In Vitro Chloroplast Protein Synthesis by the Chromophytic Alga *Olisthodiscus luteus*†

Michael E. Reith‡ and Rose Ann Cattolico*

Department of Botany, University of Washington, Seattle, Washington 98195

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ABSTRACT: The chloroplasts of chlorophytic and chromophytic plants exhibit significant morphological and biochemical differences. Presently, it is impossible to compare the influence of ctDNA on the structure and function of organelles within these two phylogenetic groups for no data exist in the literature on the profile of protein products synthesized by a chromophytic plastid. In this paper, the chloroplast DNA coded proteins of the chromophytic plant *Olisthodiscus luteus* are investigated by labeling isolated chloroplasts in vitro. Isolated plastids of excellent morphological condition are pulse labeled with [^{35}S]methionine. Approximately 100 proteins are detected by two-dimensional gel electrophoresis and fluorography. However, these isolated plastids have a number of unusual characteristics: (1) they are photosynthetically inactive; (2) in vitro protein synthesis is light independent; (3) all proteins synthesized in vitro are membrane associated.

The physical constraints of ctDNA[1] homogeneity and size require the use of both nuclear and chloroplast coded gene products for the biogenesis and functional regulation of the plastid. Moreover, the genetic and protein synthetic systems that are under the control of these two cooperating organelles must be highly coordinated if this interaction is to succeed.

To date, information on the spectrum of ctDNA products has been obtained exclusively from investigations of chlorophytic plants including algae and land plant representatives. It has been demonstrated that the ctDNA of this taxonomic group codes for one to three ribosomal RNA cistrons, nearly 50 tRNA species, and potentially about 100 proteins [see Bohnert et al. (1982) and Bottomley & Bohnert (1982) for review]. Of the large potential protein contribution, only 34 polypeptides, which include 20 ribosomal proteins (Schmidt et al., 1983), have been positively identified with respect to function.

Unfortunately, no data on ctDNA coding profiles for a major taxonomic group of plants, the Chromophyta, are available [see Cattolico et al. (1985) for review]. These nongreen plants are important for they substantially contribute to the total global carbon fixed in our environment. A number of characteristics (Gibbs, 1981; Whatley & Whatley, 1981; Coleman, 1981; Kuroiwa et al., 1981; Dodge, 1973) distinguish chromophytic plants from the Chlorophyta. These include (a) pigment composition (chlorophytes contain chlorophylls *a* and *b* whereas chromophytes contain chlorophylls *a* and *c* and sometimes phycobilins), (b) the number of membranes surrounding the chloroplast (two are present in most chlorophytes, whereas a four-membrane set occurs in a predominant number of the Chromophyta), (c) the presence or absence (chlorophytes and chromophytes, respectively) of grana, and (d) the arrangement of ctDNA into a nucleoid structure (ctDNA in chlorophytes is present in multiple small nucleoids whereas many chromophytic genera studied have the ctDNA localized in a single ring-shaped nucleoid). The observed differences in chloroplast structure and biochemical components suggest that diversity in ctDNA protein products may occur between these plant groups.

This paper represents the first attempt to analyze the profile of ctDNA-coded proteins produced by a chromophytic plant. The biflagellate unicellular alga *Olisthodiscus luteus* (Cattolico et al., 1976) provides an attractive system for these studies. It contains approximately 30 plastids per cell (Cattolico, 1978a) and is naturally wall-less, and chloroplast division occurs synchronously when cells are maintained on a 12 h light/12 h dark cycle. Extensive analyses (Aldrich & Cattolico, 1981; Aldrich et al., 1982; Ersland et al., 1981) have demonstrated that each chloroplast contains a homogeneous population of approximately 30 DNA molecules that have a M_r of 97×10^6.

The experimental method developed by Ellis and co-workers (Ellis, 1977; Ellis et al., 1977) has been used extensively in the determination of the ctDNA-coded protein products of higher plants. This technique utilizes isolated intact chloroplasts, which are incubated in the presence of labeled amino acids. Since light provides the energy for this reaction, only those proteins newly synthesized by the chloroplast will become labeled. These proteins can then be separated by gel electrophoresis and visualized by autoradiography.

Applying this technique to isolated *Olisthodiscus* chloroplasts, we have observed approximately 100 labeled chloroplast proteins. This number is similar to that observed by Ellis and co-workers (1977) for isolated pea chloroplasts. However, a number of unexpected labeling characteristics have been observed to occur in isolated *Olisthodiscus* plastids. An analysis of those factors that may significantly affect in vitro chloroplast biosynthetic capacity is discussed in this paper. A comparison of in vitro and in vivo labeled *Olisthodiscus* ctDNA coded

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‡ Present address: Department of Botany, University of Toronto, Toronto, Ontario, Canada M5S-1A1.

[1] Abbreviations: BSA, bovine serum albumin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ctDNA, chloroplast DNA; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid disodium salt; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; PVP, poly(vinylpyrrolidone); TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; RuBPCase, ribulosebisphosphate carboxylase; Tris, tris(hydroxymethyl)aminomethane.

