV in decreasing amounts of WT thylakoid membranes corresponding to 20, 10 and 5% of the amount of Δ*petA* thylakoid membrane.

specifically the amount of each cyt b_6/f subunit remaining in the thylakoid membranes (Figure 2). Cyt *f* was clearly detectable in Δ*petB* and Δ*petD* (Figure 2A, lanes 2 and 4) as were cyt b_6 in Δ*petA* and Δ*petD* (Figure 2B, lanes 3 and 4) and suIV in Δ*petA* (Figure 2C, lane 3). When thylakoid membranes from these strains were compared with decreasing amounts of WT thylakoid membranes we found that ~10% of cyt *f* remained in the absence of either cyt b_6 or suIV (results not shown). When a similar experimental comparison was performed between the WT and Δ*petA*, we found that ~5% of both suIV and cyt b_6 remained in the absence of cyt *f* (see lower right hand corner of Figure 2). Similarly Δ*petD* transformants displayed ~5% of the WT content in cyt b_6 but we could hardly detect any suIV (<0.5%) in Δ*petB* transformants (Figure 2C, lane 2). Last, we detected only trace amounts of the Rieske protein (Figure 2D) and no *petG* product (Figure 2E) in the transformants.

Synthesis of cyt f, cyt b_6 and suIV in the deletion mutants

The highly reduced level of cyt b_6/f subunits in the deletion mutants could originate from a reduced synthesis of the remaining subunits or from their lower stability—i.e. a shorter half-life—in the thylakoid membranes. Therefore we studied the rate of synthesis of the chloroplast-encoded subunits by pulse-labelling the transformant cells with [^{14}C]acetate in the presence of an inhibitor of cytoplasmic translation. Figure 3 shows an autoradiogram of the chloroplast-encoded subunits inserted in thylakoid membranes purified from cells labelled for 45 min. Quantification of the labelling is shown in Table I ('45 min' columns). Each measurement was normalized to the label incorporated in the band immediately below cyt b_6 on Figure 3. We observed that cyt b_6 labelling in Δ*petA* and Δ*petD* strains showed little change with respect to that in the WT. In contrast, the labelling of cyt *f* and of suIV was drastically reduced in the transformants. In particular suIV could not be detected in Δ*petB* transformants.

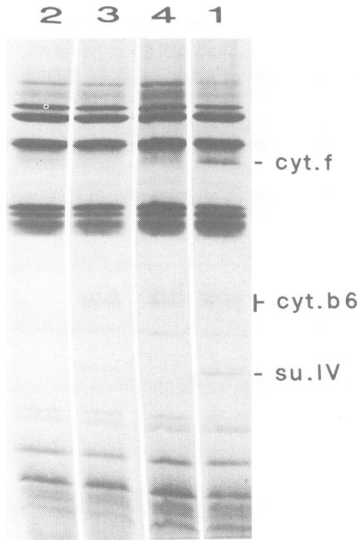

Fig. 3. Autoradiogram of chloroplast-encoded polypeptides inserted in the thylakoid membranes, viewed after urea/SDS–PAGE. Thylakoid membranes were prepared from WT cells (lane 1) and transformant cells (Δ*petB*, lane 2; Δ*petA*, lane 3; Δ*petD*, lane 4), pulse-labelled for 45 min with [^{14}C]acetate in the presence of cycloheximide (8 μg/ml).

We then shortened the time of the pulses to 5 min only. In order to keep the experimental conditions close to the actual duration of the pulse, we analysed the labelling of the whole cell content instead of purifying the membranes. This allowed us to quantify the labelling of cyt *f* and suIV (Table I, '5 min' columns) but not that of cyt b_6 which comigrates on these gels with soluble polypeptides (Figure 4). Table I shows that the relative labelling of suIV in 5 min pulses was now similar in the WT and Δ*petA* strains and close to these levels in the Δ*petB* transformant. These observations are consistent with a synthesis of suIV independent of that of cyt b_6 or cyt *f*. Comparison of the labelling patterns in 5 min and 45 min pulses shows that the stabilization of suIV depended on the presence of the other cyt b_6/f subunits. In particular, it points to a cyt b_6-dependent stabilization step occurring within <45 min.

