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Energy Supply for ATP-Synthase Deficient Chloroplasts of *Chlamydomonas reinhardii*

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The mutant *F54* of the unicellular green alga *Chlamydomonas reinhardii* is not able to perform photophosphorylation. Nevertheless, it grows on acetate and the chloroplasts accomplish most of their energy-requiring synthetic processes. However, no light-dependent chloroplast protein synthesis could be detected in intact *F54* chloroplasts isolated from a cell wall-deficient double mutant *F54·cw-15*. Exogenous ATP was not able to induce this in organello protein synthesis to an appreciable degree. In contrast, the strictly ATP-dependent protein synthesis was stimulated very efficiently by glyceraldehyde-3-phosphate, dihydroxyacetone phosphate and glycerol-3-phosphate, but strongly inhibited by 3-phosphoglycerate. These compounds can be transported across the envelope membrane by the triose phosphate translocator. Pyridoxal phosphate, a specific inhibitor of the translocator, abolished the stimulation by triose phosphates. Spermidine, which activates initiation of translation in chloroplasts, enhanced triose phosphate-stimulated protein synthesis even further. In the dark, no stimulation was observed, indicating that a light-dependent reaction was also involved in this kind of ATP production in chloroplasts. The results suggest that chloroplasts defective in photophosphorylation recruit their energy via an ATP shuttle which was shown in this study to import rather than export ATP across the chloroplast envelope.

Key words: ATP synthase deficient mutant — *Chlamydomonas reinhardii* — Chloroplasts — Protein synthesis.

Photosynthetic energy capture and CO_2 fixation are considered to be the main functions of chloroplasts. However, plastids fulfill a number of additional vital functions, such as starch and carbohydrate metabolism, fatty acid and lipid synthesis, nitrite and sulfate assimilation, as well as amino acid synthesis (Anderson 1981, Emes and Tobin 1993). These activities, which in chloroplasts are driven by photosynthetically produced energy-rich compounds, depend on the maintenance of the correct protein complement of the plastids. Protein import into plastids and especially the expression of chloroplast encoded proteins are also energy requiring processes (Cline et al. 1985, Olsen et al. 1989). Indeed, in vivo chloroplast protein synthesis is light dependent, mainly via ATP requirement, but partly also because of light-induced translational activating factors which bind to specific mRNA (Michaels and Herrin 1990, Wijk and Eichacker 1996). In synchronized cultures of the unicellular green alga *Chlamydomonas reinhardii*, chloroplast protein synthesis is maximal in the middle of the light period (Breidenbach et al. 1984, 1988). In isolated chloroplasts of this alga, methionine incorporation into chloroplast proteins is driven by light i.e. the ATP needed for protein synthesis is produced by photosynthetic processes (Leu et al. 1984). Therefore, the extent of protein synthesis in isolated chloroplasts (in organello protein synthesis) reflects the supply of chloroplasts with ATP.

Unicellular algae offer the opportunity to study mutants which have defects in the light reactions of photosynthesis, and are thus incapable of producing ATP in the chloroplast. Such mutants, for example as *Chlamydomonas reinhardii F54* which has a defective or missing coupling factor CF1, can nevertheless grow heterotrophically with acetate as a carbon source (Lemaire and Wollman 1989, Drapier et al. 1992). Their chloroplasts are green and synthesize chloroplast proteins. The question arises, how the chloroplasts in such mutants are supplied with the energy necessary to drive protein synthesis and all other chloroplast functions essential for cell life.

If isolated chloroplasts from these mutants were available, in organello protein synthesis could serve as a test by which the ability of different exogenous compounds to act as possible energy sources could be examined. In addition, the metabolic capabilities of the chloroplasts and the way that the translocation of compounds across the chloroplast envelope influences protein synthesis, could be studied in organello. Since intact, metabolically active chloroplasts cannot be isolated from *Chlamydomonas* strains with wild-type cell walls, we had to introduce the mutant *F54* phenotype into the cell wall-deficient strain *cw-15*, which thus facilitated the isolation of photosynthetically incompetent chloroplasts.

Abbreviations: CF1, coupling factor 1 of chloroplast ATP-synthase; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; Glc6P, glucose-6-phosphate; GlyP, glycerol-3-phosphate; LS, large subunit of ribulose-1,5-bisphosphate carboxylase; PEP, phosphoenol pyruvate; PGA, 3-phosphoglyceric acid; PLP, pyridoxal-5′-phosphate; Pyr, pyruvate; WT, wild-type.

Materials and Methods

Strains and cultivation—Two cell wall-deficient strains, *Chlamydomonas reinhardii cw-15 nit-2 mt−* (CC-1615) and *cw-15 (nit+) mt−* (CC-1883) were obtained from Dr. E. Harris (*Chlamydomonas* Genetics Center, Duke University, N.C. U.S.A.). CC-1615 was mainly used for chloroplast isolation, CC-1883 for crossings. These *cw-15* strains were cultivated in the medium I of Sager and Granick (1954) supplemented with 0.2% Na-acetate as described by Bolli et al. (1981). For chloroplast isolation cells were grown synchronously in 3 liter penicillin flasks with a 14 h light/10 h dark cycle at 25°C and harvested at the 6th hour of the third light period. The mutant *Chlamydomonas reinhardii F54 mt+* (CC-980) was obtained also from Dr. Harris and used for crossing. As this strain later was found to be partially reverted, the strain *F54* from Dr. D. Drapier (C.N.R.S., Institut de Biologie Physico-Chimique, Paris) was used for labeling experiments. All mutants bearing the *F54*-phenotype were cultivated at reduced light intensity of about 300 lux in TAP medium (Gorman and Levine 1965) enriched with 0.2% Na-acetate and 0.04% yeast extract (Difco).

