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# The Chloroplast ATP Synthase in *Chlamydomonas reinhardtii*

## I. CHARACTERIZATION OF ITS NINE CONSTITUTIVE SUBUNITS\*

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We have characterized the subunit composition of the chloroplast ATP synthase from *Chlamydomonas reinhardtii* by means of a comparison of the polypeptide deficiencies in a mutant defective in photophosphorylation, with the polypeptide content in purified coupling factor (CF)<sub>1</sub> and CF<sub>1</sub>·CF<sub>0</sub> complexes. We could distinguish nine subunits in the enzyme, four of which were CF<sub>0</sub> subunits. Further characterization of these subunits was undertaken by immunoblotting experiments, [<sup>14</sup>C]dicyclohexylcarbodiimide binding and analysis of their site of translation. In particular, we were able to show the presence of an as yet unidentified δ subunit in CF<sub>1</sub> from *C. reinhardtii*.

We have identified a 70-kDa peripheral membrane protein in the thylakoid membranes of *C. reinhardtii*, which is immunologically related to the β subunit of CF<sub>1</sub>. We discuss its conceivable ATPase function with respect to the Ca<sup>2+</sup>-dependent ATPase activity previously reported in the thylakoid membranes from *C. reinhardtii* (Piccioni, R. G., Bennoun, P., and Chua, N. H. (1981) *Eur. J. Biochem.* 117, 93-102).

Energy transducing membranes in bacteria, mitochondria, and chloroplasts contain a H<sup>+</sup>-dependent ATP synthase. The functional properties of the enzyme result from the association of a catalytic extrinsic sector (C)F<sub>1</sub>,<sup>1</sup> containing nucleotide binding sites, with an intrinsic sector (C)F<sub>0</sub>, responsible for proton translocation across the membranes (for a review, see Ref. 1).

The enzymes from the three sources show large similarities, but significant differences nevertheless exist between F<sub>1</sub>F<sub>0</sub> from mitochondria and, for instance, CF<sub>1</sub>·CF<sub>0</sub> from higher plant chloroplasts. Whereas the chloroplast ATP synthase is comprised of five CF<sub>1</sub> subunits, αβτδε, and three to four CF<sub>0</sub> subunits, numbered I-IV (2, 3), mitochondrial ATP synthases contain at least three additional subunits, OSCP, F<sub>6</sub> and IF<sub>1</sub>, which are loosely bound to F<sub>1</sub>.

The current terminology is somewhat confusing since se-

quence analysis of the different subunits has shown the δ subunit from CF<sub>1</sub> to be more similar to the OSCP than to the δ subunit from F<sub>1</sub>, which in turn is homologous to ε from CF<sub>1</sub> (4). Subunit δ from CF<sub>1</sub> differs, however, from the OSCP subunit in that it does not confer oligomycin sensitivity to the membrane-bound enzyme. In addition, it is retained on CF<sub>1</sub> whereas OSCP is not found in F<sub>1</sub>, after extraction of the extrinsic moiety of the complex.

Characterization of the chloroplast ATP synthase from *Chlamydomonas reinhardtii* was of particular interest for several reasons. First, detailed knowledge of the subunit composition of the complex was a prerequisite to study its assembly in several mutants defective in photophosphorylation (see accompanying paper, 28). In this respect, since CF<sub>1</sub>·CF<sub>0</sub> complexes had never been extensively purified from this algae, the exact number of subunits, particularly in CF<sub>0</sub>, remained largely unknown. In addition, although CF<sub>1</sub> from *C. reinhardtii* had been extensively studied, there was little, if any, evidence for the presence of a δ subunit and positive identification of the catalytic subunit β remained controversial (5, 6). Second, some differences in the properties of CF<sub>1</sub> from *C. reinhardtii* and from higher plants suggested structural differences: at variance with spinach CF<sub>1</sub>, the enzyme from *C. reinhardtii* was reported (7) not to be activated by heat or proteolysis. Therefore, a detailed biochemical analysis of the chloroplast ATP synthase from *C. reinhardtii* was required in order to understand how it compared with its counterparts from mitochondria and higher plant chloroplast.

### MATERIALS AND METHODS

Chloroplast mutant FUD50 altered in the ATP synthase was isolated by P. Bennoun (Centre National de la Recherche Scientifique, Paris) and characterized by Woessner *et al.* (8).

BF25/FUD50 double mutant was obtained by crossing the BF25 nuclear mutant mt- by the FUD50 chloroplast mutant mt+ according to Levine and Ebersold (9). The nuclear BF25 mutant was previously described as altered in the oxygen evolving system due to the absence of a 20-kDa protein associated with PSII (10, 11).

All strains were grown at 25 °C in Tris acetate/phosphate medium (12) at a light intensity of 300 lux. Cells were harvested in late exponential phase (4.10<sup>6</sup> cells/ml), except for pulse labeling experiments (2.10<sup>6</sup> cells/ml) which were carried out according to Delepelaire (13). In these experiments cells were incubated in the presence of an inhibitor of cytoplasmic translation/cycloheximide (8 μg/ml) for 10 min before incubation with [<sup>14</sup>C]acetate (74 kBq/ml) for 45 min at a light intensity of 400 lux. It was stopped by the addition of an excess of unlabeled acetate. Purified thylakoids were prepared according to Chua and Bennoun (14).

CF<sub>1</sub> was released from the thylakoid membranes by chloroform extraction as described by Piccioni *et al.* (5). CF<sub>1</sub>·CF<sub>0</sub> was purified using the procedure of Pick and Racker (2), slightly modified as in Refs. 15 and 16: thylakoid membranes were solubilized in the presence of 1% octylglucoside, 0.5% cholate, the 100,000 × g supernatant was then subjected to ammonium sulfate fractionation and the 37-48% fraction was subjected to sucrose gradient centrifugation in the presence of 0.1% Triton X-100.

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<sup>1</sup> The abbreviations used are: CF, coupling factor; OSCP, oligomycin sensitivity conferring protein; OEE, oxygen-evolving enhancer; DCCD, dicyclohexyl-carbodiimide; SDS, sodium dodecyl sulfate; RBUP<sub>2</sub> Case, ribulose-bisphosphate carboxylase/oxygenase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

DCCD labeling was performed either on isolated ATP synthases according to Pick and Racker (2) or on purified thylakoids of *C. reinhardtii* by incubating the membranes (0.1 mg of chlorophyll/ml) with 20  $\mu$ M [ $^{14}$ C]DCCD in a Tricine buffer, pH 8, at 0 °C for 30 min.

ATP hydrolysis was measured *in vitro* according to Lien and Racker (17).

