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Studies on the cytochrome b_6/f complex. I. Characterization of the complex subunits in *Chlamydomonas reinhardtii*

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Key words: Cytochrome b_6/f complex; Peroxidase; Thylakoid membrane; Protein assembly; Chloroplast biogenesis

We have analyzed the heme-associated peroxidase activity in thylakoid membranes from the green algae *Chlamydomonas reinhardtii* after electrophoresis in the presence of sodium dodecyl sulfate. Besides cytochrome *f* and cytochrome b_6, we observed peroxidase activity in two other bands, of 34 and 11 kDa, of unknown origin. Characterization of the b_6/f complex subunits was undertaken by means of a comparison of the polypeptide deficiencies in several b_6/f mutants with the polypeptide content of preparations enriched in b_6/f complexes. We conclude that the b_6/f complex consists of five subunits. Using site-specific translation inhibitors, we show that cytochrome *f*, cytochrome b_6 and subunit IV are of chloroplast origin, whereas the Rieske protein and probably subunit V are translated on cytoplasmic ribosomes. A model of assembly of the complex is proposed: a cytochrome moiety, comprising the subunits of chloroplast origin, is assembled in the thylakoid membranes prior to the insertion and assembly of the subunits encoded in the nuclear genome.

Introduction

In recent years, there have been several reports on the purification of b_6/f complexes from higher plants [1–4] and cyanobacteria [5]. Cytochromes *f* and b_6, and the Rieske protein have been identified among the putative subunits of the isolated complexes. The cytochromes were characterized by their redox differential absorption spectra and by heme staining upon SDS-polyacrylamide gel electrophoresis of the complex, whereas the Fe-S center-containing Rieske protein was identified by its characteristic EPR signal at $g = 1.89$. The use of antibodies against these subunits have proved useful for their identification in thylakoid membranes from different species and in subsequent preparations enriched in b_6/f complexes [6–8].

There is a controversy about the number of additional subunits in the b_6/f complex. Besides a polypeptide in the 14–18 kDa region, quoted as subunit IV and supposedly related to cytochrome b_6 [9,10], polypeptides in the 35 kDa region and polypeptides of molecular weights smaller than 10 kDa were observed in some instances [2,3,5].

Analysis of the pattern of polypeptide deficiencies in mutants lacking the b_6/f complex can be regarded as an alternative approach to the identification of the polypeptides constitutive of the complex. The absence of the main subunits of the b_6/f complex was reported in mutants from *Maize* [11] and *Lemna* [12]. Maroc and Garnier [13] described mutants from *Chlamydomonas rein-*

Abbreviations: Cyt, cytochrome; PS, Photosystem; HP, high potential; LP, low potential; α, antibody; TMBZ, 3,3′,5,5′-tetramethylbenzidine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PBS, phosphate-buffered saline; F_0, initial yield of fluorescence; F_{max}, maximal yield of fluorescence.

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hardtii lacking the two cytochromes, but they did not report on the possible absence of other polypeptides.

In the present study, we attempted to characterize the subunits of the b_6/f complex from *C. reinhardtii* through a comparison of the polypeptide content of preparations, enriched in b_6/f complexes, with polypeptide deficiencies in several photosynthesis mutants altered in this complex. We have successfully used a similar approach for the identification of the subunits of protein complexes containing the PS I and PS II reaction centers [14,15]. The sites of synthesis of the various subunits were investigated by pulse-labelling experiments in the presence of site-specific translation inhibitors. The comparative analysis of the pulse-labeled thylakoids from the mutant strains and the wild type gave some insight on the mode of assembly of the complex in the thylakoid membrane.

Materials and Methods

Mutant strains. In this study, we analysed several mutants from *C. reinhardtii*, showing altered b_6/f complexes, with respect to the wild type and, in some instances, to the FuD112 mutant lacking in PS II reaction centers [16]. The mutant strains, blocked in the electron transfer chain at the level of the b_6f complex, are listed below.

– The nuclear mutant, ac21, and the chloroplast mutant, FuD2, were isolated and characterized by Levine and Smillie [17] and Bennoun et al. [18], respectively. Contrarily to the other mutants described in this paper, the FuD2 mutant still grows in phototrophic conditions although at reduced rates as compared to the wild type.

– The chloroplast mutants, FuD4, FuD6, FuD8, and the nuclear mutant, F18, were obtained after mutagenesis with 5-fluorodeoxyuridine for the former [18], and 5-fluorouracyl for the latter [14].

The enrichment step was performed according to Schmidt et al. [19] as modified by Bennoun and Delepelaire [20]. The b_6/f mutants were screened by their high stationary level of fluorescence as described by Bennoun and Levine [21]. They display fluorescence characteristics similar to that of the FuD2 mutant previously described by Bennoun et al. [18]: same F_0 as in the wild type and a continuous fluorescence rise with no decay phase. Their F_{max} level is identical to that observed in the presence of DCMU in wild-type cells.

