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Restoration of phototrophic growth in a mutant of *Chlamydomonas reinhardtii* in which the chloroplast *atpB* gene of the ATP synthase has a deletion: An example of mitochondria-dependent photosynthesis

(mitochondrial inhibitors/suppressor mutations/phosphate translocator/ADP-ATP carrier)

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ABSTRACT The *Chlamydomonas reinhardtii* mutant FUD50 has a deletion in the *atpB* gene of the chloroplast ATP synthase [Woessner, J. P., Masson, A., Harris, E. H., Bennoun, P., Gillham, N. W., and Boynton, J. E. (1984) *Plant Mol. Biol.* 3, 177–190]. We have isolated a suppressed strain (FUD50su) that can grow under phototrophic conditions, although it still showed no synthesis of the β subunit of coupling factor 1. Thylakoid membranes of the FUD50su strain were similar to those of the original FUD50 strain, in that they both lacked all the subunits making up the chloroplast ATP synthase complex. We show that photosynthesis in FUD50su is sensitive to inhibitors such as antimycin, specific for mitochondrial electron transport. This observation indicates that photosynthesis in the FUD50su strain is achieved through an unusual interaction between mitochondria and chloroplast. Exportation of light-induced reduced compounds from the chloroplast to the mitochondria elicits ATP formation in the latter, and ATP is subsequently imported to the chloroplast. The activation of such an ATP shuttle coupled to an NADPH shuttle would thus provide the reducing power and the free energy needed for carbon assimilation in a chloroplast that lacks chloroplast ATP synthase.

Light energy is converted to chemical potential in the chloroplast of green algae and higher plants by the light-induced vectorial electron transfer resulting in the reduction of NADP⁺ and in the formation of an electrochemical gradient across the thylakoid membrane. This gradient is used for ATP synthesis by a membrane-bound enzyme, ATP synthase, as first suggested by Mitchell (1). NADPH and ATP of photosynthetic origin are then used to convert inorganic carbon (CO₂) into reduced carbon compounds through the Calvin cycle. Mutants deficient either in the chloroplast ATP synthase or in some component of the electron transport chain are, therefore, incapable of photosynthetic carbon assimilation and must be grown under heterotrophic conditions. This growth requirement has been used to screen photosynthesis mutants (2). In this study, we report on the paradoxical existence of a photosynthesis mutant able to grow under phototrophic conditions, although it lacks the chloroplast ATP synthase.

MATERIALS AND METHODS

Mutant Strains. The FUD50 strain has been shown to be deficient in chloroplast ATP synthase due to a deletion in the *atpB* gene (3). The *Chlamydomonas reinhardtii* strain FUD50su was isolated after mutagenesis of FUD50 cells

with azide as described (4). This mutagenesis was followed by several enrichment steps in which cells were cultured in minimal medium (Tris/phosphate medium with pH adjusted to 7.2 with hydrochloric acid) in the light (1000 lux). After plating, colonies able to grow on minimal medium were selected, and one of them was used in this study. Genetic analysis of FUD50su showed that FUD50 could be recovered from FUD50su⁺ × FUD50⁻ crosses. On the other hand, the transmission of the characteristics of the FUD50su strains was identical in reciprocal crosses, therefore, excluding chloroplast inheritance and was consistent with the presence of at least two nuclear mutations.

Growth. Strains were grown at 25°C either under phototrophic conditions (minimal medium at a light intensity of 1000 lux except when otherwise indicated) or in mixotrophic conditions [Tris/acetate/phosphate medium (TAP) (5) at a light intensity of 300 lux] except for the FUD50 mutant that can only grow in heterotrophic conditions. The growth rates of the various strains were estimated by counting cells with a hemacytometer.

Methods. Kinetics of the 515-nm absorption change were measured *in vivo* according to Joliot and Delosme (6). Absorption changes were induced by a strong actinic flash through a broad-band blue filter and measured by using monochromatic detecting flashes. Thylakoid membranes were purified according to the procedure of Chua and Bennoun (7). Gel electrophoresis, pulse-labeling experiments, and autoradiographs were performed as described (8). The coupling factor (CF) complex CF0-CF1 was purified by a procedure similar to that of Pick and Racker (9) except that ammonium sulfate was omitted during membrane solubilization. Oxygen exchange was measured with a platinum/KCl/silver Clark electrode (10), in the presence of 10 mM bicarbonate.