Nondenaturing gel electrophoresis and revelation of ATPase activity on gels were performed as described by Piccioni *et al.* (5). Denaturing gel electrophoresis, immunoblotting experiments, and autoradiographies were performed as described in Ref. 15.

Molecular weights of the polypeptides were estimated using high and low molecular weight calibration kits from Pharmacia LKB Biotechnology, Inc. Protein concentrations were determined using the procedure of Bensadoun and Weinstein (18).

Antibodies against ATP synthase subunits from *C. reinhardtii*, spinach, yeast mitochondria, and beef heart mitochondria were obtained by courtesy of N-H. Chua (Rockefeller University, New York, NY), J. M. Galmiche and G. Girault (Centre d'Etudes Nucleaires, Saclay), C. Leaver (University of Edinburgh), J. Lunardi and A. Dupuis (Centre d'Etudes Nucleaires, Grenoble), respectively. CF<sub>1</sub> and CF<sub>1</sub>·CF<sub>0</sub> from spinach and F<sub>0</sub>F<sub>1</sub>-enriched fractions from beef heart mitochondria were kindly supplied by J. M. Galmiche and G. Girault and by J. Lunardi, respectively.

## RESULTS

**Number of Subunits in the Chloroplast ATP Synthase from *C. reinhardtii***—In order to characterize the subunits constitutive of the chloroplast ATP synthase from *C. reinhardtii*, we first isolated the extrinsic part of the enzyme by a chloroform treatment of the thylakoid membranes. CF<sub>1</sub> was then further purified from the crude extract either by DEAE-cellulose chromatography or by nondenaturing electrophoresis. In the two cases we obtained a protein complex consisting

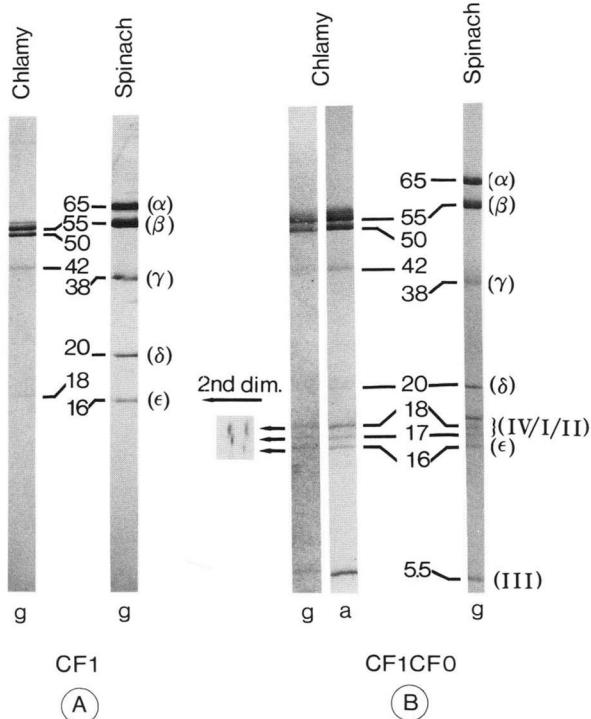


FIG. 1. Subunit composition of CF<sub>1</sub> (A) and CF<sub>1</sub>·CF<sub>0</sub> (B) preparations from spinach and *C. reinhardtii* analyzed on 12–18% acrylamide gels containing 8 M urea. Subunits in the complexes from spinach are identified by their name and apparent  $M_r$ . Subunits in the complexes from *C. reinhardtii* are indicated by their apparent  $M_r$  only. A 20-kDa subunit is observed in CF<sub>1</sub>·CF<sub>0</sub> from [ $^{14}$ C]acetate stationary labeled cells of *C. reinhardtii* after autoradiography (a) but not by Coomassie Blue staining (g). The inset corresponds to the 18–16-kDa region after two-dimensional gel electrophoresis (first dimension, 12–18% acrylamide with 8 M urea; second dimension, 7.5–15% acrylamide) of CF<sub>0</sub>·CF<sub>1</sub> preparations.

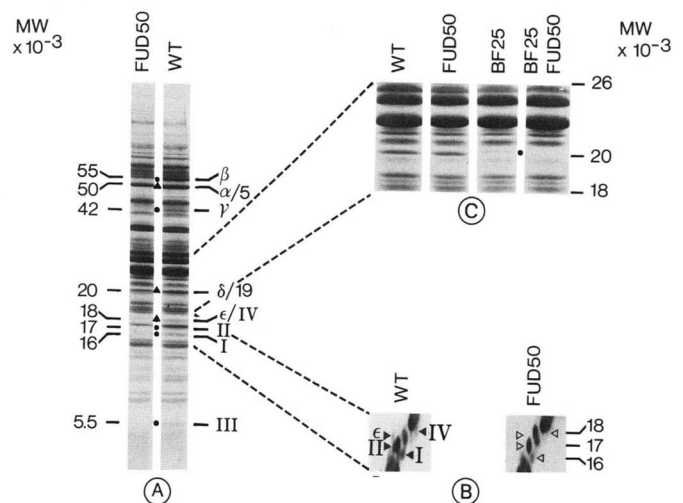


FIG. 2. A, polypeptide patterns of thylakoid membranes from the WT and FUD50 mutant lacking the chloroplast ATP synthase. Same gel system as described in the legend to Fig. 1. Identification of each ATP synthase subunit, lacking in the FUD50 mutant, and the corresponding apparent  $M_r$  are given on the left and right, respectively. 5–19 correspond to PSII polypeptides numbered following the nomenclature of Chua and Bennoun (14). B, two-dimensional gel electrophoresis after silver staining of the 18–16-kDa region of the FUD50 and WT thylakoids. First dimension, 12–18% acrylamide with 8 M urea; second dimension, 7.5–15% acrylamide gel; note the absence (white arrows) of two bands in the 18-kDa region in the FUD50 thylakoids. Also indicated are the losses of the 17- and 16-kDa polypeptides. C, enlargement of the 18–26-kDa region of a similar gel loaded with SDS-solubilized thylakoid membranes from the WT, the FUD50 mutant lacking the ATP synthase, the BF25 mutant specifically lacking in the OEE<sub>2</sub> subunit of PSII, and the double mutant BF25/FUD50. Note the presence of a minor 20-kDa band remaining in BF25 which is lacking in the double mutant.

in four subunits of apparent molecular masses, 55, 50, 42, and 18 kDa as deduced from their electrophoretic mobility on 12–18% polyacrylamide gels run in the presence of SDS and urea (Fig. 1A). Also visible is a 57-kDa polypeptide, the amount of which changes from one preparation to another. It migrates in the position of the large RBU<sub>p2</sub> Case subunit which is an expected contaminant since it is a major extrinsic-like component of the thylakoid membranes. In the absence of urea, the two bands of higher molecular weight comigrated as one band of 52 kDa (not shown). These observations are consistent with previous reports in which the four bands were attributed to the  $\alpha\beta\tau\epsilon$  subunits of CF<sub>1</sub> (5, 7). Negligible amounts of CF<sub>1</sub> were extracted by EDTA washing of the thylakoid membranes from *C. reinhardtii* (not shown), in contrast with the case of CF<sub>1</sub> from spinach. The polypeptide content of the latter complex is shown for comparison on Fig. 1A. Besides the presence of a fifth subunit in spinach, the putative  $\delta$  subunit, there was a shift in electrophoretic mobility of the bands of higher molecular weight.