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proteins is presented in the following paper (Reith & Cattolico, 1985).

Materials and Methods

Growth of Cells. Olisthodiscus luteus (Carter) was grown in an artificial seawater medium (0-3) on a 12 h light/12 h dark cycle as described elsewhere (McIntosh & Cattolico, 1978). All cultures were stringently monitored for both bacterial and fungal contamination. A solution composed of 2.0 g of nutrient broth and 1.25 g of yeast extract in 250 mL of 0-3 medium, distributed in 5.0-mL aliquotes, and autocleaved for 20 min was used for this test. Cell counts were made in a Model ZB1 Coulter cell counter. Chloroplasts were counted according to Cattolico et al. (1976).

Chloroplast Isolation. Cells in the exponential phase of growth [(1–5) × 10^4 cells/mL] were harvested between hours 4 and 6 of the light period by layering approximately 200 mL of culture onto a 20-mL 90% Percoll pad containing 50 mM HEPES, pH 7.6, and 1 mM $MgCl_2 \cdot 6H_2O$ (Price et al., 1978). The gradient was centrifuged at 165*g* for 10 min in a Sorvall HS-4 rotor. This and all subsequent procedures were carried out at 5 °C. The thin layer of cells that covers the bottom of the centrifuge bottle was recovered in a Pasteur pipette and further concentrated by spinning it at 3000*g* for 5 min. As much supernatent was removed as possible without distrubing the loose pellet. The pellet was gently resuspended in the remaining supernatent and the cell concentration adjusted to 2.3 × 10^7 cells/mL in buffer SHMB (0.33 M sorbitol, 50 mM HEPES, 1 mM $MgCl_2 \cdot 6H_2O$, and 2% BSA (fraction V, Sigma) adjusted to pH 7.6 with KOH]. After passage through a French press at 450 psi, remaining whole cells were removed by centrifuging 15 s at 2600*g*. The chloroplasts were then pelleted by accelerating to 5900*g* and braking immediately, after which they were then gently resuspended in buffer SH (0.33 M sorbitol–50 mM HEPES, pH 7.6).

Protein Synthesis. Chloroplast preparations were tested for incorporation of [^{35}S]methionine into TCA-insoluble material by adding 4–5 μCi of [^{35}S]methionine (1140 Ci/mmol) to 0.1 mL of chloroplasts (corresponding to approximately 8 μg of chlorophyll or 50 μg of protein). This reaction mixture was incubated in a 20 °C water bath with stirring. The chloroplasts were illuminated with a Dicrolite (West Coast Metals) at approximately 160 μE m^{-2} s^{-1} at the reaction vessel surface. The spectrum of this light source falls mainly within the range of photosynthetically active light (400–750 nm). Dark controls were wrapped in aluminum foil. Inhibitor addition was made 5 min prior to the introduction of label. Incubations were terminated, usually after 30 min, by spotting duplicate 50-μL aliquots on 23-mm Whatman 3MM filter paper disks. Trichloroacetic acid precipitable incorporation was determined according to the technique of Bollum (1968). To prepare samples for gel electrophoresis, 1 mL of purified chloroplasts was incubated with 40–50 μCi of [^{35}S]methionine for 30 min at 20 °C. The incubation was terminated by placing the sample on ice.

Sample Preparation. Chloroplasts labeled for electrophoretic analysis were pelleted at 15000*g* for 1 min. The chloroplasts were usually separated into membrane and soluble fractions by resuspending the pellet in 50–100 μL of solution A [0.1 M HEPES–0.1 M DTT, adjusted to pH 7.6 with KOH (Chua, 1980)] followed by vigorous vortexing. The chloroplasts were then allowed to sit on ice for 15 min. Samples were centrifuged for 10 min at 15000*g*, and the supernatent containing the soluble proteins was removed. The membrane-containing pellet was washed with 1 mL of 50 mM Tris, pH 7.6, and resuspended in a small volume of solution A. For one-dimensional SDS gels, 3 volumes of sample was mixed with 2 volumes of solution B [5% SDS, 30% sucrose, and 0.1% bromophenol blue (Chua, 1980)] and then heated in boiling water (Chua, 1980) for 30 s immediately before loading. For two-dimensional gel electrophoresis, the samples were adjusted to 9.5 M urea, 0.5% SDS, and 1% pH 3–10 ampholines (O'Farrell et al., 1977). The reaction mixture was allowed to sit for 15 min at room temperature, after which an equal volume of the NP-40-containing lysis buffer of O'Farrell (1975) was added and the samples were loaded onto gels.

