Protein synthesis by isolated chloroplasts of *Chlamydomonas reinhardii*

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Chloroplasts which have been isolated from the cell wall mutant *cw 15-2* of *Chlamydomonas reinhardii* are active in light-dependent, in organello protein synthesis. Certain characteristics of protein synthesis in this system were tested, such as time course of amino acid incorporation, sensitivities to Mg^{2+} as well as to specific inhibitors, and found to be typical of chloroplasts isolated from higher plants. The major products made were identified as the large subunit of ribulose-1,5-bisphosphate carboxylase and the 32-kDa rapidly labelled membrane protein.

Chlamydomonas reinhardii *Isolated chloroplast* *Chloroplast protein synthesis*

1. INTRODUCTION

Research on the biogenesis of chloroplasts gained much impetus in recent years from studies on in organello protein synthesis by isolated chloroplasts. This approach provided the most direct information on which proteins were made on chloroplast ribosomes. So far, the available information has been obtained from a limited number of higher plants and algae (review [1]).

The unicellular green alga, *Chlamydomonas reinhardii,* is one of the best studied organisms in terms of genetics and physiology of plant cells, and several extrachromosomal mutants have been characterized. However, certain studies have been hampered by difficulties encountered in isolating intact chloroplasts from this alga. Consequently, information regarding chloroplast protein synthesis has been obtained only indirectly: in vivo, by pulse-labelling the cells in the presence of specific inhibitors [2–6] or in vitro, using chloroplast DNA [7], free or thylakoid-bound polyribosomes [8,9] or poly(A)-free RNA [10].

We have been working with isolated chloroplast fractions prepared from the cell wall mutant *cw-15* or *Chlamydomonas* [11]. The isolated chloroplasts were capable of protein synthesis when lysed and supplemented with an ATP-regenerating system. Furthermore, the RNA extracted from these chloroplasts directed the synthesis of both soluble and membrane proteins [12]. We here describe the light-dependent in organello protein synthesis by isolated chloroplasts from Chlamydomonas, as well as the main translation products obtained in this system (A preliminary report of this work was presented at the 6th International Congress on Photosynthesis in Brussels [14]). This is the first report of such studies made with this alga, although the isolation of photosynthetically active *Chlamydomonas* chloroplasts has recently been reported [13].

2. MATERIALS AND METHODS

Cultures of *C. reinhardii cw 15-2 mt*$^+$ (Chlamydomonas Genetics Center, Duke University) were

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grown synchronously and harvested in the middle of the third light period [8]. The procedure for the isolation of the chloroplasts was modified from [11]: The cells harvested from a 2-l culture (about 2×10^7 cells/ml) were resuspended in 30 ml cold homogenization medium (375 mM sorbitol; 35 mM Hepes-KOH, pH 8.3; 5 mM $MgCl_2$; 1 mM $MnCl_2$; 2 mM K-EDTA) and broken in the Yeda pressure cell at 5 atm (5×10^5 Newton/m^2). After addition of K-EDTA to a final concentration of 8 mM, the homogenate was gently shaken for 30 min at 0°C. The homogenate was centrifuged up to $3000 \times g$ and to rest in the shortest possible time. The pellet was resuspended in 50 ml homogenization medium containing 40% Percoll and layered on 2×11 ml 60% Percoll in homogenization medium. After centrifugation at $5000 \times g$ for 20 min at 4°C, the intact chloroplasts were recovered from the interface between the two Percoll layers, incubated with 8 mM EDTA as above, and subsequently washed twice with homogenization medium. Sterile techniques were observed wherever possible.

Protein synthesis was carried out at 25°C for 20 min. The standard medium was adopted from [15] and contained: 375 mM Sorbitol; 35 mM Hepes-KOH (pH 7.7); 2 mM EDTA; 1 mM $MnCl_2$; 1 mM $MgCl_2$; 0.1 mM Na_2HPO_4; 25 μM of each amino acid except methionine; 5 μM unlabelled methionine and 100 μCi/ml [^{35}S]methionine (800–1400 Ci/mMol; NEN), unless stated otherwise. Chloroplasts were added to a concentration of 250 μg chlorophyll/ml.

