MARINE BIOTECHNOLOGY

Purification of Plastids from the Dinoflagellate *Lingulodinium*

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Abstract

Peridinin-containing dinoflagellates are a group of generally marine and photosynthetic protists whose plastids display a number of unusual features. In particular, the plastid genome may be reduced to as few as a dozen genes, and it is not clear if all these genes are expressed. To begin to characterize the plastid proteins, we attempted to purify chloroplasts from the dinoflagellate *Lingulodinium polyedrum*. We tested several different protocols and found that the organelles were inherently fragile and difficult to isolate intact. In particular, standard purification protocols as described for higher plants produced only broken plastids, as judged by complete loss of the stromal protein RuBisCO. We found that small amounts of RuBisCO could be retained in the plastids if the cells were treated with cellulase prior to lysis. Finally, we report that almost all RuBisCO was retained in plastids prepared from cells subjected to a heat shock treatment, although cellular proteins were denatured by the treatment.

Key words: chloroplast — dinoflagellate — purification — RuBisCO — PCP

Introduction

The peridinin-containing plastids found in most photosynthetic dinoflagellates have a number of particular morphologic and biochemical characteristics. For example, these plastids are surrounded by 3 membranes (Gibbs, 1981) rather than the more typically found 2 or 4. The peridinin itself is an allenic oxi-carotenoid (Jeffrey et al., 1975), which has been found to date only in photosynthetic dinoflagellates, and is found together with chlorophyll *a* bound to peridinin–chlorophyll *a* proteins (PCPs) (Prezelin and Haxo, 1976). The principal light-harvesting protein in the algae is a water-soluble PCP whose structure is clearly unrelated to any other light-harvesting proteins (Norris and Miller, 1994; Hofmann et al., 1996). An intrinsic membrane protein (iPCP) related to the chlorophyll *ab*-binding protein of higher plants is also found (Grossman et al., 1995) but is thought to be of lower abundance than the soluble form. Perhaps even more unusual is the finding that the plastids contain a form II ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), previously found only in some species of anaerobic proteobacteria (Morse et al., 1995; Rowan et al., 1996). The form II enzyme differs from the typical form I enzyme in that it is composed only of large subunits that share limited sequence identity with those of the form I enzyme (Narang et al., 1984). Importantly, oxygen competes with CO_2 more successfully for binding to the active site of the form II enzyme than to that of the form I enzyme (Whit2ney and Andrews, 1998), suggesting that special mechanisms might be required to allow this enzyme to function efficiently (Nassoury et al., 2001). Finally, the sequence of a nuclear-encoded plastid-directed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forms a monophyletic group with the plastid isoforms of cryptomonads, within an otherwise cytosolic clade, and is thus distinct from all other plastid GAPDHs (Fagan et al., 1998).

Molecular studies have demonstrated that the genome of at least some peridinin–containing dinoflagellate plastids is also quite unconventional, as it appears to be encoded on small single-gene or dual-gene minicircles (Zhang et al., 1999, 2001; Barbrook and Howe, 2000; Hiller, 2001; Barbrook et al., 2001). It has been suggested that the isolation of minicircle sequences based on polymerase chain reaction (PCR) may have reached saturation (Howe et al., 2003), and if so, the dinoflagellate plastid would contain one of the most reduced plastid genomes known. To date, the genes found in minicircles include the 16S and 23S rRNAs, 2 photosystem I (PSI) components (*psaA* and *psaB*), 6 photosystem II (PSII) genes (*psbA*, *psbB*, *psbC*, *psbD*, *psbE* and *psbI*), 2 subunits of ATP

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synthase (*atpA* and *atpB*) and two cytochrome b_6f subunits (*petB* and *petD*).