RESULTS

Biochemical Characterization. Fig. 1A shows urea/NaDodSO₄ polyacrylamide gels comparing polypeptides from the thylakoid membranes of the FUD50, FUD50su, and wild-type (wt) strains as well as polypeptides from purified *C. reinhardtii* CF0-CF1 complexes. These complexes, although showing some contamination by high molecular weight polypeptides, contained the same eight subunits described for similar complexes from spinach thylakoids (11, 12). Thylakoids from the two mutant strains had exactly the same polypeptide composition and were both deficient in the CF0-CF1 subunits (for details, see Fig. 1A). Polypeptides synthesized inside the chloroplast and inserted into the membranes were then analyzed by using purified thylakoids

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Abbreviations: CF, coupling factor; wt, wild-type.

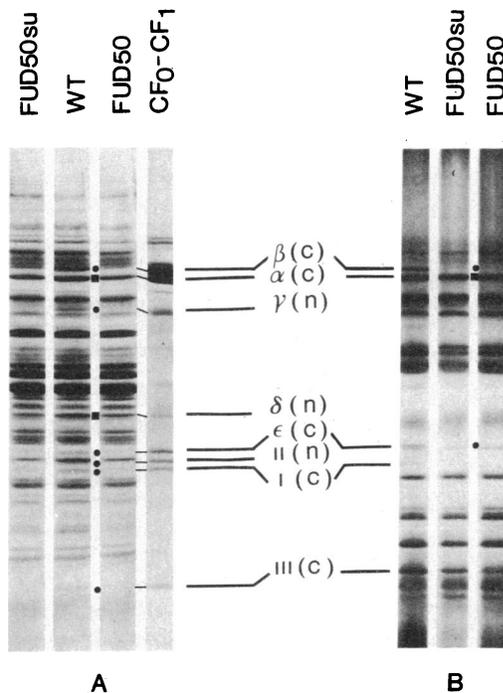


FIG. 1. (A) Polypeptides from thylakoid membranes of FUD50, FUD50su, and wt strains and from preparations enriched in CF0-CF1 were separated by urea/NaDodSO₄ polyacrylamide gel electrophoresis (and visualized by Coomassie blue staining). ●, Polypeptide deficiencies that are visible in the mutants strains (β , γ , and ϵ of CF1; I, II, and III of CF0); ■, polypeptide deficiencies that are hidden by comigrating polypeptides (α and δ of CF1). (B) Autoradiograms of chloroplast translation products, inserted in the thylakoid membranes from wt and mutant strains, viewed after urea/NaDodSO₄ polyacrylamide gel electrophoresis. ●, Polypeptides lacking in the mutant membranes; ■, polypeptide deficiencies hidden by comigrating polypeptides. n, Nuclear-encoded subunits; c, chloroplast-encoded subunits.

prepared from cells pulse-labeled in the presence of cycloheximide to inhibit cytoplasmic translation (13). Fig. 1B shows the absence of the β and ϵ subunits of CF1 in the mutants although insertion of the CF0 subunit III still occurred. The absence of the α subunit was also documented by using another gel system that separated the α subunit from the chloroplast-encoded 50-kDa subunit of the photosystem II core (data not shown). Further analysis of pulse-labeled polypeptides in whole cells showed that the two mutant strains were totally deficient in the synthesis of the β subunit whereas a slight amount of α subunit could be detected (data not shown).

Absorbance Changes at 515 nm. Light-induced absorbance changes at 515 nm were attributed, by Junge and Witt (14), to a shift in the absorption spectra of thylakoid pigments, mainly chlorophyll b and carotenoids, caused by the generation of a transmembrane electric field. It has been shown that the relaxation of the electric field in the 50- to 500-ms time range is correlated with the activity of the ATP synthase that collapses the membrane potential. A slower decay phase (time range, >1 s) is correlated with the passive leak of protons through the membrane (15, 16). Comparison of the 515-nm absorbance changes in wt and mutant strains is shown in Fig. 2. As reported by Joliot (17), the ATP synthase in the wt strain was only slightly active in the absence of a preillumination (curve A), and the relaxation of the 515-nm absorbance change was slow ($t_{1/2} = 320$ ms). Activation of the ATP synthase by a preillumination produced a dramatic decrease in the $t_{1/2}$ of the decay phase (curve B) ($t_{1/2} = 20$ ms), which was prevented by addition of tri-*n*-butyltin (curve C)