Radioactive labeling of chloroplast proteins in vivo—*Chlamydomonas* cells in the logarithmic growth phase were harvested by centrifugation at $200 \times g$ at room temperature, washed once and then resuspended in sterile growth medium without sulfate and without trace elements to give a density of 5×10^7 cells ml^{-1}. After 30 min of incubation at dim light on a shaker 10 $\mu g\ ml^{-1}$ cycloheximide were added. The cells were further incubated for 10 min, then 100 $\mu Ci\ ml^{-1}$ [^{35}S]sulphate (3.7 MBq ml^{-1}; Amersham International, U.K.) were added, followed by incubation overnight (15 h). Separation into membranes and supernatant was as described below in "test for CF1 subunits".

Genetic crossings—Gametes were produced by growing *Chlamydomonas reinhardii cw-15 mt−* (CC-1883 or CC-1615) and *F54 mt+* (CC-980) first on agar plates containing medium I of Sager and Granick (1954) with 1/10 of nitrogen source and 0.2% Na-acetate. Then the cells were suspended in 2 ml of the same liquid medium, but without a nitrogen source and shaken gently for 1 h in dim light. Further methods for mating and tetrad analysis were essentially as described by Harris (1989) and are based on Levine and Ebersold (1960).

Selection of cw-15 F54 double mutants—To test the offspring from the above crosses for cell wall deficiency, 1 ml liquid culture was mixed with 50 μl 10% Triton X-100 and the disintegration of the cells was observed microscopically under phase contrast.

Mutants, which by Triton-test were considered to be of *cw-15* phenotype and which were found to contain *F54*-chloroplasts (see below), were further tested for ease of cell breakage in the Yeda press. Synchronously cultured cells (200 ml) were harvested at the 6th hour in the third light period, and resuspended in 16 ml isolation medium (see "chloroplast isolation" below). Portions (2 ml) of this cell suspension were broken in the Yeda press at 4.0, 4.5, 5.0, 5.5, 6.0 and 8.0×10^5 Pa. Strains containing only 20% of intact cells after breakage at 5.0×10^5 Pa, as judged by microscopy, were well suited for chloroplast isolation.

The simplest procedure to screen for the *F54* phenotype, which is a photosynthetic defect, would have been to look for the inability to grow under phototrophic conditions or for enhanced fluorescence of the colonies as compared to wild type. However, in our hands even the parent strain *F54* showed some residual growth in the absence of acetate, and fluorescence intensities were not distinctly different. Therefore, we tested directly for the inability to synthesize the α-subunit of CF1. *Cw-15* strains to be tested were cultivated in 35 ml TAP medium containing 0.2% Na-acetate and 0,04% yeast extract. Cells were washed once and resuspended in 1–2 ml MES buffer (20 mM MES (pH 6.0), 10 mM NaCl). The cells were broken in the French press at 3.6 MPa, and the cell homogenate separated into membranes and supernatant by centrifugation for 30 min at $39,000 \times g$ and 4°C (Sorvall SS-34 rotor). Membranes were washed twice and then resuspended in the MES buffer to obtain the initial volume. A volume corresponding to 5 μg chlorophyll of the membrane suspension and the same volume of the supernatant were loaded on a 12–18% SDS polyacrylamide gel. After electrophoresis the proteins were transferred to nitrocellulose and immunostained with antiserum against total CF1 of *Chlamydomonas*. Alternatively, antisera directed preferentially against either the CF1-α or CF1-β subunits of *Chlamydomonas* or of spinach were used (see below).

Chloroplast isolation—The method based on the procedure described by Mendiola-Morgenthaler et al. (1985) and Su and Boschetti (1994) was essentially as follows; 200 $\mu g\ ml^{-1}$ chloramphenicol were added to 3 liters of synchronized cell culture 20 min before harvesting. All subsequently used media also contained 200 $\mu g\ ml^{-1}$ chloramphenicol, except the final washing. The cell pellet was first rinsed and then resuspended to a final volume of 25 ml in isolation medium composed of 250 mM sorbitol, 35 mM HEPES-KOH (pH 7.8), 1 mM $MnCl_2$, 5 mM $MgCl_2$, 2 mM EDTA-KOH (pH 7.8) and 200 $\mu g\ ml^{-1}$ chloramphenicol (stock solution of 100 mg ml^{-1} in 70% ethanol). The cell suspension was maintained for 3 min at 5.2×10^5 Pa in the Yeda pressure cell before the cells were broken. The homogenate was adjusted to 35 ml with isolation medium, the EDTA concentration raised to 10 mM (0.5 M K-EDTA stock solution, pH 7.8), and the suspension gently agitated (no stirrer) during 20 min on ice. After centrifugation at 5,000 rpm ($3,000 \times g$) for 10 seconds (Sorvall SS-34 rotor, rapid decceleration), the green pellet was carefully resuspended in 25 ml isolation medium and then mixed with an equal volume of 80% Percoll (36 ml Percoll+9 ml 5 times concentrated isolation medium). Twenty-five ml of this mixture were layered on top of 12 ml 60% Percoll, and centrifuged in a Sorvall HS-4 swingout rotor for 20 min at 4°C and 5,000 rpm ($4,800 \times g$). The supernatant was aspirated and the green chloroplast band at the boundary between the two Percoll layers was carefully collected using a pipette with a wide tip. The volume was brought to 35 ml with isolation medium without chloramphenicol and the chloroplasts were sedimented for 10 seconds at 5,000 rpm ($3,000 \times g$) and 4°C in the Sorvall SS-34 rotor with rapid braking. The pelleted chloroplasts were washed twice again without chloramphenicol, once with a 1 : 1 mixture of isolation and incubation medium and then with incubation medium alone (250 mM sorbitol, 35 mM HEPES-KOH (pH 7.8), 1 mM $MnCl_2$, 0.5 mM $MgCl_2$, 0.1 mM Na_2HPO_4, 2 mM EDTA-KOH (pH 7.8)). The final pellet was resuspended in the smallest possible volume of incubation medium (2–4 mg ml^{-1} chlorophyll) and used immediately.