Gel Electrophoresis. One-dimensional gel electrophoresis was performed on 10%–20% linear-gradient polyacrylamide gels in the buffer system of Laemmli (1970). The 0.8 mm thick gels were run in an apparatus similar to that of Matsudaira & Burgess (1978). Two-dimensional gel electrophoresis was done according to the technique of O'Farrell et al. (1977) with pH 3–10 ampholines in the first dimension and a 10%–16% exponential acrylamide gradient in the second. First-dimension gels were run in 2.5 mm × 130 mm glass tubes. Second-dimension gels were 170 × 158 × 0.75 mm in size. After electrophoresis, gels were stained with Coomassie blue and prepared for fluorography with Enhance (New England Nuclear). Dried gels then were exposed to prefogged (Laskey & Mills, 1975) X-ray film (Kodak X-Omat R) at −70 °C. One-dimensional gels were exposed for the equivalent of 10^5 dpm for 24 h while two-dimensional gels were exposed for the equivalent of 10^6 dpm for 24 h.

Electron Microscopy. Isolated chloroplasts were prepared for electron microscopy by the addition of glutaraldehyde and osmium tetroxide to final concentrations of 2.0% and 1.0%, respectively. Samples were embedded, sectioned, and stained as described previously (Barlow & Cattolico, 1980) and viewed in a Phillips 300 electron microscope.

Miscellaneous Assays. For assays of photosynthetic capability, resuspended chloroplasts were adjusted to 2 mM EDTA, 1 mM $MnCl_2$, 5 mM $Na_4P_2O_7$, and 0.5 mM KH_2PO_4 (Jensen & Bassham, 1966). CO_2-dependent O_2 evolution was measured with a YSI Model 53 biological oxygen electrode after addition of $NaHCO_3$ to a concentration of 10 mM. To measure carbon dioxide fixation, 50 μCi of $NaH^{14}CO_3$ was added to 0.5 mL of isolated chloroplasts after a 5-min preillumination. Fifty microliter aliquots were then removed and processed according to Rathnam & Edwards (1976).

Protein was assayed by the method of Lowry et al. (1951). Chlorophyll was determined according to Arnon (1949), using extinction coefficients calculated by Dr. S. Gibbs, McGill University (personal communication), where chlorophyll *a* (μg/μL) = $11.02A_{663} - 0.18A_{630}$.

Results

Chloroplast Isolation Technique. Several criteria must be met to obtain high-quality chloroplasts from *Olisthodiscus*. Due to the fragile, wall-less nature of this organism, standard centrifugation and resuspension techniques often leave many of the cells badly damaged, thus exposing the plastids to the high ionic strength of the growth medium. To recover whole cells as gently as possible, a modification of the technique of Price et al. (1978) was developed. In this method, cells are centrifuged through a Percoll–seawater pad. As a result, nearly all the algal cells recovered are still actively swimming upon resuspension in breaking buffer.

Breaking buffer composition was the second major factor that significantly affected isolated chloroplast integrity. When amino acid incorporation was used to measure chloroplast quality, it was shown (Table I) that among the low molecular weight sugars sorbitol was a much more effective osmoticum

Table I: Effect of Buffer Complement on in Vitro Plastid Protein Synthesis

	sugars[a]		
	0.3 M sucrose	0.3 M mannitol	0.3 M sorbitol
light	1964	2778	3597
dark	1453	2782	4203
no chloroplasts	242	555	666
	high molecular weight molecules[b]		
	2% Ficoll	2% PVP	2% BSA	2% BSA/SH
light	3203	7012	48 988	48 421
dark	3255	7070	41 489	48 985
no chloroplasts	1863	976	9 182	1 260

[a] Plastids were isolated in buffers containing 50 mM HEPES (pH 7.6), 1 mM $MgCl_2 \cdot 6H_2O$, and the indicated sugar. A total of 0.1 mL of chloroplasts was incubated with 5 μCi of [^{35}S]methionine for 30 min. Data are expressed as cpm incorporated. [b] Plastids were isolated in buffers containing 0.3 M sorbitol, 50 mM HEPES (pH 7.6), 1 mM $MgCl_2 \cdot 6H_2O$, and the indicated high molecular weight compound. In the final experiment, chloroplasts were resuspended in buffer SH (0.3 M sorbitol–50 mM HEPES, pH 7.6) rather than in the original isolation buffer.

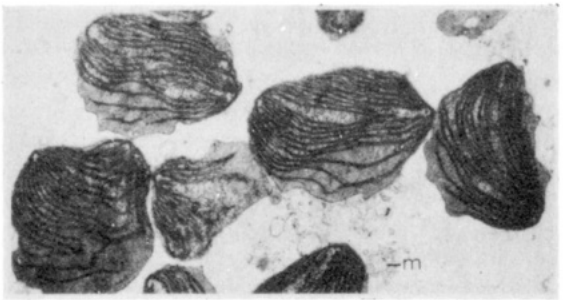

FIGURE 1: Electron micrograph of isolated *Olisthodiscus* chloroplasts: m, mitochondria; magnification 15600×.

than either mannitol or sucrose and that sorbitol combined with BSA rather than Ficoll or PVP (Table I) gave the best protection to plastids during cellular disruption. However, it was necessary to remove the chloroplasts from this BSA-containing buffer to minimize background incorporation (Table I) during amino acid labeling.