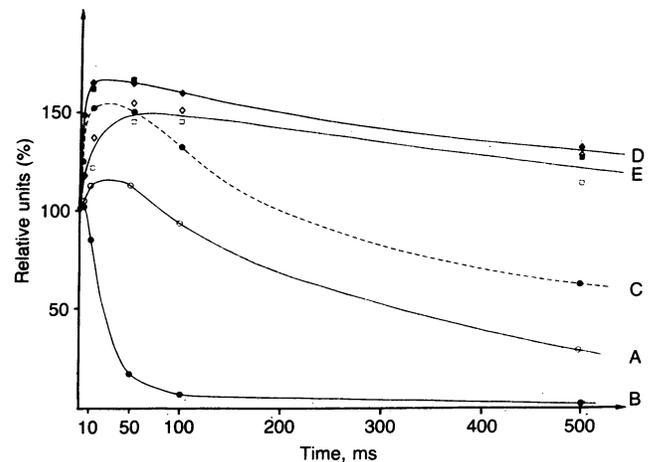


FIG. 2. Light-induced absorbance changes in wt (●, ○), FUD50 (■, □), and FUD50su (◆, ◇) strains, measured at 515 nm, with (●, ■, ◆) or without (○, □, ◇) a 30-s preillumination with broad red light. Broken line corresponds to an experiment that used wt cells in the presence of 0.5 μ M tri-*n*-butyltin. The results are expressed in relative units referring to the amplitude of the rapid-rise phase of the field indicating a change detected 300 μ s after an actinic flash.

($t_{1/2} = 700$ ms), an inhibitor of the ATP synthase (17). Because of the absence of CF0-CF1 in the FUD50 mutant, the relaxation of the transmembrane electric field displayed the same slow decay phase independent of preillumination (curves D and E, $t_{1/2} = 2700$ and 2500 ms, respectively) (6). The relaxation of the transmembrane electric field in the FUD50su strain was similar to that in the FUD50 strain and was also independent of a preillumination (curves D and E). These results confirmed the absence of ATP synthase activity in the thylakoid membrane of the FUD50su strain.

Growth Rate Characteristics. Under phototrophic conditions, the generation time of the wt strain decreased with increasing light intensities from 12.5 hr at 1000 lux to 9 hr at 2000 lux and to 8.5 hr at 4000 lux. In contrast, high light intensities had a deleterious effect on the growth of the FUD50su strain. A similar effect has been observed (18) by using mutants of *C. reinhardtii* deficient in ATP synthase, when grown in mixotrophic conditions. This deleterious effect of high-intensity light, in such mutants, is likely due to the light-induced accumulation of a high electrochemical gradient that may induce photoinhibition (see for review ref. 19). Therefore, the similar behavior of FUD50su cells further substantiates the absence of a membrane bound ATP synthase that was indicated by the slow relaxation of the 515-nm electrochromic shift. The generation time of the FUD50su strain was 32 hr at 1000 lux and 60 hr at 2000 lux, whereas growth was almost abolished at 4000 lux. At the optimal light intensity for the FUD50su strain (1000 lux), there was a growth rate ratio of 2.6 between the wt and FUD50su strains (see Fig. 3).

Sensitivity in FUD50su Strain Toward Inhibitors of the Mitochondrial Electron Transport Chain. Although deficient in chloroplast ATP synthase, the FUD50su strain was able to grow under phototrophic conditions and thus to synthesize carbon compounds through the Calvin cycle, which requires both ATP and NADPH. Therefore, the question arises as to how this strain can supply the chloroplast with ATP.

In algal cells, photosynthetic and mitochondrial electron transport are coupled to ATP production. To check whether mitochondria were involved in carbon metabolism in the FUD50su strain, we tested the effect of mitochondrial inhibitors on growth rates, on photosynthesis, and on respiration in wt and mutant strains grown under phototrophic conditions.

Antimycin is known to block the mitochondrial respiratory chain at the Q_0 site of the cytochrome b/c_1 complex (20). Accordingly, we observed that $0.1 \mu\text{M}$ antimycin totally inhibited the heterotrophic growth of *C. reinhardtii*. The effect of antimycin on phototrophic growth of the wt and FUD50su is shown in Fig. 3. Whereas growth of wt cells was not altered by antimycin, that of FUD50su cells was totally inhibited when the inhibitor was present either at the beginning culture (Fig. 3) or in the mid-exponential phase (data not shown). The same pattern of sensitivity was observed with $1 \mu\text{M}$ myxothiazol, which blocks the respiratory chain at the Q_1 site of the cytochrome b/c_1 complex (21) (data not shown). Rotenone, which blocks the oxidizing side of the iron sulfur centers of the mitochondrial complex I (22), had no inhibitory effect on photosynthetic growth in either strain. Data indicating that photosynthesis is controlled by mitochondrial respiration in the FUD50su strain are presented in Table 1. A 60% inhibition of O_2 consumption in darkness was observed in the presence of antimycin in wt and FUD50su strains. This was indicative of a block in mitochondrial respiration for both strains. That some O_2 consumption was antimycin insensitive is consistent with the existence of chlororespiration (23) and of an alternative respiration pathway in the mitochondria (24). It has been shown that these two pathways were antimycin insensitive and corresponded each to $\approx 20\%$ of the total O_2 consumption (23, 25).