The genetic analysis of the mutants was performed according to Levine and Ebersold [22]. We analysed an average of 12 tetrads per cross and checked the nuclear segregation of the mating type. For chloroplast mutations, the transmission was almost exclusively from the mating type + parental strain, while a 2:2 segregation was observed for all nuclear mutations.

Methods. All the strains were grown at 25°C in Tris-acetate-phosphate medium at a light intensity of 300 lx. The cells were harvested in late exponential phase ($4 \cdot 10^6$ cells/ml), except for pulse-labelling experiments ($2 \cdot 10^6$ cells/ml).

Pulse labelling was carried out according to Delepelaire [23]. Purified thylakoid membranes were prepared according to Chua and Bennoun [24]. Preparations enriched in b_6/f complex were obtained by a procedure similar to that of Pick and Racker [25], using thylakoid membranes from the wild type or FuD50 mutant lacking the chloroplast ATPase [26], solubilized in the absence of $(NH_4)_2SO_4$ by a mixture of 1% octylglucoside and 0.5% cholate. After fractionation of the supernatant by $(NH_4)_2SO_4$ precipitation, and sucrose gradient centrifugation, the upper reddish brown band was collected and analysed for heme content after SDS-polyacrylamide gel electrophoresis.

Electrophoresis were run in the Laemmli system [27] using 7.5–15% acrylamide gradients (referred to as a 'conventional gel system' in the present paper) or 12–18% acrylamide gradients in the presence of 8 M urea [28]. Heme staining was performed according to Thomas et al. [29]. Polypeptides were stained by Coomassie blue or detected by autoradiography of the dried gels using Agfa-Gevaert industrial P films. Molecular weights of the polypeptides were estimated using a low molecular-weight calibration kit from Pharmacia.

Electrophoretic transfers were carried out according to Towbin et al. [30]. Protein saturation of the nitrocellulose sheets was performed using either 0.2% tween and 0.25% gelatin in phosphate-buffered saline or 5% low-fat milk in phosphate-buffered saline. Binding of the α-Rieske and α-b_6, generously provided by G. Hauska, was detected using radioiodinated protein A according to Burnette [31].

Results

In order to characterize the heme deficiencies in the b_6/f mutants we first compared the heme content of the thylakoid membranes from *C. reinhardtii* and spinach by TMBZ staining of urea gels (Fig. 1A). Both types of thylakoid displayed the same two bands of apparent molecular weight, 41 000 and 23 000, in this gel system. In conventional SDS-polyacrylamide gel electrophoresis they had an apparent molecular weight of 38 000 and 25 000 (not shown). On the other hand we noted the presence of two stained bands in *C. reinhardtii*, quoted h_1 and h_2 on Fig. 1A, of apparent molecular weights, 34 kDa and 11 kDa, respectively. In spinach thylakoids, h_2 was never observed whereas a faint staining in the position of h_1 could be detected in some instances. The stained bands of 38 and 25 kDa (41 and 23 kdA, respectively, in urea gels) have been previously attributed in spinach to Cyt f and Cyt b_6, respectively [1]. Accordingly, purified b_6/f complexes from *C. reinhardtii* contained these two bands (Fig. 1A), whereas most of the b_6/f mutants showed no heme staining in these electrophoretic positions (Fig. 1B). In contrast, h_1 and h_2 were absent in the b_6/f complex preparations but they remained in the various b_6/f mutants that we have characterized. These bands were still visible in mutants lacking in PS II reaction centers (FuD112 on Fig. 1B). Therefore they do not correspond to Cyt b-559_{HP} which is structurally associated with PS II reaction centers [32] and is lacking in PS II mutants (Wollman, F.-A., unpublished results).