For the measurements of amino acid incorporation aliquots of 20 μl were precipitated on glass fiber filters (Whatman GFA) with 10% trichloroacetic acid. The filters were washed with 5% trichloroacetic acid, ethanol and acetone and the radioactivity measured by liquid scintillation spectrometry.

Products of in organello protein synthesis were analyzed by SDS-gel electrophoresis in the system in [16] on linear polyacrylamide gradient gels. After staining with Coomassie brilliant blue R250 the gels were treated with Enlightning (NEN), dried and exposed to X-ray film (Kodam X-Omatic AR).

The 32-kDa membrane polypeptide was identified by limited proteolysis [17]. The large subunit of the ribulose-1,5-bis-phosphate carboxylase was identified by direct immunoprecipitation [18] with specific rabbit antiserum prepared against this protein from *Chlamydomonas*.

3. RESULTS AND DISCUSSION

When the cells of the mutant *cw-15* of *C. reinhardii* are broken up by low pressures (in 5 atm) in the Yeda press, intact chloroplast can be isolated from the homogenate and separated from unbroken cells, stripped chloroplasts and other particles by centrifugation on step gradients of Percoll. The final chloroplasts preparations contained 70 S- and essentially no 80 S-type ribosomes, although marker enzyme activities for mitochondria and endoplasmic reticulum were still detectable. About 80–85% of the particles were intact, based on the O_2-evolution in the presence of ferricyanide [19]. The chloroplasts were also active in bicarbonate-dependent photosynthetic O_2-evolution and in CO_2-fixation (in preparation).

In the course of the isolation of the chloroplasts, we have routinely used media containing 5 mM $MgCl_2$ since this reduced clumping of the particles during fractionation. Using this medium, we found rather low rates of incorporation of radioactive amino acids. However, the addition of ATP greatly stimulated the incorporation, and furthermore, the stimulation was dependent on light [14]. This stimulation was due to chelation of free Mg^{2+} by the nucleotide. When the incubations were carried out in media containing different concentrations of $MgCl_2$ and in the absence of ATP, the incorporation of radioactive amino acid showed optimum rates up to 1 mM $MgCl_2$, similar to the optimum found for pea chloroplasts [15]. Above 1 mM $MgCl_2$, there was a drastic inhibition of incorporation, with values falling to about 10–15% of maximum when more than 2 mM $MgCl_2$ was present in the incubation medium (fig.1).

The inhibition by higher concentrations of free Mg^{2+} could be overcome by the addition of chelators such as ATP or EDTA to the incubation mixture (table 1). In the standard incubation mixture containing 1 mM $MgCl_2$ as well as 1 mM $MnCl_2$ and 2 mM EDTA, all the Mg^{2+} was essentially bound. We also found that low concentrations of inorganic phosphate (0.1 mM) caused about 50% stimulation of protein synthesis.

Protein synthesis by intact chloroplasts was

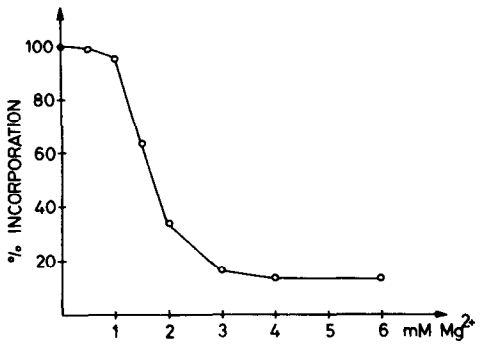

Fig.1. Influence of Mg-concentration on amino acid incorporation by isolated *Chlamydomonas* chloroplasts in standard medium with varying Mg^{2+}-concentrations.