In our experimental conditions, steady-state photosynthetic O_2 evolution was significantly lower in the FUD50su strain than in the wt. This was expected from the coupling of photosynthetic electron transport and light-induced electrochemical potential. The latter accumulates to considerably higher levels in the mutant than in the wt at the light intensity used in the experiment (≈ 20 photons per reaction center per s). On the other hand, a dramatic decrease in O_2 evolution

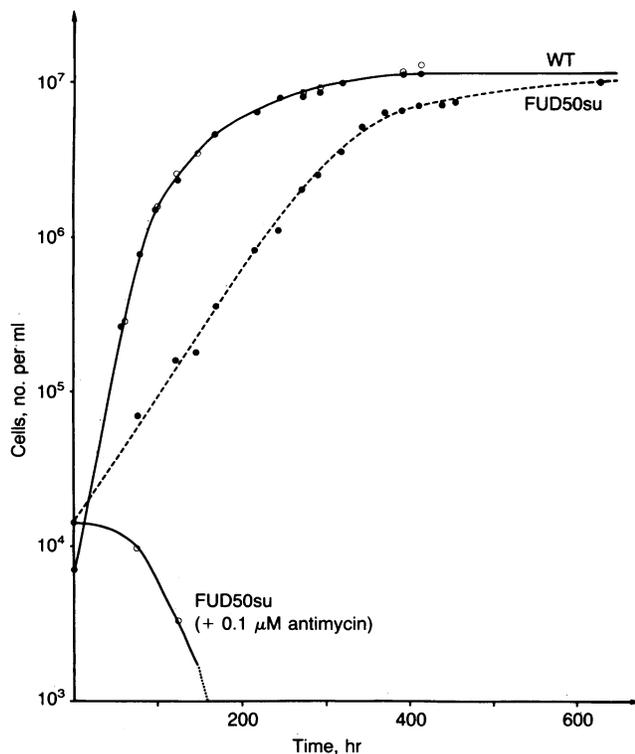


Fig. 3. Growth curves of the FUD50su strain (generation time, 32 hr) in comparison to the wt strain (generation time, 14.5 hr) in phototrophic conditions at 1000 lux. The cultures were grown in the presence (○) or in the absence (●) of $0.1 \mu\text{M}$ antimycin.

Table 1. Relative rates of O_2 consumption and evolution measured with a Clark electrode

| Strain | Respiration, relative units | Net photosynthesis, relative units |
|---------------------------------------|-----------------------------|------------------------------------|
| wt | 100 | 100 |
| wt + $0.1 \mu\text{M}$ antimycin | 44 | 98 |
| FUD50su | 105 | 30 |
| FUD50su + $0.1 \mu\text{M}$ antimycin | 44 | 3 |

Results were expressed in relative units (wt strain = 100%). Respiration yield was $31.5 \mu\text{mol}$ of O_2 consumed per hr per mg of chlorophyll in the wt strain. O_2 evolution was $81 \mu\text{mol}$ of O_2 produced per hr per mg of chlorophyll in the wt strain.

was observed in the FUD50su upon addition of $0.1 \mu\text{M}$ antimycin (90% inhibition), whereas no effect was observed in the wt strain. Thus, photosynthetic O_2 evolution was sensitive to inhibition of mitochondrial electron transport in the case of the mutant only.