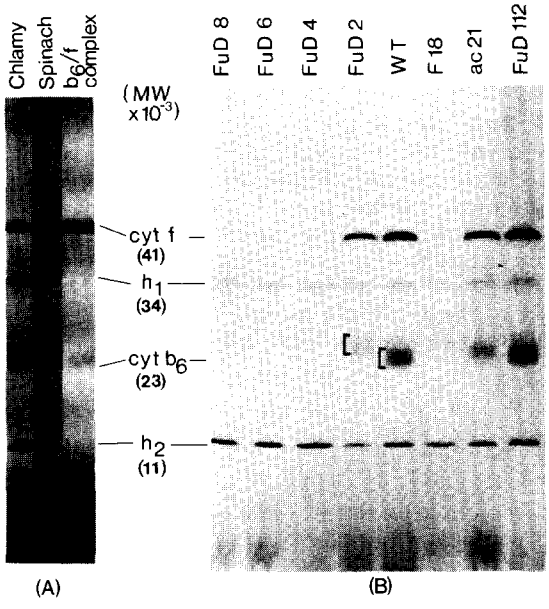

Fig. 1. Heme staining of polypeptides from purified thylakoids and from a b_6/f complex preparation, using urea SDS polyacrylamide gel electrophoresis (A) comparison of a b_6/f complex preparation with thylakoid membranes from the wild type of *C. reinhardtii* and from spinach. Cyt f and Cyt b_6 are observed in the three samples. Note the presence of two heme stainable bands. h_1 and h_2, in thylakoid membranes from *C. reinhardtii*. (B) comparison between thylakoids from different strains of *C. reinhardtii*. Cyt f and Cyt b_6 are still visible in the ac21 mutant, but are absent in the FuD4, FuD8 and F18 mutants. Traces of Cyt f are detected in the FuD6 mutant. The FuD2 mutant contains Cyt f and a modified Cyt b_6. Note the presence of h_1 and h_2 in all strains, including the FuD112 mutant lacking PS II reaction centers. WT, wild type.

Three types of b_6/f mutant could be distinguished on the basis of their heme content:
- mutants devoid of Cyt f and Cyt b_6, like FuD4, FuD8 and F18, or showing only traces of Cyt f, like FuD6;
- mutants displaying a heme-staining pattern similar to that in the wild type, like ac21;
- mutants showing a modification in the electrophoretic position of one of the two cytochromes. This was the case of the FuD2 mutant which contains a somewhat modified Cyt b_6.

We first analysed the group of mutants devoid of both cytochromes. After Coomassie blue staining of urea gels (Fig. 2), we noted the absence of the 41 kDa polypeptide, corresponding to Cyt f, in the four mutants thought to lack the b_6/f complex on the basis of their heme-staining pattern. This group of mutants showed two other deficiencies in the 23 and 19.5 kDa positions (see inset at the right-hand corner of Fig. 2). The former deficiency corresponds to the position where Cyt b_6 was detected by the heme-staining procedure used in Fig. 1. No other modifications were observed among the population of polypeptides of still lower molecular weight, which is best resolved with this gel system.

We then performed immunoblotting experiments in order to check the absence of the Rieske protein in these mutants. This is shown on Figs. 3 and 4, for the F18 mutant taken as typical of the

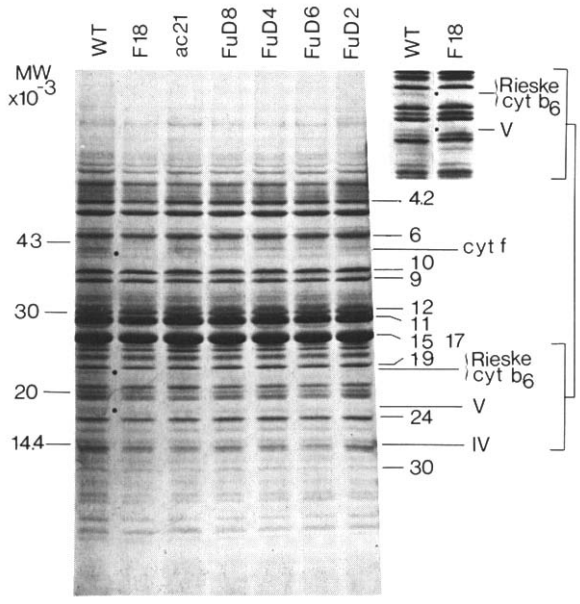

Fig. 2. Polypeptide patterns of thylakoid membranes from wild type (WT) and b_6/f mutant strains from *C. reinhardtii*, using urea-SDS polyacrylamide gel electrophoresis after Coomassie blue staining. Polypeptides are numbered according to Bennoun et al. [15]. Besides the absence of Cyt f in several mutants, two polypeptide deficiencies are observed in the 23 and 19 kDa regions. Comparison of these regions in the F18 and wild type thylakoid membranes is shown the insert at the right upper corner.

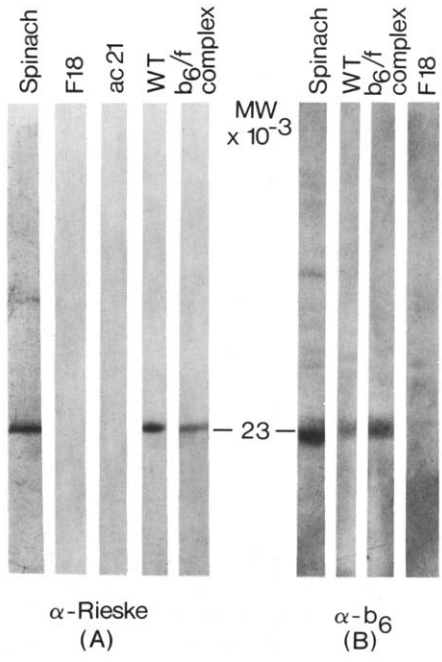

Fig. 3. Immunoblots on a b_6/f complex preparation and on thylakoid membranes from wild type (WT) and b_6/f mutant strains after urea-SDS polyacrylamide gel electrophoresis. Using α-Rieske protein (A) and α-Cyt b_6 (B) coupled to radioiodinated protein A. Note that 23 kDa bands are labeled with the two antibodies in spinach and *C. reinhardtii* wild-type thylakoids as well as in the b_6/f complex preparation. No such labeling is observed in the F18 mutant. The ac21 mutant shows no 23 kDa labeling with the α-Rieske only.