In marked contrast to suIV, cyt *f* labelling was still low in 5 min pulses in the Δ*petB* and Δ*petD* transformants (Figure 4, lanes 0). It remained close to that observed in 45 min pulses (Table I). This observation suggested that suIV and cyt b_6 were required to produce high rates of synthesis of cyt *f* rather than to improve its stability in the thylakoid membranes.

Turnover of cyt f, cyt b_6 and suIV in the deletion mutants

Cells, pulse-labelled for 5 min with [^{14}C]acetate in the presence of an inhibitor of cytoplasmic translation, were then chased by addition of 10 vols of TAP medium. This allowed a rapid dilution of both the radiolabelled acetate and the inhibitor of cytoplasmic translation. Figure 4 shows the result of such an experiment where an increase in the turnover of suIV is clearly visible from the WT situation (panel A) to the Δ*petA* (panel C) then Δ*petB* (panel B) situations. Although the amount of label incorporated in cyt *f* dropped considerably in the Δ*petB* and Δ*petD* transformants, the poorly labelled band in the cyt *f* position was still visible after long chase times (panels B and D). The behaviour of

Table I. Relative rates of synthesis (% of WT) for cyt b_6, suIV and cyt *f* measured in 45 min and 5 min pulse-labelling experiments

	cyt b_6		suIV		cyt *f*	
	45 min	5 min	45 min	5 min	45 min	5 min
WT	100	nd	100	100	100	100
Δ*petA*	112	nd	35	105	0	0
Δ*petB*	0	nd	0	75	15	19
Δ*petD*	73	nd	0	0	15	20

nd, not determined.