In organello protein synthesis—Per assay 100 μl incubation medium (see above) containing chloroplasts equivalent to 20–40 μg chlorophyll, 25 μM of each amino acid except methionine, 10 μCi [^{35}S]L-methionine (370 kBq; NEN), and other components as indicated in the text, were placed in an Eppendorf tube. The samples were incubated at 25°C for 20 min in a glass water bath which was illuminated from two sides at a distance of 30 cm with a photolamp (Osram R125, 300 W). The reaction was stopped by freezing the tubes in liquid nitrogen.

Electrophoretic analysis of proteins—The radiolabeled samples of 100 μl volume were solubilized at 90°C for 5 min with 50 μl of a solution containing 9% SDS, 4.5% 2-mercaptoethanol, 18%

sucrose, 30 mM Tris-HCl (pH 7.6), and 0.06% Bromphenole Blue, and loaded on a SDS polyacrylamide gel (12–18%). The proteins were separated according to Laemmli (1970). The gels were either stained with Coomassie Brilliant Blue R250, then treated with Amplify (Amersham Int., U.K.), and dried and exposed to X-ray film, or used directly for immunoblotting.

The radioactivity in the protein bands was quantified according to Rothen et al. (1997) either on the dried gel using a PhosphorImager (Molecular Dynamics) or by scanning the X-ray film and analyzing the digitalised image with Image Quant (Molecular Dynamics).

For immunoblotting, the proteins were electrophoretically transferred from the gel onto nitrocellulose (BA 85 0.45 μm, Schleicher & Schüll). The primary polyclonal rabbit antiserum directed against *Chlamydomonas* chloroplast coupling factor CF1 proteins was from our laboratory and did not crossreact with LS. The antisera directed specifically or preferentially against the CF1-α and CF1-β subunits of *Chlamydomonas* were kind gifts from Dr. G. Girault, Gif-sur-Yvette, France, and Dr. A.T. Jagendorf, Cornell University, U.S.A., and those of spinach were kindly given by Dr. G. Girault.

Two-dimensional gel electrophoresis—Analysis of CF1 subunits by two-dimensional isoelectric focussing-SDS-PAGE was performed according to the procedure described by Clemetson et al. (1992) with the following modifications: 100 μl of the sample supplemented with 10 μl 20% SDS and 10 μl 2-mercaptoethanol were heated for 2 min at 90°C. Then 140 μl of a solution containing 9.5 M urea, 2% Servalyte 3-7 (Serva, Heidelberg), 8% Nonidet P-40 were added, and the mixture loaded on the first dimensional gel for isoelectric focussing (pH 3-7), and run for 16 h at 260 V. The second dimensional SDS-gel contained a linear 12–18% polyacrylamide gradient. The gels were stained or immunoblotted as described above.

Results

Photosynthesis-incompetent and cell wall-deficient double mutants of Chlamydomonas reinhardii—The mutant strain *Chlamydomonas reinhardii F54* is not able to synthesize the chloroplast-encoded α-subunit of CF1 and shows impaired photosynthetic ATP production (Lemaire and Wollman 1989). The mutation is confined to the nuclear genome, and affects the translation, but not the transcription, of the chloroplast-encoded gene for CF1 α-subunit, as the mRNA for this protein was found to be present in mutant cells (Drapier et al. 1992). To obtain a double mutant strain with *F54* phenotype from which intact chloroplasts can be isolated, we crossed *Chlamydomonas reinhardii F54 mt+* with the cell wall-deficient strain *cw-15 mt−*. As cell wall-deficient strains mate rather poorly, and since *F54* grows slowly and has a tendency to revert, the recovery of double mutants was rather low. From 72 tetrads 10 double mutants *F54·w-15* could be isolated from which only one (No. *619f*) was stable over three years. The chlorophyll content of this double mutant was about 2×10^{-6} μg cell^{-1} and did not differ significantly from that of either of the parent strains.

To characterize their protein pattern, the mixotrophically grown parent strains *cw-15* and *F54*, and the double mutant *F54·w-15* (strain *619f*) were labeled in vivo with [^{35}S]sulphate in the presence of cycloheximide. The cells were homogenized, and the proteins were analyzed first by one-dimensional SDS-PAGE (Fig. 1A). As the apparent molecular weight of the large subunit of ribulose-1,5-bisphosphate carboxylase (LS) is identical to that of the α-subunit of CF1 (filled arrow) and very similar to that of the β-subunit (open arrow), the cell homogenate was separated into a supernatant fraction accumulating the soluble LS, and into washed membranes containing (in *cw-15*) the α- and β-subunits. The latter fraction was still contaminated with traces of LS. The identity of the CF1-subunits was confirmed by Western blot analysis using antisera directed preferentially against α- or β-subunits or by an antiserum raised in our laboratory against isolated CF1 of *Chlamydomonas*. By contrast, 2-dimensional electrophoresis (Fig. 1B) allowed a complete separation of LS (outside the pH range represented in Fig. 1B) and the α- and β-subunits and their identification by the isoelectric points.