Finally, the method of cellular disruption was shown to be of significant importance in these studies. Cells broken by Dounce homogenization, shaking with glass beads, sonication, or centrifuging through small-mesh nylon screens generally resulted in chloroplasts of low and inconsistent quality. Moreover, a large number of contaminating whole cells was often left by these techniques. Of the numerous methods tested, disruption of the cells by shear force in a French press was most effective. A specific cell concentraton (2.3×10^7 cells/mL) and breaking pressure (450 psi) were determined by trial and error to be most effective in producing chloroplasts of high quality.

Quality of Isolated Plastids. Throughout this investigation, a number of morphological and biochemical techniques were used to probe the quality of chloroplasts isolated from *Olisthodiscus*. Depending on the method used, significant differences in the capabilities of the isolated plastids were obtained.

Phase-contrast microscopy indicated that 60%–70% of the chloroplasts were highly refractile. The criterion of refractility has often been used as a measurement of chloroplast intactness. When plastid preparations were analyzed by electron microscopy (Figure 1), the plastids were undamaged (i.e., no loosening of stacked thylakoids or membrane blebbing). Few contaminating mitochondria and no nuclei were present in these micrographs. The innermost pair of the four limiting

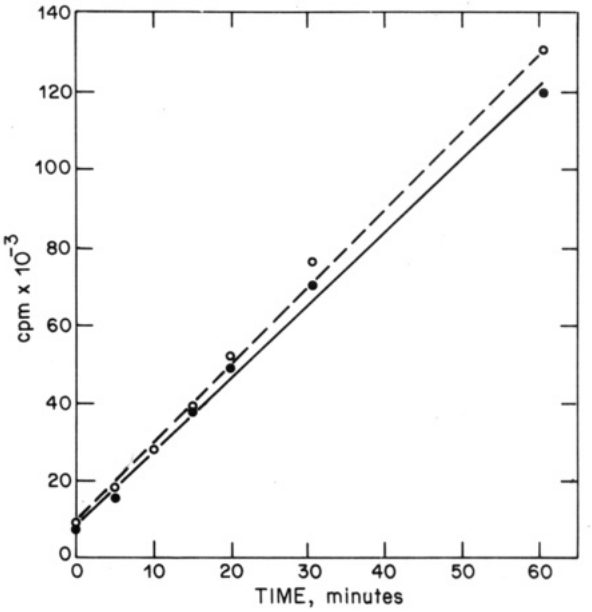

FIGURE 2: Incorporation of [^{35}S]methionine by isolated chloroplasts in the presence (○) or absence (●) of light. Each point is a mean of two replicates. Variation between replicates was no greater than 5%.

Table II: Effect of Inhibitors on in Vitro Protein Synthesis[a]

conditions	% of light control[b]
dark	94
light + chloramphenicol (50 μg/mL)	8
dark + chloramphenicol (50 μg/mL)	8
light + cycloheximide (1 μg/mL)	106
dark + cycloheximide (1 μg/mL)	105
light + RNase (30 μg/mL)	103
light + DCMU (5 μM)	107
light + CCCP (5 μM)	99
light + energy-generating system[c]	97
dark + energy generating system	104
light + 2% Triton X-100 treatment[d]	<1

[a] A total of 0.1 mL of isolated chloroplasts in buffer SH (0.3 M sorbitol–50 mM HEPES, pH 7.6) was incubated with 5 μCi of [^{35}S]-methionine for 30 min under the conditions described. Each data point is the mean of two replicate samples. [b] The light control contained 44 800 cpm. [c] The energy-generating system consisted of 2 mM ATP, 5 mM creatine phosphate, and 100 μg/mL creatine phosphokinase. [d] Chloroplasts were incubated for 30 min in the light and then solubilized with 2% Triton X-100. After being centrifuged at 15000*g* for 15 min, the pellet was tested for incorporation.

plastid membranes of an *Olisthodiscus* chloroplast appeared to be intact in most isolated plastids. However, the intactness of the outermost pair of membranes was more difficult to assess.

The [^{35}S]methionine incorporation studies done to monitor plastid functionality (Figure 2) yield an interesting obse vation. Chloroplasts incorporate this polypeptide precursor with equal efficiency whether the plastids are in the light or maintained in the dark during the incubation period. A linear rate of precursor incorporation is seen to occur for at least 60 min. Similar data are obtained when either [^{3}H]arginine or [^{14}C]leucine is used as the labeled amino acid.