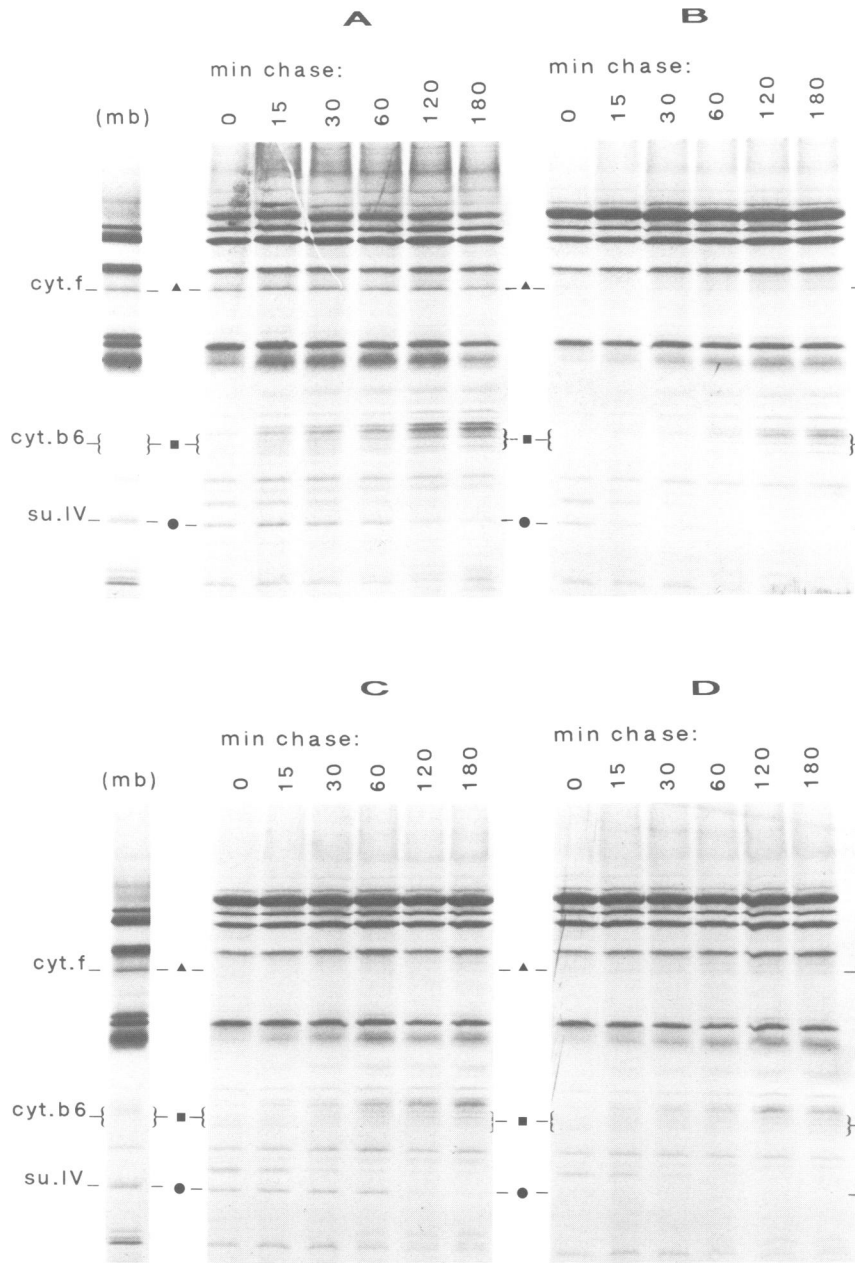

Fig. 4. Autoradiograms of a urea–SDS gel showing a pulse–chase labelling of the chloroplast-encoded polypeptides from WT cells (panel A); Δ*petB* cells (panel B); Δ*petA* cells (panel C) and Δ*petD* cells (panel D). Cells were pulse-labelled for 5 min with [^{14}C]acetate in the presence of 8 μg/ml cycloheximide and then chased for 0, 15, 30, 60, 120 or 180 min. Electrophoretic migration points of cyt *f* (▲), cyt b_6 (■) and suIV (●) are indicated. Note the appearance of unrelated comigrating proteins in the cyt b_6 region during the chase. mb, thylakoid membranes from the WT strain.

Table II. Relative labelling of cyt *f* and suIV in the course of a pulse–chase experiment

		Time of chase (min)				Half-life
		0	60	120	180	(min)
cyt *f*	WT	100	97	90	81	>120
	Δ*petB*	100	100	69	56	>120
	Δ*petD*	100	69	60	46	>120
suIV	WT	100	113	43	33	111
	Δ*petA*	100	40	11	5	45
	Δ*petB*	100	8	3	0	16