The strong emphasis on dinoflagellate plastid genes has been driven by an interest in the evolutionary history of dinoflagellate plastids (Zhang et al., 2000). In contrast, our understanding of the plastid proteome is still at a very basic level. Indeed, the primary interest in plastid proteins has been the relation between the circadian rhythms of carbon fixation and oxygen evolution and clock-controlled regulation of plastid protein synthesis (Fagan et al., 1999; Le et al., 2001; Nassoury et al., 2001). A number of plastid proteins have been identified and characterized, but so far all have been found to be nuclear-encoded (Hiller et al., 1995; Morse et al., 1995; Le et al., 1997; Fagan et al., 1999). Nuclear-encoded plastid-directed proteins can be readily identified by virtue of an unusual leader sequence containing 2 hydrophobic regions surrounding a region rich in hydroxylated amino acids, apparently a requirement of the targeting mechanism into triple membrane bound plastids (Nassoury et al., 2003).

Plastid purification from dinoflagellates has been reported only once (Asano et al., 1998). This report showed that purified organelles had retained protein kinase activity, but it did not evaluate plastid integrity or the degree of purification. We have found the issue of plastid integrity to be the most important during our attempts to develop a purification protocol. We have therefore developed a sensitive immunologic assay for plastid integrity using measurements of known protein marker retention. To date the only molecular markers identified in the *Lingulodinium* chloroplast are RuBisCO (Morse et al., 1995), PCP (Le et al., 1997) and GAPDH (Fagan et al., 1999). We have exploited the previously prepared anti-RuBisCO and anti-PCP antibodies (Nassoury et al., 2001) to follow retention of the stromal marker RuBisCO and the thylakoid lumen marker PCP during purification. The results of these immunologic assessments were also confirmed by 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE), the type of analysis likely to be of particular utility in proteomic characterization of soluble stromal as well as lumenal and peripheral thylakoid proteins in plastid preparations (Peltier et al., 2000). Our studies underscore the importance of organelle integrity and document the plastid protein profiles resulting from several different purification protocols.

Materials and Methods

Cell Culture. *Lingulodinium polyedrum* (previously *Gonyaulax polyedra*) was obtained from the Provasoli-Guillard Culture Center for Marine Phytoplankton (strain 1936, Booth Bay Harbor, Maine) and grown in a modified seawater medium (f/2) (Guillard and Ryther, 1962) at constant temperature (16°C) in 12:12 light-dark cycles using cool white fluorescent light at an intensity of 40 μmol $m^{-2}s^{-1}$. The beginning of the light period was defined as time 0 (LD 0), and the beginning of the dark period as LD 12. Cultures were grown to a cell density of 5000 to 6000 cells ml^{-1} as measured by counting using a hemocytometer. The cell cultures are unialgal but not axenic, and the prokaryotes in the cultures have not been defined.

Plastid isolation protocols. All plastid purification protocols used cells harvested by filtration on Whatman 541 paper and washed 3 times with sterile culture medium before use to remove bacteria. The standard plastid purification protocol, (*protocol 1*) as used for higher plants (Schuler and Zielinski, 1989), involved resuspension of the cells in isolation buffer A (100 mM Tris-HCl buffer, pH 7.5, containing 0.3 M sucrose, 10 mM EDTA, and 14 mM ß-mercaptoethanol) and a 5-minute incubation at 2000 psi in an ice-cold N_2 bomb (Parr Instruments). The cells were lysed as the pressure was released slowly enough to produce discrete drops of liquid. This crude extract was centrifuged at 120*g* for 3 minutes to remove unbroken cells and cell debris. Five milliliters of this clarified lysate was then loaded on a 15-ml 10% to 30% linear Percoll (Amersham-Pharmacia Biotech) gradient in isolation buffer atop a 60% Percoll cushion and centrifuged at 6000*g* for 25 minutes at 4°C. The pigmented bands were isolated and the Percoll was removed by centrifugation after dilution with 10 volumes of isolation buffer. These preparations were analyzed using antibodies to monitor retention of marker proteins and 2-dimensional gel electrophoresis to characterize the protein complement as described below.