CF<sub>1</sub>·CF<sub>0</sub> complexes from *C. reinhardtii* were purified after octylglucoside/cholate solubilization of the thylakoid membranes (for details see “Materials and Methods”). After checking the distribution of ATPase activity among the fractions of the sucrose gradient we analyzed the polypeptide content in the fraction of maximal specific activity (2–5  $\mu$ mol of P<sub>i</sub>/min/mg of protein, corresponding to Ca<sup>2+</sup>-ATPase assay in the presence of 0.5 mg/ml trypsin (17)) in comparison with that in CF<sub>1</sub>·CF<sub>0</sub> from spinach purified by the same procedure (Fig. 1B). The latter complex displays the nine subunits already characterized by several groups (2, 3), namely five CF<sub>1</sub> subunits ( $\alpha$ ,  $\beta$ ,  $\tau$ ,  $\delta$ , and  $\epsilon$ ) and four additional polypeptides constitutive of CF<sub>0</sub> (I, II, III, and IV). Coomassie Blue staining

TABLE I  
Identification of the subunits of the chloroplast ATP synthase from *C. reinhardtii*

Subunit	Apparent $M_r$	Site of translation <sup>c</sup>	Presence in		Accumulation in FUD50 cells	Membrane association in FUD50 <sup>a</sup>	DCCD binding in wild type	Immunological cross-reactivity
			CF <sub>1</sub>	CF <sub>1</sub> -CF <sub>0</sub>				
	<i>kDa</i>							
$\beta$	55 (55)	CHL	+	+	—	—	—	+
$\alpha$	50 (65)	CHL	+	+	—	—	—	+
$\tau$	42 (38)	CYT	+	+	—	ND <sup>d</sup>	—	ND
$\delta$	20 (20)	CYT	—	+	—	ND	—	+
$\epsilon$	18 (16)	CHL	+	+	—	—	—	ND
IV	18 (18.5)	CHL	—	+	—	+	—	ND
II	17 (17)	CYT	—	+	—	ND	—	ND
I	16 (18)	CHL	—	+	—	+	—	ND
III	5.5 (5.5)	CHL	—	+	—	+	+	ND

<sup>a</sup> Detected as <sup>14</sup>C-pulse-labeled polypeptides in thylakoid membranes.

<sup>b</sup> Corresponding  $M_r$  in spinach given in parentheses.

<sup>c</sup> CHL, translated in the chloroplast; CYT, translated in the cytoplasm.

<sup>d</sup> With antibodies against the corresponding spinach subunits.

<sup>e</sup> ND, not done.

(g) of the polypeptides in CF<sub>1</sub>-CF<sub>0</sub> complexes from *C. reinhardtii* resolved the four CF<sub>1</sub> subunits already identified on Fig. 1A and only three other subunits of 17, 16, and 5.5 kDa, most likely CF<sub>0</sub> subunits. However, two-dimensional gel electrophoresis of the CF<sub>1</sub>-CF<sub>0</sub> complex resolved an additional subunit comigrating with the 18-kDa CF<sub>1</sub> polypeptide (see inset in Fig. 1B). Autoradiography (a) of a similar preparation purified from cells grown in the presence of [<sup>14</sup>C]acetate allowed us to detect another additional band of 20 kDa (Fig. 1B). We note that contamination by the large RBU<sub>p2</sub> Case subunit is also visible in this preparation. Thus, we could distinguish nine subunits in the ATP synthase from *C. reinhardtii* as it was the case for the chloroplast ATP synthase from spinach.

Since CF<sub>1</sub>-CF<sub>0</sub> preparations might be either depleted in some genuine subunits of the complex or contaminated by copurifying polypeptides, we looked for an independent estimate of the subunit composition of CF<sub>1</sub>-CF<sub>0</sub>. This was achieved by the analysis of the pattern of polypeptide deficiencies in the thylakoid membranes of the FUD50 mutant (Fig. 2A) which bears a deletion in the chloroplast *atpB* gene coding for the  $\beta$  subunit and is therefore devoid of chloroplast ATP synthase (8, 19). Consistent with our identification of the subunits enriched in purified CF<sub>1</sub>-CF<sub>0</sub> were the absence of five bands of 55, 42, 17, 16, and 5.5 kDa (indicated by ● in Fig. 2A) and the deficiencies in the 50-, 20-, and the 18-kDa regions (indicated by ▲ in Fig. 2A), where comigrations are known to occur (20). Two-dimensional gel electrophoresis of the FUD50 thylakoids allowed us to observe the absence of the 50-kDa polypeptide (data not shown) and of two polypeptides, after silver staining, in the 18-kDa region (see Fig. 2B). Also visible in Fig. 2B are the absences of the 17- and 16-kDa polypeptides in the FUD50 mutant. Therefore, out of the nine subunits found in purified CF<sub>1</sub>-CF<sub>0</sub> complexes, all but one, in the 20-kDa region, could be identified independently as part of the chloroplast ATP synthase. The latter subunit was expected to migrate in the same 20-kDa region as the OEE<sub>2</sub> subunit of PSII (10). However, the two-dimensional gel system did not resolve two polypeptides in this molecular weight region. Therefore we constructed a double mutant by crossing the FUD50 mutant with the BF25 mutant specifically lacking in the OEE<sub>2</sub> subunit. A comparison of the 18–26-kDa region in the polypeptide patterns of the thylakoid membranes from the WT, FUD50, BF25, and FUD50/BF25 strains is shown on Fig. 2C. The BF25 mutant showed a much lower staining in the 20-kDa region as compared to that in the WT or the

FUD50. However, a 20-kDa polypeptide was still visible. In contrast, the double mutant FUD50/BF25 was totally lacking in 20-kDa polypeptides. This comparison demonstrates that the 20-kDa band observed in the WT resulted from the comigration of two polypeptides, a PSII subunit lacking in the BF25 mutant and an ATP synthase subunit lacking in the FUD50 mutant. Thus the analysis of both the polypeptide content in CF<sub>1</sub>-CF<sub>0</sub> preparations from *C. reinhardtii* and the polypeptide deficiencies in ATP synthase mutants are in good agreement and lead to the identification of nine subunits constitutive of the protein complex.

