SYNTHESIS OF POLYPEPTIDES OF THE CHLOROPHYLL–PROTEIN COMPLEXES IN ISOLATED CHLOROPLASTS OF *EUGLENA GRACILIS*

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1. Introduction

A novel purification procedure has been described for obtaining intact *Euglena gracilis* chloroplasts in gradients of Percoll with a high and strictly light-dependent capacity for protein synthesis [1]. We have now further tested such chloroplasts with respect to the synthesis of thylakoid membrane proteins in connection with a general project on the genetic information potential of chloroplast DNA [2].

Three major chlorophyll–protein complexes have been described in higher plants [3–8], *Chlamydomonas* [9] and *Caulerpa cactoides* [10] but none so far for *Euglena gracilis*. Here we characterize three chlorophyll–protein complexes, namely CP_1, CPa and LHCP (in the nomenclature of [3]), which we obtain upon dissociation of washed thylakoid membranes of *Euglena*. Furthermore, we show that one major and three minor proteins associated with CP_1 and two major and three minor proteins associated with CPa are synthesized by isolated chloroplasts. Two major proteins of LHCP seem not to be synthesized within the chloroplast. The results are compared with data obtained by others with *Chlamydomonas* and with higher plants.

2. Materials and methods

Euglena gracilis Z was grown photoheterotrophically on the modified Hutner's media of [11] with vitamin B_{12} at 50 ng/l.

Chloroplasts were isolated by the basic procedure in [1] with the following modifications:

(i) For digestion, the cells were resuspended in 0.38 M sorbitol–50 mM potassium phosphate (pH 7) containing 5 mg/ml trypsin (bovine pancreas type III, Sigma);

(ii) For lysis, the cells were resuspended in 0.33 M sorbitol, 0.5 mM Na_2EDTA–25 mM Hepes (pH 7.4) containing 1 mg/ml trypsin inhibitor (from egg white, Boehringer) and were broken by low speed homogenization in a Waring blendor for 3–5 s;

(iii) For the density centrifugation step we utilized a linear gradient of 10–80% Percoll (Pharmacia).

Light-dependent protein synthesis by the isolated chloroplasts was done as in [12] in the presence of 10–20 μCi [^{35}S]methionine (>600 Ci/mmol) or [^{3}H]leucine (125 Ci/mmol) (Amersham) per ml chloroplasts at 20°C with gentle shaking under illumination with red light. Red light was obtained with a red filter with a transmittance of 100% at wavelengths >640 nm. Total protein synthesis was determined according to [13].

Thylakoids from radioactively labelled chloroplasts were washed as in [14] to remove the soluble components of the chloroplast and peripheral proteins from the thylakoid membranes. Finally, the thylakoids were resuspended in 100 mM tricine–KOH (pH 7.8) at 2.5 mg chl/ml final conc. Thylakoids were stored at −20°C.

Discontinuous SDS–polyacrylamide gel electrophoresis was according to [15] on slab gels 2.0 mm thick. In the first dimension the stacking gel (1 cm) contained 4% acrylamide while the running gel

Abbreviations: chl, chlorophyll; CP_1, P700–chlorophyll *a*–protein; CPa, photosystem (PS) II-associated chlorophyll *a*–protein; LHCP, light-harvesting chlorophyll *a/b* protein; FC, free chlorophyll; kd, kilodaltons

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(11 cm) contained 8% acrylamide. The second dimension gel contained also a 1 cm stacking gel and a 19 cm 10–15% linear gradient of acrylamide in the running gel.

The chloroplast membranes were solubilized at 4°C in enough sample buffer (0.3 M Tris–HCl (pH 8.8) containing 10% glycerol and 1% SDS, at 1 X conc.) to give a final SDS:chl weight ratio of 10:1 (w:w) and 1 mg chl/ml. The solubilized thylakoids were applied immediately to the first dimension gel. The electrophoretic run was performed at 4°C for 5.5 h at 12.5 mA constant current. The gel and the upper and lower reservoir buffers were cooled to 4°C prior to the run in the first dimension.

For the second dimension run, we incubated a strip of the 8% gel, containing the 4 chl zones, in 2% SDS-stacking buffer at room temperature for 1.5 h. Afterwards, the gel strip was incubated at 60°C for 5 min and immediately embedded in the stacking gel of the second dimension with 1% agarose (in stacking buffer and 0.1% SDS). Electrophoresis in the second dimension was performed at 30 mA constant current. The dried gels were exposed directly to X-ray film (Typon Typox TX Kodak) for 5–7 days at room temperature.