In the parent mutant *F54* and in the double mutant *F54·cw-15* (*619f*) no α-subunit was found on 2-D gels neither immunologically nor by radioactive labeling, demonstrating that in the double mutant the chloroplasts were indeed of the desired phenotype *F54*. Interestingly, in these mutants the β-subunit was found, by immunostaining as well as by radioactive labeling, almost exclusively in the supernatant, whereas in the phenotypically wild-type chloroplasts of *cw-15*, the β-subunit accumulated as expected mainly in the membrane fraction. It seems that in the absence of α-subunits the newly formed β-subunits are not able to become incorporated in a thylakoid membrane-associated protein complex. Whether this behavior of the β-subunit relates to the appearance of faint, immunologically cross-reacting bands of higher molecular weight in *F54*, but not in *cw-15* strains, is not clear (Fig. 1A).

Comparison of in organello protein synthesis in isolated wild-type and F54-chloroplasts—With the double mutant and the *cw-15* strains we were able to isolate two different types of chloroplasts: (a) the phenotypically wild-type chloroplasts from *Chlamydomonas reinhardii cw-15* (designated in the following as WT-chloroplasts) and the photosynthetically incompetent *F54*-chloroplasts from the double mutant *F54·cw-15*.

Previously, we have shown that intact chloroplasts isolated from synchronized cultures of *Chlamydomonas reinhardii cw-15* at the middle of the light period (L6) were able to perform light dependent protein synthesis as measured by incorporation of [^{35}S]methionine into chloroplast proteins (Mendiola-Morgenthaler et al. 1985). The radiolabeled proteins were presumably the products only of translational elongation in preformed polysomes. Addition of 0.4 mM spermidine, however, stimulated initiation of translation to some extent (Blättler et al. 1992). In Fig. 2A and 2B we compared the light-dependent in organello protein

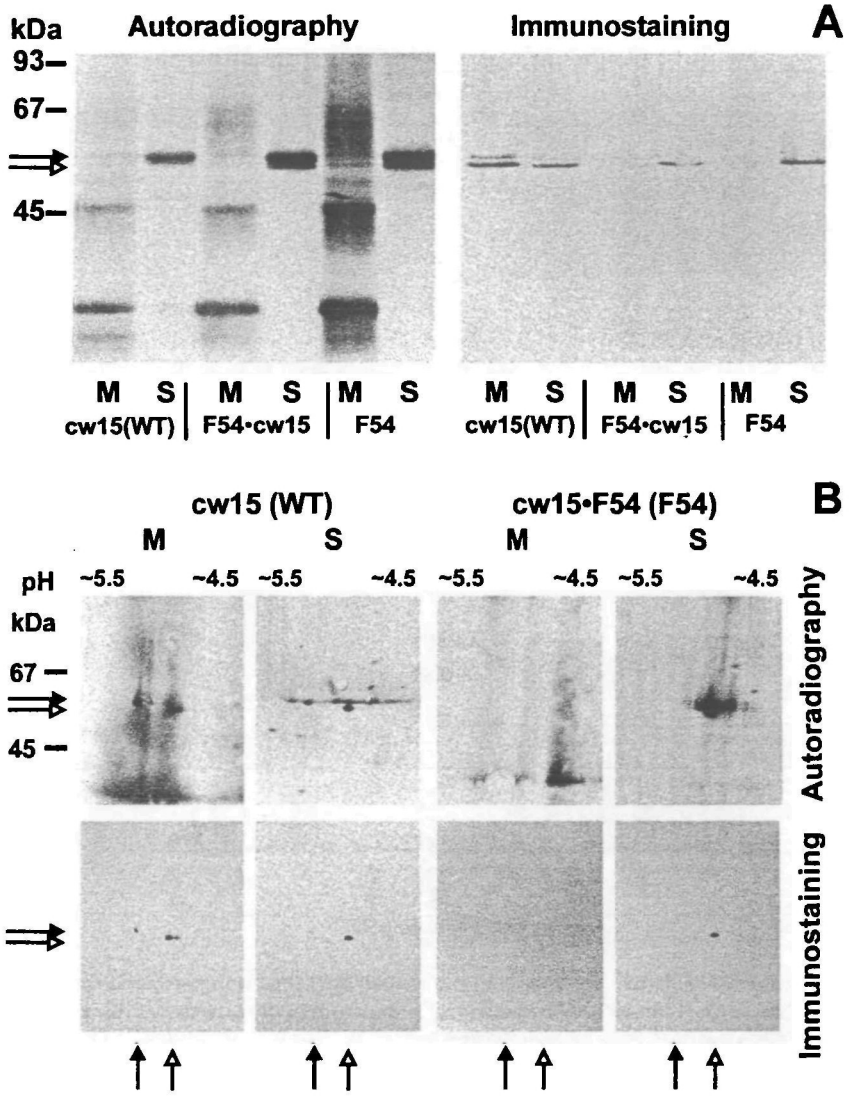

Fig. 1 Electrophoresis of proteins from parental strains, *cw-15* and *F54*, and from the double mutant *cw-15·54* (*619f*). After labeling mixotrophic cultures in vivo with [^{35}S]sulphate in the presence of cycloheximide the cells were concentrated by centrifugation, disrupted in the French press at 3.6 MPa, and the homogenate separated into membranes (M) and supernatant (S) by centrifugation at 39,000 × *g* for 30 min. Membranes were washed twice. (A): One dimensional SDS-PAGE. Membranes containing 5 μg chlorophyll (corresponding to 65 μg protein) or an equivalent amount of the supernatants (20 μg protein) were loaded per lane. (B): Two dimensional separations of strains *cw-15* and *cw-15·54* (*619f*) by isoelectric focussing/SDS-PAGE. Open arrow: β-subunit of CF1; filled arrow: α-subunit of CF1 which migrates to the same apparent molecular weight as LS. The antiserum against CF1 of *Chlamydomonas reinhardii* used for immunostaining was shown not to crossreact with LS.