DISCUSSION

Mutants of *C. reinhardtii* deficient in the chloroplast ATP synthase complex do not grow on minimal medium in the light. Among these mutants, the FUD50 was characterized as displaying a deletion of ≈ 2.3 kilobase pairs in the Bam10/Eco11 fragment overlapping region of the chloroplast DNA (3). This region has a total size of 5.3 kilobase pairs and was shown to contain the *atpB* gene coding for the β subunit of the ATP synthase (26). Consequently, the FUD50 strain was lacking in *atpB* mRNA, while *atpA* mRNA, corresponding to the transcript of the gene coding for the α subunit, was still present (J. P. Woessner, personal communication). Thus the possibility that the FUD50su strain, which recovered the ability to grow under phototrophic conditions, would originate from a reversion of the FUD50 mutation could be rejected. In addition, this strain carried nuclear but not chloroplast mutation (other than the FUD50 mutation). The biochemical characterization of the thylakoid proteins from the FUD50su showed that it was still deficient in all CF0-CF1 subunits and in particular in the synthesis of the β subunit. Therefore, we could conclude that restoration of growth in phototrophic conditions was not due to a restoration of synthesis of the chloroplast β subunit or to a rerouting of the mitochondrial β subunit into the chloroplast, which would allow assembly of the CF0-CF1 complex. On the other hand, the absence of ATP synthase activity, as demonstrated by the slow relaxation of the transmembrane electric field, argued against an activation of an alternative ATP synthase in the thylakoid membranes, for instance associated with the ATPase activity detected by Piccioni *et al.* (27) in *C. reinhardtii*.

The sensitivity of the phototrophic growth of the FUD50su strain toward specific inhibitors of mitochondrial electron transport, such as antimycin or myxothiazol, showed that this growth restoration resulted from a close interaction between mitochondria and chloroplast. We note that antimycin was without effect on the phototrophic growth of the wt cells, which is consistent with indirect ATP export from the chloroplast to the cytosol through the phosphate translocator (24).

No significant increase in membrane contact between chloroplast and mitochondria could be detected in the mutant in comparison to the wt strain (J. Olive, personal communication). We were then led to consider a metabolic pathway in which ATP was supplied to the chloroplast by the mitochondria. In Fig. 4, we describe the pathways that were the most consistent with our experimental observations, namely rotenone insensitivity, antimycin and myxo-

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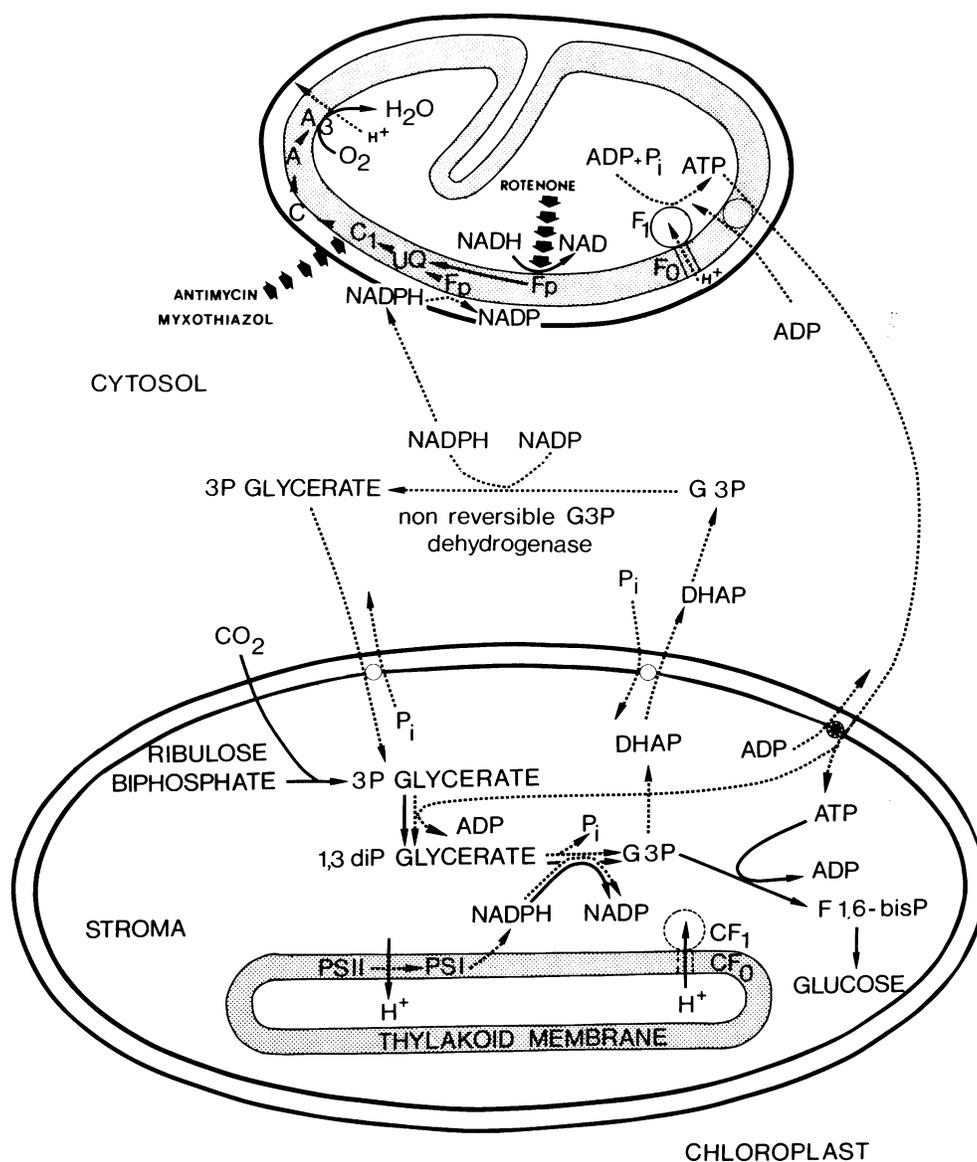