CO_2-dependent O_2 evolution by the isolated chloroplasts was carried out with a Rank model oxygen electrode. The assay mixture contained 0.2 ml chloroplasts corresponding to ~100 μg chl in 1 ml final vol. of assay medium (0.350 M sorbitol, 2 mM isoascorbate, 10 mM $NaHCO_3$, 3–5 mM Na-phosphate, 50 mM Hepes–NaOH, pH 7.6). The assay solutions were flushed with nitrogen before the addition of the chloroplasts. Determinations were made at 20°C. Chloroplasts were illuminated with a single 150 W spot light mounted 15 cm away. Net oxygen evolution of whole cells was measured directly in photoheterotrophic medium.

Chlorophyll was measured according to [16]. The room temperature absorption spectra of the chl–protein complexes were recorded by directly measuring the corresponding excised gel segments in a Perkin-Elmer spectrophotometer model 552.

3. Results and discussion

Before attempting any study of the chl–protein complexes of *Euglena gracilis*, it was important to show that chloroplasts from photoheterotrophically grown and vitamin B_{12}-deficient cells have a functional photosynthetic system, e.g., retained the capacity for CO_2-dependent O_2 evolution. The data in table 1 show that the rate of O_2 evolution of isolated chloroplasts is 55% of that of whole cells. These values favorably compare with data in [17] also using *Euglena gracilis* chloroplasts which had not undergone a purification step in a Percoll gradient. Furthermore, we observe that the incorporation of labelled amino acids into acid-precipitable proteins is strictly light-dependent. A more detailed characterization of the proteosynthetic activity of *Euglena* 'Percoll' chloroplasts appears in [1].

Washed thylakoids were solubilized as specified and the proteins analysed by gel electrophoresis. In fig.1 we show the photographs of the unstained (A) and the Coomassie brilliant blue-stained (B) gels. This is the first demonstration of 4 electrophoretically resolved green bands from *Euglena* thylakoids which most likely correspond to CP_1, CPa, LHCP and FC using the nomenclature of [3]. Evidence that 3 of the green bands represent distinct chl–protein complexes is presented in fig.1B,1C. Except for the fastest moving green zone of free pigments, the top 3 green bands in 1A stain for protein with Coomassie brilliant blue (1B) and their room temperature absorption spectra in 1C display characteristic maxima at 677 nm (CP_1), 673 nm (CPa) and 467, 653 and 672 nm (LHCP) similar to reported values [3]. The spectrum for the free pigment zone is not shown. Under our experimental conditions we did not observe the possible multimeric forms of LHCP reported [3–5,7]. In *Euglena*, with a high chl *a*:*b* ratio, the content of LHCP may be relatively low [18].

A major goal of this work was to see whether any

Table 1
Light-dependent protein synthesis (A) and CO_2-dependent O_2 evolution (B) in isolated 'Percoll' chloroplasts of *Euglena gracilis*

(A)	Incorporation (cpm/μg chl . 30 min)	
	[^{3}H]leucine	[^{35}S]methionine
Light	5742	18 385
Dark	56	86
(B)	μmol O_2/mg chl . h	
Chloroplasts	21.7	
Whole cells	38.2	

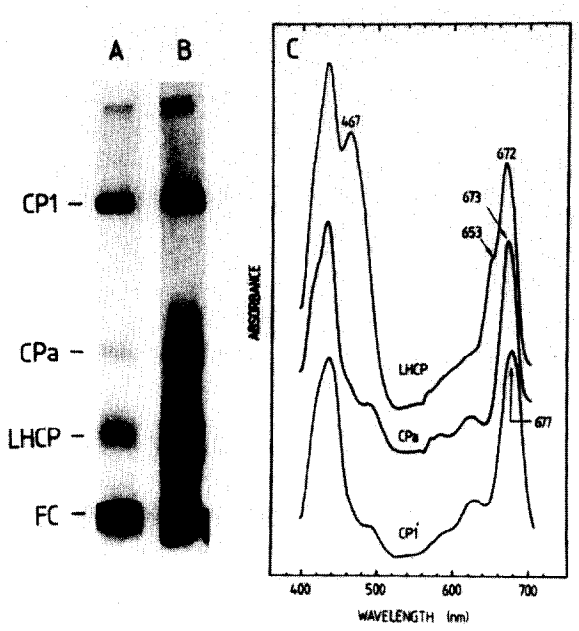

Fig.1. Gel electrophoresis and absorption spectra of chl–protein complexes: (A) unstained gel; (B) Coomassie brilliant blue-stained gel; (C) room temperature absorption spectra of gel segments containing the chl–protein complexes.