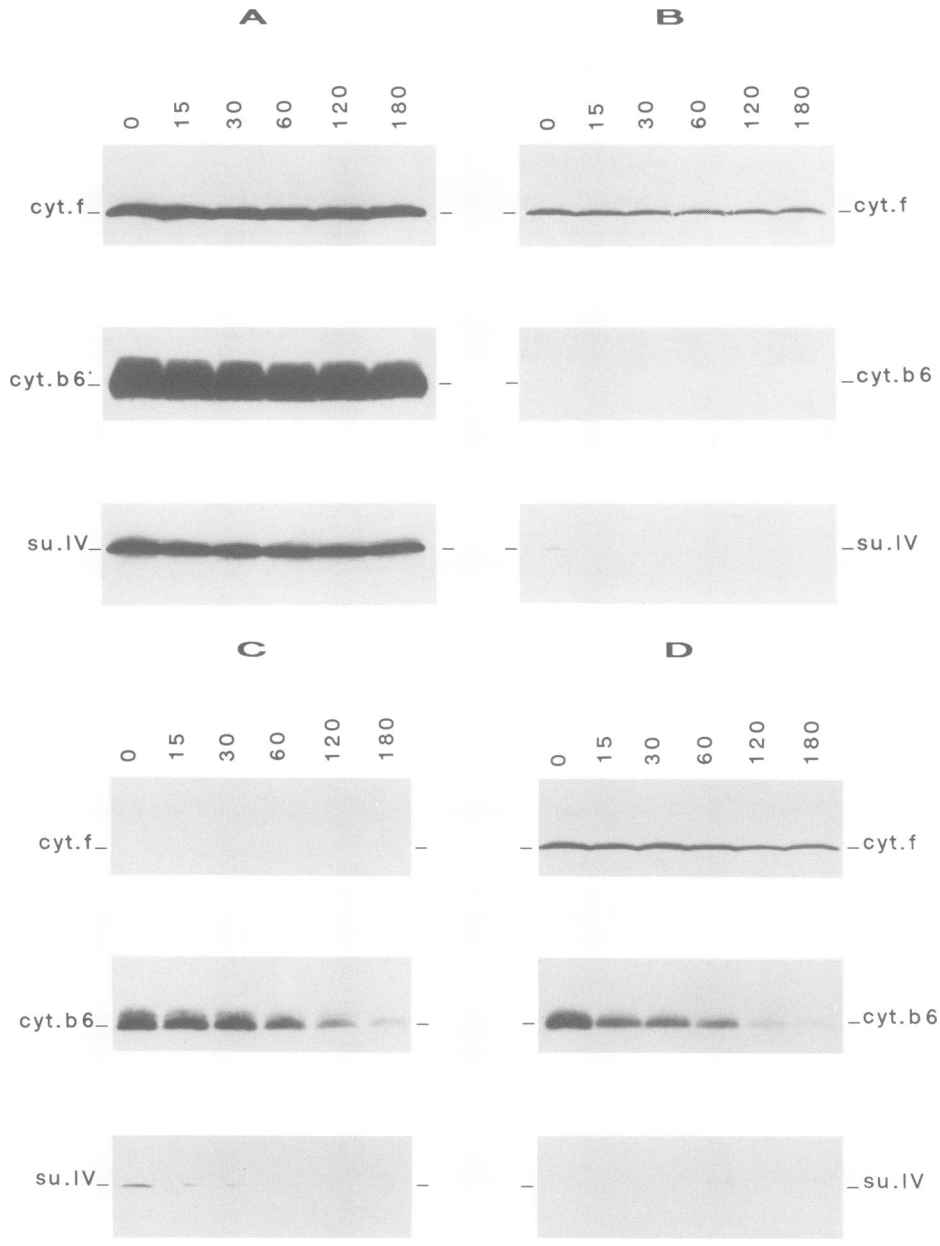

Fig. 5. Immunoblots of whole cell proteins, upon incubation for variable times with chloramphenicol, an inhibitor of chloroplast protein translation. Polypeptides from WT cells (**A**), Δ*petB* cells (**B**), Δ*petA* cells (**C**) and Δ*petD* cells (**D**). Cells were placed for 0, 15, 30, 60, 120 or 180 min in the presence of chloramphenicol (100 μg/ml), then subjected to SDS−PAGE, electroblotted onto nitrocellulose and reacted with antisera against cyt *f*, cyt b_6 (N-terminal part) or suIV (N-terminal part).

cyt b_6 could not be conveniently assessed in these experiments owing to the diffuse aspect of the labelled band at early chase times and to the appearance of unrelated comigrating proteins over longer periods of chase.

The relative amounts of cyt *f* and suIV remaining in the different strains were then quantified using a PhosphorImager. The label incorporated in each band was normalized to a constant labelling on P6, the band immediately above cyt *f*. This normalization allowed us to correct for variations from one load to another (in particular, a general increase in apparent labelling is clearly visible in samples corresponding to the longest times of chase on panel D). The results are given Table II together with the resulting half-lives of the two subunits in each instance.

The small fraction of mature cyt *f* synthesized and inserted in the thylakoid membranes of the deletion mutants proved very stable since it was still detectable in Δ*petD* and Δ*petB* transformants after a 180 min chase period. In the two cases cyt *f* half-life remained longer than 120 min. This contrasted with the extensive destabilization of suIV which displayed an 8-fold increase in turnover in the absence of cyt b_6.