first group of mutants in the above classification. In spinach as well as in the wild-type of *C. reinhardtii* the α-Rieske labeled a single band of 23 kDa in the urea gel system. No labeling was observed in the F18 mutant (Fig. 3A). When similar experiments were performed with a α-Cyt b_6, a 23 kDa band was also labeled in spinach and in the wild type of *C. reinhardtii*. This label was absent in the F18 mutant (Fig. 3B). That these observations revealed a comigration of the Rieske protein and Cyt b_6 in this gel system, and not a cross-reaction of the antibodies, is illustrated on Fig. 4: in conventional gel systems (i.e., in the absence of urea) the two proteins migrated at different positions giving rise to a label in the 25 kDa region with the α-Cyt b_6 and in the 19 kDa region with the α-Rieske. The absence of radiolabeled bands in these positions in the F18 thylakoids confirmed the absence of the two antigens in this type of mutants.

In agreement with their heme staining pattern, the ac21 and FuD2 mutants contained Cyt f, corresponding to the 41 kDa polypeptide in the urea gel shown on Fig. 2. However, the 23 kDa band was lacking in the ac21 mutant and was present in the FuD2 mutant. Immunoblotting experiments (Figs. 3 and 4) showed that the Rieske protein was lacking in the ac21 mutant and was present in the FuD2 mutant, whereas Cyt b_6 was present in both strains with a modified electrophoretic position in the case of the FuD2 mutant. These observations indicated that the 23 kDa band, revealed by Coomassie blue staining of urea gels, corresponded to the Rieske protein only. Therefore, it should be emphasized that Cyt b_6 is not stained efficiently enough to be detected among the polypeptides of the thylakoid membrane.

As was the case with the mutants lacking the

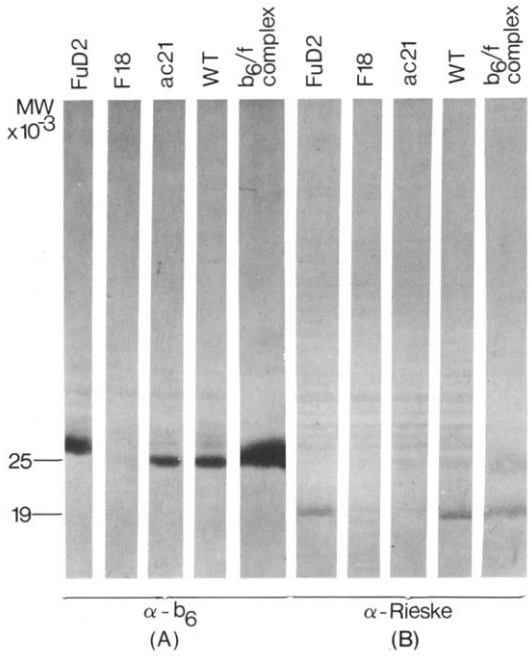

Fig. 4. Same as Fig. 3 after SDS polyacrylamide gel electrophoresis. Distinct bands are labeled by the α-Rieske protein (19 kDa) and the α-cytb6 (25 kDa). Note the absence of labeling with α-Rieske protein in the ac21 mutant and the absence labeling by the α-Rieske protein and α-Cyt b_6 in the F18 mutant. WT, wild type.

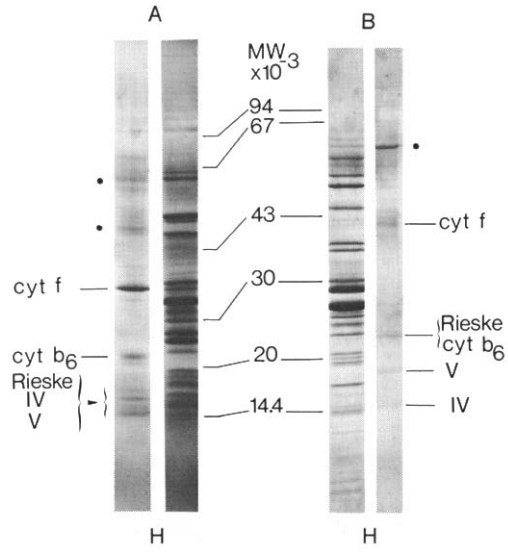

Fig. 5. Comparison of the polypeptide patterns in preparations enriched in b_6/f complexes and in thylakoid membranes from the wild-type strain. (A) Heated samples after SDS polyacrylamide gel electrophoresis. (B) Heated samples after urea-SDS-polyacrylamide gel electrophoresis. The asterisk indicates contaminant polypeptides of high molecular weight, the amount of which varied from one preparation to another.

whole b_6/f complex, thylakoids from the ac21 mutant were lacking in a 19.5 kDa polypeptide in the urea gel system (Fig. 2). This polypeptide was still present in the FuD2 thylakoids, although showing a modified electrophoretic mobility in some experiments.