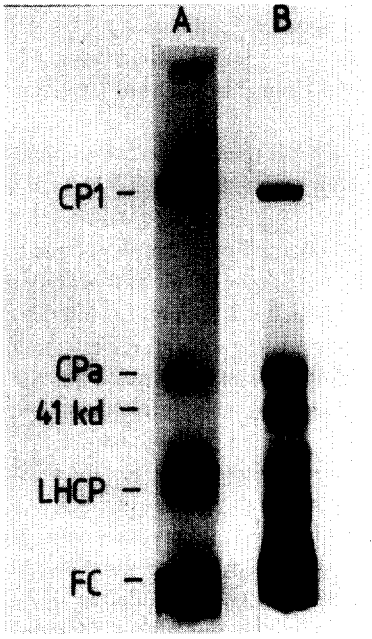

Fig.2. Identification of the chl–protein complexes synthesized by isolated chloroplasts: (A) unstained gel; (B) autoradiograph.

of the proteins of the chl–protein complexes were synthesized *in organello*, i.e., by the isolated chloroplasts. In fig.2 we show the unstained gel (A) and the corresponding autoradiograph (B). CP_1 and CPa are clearly radioactively labelled, however, we can draw no clear conclusions for LHCP since the corresponding region in 2B has a relatively high radioactive background. Since no distinct zone corresponds to LHCP in 2B, we tentatively conclude that also in *Euglena* the proteins of LHCP are synthesized in and imported from the cytoplasm. With respect to CP_1, our results align with reports that isolated spinach and *Sorghum* chloroplasts synthesize CP_1 [19,20] respectively. However, CP_1 was not synthesized in isolated pea chloroplasts [21].

CPa is considered to contain the reaction center of PS II [7,9,15]. Our results are direct evidence that protein(s) associated with CPa are synthesized within isolated chloroplasts. These results also align with the report that chloramphenicol inhibits the synthesis of 2 major polypeptides in a PS II-active membrane fraction of *Chlamydomonas* [22]. Similarly, synthesis of polypeptides 5 and 6, considered to be part of PS II in *Chlamydomonas* [9], was inhibited by chloramphenicol [23].

To gain insight into the polypeptide composition of CP_1, CPa and LHCP, we incubated a gel strip similar to that in fig.1A in 2% SDS for 1.5 h, then at 60°C for 5 min to dissociate the chl–protein complexes. The gel strip was placed on top of a second gel and re-electrophoresed as specified. The Coomassie brilliant blue-stained gel (fig.3A) reveals that upon dissociation CP_1 yields a major protein of 73 kd and at least 3 minor proteins of 47, 30 and 15 kd. We could also discern a faint green band of ~115 kd most likely representing undissociated CP_1. CPa yields two major stainable proteins of 57 and 48 kd and a minor component of 41 kd. The LHCP yields two major proteins of 26 and 23 kd. With respect to major proteins our results are comparable with those obtained for CP_1 [7,24], CPa [5,7,9] and LHCP [7,20,24–26]. Minor protein components are still poorly characterized [7,24,27] and one should be aware, that no uniform terminology exists. A comparative analysis is beyond the scope of this paper.

The autoradiograph (fig.3B) reveals a number of interesting features:

(i) The major protein of CP_1 (73 kd) is very weakly labelled while the 15 kd protein shows a relatively strong radioactive signal. The 47 and 30 kd -