This unusual result was further investigated with inhibitor probes. Addition (Table II) of chloramphenicol reduces incorporation by 92% whereas cycloheximide has no effect on protein synthesis. These observations are unaffected by the presence or absence of light during the incubation period. These data give evidence that protein synthesis occurs on plastid 70S ribosomes and not on contaminating 80S species. The fact that RNase has no effect (Table II) on methionine

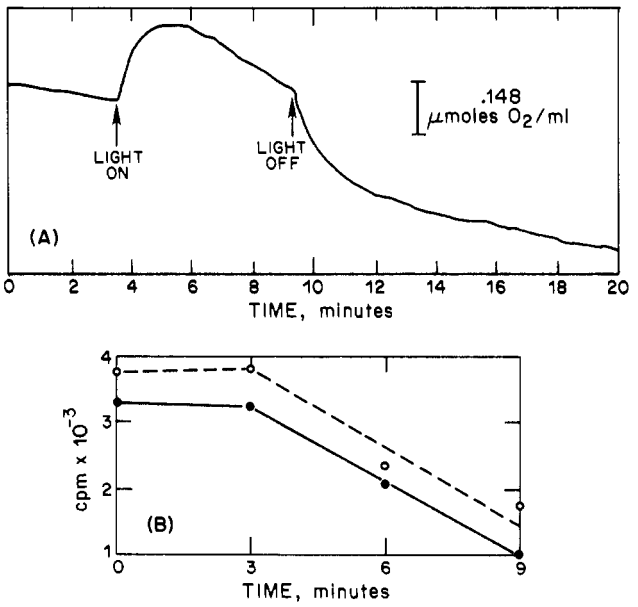

FIGURE 3: Photosynthetic activity of isolated chloroplasts: (A) oxygen evolution; (B) incorporation of $NaH^{14}CO_3$; (○) light incorporation; (●) dark incorporation. Each point is a mean of two replicates. Variation between replicates was no greater than 7%.

incorporation demonstrates that the chloroplasts must have a substantial degree of morphological integrity. Penetration by this enzyme into the plastid would cause a severe depression in protein synthesis within the isolated organelle. This result also indicates that contaminating cytoplasmic polysomes are not involved in the observed protein synthesis since these organelles are susceptible to degradation by RNase. If chloroplasts that have incorporated label for 30 min are solubilized in 2% Triton X-100 and centrifuged (15000*g*, 15 min), less than 1% of the incorporated label is retained in the pellet (Table II). This result indicates that the observed incorporation is not attributable to contaminating bacteria (Parenti & Margulies, 1967).

The presence of DCMU, an inhibitor of electron transport, causes no reduction (Table II) in either light or dark incorporation capacity of the plastids. This lack of inhibition is consistent with the observation that photosynthetic energy capture does not play a role in supplying the energy needed for protein synthesis. The inhibitor of both oxidative and photosynthetic phosphorylation, CCCP (Heytler, 1963), also fails (Table II) to decrease incorporation. These results exclude the possibility that energy is supplied through phosphorylation events either in the chloroplasts or by contaminating mitochondria. The addition of ATP to the incubation mixture also has no effect (Table II). Since mannitol is the major photosynthetic storage product in *Olisthodiscus* (Hellebust, 1965), it is possible that the osmoticum component, sorbitol, might serve as an energy source. However, chloroplasts incubated in the presence of this labeled sugar alcohol had negligible incorporation. Moreover, no difference in the dark-labeling response was observed if chloroplasts were isolated in osmotica other than sorbitol. Though we have no direct data, these results imply that a storage product must supply the ATP necessary for chloroplast protein synthesis.

Finally, though isolated plastids retain Hill reaction capability, these chloroplasts evolve O_2 (Figure 3A) and fix CO_2 (Figure 3B) for only approximately the first minute of the incubation period. Thereafter, the chloroplasts respire, and CO_2 incorporation quickly drops to background levels. Maintenance of long-term CO_2 fixation or O_2 evolution was not enhanced by any buffer additions or isolation techniques

Table III: Distribution of Radioactivity among Soluble and Membrane Fractions of in Vitro Labeled Isolated Chloroplasts[a]

fraction	protein (μg)	% of total protein	cpm (×10⁻⁵)	% of total cpm	cpm/μg of protein
soluble	470	55	1.2	5	260
membrane	390	45	21.0	95	5380
	860[b]		22.2[b]		

[a] One milliliter of isolated chloroplasts was incubated with 50 μCi of [^{35}S]methionine for 30 min. The chloroplasts were then separated into soluble and membrane fractions and aliquots were taken to determine the amount of incorporation and total protein content. [b] Total.

tested. Experiments in which *Olisthodiscus* and spinach chloroplasts were mixed have shown no depression of spinach plastid O_2 evolution capacity, thus eliminating the possibility of a diffusable inhibitor such as that seen in isolated pine plastids (Martin & Bassham, 1980). Moreover, the addition of catalase to the reaction mixture in an effort to control (Robinson et al., 1980) possible H_2O_2 production by broken plastids also had no beneficial effect on oxygen evolution in the *Olisthodiscus* chloroplast preparations. Thus, while isolated *Olisthodiscus* chloroplasts are capable of in vitro protein synthesis, they are photosynthetically inactive.