That cyt *f* and suIV displayed such contrasting stabilities immediately after their synthesis, was reinvestigated for the bulk of pre-existing subunits in the transformants. To this end chloroplast translation was abolished by addition of chloramphenicol *in vivo* and the proportion of chloroplast-encoded cyt b_6/f subunits remaining in the transformants at various time points after addition of the inhibitor was determined by immunodetection. The results are shown in Figure 5; they confirm, and extend to cyt b_6, the observations drawn from the pulse−chase study: cyt *f* remained long-lived in the absence of cyt b_6 or suIV,

whereas cyt b_6 and suIV had much shorter life-times when not assembled in a mature complex.

Discussion

In an earlier study with various mutants lacking cyt b_6/f complex activity (Lemaire *et al.*, 1986) we showed that its major chloroplast-encoded subunits displayed a concerted accumulation in the thylakoid membranes of *C.reinhardtii*. This cytochrome moeity of the complex could accumulate to >50% of the amount found in WT membranes in the absence of the nuclear-encoded subunits, i.e. the Rieske protein and suV. The mechanism by which the chloroplast-encoded subunits accumulated simultaneously remained elusive. We therefore constructed three distinct deletion mutants selectively deprived of either cyt *f*, cyt b_6 or suIV. These mutants allowed us to characterize the expression of three pairs of chloroplast-encoded subunits in the absence of the third chloroplast-encoded subunit of the complex: deletion of any one of the three genes severely decreased the accumulation of the other cyt b_6/f subunits.

Stabilization of either cyt b_6 or suIV requires expression of the other chloroplast-encoded subunits of the cyt b6/f complex

In the absence of cyt *f* or suIV, the transcript levels and the rates of synthesis of cyt b_6 remained similar to that in the WT. Likewise, the transcript levels and the rates of synthesis of suIV showed little change in the absence of cyt *f* or cyt b_6. Therefore the first steps in the expression of cyt b_6 or suIV, including the translational step, show no dependence on the expression of the other major subunits of the complex. However, the half-lives of cyt *b* and suIV were dramatically shortened in the deletion mutants. The deficiencies in cyt b_6 or suIV in such mutants originated from some post-translational event preventing their stabilization in the thylakoid membranes. In particular, stabilization of suIV markedly depended on the presence of cyt b_6: its life-time was much shorter in the absence of cyt b_6 than in the absence of cyt *f*. We note that suIV accumulated to the same limited amount as cyt b_6, i.e. 5%, in the transformant lacking cyt *f*. In addition we observed that a fraction of suIV remaining in Δ*petA* transformants migrated with cyt b_6 upon centrifugation of solubilized thylakoid membranes on sucrose density gradient (results not shown). These various observations argue for the formation of a subcomplex cyt b_6/suIV of limited stability in the absence of cyt *f*. It should be noted that cyt b_6 and suIV are respectively homologous to the N-terminal and C-terminal parts of cyt *b*, a major subunit of cyt *bc* complexes in photosynthetic bacteria and mitochondria (Cramer *et al.*, 1991). Three-dimensional models for the folding of the transmembrane α-helices of cyt *b* have been proposed: they were based on the distribution of mutated residues altering the two quinone binding pockets in cyt *bc* complexes from *Rhodobacter sphaeroides* (Crofts *et al.*, 1992) or on intragenic suppressor mutations reverting cyt *b* primary mutations responsible, for instance, for inhibitor resistance in *bc* complexes from yeast mitochondria (di Rago *et al.*, 1990). Taken together, these models point to a packing of helix pairs H1/H5 and H3/H6, which are split between cyt b_6 and suIV in cyt b_6/f complexes. In addition, the model drawn from *R.sphaeroides* suggests that the two helices H5 and H6, which are part of suIV in cyt b_6/f complexes, are more peripheral and distant from one another than the four cyt b_6 helices, H1−H4. These helix-packing features may explain why an interaction of suIV with cyt b_6 would have such critical consequences on its folding in a protease-resistant form.