In order to understand if the b_6/f complex consisted of only the four polypeptides deficient in the b_6/f mutants, we attempted to purify the complex from *C. reinhardtii*. Preparations enriched in b_6/f complexes showed a b_6/f ratio of 1.76 (using millimolar extinction coefficients, $\epsilon_{543-553}$, of 21 for Cyt f [33] and $\epsilon_{563-570}$ of 14 for Cyt b_6 [34]). Similar experiments performed on intacts thylakoids yielded a b_6/f ratio of 1.94. Therefore, our b_6/f complex preparations showed a slight deficiency ($\approx 10\%$) in Cyt b_6. The polypeptide content of this preparation is shown on Fig. 5. In conventional SDS-polyacrylamide gel electrophoresis (Fig. 5A), Cyt f appeared as a main band in the 38 kDa region. Among the other polypeptides present in this preparation were a 25 kDa polypeptide, comigrating with the heme staining due to Cyt b_6 and recognized by α-Cyt b_6 (Fig. 4A), a 19 kDa polypeptide and a doublet of about 16 kDa. The 19 kDa band was labeled by the α-Rieske (Fig. 4B). A rather different pattern was observed in urea gels (Fig. 5B): besides the 41 kDa band, attributed by heme staining to Cyt f (Fig. 1), we observed only three bands, of apparent molecular weights of 23000, 19500 and 16000. As discussed above, Cyt b_6 and the Rieske protein migrated in the position of the 23 kDa band (see Figs. 1 and 3). Other polypeptides of high molecular weight, thought to be contaminants of the b_6/f complex, since their amounts varied from one preparation to another, are clearly visible in the two gel systems of Fig. 5A and B.

Thus the isolated b_6/f complex contained the four polypeptides lacking in the b_6/f mutants. However, a fifth polypeptide, of 16 kDa in urea gels, was also visible in these preparations (see Fig. 5B). It could be either a contaminant or a

constitutive subunit of the complex. In the latter case, the absence of modifications in the 16 kDa region of the polypeptide patterns of the mutants should be attributed to a comigration of several polypeptides, masking the absence of the b_6/f subunit.

In an attempt to distinguish between comigrating polypeptides in this region, on the basis of possible differences in their origin of synthesis, we performed pulse labeling experiments in the presence of site-specific translation inhibitors on whole cells of the wild type and b_6/f mutants of *C. reinhardtii*. These experiments were also designed to determine the site of synthesis of the four subunits deficient in most of the mutants, and could be used in the identification of subunit IV, known as a chloroplast-encoded subunit from the work of Alt et al. [7]. To this end, we have isolated thylakoid membranes from cells pulse-labeled for 45 min with ^{14}C-acetate in the presence of cycloheximide (Fig. 6), which inhibits cytoplasmic translation, or chloramphenicol (Fig. 7.), which inhibits chloroplast translation. It is worth mentioning that these experiments give access to the insertion of individual subunits of the complex in the thylakoid membranes of the mutants, even though they would not assemble or accumulate to the same extent as in the wild-type membranes [35,16].

Three subunits of the b_6/f complex – namely Cyt f, Cyt b_6 and subunit IV – have been reported as chloroplast translates in higher plants [7]. The chloroplast translates in the thylakoids of *C. reinhardtii* are shown on Fig. 6. The use of conventional gels (left of Fig. 6) allowed us to observe the insertion of Cyt b_6, during the time of the pulse, in all the strains we have analysed. Only did the FuD2 mutant show insertion of an abnormal Cyt b_6, with an apparent molecular weight about 1 kDa larger than usual. Cyt f was lacking from the thylakoid membranes of the FuD4 mutant, but it was inserted in the thylakoids from the other strains in which the complex does not accumulate (see for instance the F18 or FuD6 mutants in Fig. 6). The urea gel system on the right of Fig. 6 shows the absence of insertion of a 16 kDa polypeptide in the FuD6 thylakoids. Insertion of this 16 kDa polypeptide occurred in the other mutant strains (not shown). Thus, the 16