In order to further correlate these nine subunits with those which have been identified in ATP synthases from other sources, we studied their immunological cross-reactivity with well-defined antibodies, their site of translation, and their pattern of DCCD labeling. These results are described below and summarized in Table I.

**Immunological Cross-reactivity**—We have used several distinct antibodies raised against the  $\beta$  subunit of the ATP synthase from different sources (yeast mitochondria, beef heart mitochondria, and spinach chloroplast) in order to assign the CF<sub>1</sub> subunits of 55 and 50 kDa in *C. reinhardtii* to either the  $\alpha$  or  $\beta$  subunit. On Fig. 3A are compared immunoblots using antibodies against the  $\beta$  subunit from beef heart mitochondria and against the  $\alpha$  and  $\beta$  subunits from *C. reinhardtii* chloroplast. The anti- $\beta$  antibody recognized the 55-kDa band in the WT strain of *C. reinhardtii* which was absent in the  $\beta$ -less mutant FUD50. In addition, a 70-kDa polypeptide was recognized in the two strains. Its significance will be discussed in a separate section. The same observation was made with anti- $\beta$  antibodies from yeast mitochondria and from spinach (data not shown), thereby indicating that the  $\alpha$  subunit of *C. reinhardtii* CF<sub>1</sub> migrated ahead of the  $\beta$  subunit in urea gels. In contrast, the  $\alpha$  subunits had lower electrophoretic mobilities than the  $\beta$  subunits in (C)F<sub>1</sub> from spinach chloroplast or from beef heart mitochondria. Using the antibody against the ( $\alpha$  +  $\beta$ ) subunits from *C. reinhardtii*, we observed a labeling of only the  $\beta$  subunit from beef heart mitochondria F<sub>1</sub> (see Fig. 3A). We consistently observed that the  $\alpha$  subunits from F<sub>1</sub> (respectively CF<sub>1</sub>) were not immunodetected by antibodies raised against the  $\alpha$  subunit of CF<sub>1</sub> (respectively F<sub>1</sub>) (data not shown). Thus an immunological cross-reaction was observed between all  $\beta$  subunits but not between all  $\alpha$  subunits.

No cross-reaction in CF<sub>1</sub>-CF<sub>0</sub> complexes was observed using an antibody raised against the OSCP subunit of beef heart

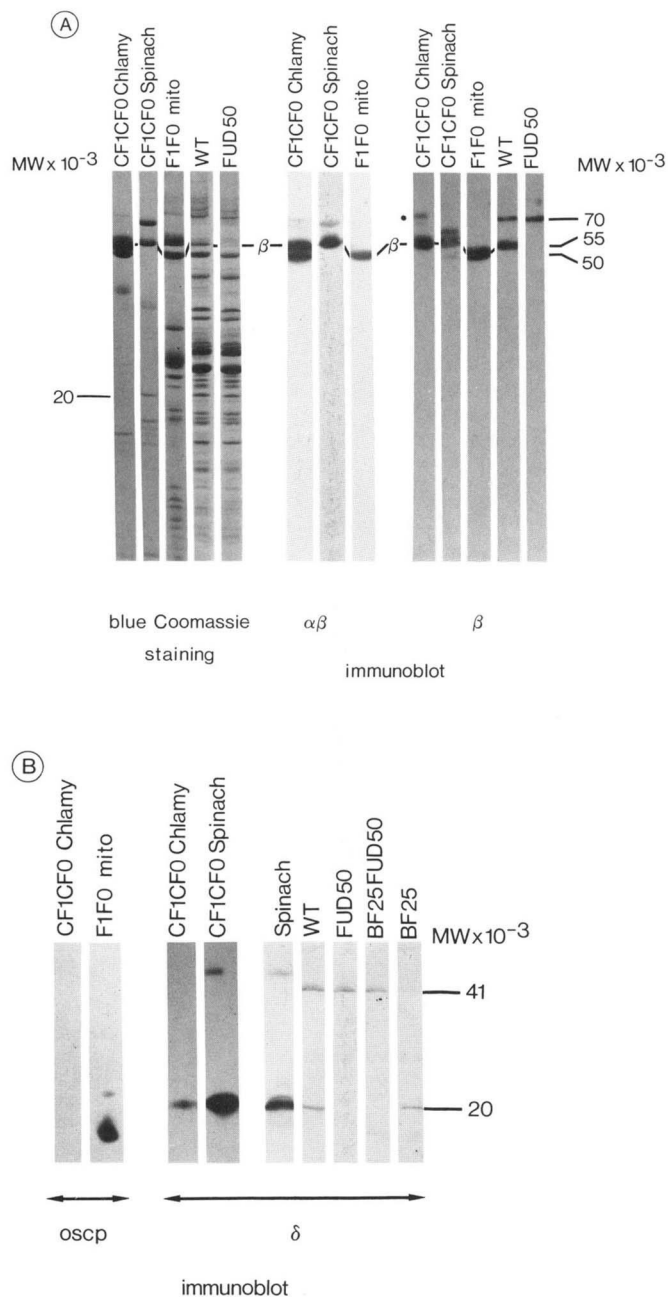


FIG. 3. *A*, ATP synthases from various sources and thylakoid membranes from WT and FUD50 after urea/SDS electrophoresis and Coomassie Blue staining or subjected to immunoblotting and probed with antibodies directed against  $\alpha/\beta$  or  $\beta$  subunits. Antibody recognition patterns of the 55-kDa bands in the different samples (-----) indicate that they correspond to the  $\beta$  subunit in chloroplasts from spinach and *C. reinhardtii* but to the  $\alpha$  subunit in beef heart mitochondria. Indicated by an asterisk (\*) is the 70-kDa polypeptide from *C. reinhardtii* which is present in CF<sub>0</sub>·CF<sub>1</sub> preparations and in the WT and FUD50 thylakoids. *B*, ATP synthases from various sources and thylakoid membranes from spinach, WT, FUD50, BF25, and BF25/FUD50 after urea/SDS electrophoresis, subjected to immunoblotting, and probed with antibodies directed against the OSCP or  $\delta$  subunits. A cross-reaction between 20-kDa polypeptides in spinach and *C. reinhardtii* is observed in purified complexes in WT and BF25 thylakoid membranes, but is absent in the FUD50 and BF25/FUD50 thylakoid membranes which lack the ATP synthase. Also visible is a cross-reaction with a 41-kDa polypeptide of unknown origin, the presence of which is not related to the presence of the ATP synthase in *C. reinhardtii*.