Analysis of Labeled Chloroplast Proteins. To analyze the proteins that the isolated plastid contributes toward its own biogenesis, the chloroplasts were separated into soluble and membrane components after labeling. As seen in the representative experiment of Table III, the membrane (45%) and soluble (55%) fractions contain approximately equivalent proportions of the total chloroplast proteins. If these two fractions are analyzed by SDS–polyacrylamide gel electrophoresis and the separated polypeptides stained with Coomassie blue, it was observed (data not shown) that the soluble and membrane fractions differ in the pattern and distribution of their respective protein components. However, when these same two fractions are analyzed for label distribution (Table III), the membrane fraction contains virtually all (95%) of the label whereas the soluble fraction is labeled to only a small (5%) extent. Nearly identical patterns of labeled proteins are obtained when soluble and membrane components are compared by autoradiographic analyses. This result indicates that the small number of counts recovered in the soluble fraction probably represents low-level contamination by membrane protein. The fact that only membrane components are labeled suggests that protein synthesis in isolated chloroplasts may be significantly altered compared to that occurring in an in vivo system.

If the labeled membrane associated chloroplast proteins are separated by two-dimensional gel electrophoresis (O'Farrell et al., 1977), approximately 100 radioactive proteins can be distinguished (Figure 4). The peptides range in size from approximately 12000 to greater than 100000 daltons. Nearly all these polypeptides electrofocus in the range of pH 5–8. Of those few proteins that run at the basic end of the gel, many are of low molecular weight (<25000) and may include ribosomal proteins. A number of the proteins synthesized by isolated chloroplasts migrate to the same positions as known soluble proteins (Reith & Cattolico, 1985), supporting the suggestion that abnormalities in the translation system of the isolated plastids result in soluble proteins remaining associated with membranes.

DISCUSSION

The purpose of this study was to obtain information on the ctDNA protein coding profile of a chromophytic plant. *Ol-*

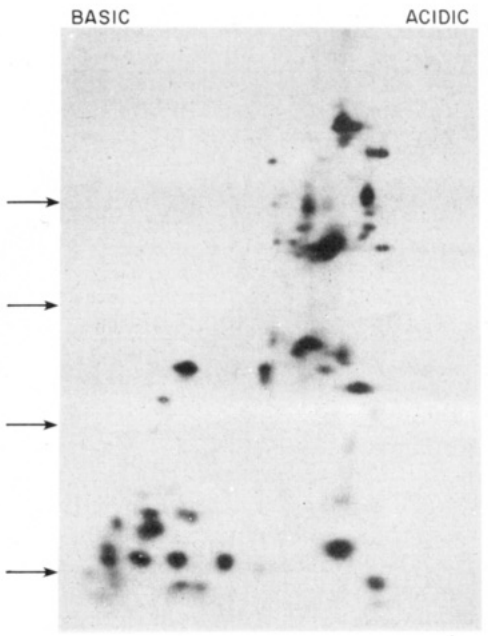

FIGURE 4: Autoradiograph of labeled membrane proteins from isolated chloroplasts following separation by two-dimensional gel electrophoresis. Arrows indicate molecular weight markers: BSA (68 000), ovalbumin (45 000), carbonic anhydrase (29 000), and myoglobin (17 000).

isthodiscus chloroplasts isolated in the presence of sorbitol and BSA are highly refractile when viewed under phase-contrast illumination. However, this study and those of other investigators have shown that this morphological criterion may not ensure that the isolated plastids are completely functional. For example, Salisbury and co-workers (Salisbury et al., 1975) have shown that highly refractile chloroplasts from *Euglena* are capable of in vitro protein synthesis yet do not fix CO_2 or evolve oxygen. Moreover, the level of cytochrome *c*-552 in these plastids (Freysinnet et al., 1979) was only 5% of that found in intact cells. It was suggested that this 10 000-dalton protein, which acts as an electron carrier between cytochrome *f* and P-700, was lost from the chloroplasts during isolation, thus resulting in the loss of photosynthetic capacity. Loss of chloroplast components during isolation has also been reported by Tao & Jagendorf (1973). A total of 80%–90% of their isolated pea chloroplasts was "opaque and shiny" by phase-contrast microscopy and could not reduce ferricyanide until hypotonically disrupted. However, these chloroplasts had retained only 37% of the RuBPCase activity determined for whole cells by immunoprecipitation techniques. Thus, refractility of isolated plastids does not preclude the loss of important components from the chloroplast.

The chloroplasts of higher green plants are surrounded by a double membrane. These two membranes are (Heldt & Sauer, 1971) differentially permeable, to small metabolites, ions, and high molecular weight molecules. However, the chloroplasts of most chromophytes including *Olisthodiscus* are surrounded by four membranes—an inner double membrane or chloroplast envelope and an outer double membrane or chloroplast endoplasmic reticulum. In the chromophytic alga *Ochromonas danica*, transport vesicles are present between the chloroplast ER and the chloroplast envelope (Gibbs, 1979), indicating that the membranes of both chloroplast ER and chloroplast envelope may be involved in the regulation of molecular transport in and out of the algal plastid. The fact that the inner and outer membrane sets of the chloroplast are distinct is suggested by the observation (Magnussen & Gibbs, 1980) that during *Olisthodiscus* plastid replication the division of these membrane sets is temporally separated.