synthesis of *F54*-chloroplasts with that of WT-chloroplasts. The ATP-synthase deficient *F54*-chloroplasts were not able to incorporate [^{35}S]methionine into chloroplast proteins in a light-dependent manner either in the presence or absence of spermidine. In WT-chloroplasts, however, light stimulated methionine incorporation about 25-fold, the most prominently labeled bands being LS (55 kDa) and D1 protein (32 kDa). Spermidine had an additional 1.5-fold stimulatory effect in the light.

Effect of ATP and EDTA on in organello protein synthesis—As *F54* chloroplasts lack the ATP synthase, their low rate of in organello protein synthesis is most probably due to limited resources of ATP. In Fig. 3 the incorporation of radioactivity into the protein band D1 is shown as a function of added ATP or K-EDTA. In wild-type chloroplasts addition of up to 2 mM ATP had little effect, but higher concentrations inhibited the protein synthesis. Increasing amounts of EDTA, however, stimulated protein

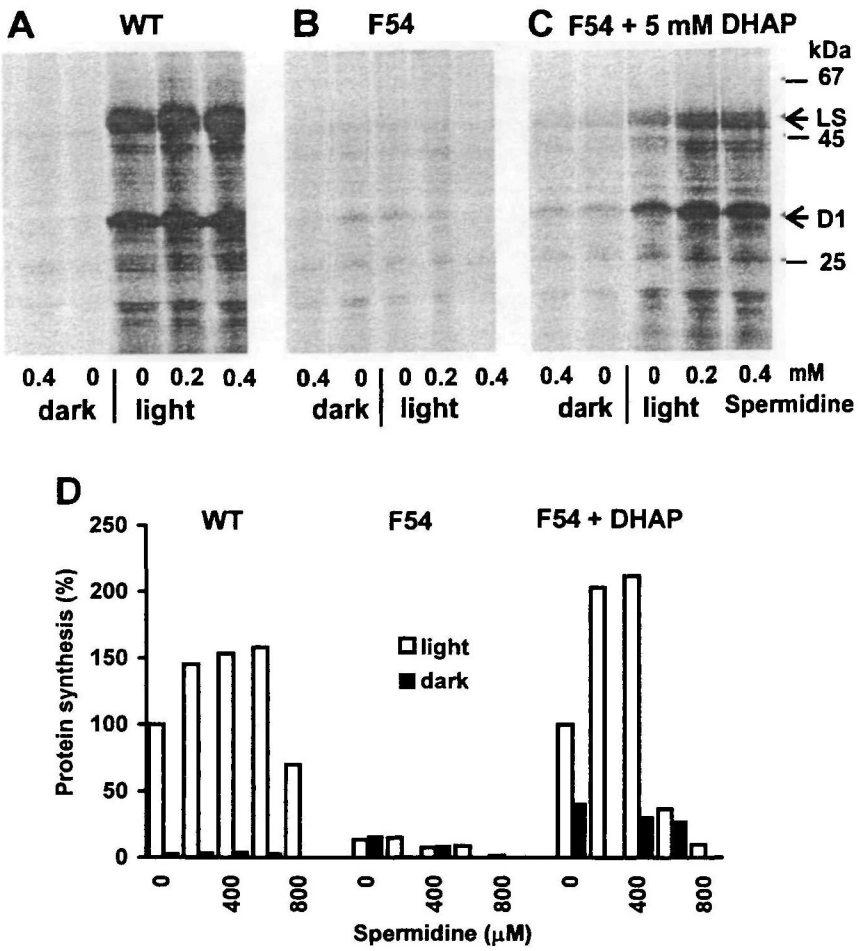

Fig. 2 In organello protein synthesis in isolated chloroplasts demonstrated by [^{35}S]methionine incorporation into chloroplast proteins. Isolated chloroplasts were incubated with [^{35}S]methionine in the light or dark and in the presence of the indicated concentrations of spermidine. A: Autoradiograms after SDS-PAGE of solubilized WT-chloroplasts from *C. reinh. cw-15* (30 μg chlorophyll per lane, corresponding to ca. 400 μg protein). B: Same as A, but with *F54*-chloroplasts from double mutant. C: As B, but chloroplasts incubated in the presence of 5 mM DHAP. D: Quantitative evaluation by PhosphorImager of in organello [^{35}S]methionine incorporation into the D1 protein. Incubation conditions were as described above. The stimulation (over background) of methionine incorporation into the D1 protein by light in the absence of spermidine was taken as 100% for WT-chloroplasts, and in the absence of spermidine, but in the presence of 5 mM DHAP for *F54*-chloroplasts. In WT, 100% corresponded to a 40-fold stimulation, and in *F54*+DHAP to a 7-fold stimulation of methionine incorporation over the dark control (without DHAP and spermidine).