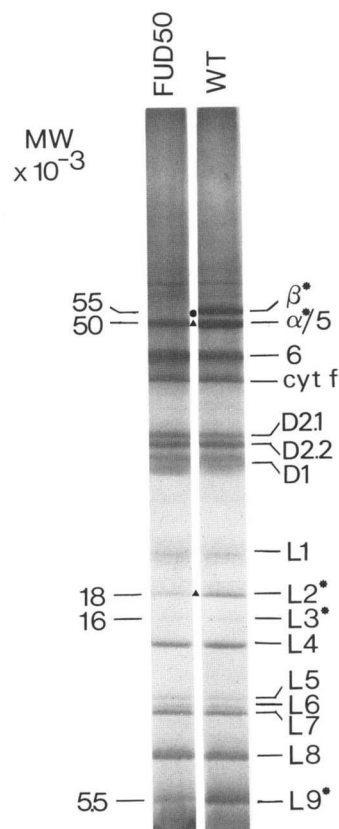


FIG. 4. Autoradiogram of chloroplast translates in thylakoid membranes from the WT and F mutant. Cells were pulse-labeled for 45 min with [<sup>14</sup>C]acetate in the presence of cycloheximide. Molecular weights are indicated in positions corresponding to ATP synthase subunits of chloroplast origin (\*). Polypeptides are named after Delepelaire (13). L2, L3, and L9 correspond respectively, to subunits IV, I, and III.

mitochondria (see left side of Fig. 3B). In contrast, we observed a weak but significant cross-reaction of a 20-kDa polypeptide with the antibody against the  $\delta$  subunit from spinach CF<sub>1</sub>. This was visible either in the CF<sub>0</sub>·CF<sub>1</sub> complex or in the thylakoid membranes from WT and BF25 strains. In contrast, no cross-reaction was detected in the FUD50 and BF25/FUD50 mutants, both of which lack the ATP synthase. The anti- $\delta$  antibody also recognized a polypeptide of higher molecular weight both in spinach and in *C. reinhardtii*. This polypeptide is not an ATP synthase polypeptide since recognition still occurred in the mutants lacking ATP synthase. Thus, the ATP synthase subunit of 20 kDa, which comigrates with the OEE<sub>2</sub> subunit upon electrophoresis of thylakoid membranes from *C. reinhardtii* on urea gels (see Fig. 2C), is the  $\delta$  subunit of CF<sub>1</sub>.

*Chloroplast Translates*—Previous work on the chloroplast ATP synthase from higher plants have shown that the  $\alpha\beta\epsilon$  subunits of CF<sub>1</sub> and subunits I, III, and IV of CF<sub>0</sub> are encoded in the chloroplast genome (3) and therefore translated on chloroplast ribosomes. Fig. 4 shows the chloroplast translates associated with the thylakoid membranes in the FUD50 and WT strains of *C. reinhardtii*. The FUD50 thylakoids lacked a 55-kDa band which was immunodetected as the  $\beta$  subunit (indicated by ● on Fig. 4) and were deficient in 50- and 18-kDa bands (indicated by ▲ in Fig. 4). Two-dimensional gel electrophoresis (Fig. 5) showed that comigration occurred in the 50-kDa region in the WT: one spot being the PSII core subunit "5" (20) also of chloroplast origin and the other the ATP synthase subunit lacking in the FUD50 thylakoids and

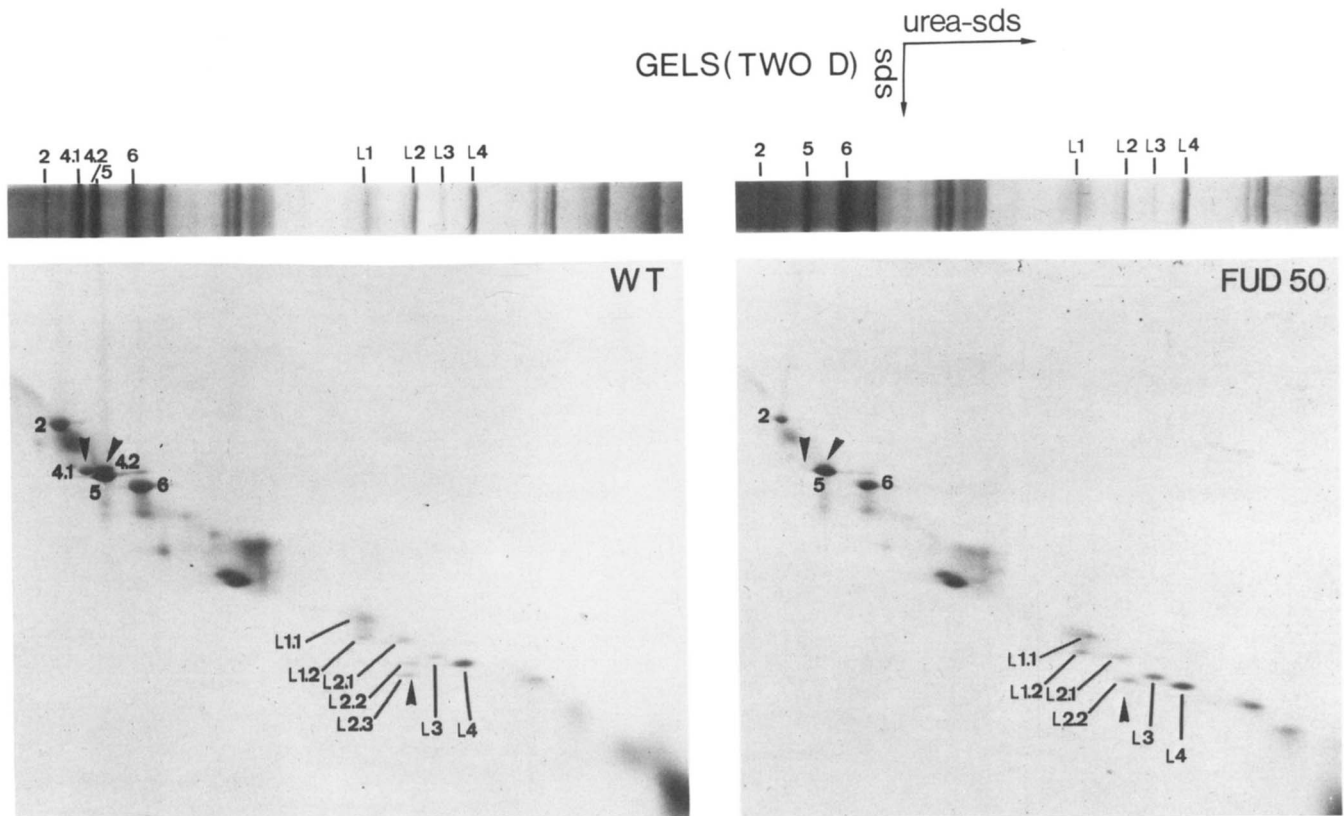


FIG. 5. Same as Fig. 4 using two-dimensional SDS-gel electrophoresis. First dimension, 12–18% acrylamide gel with 8 M urea. Second dimension, 7.5–15% acrylamide gel. Note the absence (arrows) of bands 4.1 and 4.2 ( $\alpha$  and  $\beta$ ) and L2.3 ( $\epsilon$ ) in the FUD50 thylakoids. In contrast, CF<sub>0</sub> subunits IV (L2.1) and I (L3) are clearly visible.