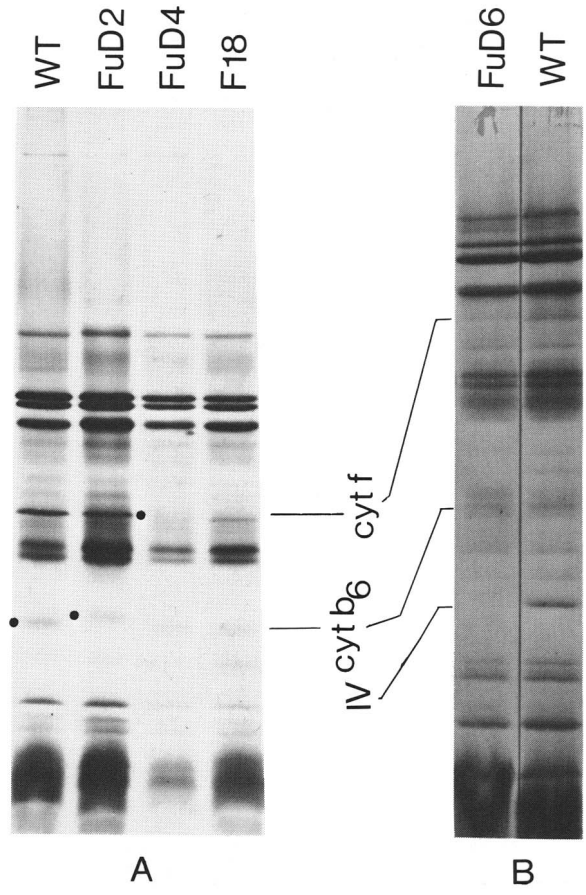

Fig. 6. Autoradiograms of chloroplast translates, inserted in the thylakoid membranes from wild-type (WT) and b_6/f mutant strains, viewed after SDS polyacrylamide gel electrophoresis (A) and urea-SDS polyacrylamide gel electrophoresis (B). Cells were pulse-labeled for 45 min with [^{14}C]acetate, in the presence of cycloheximide. Note the modified position of Cyt b_6 in the FuD2 mutant and the absence of Cyt f and of subunit IV in the FuD4 and FuD6 mutants, respectively.

kDa polypeptide that we observed in the polypeptide pattern of the b_6/f complex (Fig. 5) is probably of chloroplast origin. Therefore, it should correspond to subunit IV in the b_6/f complex of *C. reinhardtii*.

The polypeptides, translated on cytoplasmic ribosomes and inserted in the thylakoid membranes during the time of our pulse labeling, are shown on the urea gel of Fig. 7. There was no detectable insertion of the 23 kDa polypeptide, corresponding to the Rieske protein, in the ac21 nuclear mutant which contains Cyt f and Cyt b_6. On the contrary the 23 kDa band was visible in

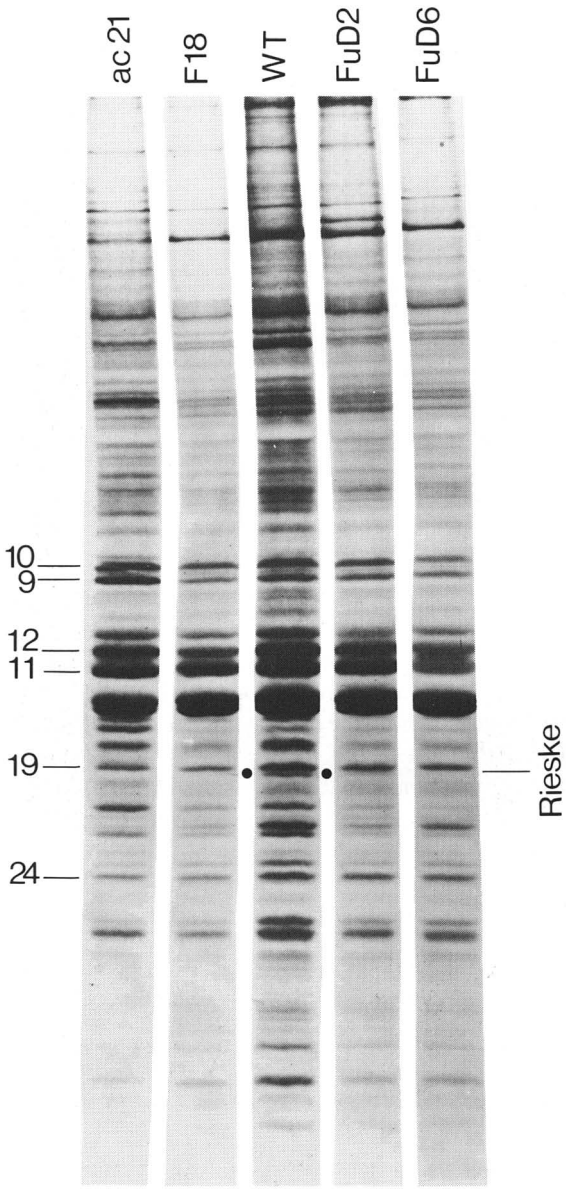

Fig. 7. Autoradiograms of cytoplasmic translates, inserted in the thylakoid membranes from the wild-type (WT) and b_6/f mutant strains, viewed after urea-SDS polyacrylamide gel electrophoresis. Note the absence of the Rieske protein in all mutants but FuD2.

the pulse-labeled thylakoids from the FuD2 mutant, as expected from the immunoblotting experiments and from its polypeptide pattern after Coomassie blue staining. More striking is the observation that the Rieske protein was not inserted in the thylakoid membranes from any of the other mutants (see for instance F18 and FuD6 in Fig. 7).