In *Olisthodiscus* as well as many other chrysophytes, the chloroplast membrane that faces the cytosol is contiguous with the cytoplasmic ER network (S. B. Barlow and R. A. Cattolico, unpublished results; Gibbs, 1962). This arrangement is vastly different from that seen in higher plant or green algae where no association between the limiting chloroplast membrane or cytosol ER has been observed to occur. Removal of the chromophytic chloroplast from its normal cellular position could irreversibly damage the organelle membrane system. Thus, even though refractility is maintained in the isolated plastid, damage to one or more of the composite membrane sets could disrupt the entrance and/or retention of specific molecules necessary for normal plastid function.

The isolated *Olisthodiscus* chloroplasts incorporate labeled methionine into protein at a high rate [6.4×10^{-15} M methionine (μg of chlorophyll)$^{-1}$ h^{-1}]. This compares well with rates of 6.1×10^{-15} M methionine (μg of chlorophyll)$^{-1}$ h^{-1} reported for spinach and 14×10^{-15} M methionine (μg of chlorophyll)$^{-1}$ h^{-1} for *Euglena* plastids isolated from protoplasts of vitamin B_{12} stressed cells (Price & Reardon, 1982). However, protein synthesis in isolated *Olisthodiscus* plastids is unusual in that (a) it is not light dependent and (b) all proteins synthesized are associated with membranes. Since *Olisthodiscus* is an obligate photoautotroph (Cattolico et al., 1976), the observation that protein synthesis in isolated plastids is light independent was unexpected. In vivo, chloroplast protein synthesis has been shown to be light dependent (Reith & Cattolico, 1985), and chloroplast division is also disrupted unless proper light intensity and photoperiod are maintained (Cattolico, 1978a). The energy source used for protein biosynthesis by isolated plastids, however, is unknown. Since isolated *Olisthodiscus* chloroplasts synthesize proteins in the dark at a rate comparable to that seen for isolated spinach chloroplasts in the light, it is unlikely that ATP pools within the organelle would be large enough to maintain protein synthesis for the duration of a 1-h incubation period. Our experiments have demonstrated that neither photosynthetic phosphorylation nor the sorbitol osmoticum provide energy to the protein synthetic system. Although the chloroplasts and mitochondria of *Olisthodiscus* have been found to be intimately associated in vivo (Cattolico et al., 1976), the observation that CCCP does not eliminate the protein synthetic response suggests that energy produced in the mitochondria (by oxidative phosphorylation) is not available to the chloroplast for protein synthesis. It is possible that the isolated chloroplasts generate ATP by the breakdown of storage products through the process of chlororespiration (Bennoun, 1982). Mannitol, which can represent 90% of the exported photosynthetic product of an *Olisthodiscus* cell (Hellebust, 1965), may well serve as this energy source. Interestingly, similar light-independent protein synthesis has been reported to occur in isolated *Acetabularia* chloroplasts (Goffeau & Brachet, 1965) as well as in isolated diatom plastids (A. C. Vasconcelos, personal communication). The source of energy for protein synthesis in the isolated chloroplasts of these organisms also remains unknown.

It was quite unexpected to find that virtually all the proteins labeled during a 30-min pulse by isolated *Olisthodiscus* chloroplasts were membrane associated. When the electrophoretic pattern of these proteins is compared to the patterns generated by membrane and soluble proteins labeled in vivo in the presence of cycloheximide (Reith & Cattolico, 1985), at least a dozen of the peptides synthesized by isolated chloroplasts comigrate with soluble, in vivo synthesized proteins.

This observation leads us to believe that some aspect of normal translation is deficient in our isolated chloroplasts. Ribosomes in both higher plant and algal chloroplasts have been shown (Chua et al., 1973) to be associated with thylakoid membranes. These ribosomes may interact with the membranes in several different ways. Some ribosomes are linked to the membrane through electrostatic interactions, others by nascent protein chain association, and still others by a combination of these two (Margulies & Michaels, 1974). It is presently unknown whether bound ribosomes produce only those proteins that will be inserted into membranes or whether stromal polypeptides are also synthesized. Likewise, it is unclear whether free ribosomes produce only soluble proteins or whether membrane proteins may also be synthesized. If stromal proteins are produced on bound ribosomes and if some aspect of protein manufacture or release is not properly functioning, it would not be unexpected to find most of the radioactivity associated with the membranes of an in vitro, chloroplast protein synthesizing system.

The labeling of isolated chloroplasts in vitro has proven to be a very useful technique for the identification of ctDNA-coded proteins in higher plants. However, few algal species have been found to be amenable to the use of this technique. Although the multiplastidic chlorophytic algae *Acetabularia* (Goffeau & Brachet 1965) and *Euglena* (Price & Reardon, 1982) have been examined, to date, studies of chromophytic plant species have never been attempted. Most likely the complexity of the chromophytic plastid structure, the intimate association of the chromophytic chloroplast with cytosol components, and the lack of general information on the molecular biology of these plants represent significant stumbling blocks in the analysis of these systems. As a result, very little is known of the chloroplast protein synthetic capacity of chromophytic plants even though nongreen plant species may account for a remarkable 35% of the CO_2 fixed on our planet (Leith, 1975).