identified as the  $\alpha$  subunit of CF<sub>1</sub> by immunological experiments (see above). These observations are consistent with the localization of the genes of  $\alpha$  and  $\beta$  subunits on the chloroplast genome in *C. reinhardtii* (8, 21). As previously described by Delepelaire (20), three polypeptides of chloroplast origin (namely L2.1, L2.2, and L2.3) comigrated in the 18-kDa band, one of which L2.3 being an ATP synthase subunit since it was lacking in the FUD50 thylakoids (see Fig. 5). Therefore, as CF<sub>1</sub> complexes contained such a 18-kDa polypeptide, we assigned this one to the  $\epsilon$  subunit.

The complexity of the pattern of polypeptides synthesized on cytoplasm ribosomes prevented us from identifying directly the nuclear-encoded ATP synthase subunits. However, as no chloroplast translates were observed in the 20- and 42-kDa positions, we conclude that the former identified by immunological reaction (see Fig. 3B, left) as  $\delta$ , is nuclear-encoded as it is the case in spinach, whereas the latter corresponded to the nuclear-encoded  $\tau$  subunit.

We did not expect that chloroplast translates corresponding to CF<sub>0</sub> subunits would be necessarily missing from FUD50 thylakoid membranes since these subunits belong to an integral membrane-protein complex and may be inserted in the membrane even though they would not accumulate in the absence of the peripheral part of the enzyme. Out of the four polypeptides of 18, 16, 17, and 5.5 kDa, which were possible CF<sub>0</sub> subunits (Fig. 1) and did not accumulate in the FUD50 thylakoids (Fig. 2), only the 17-kDa polypeptide did not migrate in the position of a chloroplast translate and was then attributed to subunit II of CF<sub>0</sub>. The others migrated in the same position as three chloroplast translates, L2.1, L3, and L9, respectively (Figs. 4 and 5).

**[<sup>14</sup>C]DCCD Labeling**—As has been previously reported for ATP synthases from spinach chloroplast and beef heart mitochondria (2, 22), subunit III of (C)F<sub>0</sub> can be identified by [<sup>14</sup>C]DCCD labeling (see Fig. 6). Among the half-dozen polypeptides labeled in the WT thylakoid membranes, only the 5.5-kDa one was present in purified CF<sub>1</sub>·CF<sub>0</sub> and missing from the thylakoid membranes of the FUD50 mutant (Fig. 6). Therefore the chloroplast translate L9 (5.5 kDa) corresponded to subunit III of CF<sub>0</sub>. The chloroplast translates L2.1 (18 kDa) and L3 (16 kDa) were attributed to the two remaining CF<sub>0</sub> subunits which are chloroplast encoded in spinach (3) and were arbitrarily named subunit IV and I, respectively.

**The 70-kDa Protein**—Most of the CF<sub>1</sub>·CF<sub>0</sub> preparations in *C. reinhardtii* contained an additional polypeptide of apparent molecular mass, 70 kDa, which cross-reacted with antibodies directed against the  $\beta$  subunit (indicated by \* on Fig. 3). This polypeptide was found together with CF<sub>1</sub> in the crude extract from WT thylakoid membranes when treated with chloroform or subjected to an alkaline shock (not shown), and was still present in FUD50 thylakoid membranes (Fig. 3, right, \*).

Piccioni *et al.* (5) have described in *C. reinhardtii* the presence of an additional latent ATPase activity on the thylakoid membranes of a mutant lacking ATP synthase activity. This activity could be recovered in the soluble phase after chloroform treatment of thylakoid membranes. In order to understand if the 70-kDa protein was responsible for this activity, crude extracts released by chloroform treatment of WT and FUD50 thylakoid membranes, were analyzed by nondenaturing gel electrophoresis according to Piccioni *et al.* (5). Silver staining revealed three bands in the WT extract (Fig. 7), indicated by 1, 2, and 3 in order of increasing

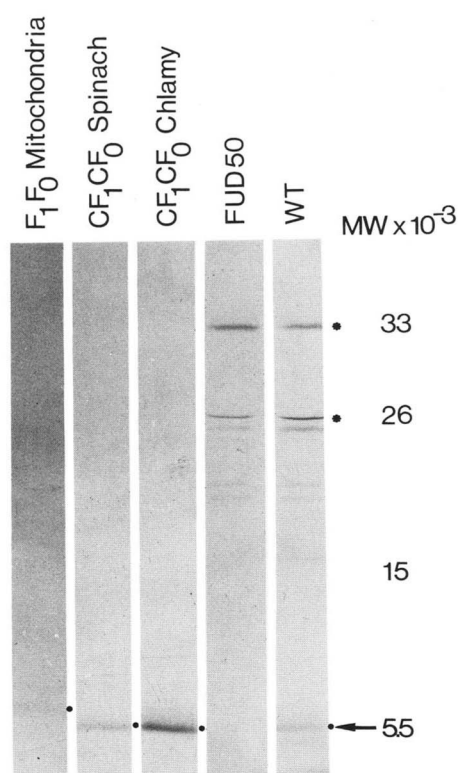


FIG. 6. Autoradiogram of a urea-SDS gel loaded with [ $^{14}\text{C}$ ] DCCD-labeled ATP synthases from various sources and thylakoid membranes from WT and FUD50 strains. Note the presence of a 5.5-kDa labeled band in purified complexes and in WT thylakoid membranes, but its absence in FUD50 thylakoid membranes. Asterisk (\*) points to several labeled membrane polypeptides of unknown function present in the WT and FUD50 strains.

electrophoretic mobility. Only one band, in the position of band 3, was found in the FUD50 extract (Fig. 7). An additional diffuse band was occasionally observed ( $\star$  on Fig. 7A).