synthesis of D1 in wild-type chloroplasts up to 2-fold, an effect which we cannot as yet explain. In contrast, in *F54*-chloroplasts EDTA had no effect, but low amounts of ATP (1–2 mM) stimulated in organello protein synthesis slightly, while 5 mM ATP inhibited labeling as in wild-type chloroplasts. Obviously, *F54*-chloroplasts were not saturated with ATP and were able to import ATP at a very low rate when it was present at low concentrations in the surrounding medium. As the in organello protein synthesis in *Chlamydomonas* chloroplasts is drastically inhibited by Mg^{2+} concentrations higher than 0.5 mM (Leu et al. 1984), some stimulatory effect could be considered to be caused by Mg^{2+}-chelating properties of ATP ($K_B = 1 \times 10^5\ M^{-1}$ [Martel 1971]) which might lower an eventual excess of free Mg^{2+} in the assay. However, since EDTA has no effect on protein synthesis in *F54* chloroplasts (Fig. 3B), this possibility is very unlikely.

Stimulation of protein synthesis in ATP synthase deficient F54-chloroplasts by components of the carbohydrate metabolic pathway—Since the stimulation of protein synthesis by ATP in organello was not very effective, a number of compounds involved in carbohydrate turnover were tested. Compounds of interest should be able to be translocated across the chloroplast envelope and used in the chloroplast to form ATP. More efficient than the ATP/ADP-translocator in the chloroplast envelope of higher plants is

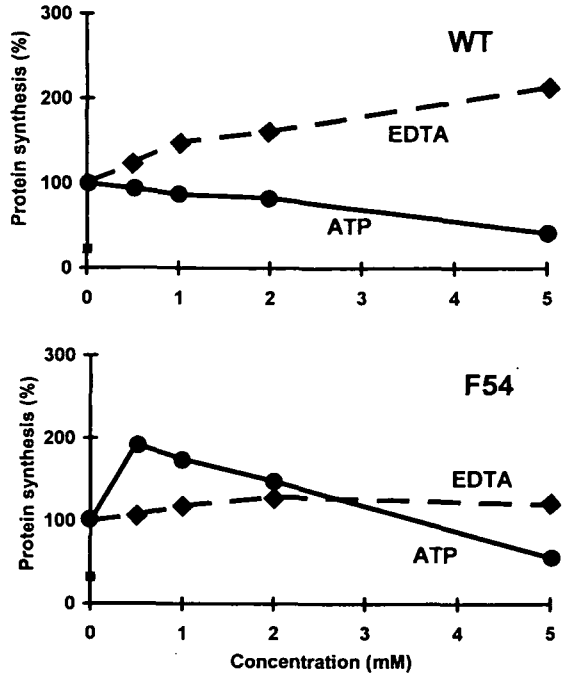

Fig. 3 Effect of increasing ATP and EDTA concentrations on in organello [^{35}S]methionine incorporation into the D1 protein by WT-chloroplasts and *F54*-chloroplasts. Radioactivity in D1 was measured after SDS-PAGE by PhosphorImager using ImageQuant software. 100% was defined as the level of stimulation of methionine incorporation into the D1 protein by light in the absence of additives. Filled square: dark control. Essentially the same graph was obtained when the radioactivity in the LS-band or in all of the chloroplast proteins was measured (not shown).

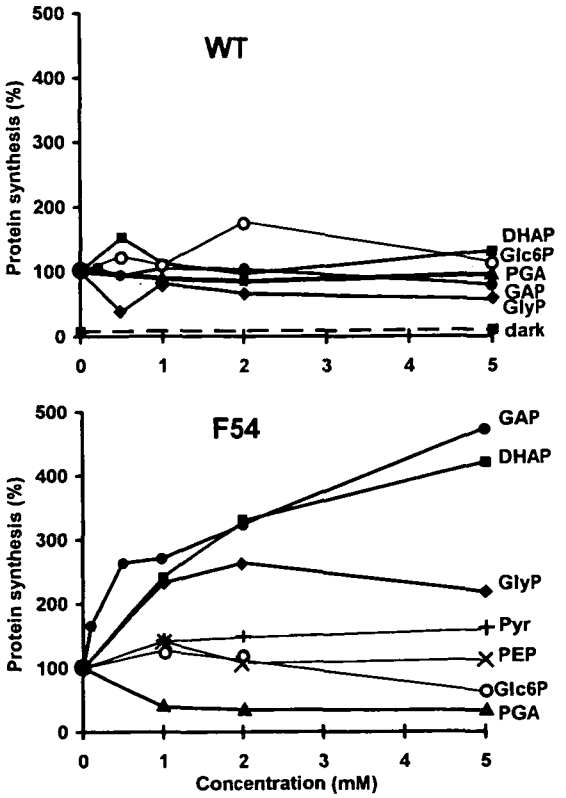

Fig. 4 Effect of increasing concentrations of various metabolites on in organello [^{35}S]methionine incorporation into the D1 protein by isolated WT-chloroplasts and *F54*-chloroplasts in the light. Radioactivity in D1 was measured after SDS-PAGE by PhosphorImager using ImageQuant software. 100% was defined as in Fig. 3. Here, 100% corresponded to a 40-fold stimulation over the dark control in WT-chloroplasts, and to 1.15-fold in *F54*-chloroplasts. Filled square/broken line: dark control.

the triose-phosphate/phosphate/phosphoglyceric acid translocator (triose-phosphate translocator) (Flügge and Heldt 1991, Flügge 1995). It is most specific for C_3-compounds containing a phosphoric ester group in position 3.