Although a 19.5 kDa polypeptide was clearly missing in the mutant thylakoids after Coomassie blue staining (Fig. 2), we observed no deficiencies in labeled polypeptides in this region in urea gels whether the pulse labeling experiments were performed in the presence of chloramphenicol or in the presence of cycloheximide (see Figs. 6 and 7). It was therefore impossible to determine, in these experiments, the site of synthesis of the fifth subunit of the b_6/f complex from *C. reinhardtii*. However, indirect evidence for its nuclear origin arises from the absence of labeled polypeptides in the 19.5 kDa region in the thylakoids from the wild type when pulse labeled with cycloheximide (Fig. 6).

Discussion

Polypeptides showing peroxidase activity in the thylakoid membrane of . reinhardtii

As previously reported by Maroc and Garnier [13], Cyt *f* and Cyt b_6 from *C. reinhardtii* were easily identified among thylakoid polypeptides after TMBZ staining of polyacrylamide gels. We showed that thylakoid membranes of *C. reinhardtii* contained two other stained bands, quoted h_1 and h_2 in this paper, distinct from Cyt *f* and Cyt b_6, since they remained in the b_6/f mutants. Although the procedure might detect peroxidase activities due to prosthetic groups other than the hemes, we looked for a possible correspondance between h_1 and h_2 and Cyt *b*-559, Hp or LP, and Cyt *b*-560, recently detected by Bendall and Sanguansermsri (personal communication). We can rule out the first possibility, since we observed h_1 and h_2 in thylakoid membranes from a mutant lacking in PS II reaction centers although PS II mutants are devoid of Cyt $b\text{-}559_{HP}$. These PS II mutants would also lack Cyt *b*-560, but would still contain significant amounts of Cyt $b\text{-}559_{LP}$ (Bendall and Sanguansermsri, personal communication). Therefore, among the cytochromes already identified in the thylakoid membranes from *C. reinhardtii*, Cyt $b\text{-}559_{LP}$ is the more likely to correspond to either h_1 and h_2. Such correlations clearly require further investigations. Still another

possibility is the participation of h_1 and h_2 in the chlororespiratory pathway described by Bennoun [36] in *C. reinhardtii*. The activity of the putative oxidase, responsible for the reoxidation of the plastoquinone pool in this pathway, was reported to be independent from the presence or absence of b_6/f complexes [37]. h_1 and h_2 might then be related to the oxidase itself or to a cytochrome complex, specific of the chlororespiratory pathway.

Characterization of the subunits of the b_6/f complex

We have identified five subunits in the b_6/f complex from the green algae *C. reinhardtii*. The complex comprised Cyt *f* and Cyt b_6, which are chloroplast translates, the Rieske protein, translated on cytoplasmic ribosomes, and two smaller subunits. That Cyt b_6/f complexes from *C. reinhardtii* contain three non-heme subunits in addition to Cyt *f* and Cyt b_6 has also been observed by Bendall and Sanguansermsri (personal communication). The electrophoretic properties of the two cytochromes and of the Rieske protein showed minor differences in Spinach and *C. reinhardtii*. These observations arose from the comparison of the positions of the heme-stained bands and from immunoblotting experiments performed on the thylakoids from the two organisms. The apparent molecular weights of these subunits varied from one gel system to another. Therefore we consider that the differences in their molecular weights as reported by different groups (reviewed in Ref. 38) may not be significant. In conventional SDS-polyacrylamide gel electrophoresis, Cyt *f* was found in the 33–38 kDa region, Cyt b_6 was in the 19–25 kDa region and the Rieske protein was about 19–20 kDa. However, we observed marked changes in this electrophoretic pattern when 12–18% acrylamide gradients in the presence of 8 M urea were used. Cyt *f* migrated with a higher apparent molecular weight (41 kDa), whereas Cyt b_6 and the Rieske protein comigrated with an apparent molecular weight of 23 kDa. This is worth mentioning, since two other subunits of the b_6/f complex, with apparent molecular weights, 19.5 and 16 kDa, were resolved in this type of gel and could be misquoted as the Rieske protein and subunit IV.

It has been previously reported that subunit IV from spinach has an apparent molecular of about 17 kDa (2) and is encoded by the chloroplast genome [7]. Out of the two small subunits that we identified in the b_6/f complex from *C. reinhardtii*, only the 16 kDa, resolved in urea gels, was shown unambiguously to be translated on chloroplast ribosomes. Therefore, its correspondence with subunit IV from spinach is very likely.