FIG. 4. Proposed metabolic pathway eliciting mitochondrial ATP supply to the chloroplast in the FUD50su strain. Broken lines indicate (i) the NADPH shuttle allowing exportation of NADPH in the cytosol by way of the phosphate translocator and oxidation of exogenous NADPH by the mitochondrial NADPH dehydrogenase and (ii) the ATP shuttle allowing exportation of ATP by way of the mitochondrial ADP-ATP carrier and importation of ATP by way of the hypothetical chloroplast ADP-ATP carrier (*). Solid lines indicated the metabolic pathway of the Calvin cycle. The ATP synthase in the chloroplast (dashed lines) is missing in the FUD50su strain. Fp, flavoprotein; F1,6-bisP, fructose 1,6-bisphosphate; G3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; PSI and PSII, photosystems I and II, respectively; 1,3 diP glycerate, 1,3-bisphosphoglycerate; 3P glycerate, 3-phosphoglycerate; UQ, ubiquinone pool; C₁, C, A, A₃, cytochromes c₁, c, a, and a₃, respectively.

thiazol sensitivity, and a lower limit of 2.6 in the growth rate ratios between the FUD50su and the wt strain grown under phototrophic condition.

In this scheme, reducing power that is required by the mitochondria to produce ATP can be exported from the chloroplast in a series of reactions involving, in particular, export of dihydroxyacetone phosphate (28) and dehydrogenation by the highly active nonreversible glyceraldehyde-3-phosphate dehydrogenase in the cytosol (29) with concomitant reduction of cytoplasmic NADP⁺ that can be used as an exogenous reductant at the outer surface of the inner mitochondria membrane (for a review see ref. 30). In such a pathway, mitochondrial oxidation of NADPH is rotenone insensitive and antimycin and myxothiazol sensitive (30). ATP can be exported from the mitochondria by the conven-

tional ADP-ATP carrier (for reviews see refs. 31 and 32). There is usually no such active ATP-ADP carrier in the chloroplast envelope although it was shown to appear at a particular stage of pea chloroplast development (33). This raises the possibility that an ADP-ATP carrier exists in the chloroplast envelope from the wt strain of *C. reinhardtii*, the activity of which would be modulated during the cell cycle. Thus, the activation of such a carrier in the FUD50su (Fig. 4) would be responsible for the importation of ATP into the chloroplast, thus explaining the observed mitochondrial-dependent photosynthesis in the FUD50su mutant. Alternatively, the mutations carried by the FUD50su could result in a modification of the signal sequence of the mitochondrial ADP-ATP carrier allowing its incorporation in the mitochondrial inner membrane as well as in the chloroplast envelope.

Of the two molecules of ATP produced by the mitochondria in the rotenone-insensitive pathway (34, 35), one is required to regenerate 1,3-bisphosphoglycerate. The net exchange in the FUD50su would then be of one molecule of ATP produced in the mitochondria per molecule of photo-synthetically reduced NADP⁺.

In the Calvin cycle, assimilation of one molecule of CO₂ requires three molecules of ATP and two molecules of NADPH. In the FUD50su strain, by using the 1:1 ATP/NADPH molar exchange, five molecules of NADP⁺ have to be reduced to allow assimilation of one molecule of CO₂. Thus, in our scheme, assimilation of one molecule of CO₂ requires 2.5 times more reduced NADP⁺ of photosynthetic origin in the FUD50su than in the wt strain. This ratio is consistent with the optimal 2.6 ratio in the growth rates that we have observed between the two strains.

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