As shown in Fig. 2C the addition of dihydroxyacetone phosphate (DHAP) to the assay medium very efficiently stimulated in organello protein synthesis by *F54*-chloroplasts in the light, but not in the dark. Spermidine had a further stimulatory effect as was shown also in WT-chloroplasts. The quantitative evaluation of the effects of increasing concentrations of various glycolytic metabolites on methionine incorporation into D1-protein by isolated chloroplasts is represented in Fig. 4. In the light, DHAP and glyceraldehyde-3-phosphate (GAP) were found to stimulate methionine incorporation into the chloroplast D1-protein in isolated *F54*-chloroplasts by about 4-fold (Fig. 4). Glycerol-3-phosphate (GlyP), although a good stimulatory compound, was less effective than GAP or DHAP. As DHAP is more stable than GAP, in most experiments DHAP was used. Addition of 3-phosphoglyceric acid (PGA) strongly inhibited in organello protein synthesis in *F54*-chloroplasts, while glucose-6-phosphate (Glc6P), phosphoenol pyruvate (PEP) and pyruvate (Pyr) showed almost no effect. Obviously, a positive or negative effect on the energy supply of *F54*-chloroplasts was exerted only by those metabolites which can be transported by the triose-phosphate translocator. Furthermore, its specificity in *Chlamydomonas* chloroplasts resembles that of higher C3-plant chloroplasts and the translocator seems to work in both directions, since in the mutant *F54* it catalyses also the uptake of the metabolites which is essential for energy supply in the photosynthetically defective chloroplasts.

In WT-chloroplasts, the effect of the above mentioned glycolytic metabolites on in organello protein synthesis (Fig. 4) was much less dramatic. Notably, the stimulation by GAP and DHAP was not very pronounced. As WT-chloroplasts produce ATP by photosynthesis, they probably do not depend much on exogenous energy supply. Interestingly, external PGA up to 5 mM in the dark and 10 mM in the light had no inhibitory effect.

The question arose whether the in organello synthesis

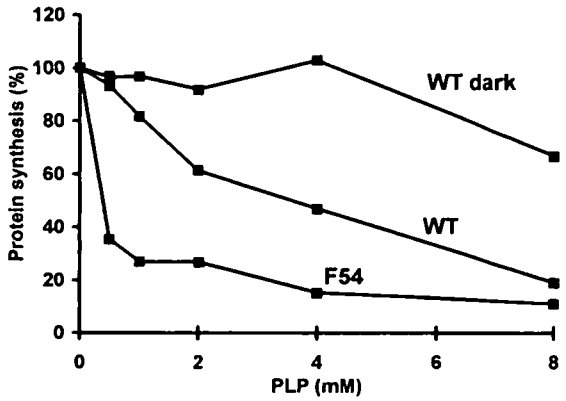

Fig. 5 Effect of PLP, a specific inhibitor of the triose phosphate translocator, on in organello synthesis of D1 protein in WT- and *F54*-chloroplasts. WT-chloroplasts were incubated with 5 mM GAP and increasing concentrations of PLP in the light or dark, *F54*-chloroplasts in the light and in the presence of 5 mM DHAP (5 mM GAP gave similar results). After SDS-PAGE, radioactivity measured by PhosphorImager in the D1 protein bands was standardized individually for each incubation series to the value without PLP (=100%). In the absence of PLP, the WT-chloroplasts used in this experiment incorporated methionine into the D1 protein to 15% in dark+GAP, and to 92% in light−GAP as compared to 100% in light+GAP.

of individual proteins was differentially affected in the presence of stimulatory compounds. We quantitated by PhosphorImager the radiolabeling of a number of individual protein bands on SDS-polyacrylamide gels. Increasing concentrations of triose-phosphate stimulated the labeling of all proteins to the same degree, indicating that the stimulatory compounds exert a general effect on translation (data not shown).

Effect of inhibitors of the triose-phosphate translocator on in organello protein synthesis—The central role of the triose-phosphate translocator in providing photosynthetically incompetent chloroplasts with ATP is further substantiated by inhibition studies. Pyridoxal-5'-phosphate (PLP) is known to specifically inhibit this translocator in higher plants (Bogner et al. 1982). In the experiment illustrated by Fig. 5, in organello protein synthesis in *F54*-chloroplasts was induced by 5 mM DHAP and light, but that of WT-chloroplasts was induced only by light. Increasing amounts of PLP immediately blocked the reaction in mutant chloroplasts, while in WT-chloroplasts inhibition occurred only at higher PLP-concentrations when P_i uptake and/or PGA export eventually became limiting. For comparison, the effect of PLP on protein synthesis in dark-incubated chloroplasts is also shown. The results agree with the idea that in WT-chloroplasts the ATP needed for all kinds of biochemical processes is produced internally, mostly by photophosphorylation, and independently from import of metabolites.

Effect of light on in organello protein synthesis—The effect of light on in organello protein synthesis is quantitatively represented in Fig. 2D. In WT-chloroplasts light is necessary and sufficient for a strong stimulation of methionine incorporation into the D1 protein, which can be explained by the light dependent ATP synthesis. In photosynthetically incompetent *F54*-chloroplasts, however, both DHAP and light are necessary for efficient protein synthesis. Spermidine at about 0.4 mM induces an additional stimulation.