Immunoblotting experiments were performed on these nondenaturing gels using anti- $\beta$  antibodies. All three bands were labeled (Fig. 7, see bands 1 and 2 in the WT extract and band 3 in the FUD50 extract). Polypeptide composition in each band was analyzed by carrying out a second electrophoresis in the presence of urea/SDS (Fig. 7C). Silver staining reveals that bands 1 and 2 were made of  $\text{CF}_1$  subunits. Band 3 contained only the 70-kDa polypeptide. Finally, bands 1 and 2 showed  $\text{Ca}^{2+}$ -dependent ATPase activity on nondenaturing gels, whereas band 3 did not (not shown).

#### DISCUSSION

We were able to resolve nine subunits in the chloroplast ATP synthase from *C. reinhardtii*. That these polypeptides are genuine ATP synthase subunits is supported by the perfect complementarity between the polypeptide deficiencies observed in thylakoid membranes in an ATP synthase mutant and the enrichment in these polypeptides in purified fractions showing ATPase activity.

The experimental evidence supporting the identification of each ATP synthase subunit is summarized in Table 1. Comparison with the chloroplast ATP synthase from spinach shows that despite some differences in apparent molecular weights, particularly between the  $\alpha$  subunits, the subunits in the two cases were very similar. In particular, our study using site-specific inhibitors of translation, indicated that the origin of synthesis of the chloroplast ATP synthase subunits is conserved between *C. reinhardtii* and higher plants (3).

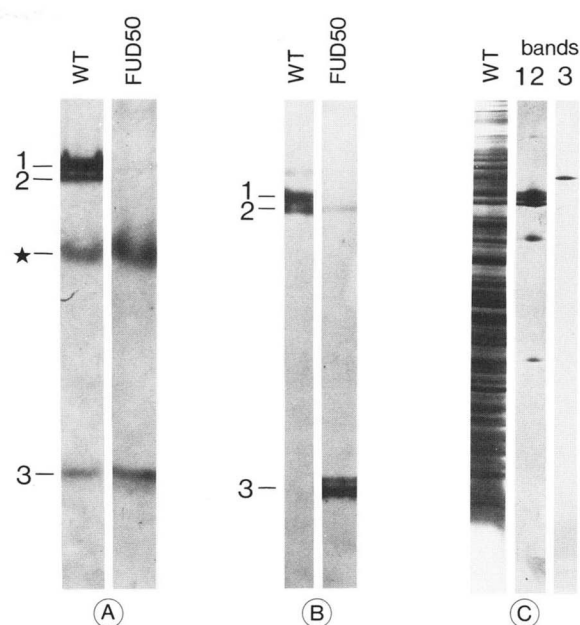


FIG. 7. Nondenaturing electrophoresis (6% acrylamide gels) of proteins extracted by a chloroform shock from WT and FUD50 thylakoid membranes. A, silver staining; B, immunoblots with antibodies against the  $\beta$  subunit of  $\text{CF}_1$ . Three bands, 1–3, are recovered from WT, whereas only the region of band 3 remains visible in the case of FUD50. Asterisk (\*) indicates a band occasionally observed after silver staining in these experiments. C, urea/SDS electrophoresis of bands 1–3 (silver staining). Bands 1 and 2 contain  $\text{CF}_1$  subunits, but band 3 contains a single 70-kDa polypeptide.

We note that  $\text{CF}_0$  subunits were inserted in the membranes of the FUD50 mutant independent from the presence of  $\text{CF}_1$  subunits. However, their accumulation was prevented in the mutant. Such insight on the biogenesis of the chloroplast ATP synthase in *C. reinhardtii* is further developed in the accompanying paper (28) where eight ATP synthase mutants were analyzed.

On the basis of immunological cross-reactions, we concluded that the  $\alpha$  subunit of *C. reinhardtii*  $\text{CF}_1$  had a lower apparent molecular weight than the  $\beta$  subunit in urea gels. This is at variance with the behavior of the  $\alpha$  and  $\beta$  subunits from spinach  $\text{CF}_1$  or yeast and beef heart  $\text{F}_1$ , when probed with the same set of antibodies. Our conclusion is in agreement with that of Piccioni *et al.* (5) and argues for either differences in molecular weights or covalent modification between the  $\alpha$  subunits of  $\text{CF}_1$  in spinach and *C. reinhardtii*. The conflicting observation reported by Merchant and Selman (6) was likely to arise from the use of a gel system lacking urea. We observed that whereas the  $\beta$  subunits from several chloroplast and mitochondrial ATP synthases showed similar apparent molecular weights and cross-reacted with  $\beta$  antibodies from all sources, the  $\alpha$  subunits from  $\text{CF}_1$  and  $\text{F}_1$  showed no immunological cross-reaction. This is consistent with the higher sequence conservation of  $\beta$  than  $\alpha$  subunits in the ATP synthase from different sources (23). Complete sequence of the  $\alpha$  subunit from  $\text{CF}_1$  in *C. reinhardtii*, when available, will help clarifying the possible differences with its counterpart from spinach  $\text{CF}_1$ .

We were able to get a positive identification of the  $\delta$  subunit of  $\text{CF}_1$  from *C. reinhardtii* by comparing thylakoid membranes from several mutant strains as well as by immunological cross-reaction with an anti- $\delta$  antibody from spinach, using purified  $\text{CF}_1\text{-CF}_0$  complexes and thylakoid membranes when they contained the ATP synthase. As previously reported by others

(5, 7), the  $\delta$  subunit was never found in CF<sub>1</sub> extracted from chloroform-treated thylakoid membranes from *C. reinhardtii*. This treatment, when applied to higher plant thylakoids is known to produce a CF<sub>1</sub> complex largely depleted in  $\delta$  subunit (24). However, the lability of  $\delta$  does not totally prevent its detection in the chloroform crude extract from higher plant thylakoids (24). This difference in detection of  $\delta$  between CF<sub>1</sub> from higher plants and that from *C. reinhardtii*, together with the lower EDTA sensitivity of CF<sub>1</sub> extraction in the latter, could be indicative of some modified CF<sub>1</sub>·CF<sub>0</sub> interaction in the algal thylakoid membranes. It resembled that of F<sub>1</sub> which cannot be released by EDTA treatment of mitochondrial particles (25). In the latter case, the OSCP subunit, which is homologous to the  $\delta$  subunit in higher plant CF<sub>1</sub> (23), is retained on the membrane upon extraction of F<sub>1</sub> (25). However, the absence of a cross-reaction in CF<sub>1</sub>·CF<sub>0</sub> preparations from *C. reinhardtii* with an antibody raised against the OSCP subunit from beef heart F<sub>1</sub>F<sub>0</sub> argues against the idea that this  $\delta$  subunit is closely related to mitochondrial OSCP. In addition, the chloroplast ATP synthase activity in *C. reinhardtii* did not show oligomycin sensitivity when assayed in intact cells (data not shown) or in open cells.<sup>2</sup> Thus, we are led to exclude that the differences observed between CF<sub>1</sub> from higher plants and *C. reinhardtii*, were based on a closer phylogenetic proximity of the latter with mitochondrial ATP synthases.