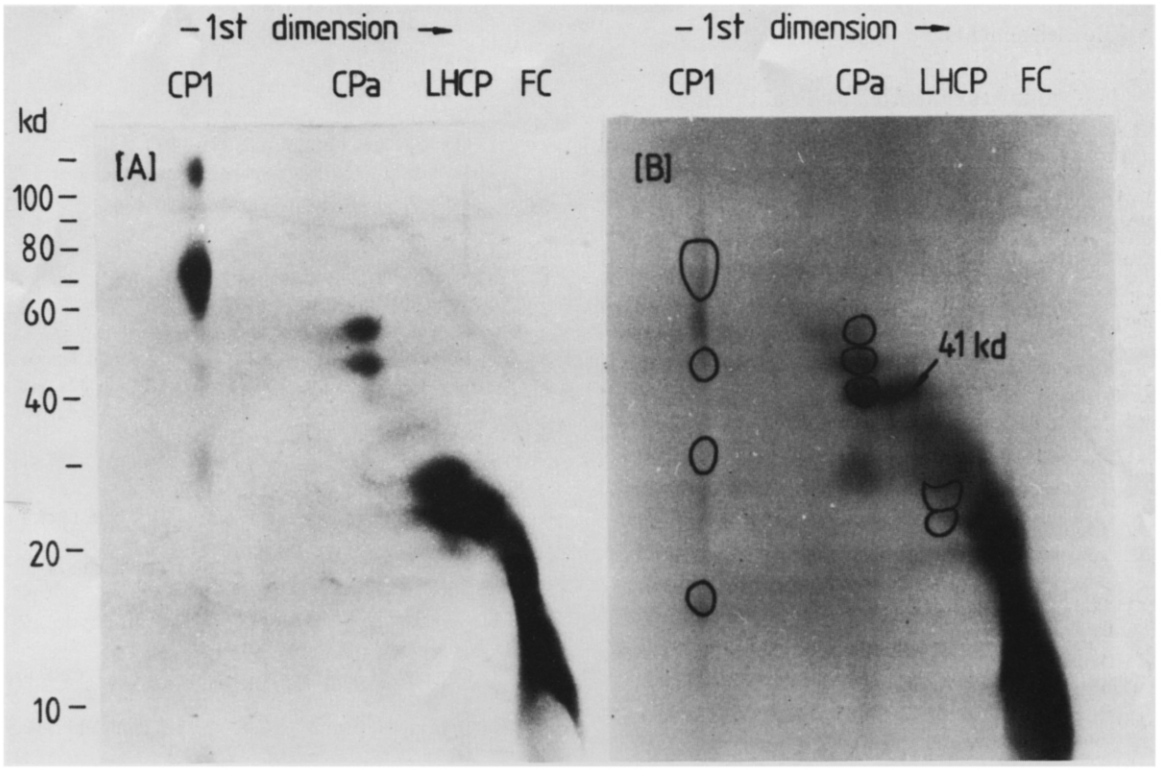

Fig.3. Two dimensional gel electrophoretic separation of dissociated chl–protein complexes and identification of protein components synthesized in isolated chloroplasts: (A) Coomassie brilliant blue-stained gel; (B) autoradiograph. Circled areas in (B) correspond to stainable proteins in (A). The arrow in (B) marks rapidly labelled protein of ~41 kd not associated with any of the chl–protein complexes. Although some of the faintly staining areas in (A) may be difficult to see, the corresponding blue spots were clearly visible on the original stained gel.

minor proteins are not labelled, however, two proteins of 58 and 28 kd not seen in the stained gel are radioactive. Thus, it seems that CP_1 is a complex of one major and several minor proteins which under the given experimental conditions show differences in their rate of synthesis.

(ii) Similarly, for CPa we find that the two major proteins (57 and 48 kd) seem to be labelled at different rates, the radioactive signal being stronger for the 48 kd protein. Most conspicuous is the high radioactivity associated with the faintly staining 41 kd protein of CPa. Two additional radioactive spots are seen corresponding to proteins of 28 and 10 kd which are not visible in the stained gel.

(iii) The two major LHCP proteins of 26 and 23 kd do not yield defined radioactive spots suggesting that they are not synthesized within the chloroplasts.

(iv) In the free pigment zone many labelled proteins accumulate ranging in size from ~10–30 kd. Unfortunately, this zone is unresolved due to an overload of proteins and therefore an evaluation is impossible.

It was reported [28] that chloroplasts from *Spirodela* synthesize a thylakoid membrane protein of 32 kd which gets very rapidly labelled but seems not to accumulate proportionately in the thylakoid. We did not observe in our experiments such a 32 kd protein but we found a membrane band protein of ~41 kd (different, however, from the 41 kd component of CPa) which is rapidly labelled (fig.2B, fig.3B) but seems not to accumulate either since a corresponding band (spot) is missing in the stained gels (fig.2A, fig.3A).

In summary these results demonstrate that chloroplasts isolated from photoheterotrophic vitamin B_{12}-deficient *E. gracilis* synthesize and assemble several proteins into specific chlorophyll–protein complexes.

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