The role of the 19.5 kDa subunit, quoted as subunit V in this paper, remains to be elucidated. Owing to the absence of a labeled band, in this region of molecular weights, in the pattern of thylakoid polypeptides translated on chloroplast ribosomes, we believe that subunit V is translated

TABLE I

POLYPEPTIDES OF CHLOROPLAST OR CYTOPLASMIC ORIGIN, ACCUMULATED (A) OR INSERTED (B) IN THE THYLAKOID MEMBRANES

+, polypeptides present; −, polypeptides below detection; ND, not determined; Chlp, chloroplast; Cytp, cytoplasmic.

	A					B				
	Cyt *f* (Chlp)	Cyt b_6 (Chlp)	IV (Chlp)	Rieske (Cytp)	V (Cytp?)	Cyt *f* (Chlp)	Cyt b_6 (Chlp)	IV (Chlp)	Rieske (Cytp)	V (Cytp?)
Wild type	+	+	ND	+	+	+	+	+	+	ND
FuD2	+	+ [a]	ND	+	+	+	+ [a]	+	+	ND
ac21	+	+	ND	−	−	+	+	+	−	ND
FuD4	−	−	ND	−	−	−	+	+	−	ND
FuD8	−	−	ND	−	−	+	+	+	−	ND
F18	−	−	ND	−	−	+	+	+	−	ND
FuD6	+/− [b]	−	ND	−	−	+/−	+	−	−	ND

[a] Polypeptide present in a modified position.
[b] Polypeptide in a markedly reduced amount.

in the cytoplasm. Preliminary experiments, using preparations enriched in b_6/f complexes after pulse labelling the cells of *C.* reinhardtii with inhibitors of chloroplast translation, favor this hypothesis. It should be noted that plastocyanin is also of low molecular weight, 10 kDa [39], and is translated in the cytoplasm [40]. However, thylakoids from the ac208 mutant from *C. reinhardtii*, lacking in plastocyanin [41], still showed a 19.5 kDa polypeptide upon urea-SDS-polyacrylamide gel electrophoresis (unpublished observation). It is then unlikely that subunit V corresponded to some plastocyanin retained in the isolated complex. That such subunits of low molecular weight may exist in b_6/f complexes from higher plants has been previously mentioned [2,5].

The assembly of the b_6/f complex

The comparison of the polypeptide content in the various mutants gave some insight on the mode of assembly of the b_6/f complexes in the thylakoid membranes of *C. reinhardtii*. We can hypothesize at least three steps in the assembly of a multiple subunit membrane protein: the insertion of the subunits in the membrane, their accumulation and subsequent assembly in a functional membrane protein complex. Our data, relevant to these processes in the case of the b_6/f complex, are gathered on Table I. The majority of the mutants that we analysed in the present paper, lack the five subunits of the complex. However, one mutant, the ac21, was deficient only in the Rieske protein and in subunit V. These observations tend to define two parts in the complex: a cytochrome moiety, comprising Cyt f, Cyt b_6 and subunit IV, which can accumulate and assemble in the membrane in the absence of the other part of the complex, consisting of the Rieske protein and subunit V. This latter part would not accumulate in the thylakoid membrane in the absence of the cytochrome moiety.

We observed that the subunits of the cytochrome moiety were inserted independently in thylakoids isolated from pulse-labeled cells of *C. reinhardtii*: for instance, Cyt b_6 and subunit IV were inserted in the absence of Cyt f in the FuD4 mutant, whereas the two cytochromes were inserted in the absence of subunit IV in the FuD6 mutant. However the inserted subunits, in the two mutants, did not accumulate to the same extent as in the wild-type thylakoids. Only did Cyt f show a slight accumulation in the absence of subunit IV in the FuD6 mutant. These observations support a concerted accumulation of the subunits by a mechanism probably involving the assembly of the newly inserted subunits in the thylakoid membranes. The other part of the complex, exemplified by the case of Rieske protein, could not be detected, by pulse labeling, in the membranes of the mutants showing no accumulation of the cytochrome moiety. This indicates that the insertion of the Rieske subunit was dependent on the preceding accumulation of the cytochrome moiety and further substantiates the existence of an assembly between to parts of the b_6/f complex.

Thus we propose the following mode of assembly of b_6/f complexes in the thylakoid membranes: as a first step, the subunits encoded in the chloroplast genome – Cyt b_6 and subunit IV which show sequence homologies with Cyt b from mitochrondria [9], and Cyt f – are inserted independently, then assembled in a cytochrome moiety which allows the subsequent insertion and concomitant assembly of the subunits encoded in the nuclear genome (the Rieske protein and subunit V). It is worth mentioning that a similar situation probably prevails in the assembly of the subunits of the PS II complex: the chloroplast encoded subunits were functionally assembled in a PS II mutant from *C. reinhardtii* devoid of most of the nuclear-encoded subunits which participate in the formation of the O_2-evolving site [15]. The reverse situation was never observed and could be easily interpreted by the fact that the O_2-evolving site subunits are peripheral membrane proteins [35]. In this respect, the case of the b_6/f complex is somewhat different, since the Rieske protein has been described as a transmembrane protein [42,43].

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