Discussion

The chloroplasts of the mutant *F54* of *Chlamydomonas reinhardii* are unable to synthesize the *a*-subunit of CF1 and lack endogenous ATP synthesis. However, the cells contain green chloroplasts with thylakoids indicating that, except for CO_2-fixation, other vital biochemical functions located in the chloroplast are operating. Obviously, in vivo and in vitro the ATP necessary to drive these processes has to be supplied from outside across the double envelope membrane. To test for ATP availability in these chloroplasts we studied in organello protein synthesis, i.e. methionine incorporation into chloroplast proteins, which is strongly dependent on the presence of ATP but not NADPH.

Although Fish et al. (1983) were able to stimulate chloroplast protein synthesis in isolated spinach chloroplasts by exogenously added ATP, our present results (Fig. 3) demonstrated that protein synthesis was rather inhibited by ATP in WT-chloroplasts, and was only slightly stimulated at low concentrations in *F54*-chloroplasts. This agrees with early reports that only a very inefficient ATP transporter exists in the chloroplast envelope (Walker 1976). In contrast, the well studied ADP/ATP translocator of the mitochondrial inner membrane is very active, but might be only distantly related to the chloroplast transporter (Schünemann et al. 1993). Only recently the gene product of a cDNA has been putatively identified as the until now unknown plastidic ADP/ATP translocator (Neuhaus et al. 1997).

The experiments illustrated in Fig. 3 and 4 suggest that even in the absence of photophosphorylation the ATP content of chloroplasts is determined mainly by internal ATP formation, rather than by the inefficient import of ATP. Compounds inducing internal ATP production are some well known carbohydrate metabolites. They exert a general, rather than a differential effect on protein synthesis, which is to be expected since they act via ATP production. Two conditions must be fulfilled for compounds from the outside to drive ATP formation in the chloroplast: (i) An import apparatus for these compounds must exist in the chloroplast envelope; (ii) enzymes must be present in the chloroplast catalyzing the metabolism of these compounds coupled with ATP formation.

In higher C3-plants GAP, DHAP, GlyP and also PGA can be transported across the envelope membrane by the very efficient triose phosphate/phosphate translocator. PEP is not a substrate for this transporter (Flügge and Heldt 1991). In root plastids, a transport of Glc6P through the envelope has also been reported (Borchert et al. 1995), although this route is normally absent in plant chloroplasts (Quick et al. 1995). Obviously, in our experiments only those metabolites which can be transported by the triose-phosphate translocator had a positive or negative effect on protein synthesis in ATP-deprived chloroplasts, pointing to the pivotal role of this transporter. Dicarboxylic acid metabolites, such as oxaloacetate, malate or glutamate, for which different transporters exist in the envelope, gave no stimulation of chloroplast protein synthesis (data not shown). After import of the phosphorylated C_3-compounds into the chloroplast, the key enzymes responsible for ATP formation might be the Calvin-cycle enzymes, NADP-glyceraldehyde-phosphate dehydrogenase together with 1,3-diphosphoglycerate kinase, which here act in the inverse direction to that in the normal Calvin cycle. Indeed, glycolytic activities within the chloroplast have been reported (Plaxton 1996). The enzyme would catalyze the oxidation of GAP to PGA coupled with the phosphorylation of ADP to ATP. DHAP or GlyP, when imported, are converted to GAP by triose-phosphate isomerase and glycerol-3-phosphate oxido/reductase, respectively. Accordingly, in our experiments the inhibition of ATP formation by PGA, i.e. inhibition of in organello protein synthesis in *F54*-chloroplasts, can easily be explained. PGA in the chloroplast will drive the back reaction, i.e. the normal Calvin-cycle reaction, whereby it becomes reduced to GAP under consumption of ATP, rather than production.

Years ago, a similar mechanism was postulated which should work in the dark to drive ATP synthesis in chloroplasts for exporting reducing equivalents (Heber 1974). In *F54*-chloroplasts, however, triose-phosphates stimulated in organello protein synthesis and, hence, ATP production only in the light (Fig. 2D). The mechanism by which light interferes with ATP synthesis is not clear. However, since the Calvin-cycle enzyme, glyceraldehyde-3-phosphate dehydrogenase, is activated by light (Müller et al. 1969, Plaxton 1996, Vivekanandan and Saralabai 1997), then the back reaction, i.e. the triose-phosphate stimulated ATP synthesis, should also be light dependent.

Our experiments suggest that this chloroplast enzyme, together with the stromal glyceraldehyde-3-phosphate dehydrogenase and triose-phosphate translocator form a shuttle mechanism for an efficient light-dependent ATP transport across the chloroplast envelope membrane. When ATP concentration is limiting in the chloroplast, GAP or DHAP are imported into the organelle and converted to ATP and PGA. The latter is reexported to the cytoplasm where it can be transformed again to GAP whereby ATP is consumed. If ATP concentration in the chloroplast is in excess, export to the cytoplasm would occur by the light-dependent reverse reaction.

In the dark, only a rather inefficient ATP import into the chloroplast occurs, eventually via a very slow ATP/ADP exchanger. This would allow for the slow heterotrophic growth of *F54* cells in the dark and would also be responsible for the slight stimulation of in organello protein synthesis in isolated *F54*-chloroplasts by low concentrations of ATP (Fig. 3).

It would be interesting to test how the metabolically active, but photosynthetically inactive, non-green plastids existing in different organs of higher plants recruit their energy from the cytoplasm. It might be that the import of energy-rich compounds across the double envelope membrane and their conversion to ATP are inherent properties of all plastids and that the energy-supply of *F54* chloroplasts described here may represent a model system for studies on isolated non-green plastids.

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