Cyt f synthesis depends on cyt b_6/suIV

The absence of cyt b_6 or suIV reduced by 90% the cyt *f* content in the Δ*petB* or Δ*petD* transformants. Surprisingly, we also observed an extensive decrease in the labelling of cyt *f* in 5 min pulses in Δ*petB* and Δ*petD* transformants. Since there was no drop in *petA* transcripts in these strains, our observation cannot be accounted for by a limitation in the amount of *petA* transcripts, nor could it be explained by an extensive post-translational degradation of cyt *f* similar to the one affecting suIV in the absence of cyt b_6. At variance with this above-described instability of suIV, we found no evidence for a significant increase in turnover of cyt *f* in the transformants whether analysed in pulse−chase studies or by immunodetection experiments of chloramphenicol-treated cells within 120 min. Taken together, these data indicate that the limited synthesis of cyt *f* observed in 5 min pulses in the absence of suIV or cyt b_6, yields individual polypeptides whose stability remains close to that in the WT. Thus cyt *f* turns out to be as stable, in the absence of cyt b_6 and suIV, as mitochondrial cyt c_1 in the absence of cyt *b* (Crivellone *et al.*, 1988). In the latter case no interaction at the translational (or early post-translational) level may occur since the two subunits are made in different compartments.

We conclude that the reduced content in cyt *f* in our transformants reflects a regulation of cyt *f* synthesis involving cyt b_6 and suIV. A number of molecular mechanisms could account for this observation. Synthesis of cyt *f* is a multistep process which encompasses translation of the *petA* message, N-terminal processing and covalent haem binding. We do not know yet whether the last two events are co-translational or in part post-translational. The regulation may then operate either during translation or very early after translation: for instance cyt *b*/suIV precomplexes may protect pre-apocytochrome *f* from immediate degradation by catalysing post-translational modification such as haem attachment or N-terminal processing. In a co-translational hypothesis, less efficient docking of the translating polysomes or premature termination of translation could occur if cyt *f* is not synthesized at its assembly site with cyt b_6/suIV. Such a situation has been suggested for the translation of cyt *b* in yeast mitochondrial membranes: a translational activating factor, CBS1, was shown to be membrane located (Michaelis *et al.*, 1991). The authors reasoned that CBS1 may allow the preferential synthesis of cyt *b* at the site of complex assembly.

In the present case, where cyt *f* appears fairly stable *per se*, its privileged synthesis at the site of assembly with the other subunits of cyt b_6/f complexes would favour its stoichiometric accumulation with that of cyt b_6 and suIV which are short-lived polypeptides if not interacting with cyt *f*. That the site of synthesis bears consequences on assembly processes has been demonstrated, for instance in experiments where distinct variants of haemagglutinin were expressed simultaneously. The proportion of resulting hetero- versus homo-oligomers depended on the relative level of expression of the variants (Boulay *et al.*, 1988): at low levels, homo-

oligomers were preferentialy made out of subunits synthesized from the same polysome, i.e. at the same site.

Thus, the biogenesis of the mature cyt b_6/f complex depends on the interplay of two types of regulations involving the constitutive subunits themselves. These two types of regulation can be distinguished on a time scale basis with respect to the rate of translation. One is a slow post-translational process, which reflects the assembly-mediated stabilization of a subunit. The other is a rapid post-translational process or even a co-translational process, by which the synthesis of a subunit depends on its immediate interaction with some other subunit of a complex. Besides the case of cyt *f* described in the present paper, such would be the case for the PSI core complex where pulse-labelling of the *psaA* product could no longer be detected in mutants blocked in the synthesis of the *psaB* product (Girard-Bascou *et al.*, 1987). In the PSII core complex, the rate of synthesis of D1 showed a marked dependence on the presence of D2, as did P5 for D1 (Erickson *et al.*, 1986; Jensen *et al.*, 1986; de Vitry *et al.*, 1989). Also, for the chloroplast ATP synthase, the β subunit controls the rate of synthesis of the α subunit (Drapier *et al.*, 1992). Thus the early control of the synthesis of a chloroplast-encoded subunit by an interacting subunit appears to be of general significance in the biogenesis of the major proteins of the thylakoid membranes.