A 70-kDa polypeptide, immunologically related to the  $\beta$  subunit of CF<sub>1</sub>, was found in the WT thylakoid membranes. Since nucleotide-binding proteins, such as kinases or ATPases show homologous regions in their primary sequences (26), the 70-kDa polypeptide could correspond to one of the thylakoid bound kinases, the activity of which has been described in *C. reinhardtii* (27) or to a minor ATPase of unknown physiological role. We note that the 70-kDa polypeptide behaved as a peripheral membrane protein since it was released from the thylakoid membranes by alkaline or chloroform shock. It was also detected in CF<sub>1</sub>·CF<sub>0</sub>-enriched fractions obtained by octylglucoside/cholate solubilization of the thylakoid membranes. However, it is not part of the chloroplast ATP synthase since it was still present in normal amounts in all the mutants defective in photophosphorylation that we have studied. These results may be related to the previous report of Piccioni *et al.* (5). These authors detected a Ca<sup>2+</sup>-dependent ATPase activity in the crude extract of chloroform-treated thylakoid membranes from either the WT of *C. reinhardtii* or the thm24 mutant lacking in CF<sub>1</sub>·CF<sub>0</sub>. In addition, when detected directly on gels after nondenaturing electrophoresis, such Ca<sup>2+</sup>-ATPase activity was found in three distinct bands, the one of lower mobility corresponding to this non-CF<sub>1</sub> ATPase. However, Piccioni *et al.* (5) were not able to identify its constitutive subunits. Using a similar experimental procedure we detected the two CF<sub>1</sub>-containing bands showing ATPase activity as described in Ref. 5. At variance with Piccioni *et al.* (5), the third band migrated faster and not slower than the CF<sub>1</sub> bands. This band showed no Ca<sup>2+</sup>-ATPase activity but contained a single polypeptide of 70 kDa, which cross-reacted with  $\beta$  antibodies. These two sets of data can be reconciled if one considers that the difference in mobility of the non-CF<sub>1</sub> band between our work and that of

Piccioni *et al.* (5) reflects a different extent of denaturation and inactivation of the Ca<sup>2+</sup>-ATPase, perhaps due to the dissociation of an oligomer of 70-kDa subunits.

These hypotheses are now under study and should open the way to an understanding of the physiological function of this enzyme in *C. reinhardtii*.

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#### REFERENCES

- Vignais, P. V. & Satre, M. (1984) *Mol. Cell. Biochem.* **60**, 33–70
- Pick, U. & Racker, E. (1979) *J. Biol. Chem.* **254**, 2793–2799
- Hennig, J. & Hermann, R. G. (1986) *Mol. Gen. Genet.* **203**, 117–128
- Walker, J. E., Runswick, M. J. & Saraste, M. (1982) *FEBS Lett.* **146**, 393–396
- Piccioni, R. G., Bennoun, P. & Chua, N. H. (1981) *Eur. J. Biochem.* **117**, 93–102
- Merchant, S. & Selman, B. R. (1983) *Eur. J. Biochem.* **137**, 373–376
- Selman-Reimer, S., Merchant, S. & Selman, B. R. (1981) *Biochemistry* **20**, 5476–5482
- Woessner, J. P., Masson, A., Harris, E. H., Bennoun, P., Gillham, N. W. & Boynton, J. E. (1984) *Plant Mol. Biol.* **3**, 177–190
- Levine, R. P. & Ebersold, W. T. (1960) *Annu. Rev. Microbiol.* **14**, 197–214
- Bennoun, P., Diner, B. A., Wollman, F.-A., Schmidt, G. & Chua, N. H. (1981) in *Photosynthesis III. Structure and Molecular Organization of the Photosynthetic Apparatus* (Akoyunoglou, G., ed) pp. 839–849, Balaban, Philadelphia
- Mayfield, S., Rahire, M., Frank, G., Zuber, H. & Rochaix, J.-D. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 749–753
- Gorman, D. S. & Levine, R. P. (1965) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 4352–4356
- Delepelaire, P. (1983) *Photochem. Photobiophys.* **6**, 279–291
- Chua, N. H. & Bennoun, P. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72**, 2175–2179
- Lemaire, C., Girard-Bascou, J., Wollman, F.-A. & Bennoun, P. (1986) *Biochim. Biophys. Acta* **851**, 229–238
- Wollman, F.-A. & Lemaire, C. (1988) *Biochim. Biophys. Acta* **933**, 85–94
- Lien, S. & Racker, E. (1971) *Methods Enzymol.* **23**, 547–555
- Bensadoun, A. & Weinstein, D. (1976) *Anal. Biochem.* **70**, 241–250
- Lemaire, C., Wollman, F.-A. & Bennoun, P. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1344–1348
- Delepelaire, P. (1984) *EMBO J.* **3**, 701–706
- Hallick, R. B. (1984) *FEBS Lett.* **177**, 274–276
- Pougeois, R., Satre, M. & Vignais, P. V. (1980) *FEBS Lett.* **117**, 344–348
- Walker, J. E., Fearnley, I. M., Gay, N. J., Gibson, B. W., Northrop, F. D., Powell, S. J., Runswick, M. J., Saraste, M. & Tybulewicz, V. L. J. (1985) *J. Mol. Biol.* **184**, 677–701
- Younis, H. M., Winget, G. D. & Racker, E. (1977) *J. Biol. Chem.* **252**, 1814–1818
- Ernster, L., Hundal, T., Norling, B., Sandri, G., Wojtczak, Grinkovich, V. A., Modyanov, N. N. & Ovchinnikov, Y. U. (1986) *Chem. Scr.* **26B**, 273–279
- Walker, J. E., Saraste, M., Runswick, M. J. & Gay, N. J. (1982) *EMBO J.* **8**, 945–951
- Wollman, F.-A. & Lemaire, C. (1988) in *Photosynthetic Production of Energy Rich Compounds* (Hall, D., Grassi, G., eds) Elsevier Scientific Publishing Co., Amsterdam
- Lemaire, C. & Wollman, F.-A. (1989) *J. Biol. Chem.* **264**, 10235–10242

<sup>2</sup> J. M. Galmiche, personal communication.