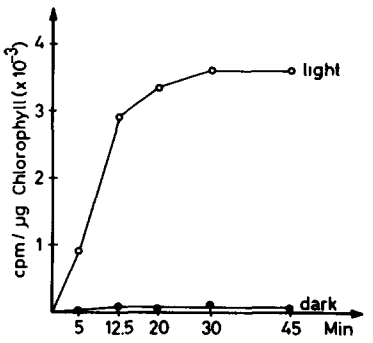

Fig.2. Time course of amino acid incorporation by chloroplasts incubated in standard medium with 70 µCi/ml [^{35}S]-methionine in light and in the dark.

strongly light-dependent, as can be clearly seen from table 1 and fig.2. No protein synthesis occurred in the dark, even in the presence of ATP or MgATP. Thus, exogenously added ATP does not replace the requirement for light. In contrast, chloroplasts which had been lysed by ultrasonication and supplemented with ATP incorporate labelled methionine about equally well in the dark or in the light. The time course of light-dependent in organello protein synthesis by *Chlamydomonas* chloroplasts resembles those so far reported for the chloroplasts of higher plants, with maximal rates occurring in the first 20 min (fig.2). The average rate of incorporation of about 4000 cpm/µg chlorophyll in 20 min compares well with that of spinach chloroplasts but is much lower than that of *Euglena* chloroplasts [20].

The effects of various inhibitors on protein synthesis by the chloroplasts were also tested (table 2). Cycloheximide had very little effect, whereas chloramphenical caused 80–85% inhibition; this indicated that protein synthesis was occurring on 70 S-type ribosomes. RNAase had a very slight effect, showing that most of the particles were intact, as confirmed by the ferricyanide dependent

Table 1

Influence of Mg^{2+}, ATP and EDTA on the rates of amino acid incorporation by isolated *Chlamydomonas* chloroplasts in the light and in the dark

Additions		Chlorophyll (cpm/µg)
None	dark	100
	light	4700
+ 4 mM Mg^{2+}	dark	90
	light	250
+ 4 mM Mg^{2+},	dark	100
+ 4 mM K-EDTA	light	3800
+ 4 mM Mg^{2+},	dark	150
+ 5 mM ATP	light	4000
Lysed chloroplasts		
+ 5 mM ATP,	dark	970
+ 4 mM Mg^{2+}	light	1060

The standard incubation mixture and conditions are described in section 2. The concentrations of the added reagents are in addition to those already present in the standard mixture. Lysed chloroplasts were obtained by short sonication of intact chloroplasts

Table 2

Effect of various inhibitors on chloroplast protein synthesis

Inhibitor	% Incorporation of [^{35}S]methionine
–	100
RNAase, 200 µg/ml	85–90
Cycloheximide, 40 µg/ml	90–100
Chloramphenicol, 100 µg/ml	15–20
DCMU, 20 µM	20–30
Methylamino-HCl, 5 mM	7–12
DCCD, 100 µM	12–25

Chloroplasts were incubated in the light for 25 min with [^{35}S]methionine in the absence or presence of inhibitor. The incubation mixture contained 5 mM $MgCl_2$ and 5 mM ATP

O_2-evolution. Inhibitors of electron transport and photophosphorylation also inhibited in organello protein synthesis, even in the presence of ATP.

Analysis of the products of protein synthesis by SDS-gel electrophoresis and fluorography revealed discrete bands (fig.3). The pattern of labelled products from in organello protein synthesis (lane 3) resembled the pattern obtained from cells which had been labelled in vivo in the presence of cycloheximide (lane 2) [12]. Among the strongly labelled products were those that banded at 55 and 32 kDa. These proteins were enriched in the soluble and membrane fractions, respectively, when the chloroplasts were lysed and centrifuged prior to electrophoresis (lanes 4,5).