Materials and methods

Cell growth conditions

WT and transformant cells were grown on Tris−acetate phosphate (TAP) medium, pH 7.2, at 25°C under 300 lux continuous illumination.

Oligonucleotides, mutagenesis and plasmids

Oligonucleotide primers for mutagenesis were synthesized using an LKB DNA synthesizer and purified according to the manufacturer's protocols. Site-directed mutagenesis was performed according to the method of Kunkel *et al.* (1987).

Plasmid pUC-atpX-AAD carrying the *aadA* cassette was kindly provided by Dr Goldschmidt-Clermont (University of Geneva). The corresponding construct has been described (Goldschmidt-Clermont, 1991).

petA, *petB* and *petD* *Hin*dIII restriction fragments were cloned in Bluescript vector KS as previously described (Büschlen *et al.*, 1991): plasmid piAH1.9 contained a 1.9 kb *Hin*dIII fragment of the *petA* gene (encoding the N-terminal part of cyt *f*); plasmids piAH3.5 and pdAH3.5 contained a 3.5 kb *Hin*dIII fragment of the *petA* gene (encoding the C-terminal part of cyt *f* and the *petD* promoter region) cloned in either orientation; plasmid pdDH1.1 contained a 1.1 kb *Hin*dIII fragment of the *petD* gene (encoding the entire sequence of suIV); plasmid pdBH1.5 contained a 1.5 kb *Hin*dIII fragment of the *petB* gene (encoding the N-terminal part of cyt b_6); plasmid pdBH1.8 contained a 1.8 kb *Hin*dIII fragment of the *petB* gene (encoding the C-terminal part of cyt b_6). Continuous *petA* and *petB* genes and production of a *petD* fragment of suitable size for transformation were obtained as described below. For each plasmid, the *Hin*dIII site that was not required for the fusion of the adjacent *pet* fragment was destroyed by exonuclease III digestion. This digestion led to plasmids piAΔH1.9, piAΔH3.5, pdAΔH3.5, pdDΔH1.1, pdBΔH1.5 and pdBΔH1.8, respectively obtained from plasmids piAH1.9, piAH3.5, pdAH3.5, pdDH1.1, pdBH1.5 and pdBH1.8. The *petA* insert of piAΔH1.9 was then excised by *Hin*dIII and *Apa*I digestion and introduced into piAΔH3.5a opened by *Hin*dIII and *Apa*I digestion, giving plasmid piWF. The *Apa*I/*Xba*I digestion of piWF produced a 4.5 kb fragment containing the whole *petA* ORF. The *petB* insert of pdBΔH1.5 was recovered by *Hin*dIII/*Xba*I digestion and introduced into pdBΔH1.8 cut by the same restriction enzymes giving the plasmid pdWB. *Apa*I/*Xba*I digestion of pdWB produced a 3.0 kb fragment containing the whole *petB* ORF. The *petD* insert of pdDΔH1.1 was recovered by *Hin*dIII digestion and introduced into *Hin*dIII-digested pdAΔH3.5, giving plasmid pdWQ. The digestion of pdWQ with *Apa*I and *Xba*I produced a 4.5 kb fragment containing the whole *petD* ORF and the intergenic *petA*/*petD* region.

Chloroplast transformation in C.reinhardtii

WT cells (mating type +) were transformed as described by Boynton *et al.* (1988) with a particle gun built in the laboratory by P.Bennoun and D.Béal. Cells were grown in liquid TAP medium to a density of 5×10^6 cells/ml. 10^8 cells were plated on TAP medium and bombarded with 1.2 μm tungsten particles coated with the appropriate DNA. Following overnight incubation in dim light at 25°C, the bombarded cells were replated on TAP in the presence of 100 μg/ml spectinomycin. Spectinomycin-resistant colonies became detectable after 2 weeks of growth. Colonies lacking cyt b_6/f activity were selected, based on their fluorescence induction kinetics after dark-adaptation. These transformants were subcloned for many rounds both on TAP-spectinomycin plates under dim light and on minimum medium plates (no acetate added) under high light. When subcloning no longer yielded any colonies able to grow on minimum medium, the transformants were considered as homoplasmic for the mutant genome. They were subsequently kept on TAP plates for further characterization.