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In Vivo Chloroplast Protein Synthesis by the Chromophytic Alga *Olisthodiscus luteus*†

Michael E. Reith‡ and Rose Ann Cattolico*
Department of Botany, University of Washington, Seattle, Washington 98195
Received September 12, 1984

ABSTRACT: Information on the ctDNA protein coding profile of the Chlorophyta, Rhodophyta, and Chromophyta might provide clues to the evolutionary mechanism(s) by which plants diverged into these three phylogenetic groups. The purpose of this study was to examine the ctDNA protein coding profile of the chromophytic plant *Olisthodiscus luteus*. Whole cells were labeled in the presence of cycloheximide, an inhibitor of cytoplasmic protein synthesis. Control experiments demonstrate that the chloroplast proteins labeled in vivo by this technique form a distinct subset of the total proteins synthesized by the cell. Approximately 50 plastid proteins (35 soluble, 15 membrane) were detected after two-dimensional gel electrophoresis and fluorography. Three ctDNA-coded proteins, the large subunit of ribulosebisphosphate carboxylase, the apoprotein of the P700–chlorophyll *a*–protein complex, and the "photogene" were identified. These proteins are also coded by chlorophytic ctDNA. Unexpectedly, the ctDNA of *Olisthodiscus* was shown to code for the small subunit of ribulosebisphosphate carboxylase. The gene for this enzyme subunit is nuclear coded in all chlorophytic plants that have been analyzed.

Biochemical and morphological studies demonstrate that large differences exist among the chloroplasts of extant plants. Rhodophytes, chromophytes, and chlorophytes all contain chlorophyll *a* as the primary photosynthetic pigment, but these superphyla contain phycobilins, chlorophyll *c*, and chlorophyll *b*, respectively, as their major accessory pigments (Stewart, 1974). Moreover, major variations in plastid structure are shown to exist among these three plant taxa when the number of membranes that limit the chloroplast, lamellar distribution within the plastid, pyrenoid occurrence, and DNA localization are compared (Dodge, 1973; Gibbs, 1981a; Kirk & Tilney-Basset, 1967; Coleman, 1979; Kuroiwa et al., 1981).

Two different mechanisms have been proposed to explain how such extensive chloroplast diversity may have evolved. By a monophyletic scheme, a single unique symbiotic event between a blue-green alga and host cell would have occurred. Following this association, divergent evolution would result in the generation of multiple chloroplast types (Cavalier-Smith, 1982; Taylor, 1979; Ellis, 1983; Bogorad, 1975). Alternatively, a number of investigators (Raven, 1970; Gibbs, 1981b; Whatley & Whatley, 1981) have suggested that chloroplasts have arisen multiphyletically. In this scheme, different (either prokaryotic or eukaryotic) photosynthetic cells entered into symbiotic association with colorless eukaryotic cells. Chloroplast diversity in existing plants would thus reflect the diversity that existed among chloroplast ancestral types.

How different is the underlying genetics of evolutionary divergent plant taxa? Although many of the ctDNA[1] genes of chlorophytic plants have been identified and mapped (Whitfeld & Bottomley, 1983), virtually no information is available on the ctDNA gene profile of chromophytic or rhodophytic plant species (Cattolico et al., 1985).

To compare coding responsibilities among plant species that have distinctly different chloroplast types, the chromophytic alga *Olisthodiscus luteus* has been chosen as our experimental system (Cattolico et al., 1976). This alga contains a ctDNA that is similar in size (97×10^6 daltons) to that of most chlorophytic plants (Aldrich & Cattolico, 1981; Ersland et al., 1981; Bohnert et al., 1982). Our studies have demonstrated that the extremely complex chromophyte plastid with its integrated organelle/cytosol membrane system presents new difficulties to the investigator over those normally encountered during the isolation and analysis of chlorophytic plant chloroplasts. Initial attempts to identify *Olisthodiscus* ctDNA coded proteins (Reith & Cattolico, 1985) utilized the technique of Ellis and co-workers (Ellis, 1977; Ellis et al., 1977), in which isolated chloroplasts are pulsed with a radioactive precursor and labeled products monitored by gel electrophoresis. Although plastids isolated from *Olisthodiscus* are both highly refractile and capable of synthesizing approximately 100 peptides, these chloroplasts appear to have lost, during the organelle isolation procedure, a number of important regulatory characteristics necessary for the proper translation of the chloroplast genome. It has been observed that (1) protein synthesis is light and ATP independent, (2) all of the labeled proteins are membrane associated, and (3) all proteins are

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‡ Present address: Department of Botany, University of Toronto, Toronto, Ontario, Canada M5S-1A1.

[1] Abbreviations: ctDNA, chloroplast DNA; CAP, chloramphenicol; CHI, cycloheximide; CP1, P700–chlorophyll *a*–protein complex; PS II, photosystem II; RuBPCase, ribulosebisphosphate carboxylase.

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