We have identified the 55-kDa product as the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase by specific immunoprecipitation (lane 6), and the 32 kDa product as the rapidly labelled

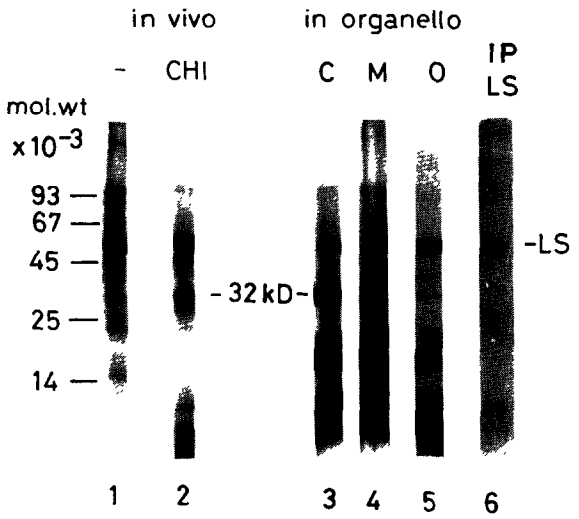

Fig.3. Products of in organello protein synthesis in *Chlamydomonas* chloroplasts, and of in vivo labelled cells. Lanes 1,2, in vivo labelled products in the absence (lane 1) or presence of 10 μg/ml cycloheximide (lane 2). Lanes 3-5, in organello labelled products of *Chlamydomonas* chloroplasts. C, total; M, membrane fraction; O, supernatant fraction. M and O fractions were obtained after lysis of chloroplasts and centrifugation at 30000×g for 20 min. Lane 6, immunoprecipitated large subunit of ribulose-1,5-bisphosphate-carboxylase from in organello labelled chloroplasts. Electrophoresis was done on a 10-20% linear gradient of polyacrylamide. kD, kDa.

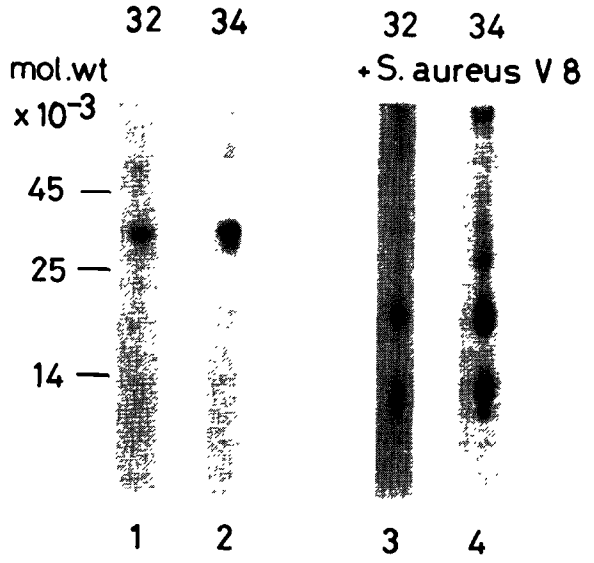

Fig.4. Identification of the in organello labelled 32-kDa polypeptide by partial proteolytic digestion. Lanes 1,2, 32-kDa in organello labelled product and 34-kDa product labelled in vitro by translation of chloroplast RNA in the reticulocyte lysate were excised and reelectrophoresed on a 15% polyacrylamide gel. Lanes 3,4, bands were excised and reelectrophoresed as in 1 and 2, in the presence of 2 μg *S. aureaus* V8 protease.

32-kDa membrane protein by its proteolytic fingerprints (fig.4). These fingerprints as well as those of the 34-kDa precursor form made by translation of chloroplast RNA in vitro [9] correspond to those published for the proteins from *Chlamydomonas* and other organisms [21]. The LS and the 32-kDa polypeptide have also been found to be the main soluble and membrane products of in organello protein synthesis by higher plant chloroplasts [1]. The identity of the heavily labelled material at 15-20 kDa is not known to us. It does not correspond to any stainable protein material and may be an artefact.

C. reinhardii has been a favorite for studying chloroplast development and autonomy. The possibility of investigating in organello protein synthesis in this alga could complement and augment the existing information on chloroplast biogenesis.

Apart from determining directly the sites of synthesis of chloroplast proteins and comparing these to other systems, the more compelling problems regarding regulation, transport and assembly of

chloroplast proteins to form functional complexes may be more conveniently studied in this organism.

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