DNA analysis

Small-scale preparations of total DNA were obtained from 30 ml TAP cultures grown under dim light at 2×10^6 cells/ml. DNA purification was as in Roffey *et al.* (1991). For hybridization with specific probes, DNA samples were separated on 0.8% agarose gels, transferred under vacuum to nylon membranes (Hybond-N, Amersham International) and hybridized to ^{32}P random-labelled probes corresponding to the restriction fragment under examination.

Protein isolation and separation

Thylakoid membranes were isolated as described by Chua and Bennoun (1975) from cultures grown at a density of 5×10^6 cells/ml. Before electrophoresis, proteins from whole cells or from thylakoid membrane preparations were solubilized in the presence of 1% SDS at 100°C for 50 s. For immunodetection of the *petG* product, the protein content of intact thylakoid membranes was precipitated in the presence of 10% TCA for 1 h at 4°C, then neutralized with 0.5 M DTT/Na_2CO_3, and solubilized in the presence of 1% SDS at 100°C for 50 s. Polypeptides were separated by either denaturing (in the presence of 8 M urea) or non-denaturing SDS−PAGE (12−18% polyacrylamide gradient).

Labelled polypeptides were detected by autoradiography of the dried gels. Quantification of the labelling was performed using either a PhosphorImager (Molecular Dynamics, USA) or a Scanjet Plus (Hewlett Packard, USA) associated with a Quantiscan program (Biosoft Inc. Cambridge, UK).

Protein transfer and immunoblotting

Proteins were electroblotted as described by Towbin *et al.* (1979) onto nitrocellulose sheets, except for the *petG* product which was transferred onto polyvinylidene difluoride (PVDF) membranes. Immunodetection was carried out using an enhanced chemiluminescence (ECL) method (Amersham International) according to the protocol supplied by the manufacturer. The anti-Rieske antiserum was prepared against the intact polypeptide separated by polyacrylamide gel electrophoresis using thylakoid membranes from a double mutant lacking the δ subunit of the chloroplast ATP synthase and the OEE2 subunit which otherwise comigrate with the Rieske protein. After electroelution from a gel slice, the Rieske protein was injected subcutaneously into New Zealand white rabbits. Sera were collected bimonthly after booster injections as previously described (de Vitry *et al.*, 1989). Preparation of anti-cyt *f* antiserum was described previously (Bulté and Wollman, 1992). Antisera raised against the *petG* product and against synthetic peptides corresponding to the C-terminal and N-terminal parts of cyt b_6 and to the N-terminal part of suIV were kindly provided by J.-L.Popot and Y.Pierre (Institut de Biologie Physico-Chimique, Paris, France).

In vivo labelling and pulse−chase experiments

Whole cells (2×10^6 cells/ml) were pulse-labelled according to Delepelaire (1983), in the presence of cycloheximide (8 μg/ml) as an inhibitor of cytoplasmic translation: [^{14}C]acetate was added at a concentration of 10^{-4} M (5 μCi/ml) for 5 min or at a concentration of 2×10^{-5} M (1 μCi/ml) for 45 min. The 5 min pulse-labelling experiment was stopped by addition of 1 vol of ice-cold sodium acetate (50 mM). For pulse−chase experiments, cells were first pulse-labelled for 5 min in the above-described conditions and then chased by addition of 10 vols of TAP medium containing 50 mM acetate. Aliquots were removed after 0, 15, 30, 60, 120 and 180 min and the chase was stopped by addition of 1 vol of chilled sodium acetate (50 mM).

Investigation of the stabilility of the accumulated subunits

Whole cells (2×10^6 cells/ml) were incubated in the presence of chloramphenicol (100 μg/ml) as an inhibitor of choroplast translation.

Aliquots were removed and chilled on ice after 0, 15, 30, 60, 120 and 180 min.

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