Several variations on a ''stirred plastid'' preparation were developed that purified plastids with low but detectable levels of RuBisCO (protocol 2). In one version of this protocol, cells were harvested by filtration and washed as described above. Cells from 4 L of culture medium were resuspended in 10 ml isolation buffer B (0.626 M Sorbitol, 0.1 M KCl, 0.1 M Hepes-NaOH, pH 7.2, 20 mM ß-mercaptoethanol, 0.1 % bovine serum albumin [BSA]) containing 0.6 g cellulase. The suspension was shaken gently at 16°C for 1 hour, and then transferred to a prechilled nitrogen bomb (Parr Instruments). The nitrogen pressure was brought to 1100 psi and released immediately. The suspension was centrifuged at 120*g* for 3 minutes, and the pellets containing still unbroken cells were resuspended in a low osmotic strength buffer (0.25 M Sorbitol, 0.1 M Hepes-NaOH, pH7.2, 0.1 M KCl, 0.1 %

BSA) and stirred vigorously with a magnetic stirrer for 20 minutes in an ice bath. This stirred homogenate was centrifuged at 120 rpm for 3 minutes to remove unbroken cells, and the supernatant was centrifuged at 1000*g* for 3 minutes. This final pellet represented the crude plastid preparation and was resuspended in 10 ml isolation buffer B. As an alternative to this procedure, cells were resuspended in 10 ml isolation buffer B containing 0.05% Saponin (ICN) instead of the low ionic strength buffer, and the suspension was stirred vigorously for 15 minutes on ice. This stirred homogenate was centrifuged as described above to produce the plastid-containing pellet.

Plastids were purified from the crude preparations by density gradient centrifugation. A stock solution of PBF-Percoll (3% [w/v] PEG 8000, 1% (w/v) BSA and 1% [w/v] Ficoll) was first prepared by dissolving the dry powders in 100% Percoll. A 15-ml 10% to 30% linear PBF-Percoll gradient in isolation buffer was laid on top of a 5-ml 40% PBF-Percoll in isolation buffer cushion. Five milliliters of the crude plastid preparation was layered on top of the Percoll gradients and centrifuged at 6,000 *g* for 25 minutes at 4°C (Beckman JS13.1 rotor). Two distinct highly pigmented bands were found in Percoll, and the lower band was collected with a Pasteur pipette. These plastid preparations, examined microscopically, typically contained more than 90% aggregated and pigmented plastids. To remove the Percoll from the plastid preparation, 3 volumes of cold washing buffer (isolation buffer without BSA) were added, gently mixed, and the plastid preparation was centrifuged at 1400*g* (Beckman JS13.1 rotor) for 15 minutes at 4°C. This process was repeated 3 times, each time with fresh washing buffer and gentle resuspension of the plastid pellet using a Pasteur pipette.

The plastids that retained the most RuBisCO were prepared using a 1-hour treatment with cellulase at 40°C to degrade the cell wall (*protocol 3*). For this ''heat shock'' preparation, cells were resuspended in 10 ml cellulase digestion buffer (0.4 M sucrose, 0.02 g/ml cellulase) per 4 L of cell culture. The suspension was incubated with gentle shaking at 40°C for 1 hour to digest the cell wall. The cells were then centrifuged and resuspended in 15 ml isolation buffer A (0.4 M sucrose, 0.1 M KCl, 0.1 M Hepes-NaOH [pH 7.2], 0.03 M ß-mercaptoethanol, 0.1% BSA), and transferred to a prechilled nitrogen bomb (Parr Instruments). The nitrogen pressure was brought to 500 psi then immediately and slowly released. All subsequent steps were carried out at 4°C. The cell homogenates were centrifuged at 120*g* for 10 minutes to pellet down unbroken cells and cell debris. The pellets were resuspended in 10 ml isolation buffer, and the bomb treatment was repeated 3 times. The combined supernatants constituted the crude plastid preparations from which plastids were purified as for the stirred plastid protocol described above.

Immunologic Assay for Plastid Integrity. At each step of the purification, 0.2% of the volume of each sample was removed for gel analysis, including the initial total protein extract, the crude extract after centrifugation at 120*g* (to remove whole cells and large cellular debris), the PBF-Percoll purified plastids, and the supernatant remaining above PBF-Percoll gradient. These samples were dissolved in sodium dodecylsulfate (SDS) sample buffer (Laemmli, 1970) to a final volume of 100 µl, from which 10 µl was electrophoresed on 10% polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes. The different fractions are directly comparable because they contain similar percentages of the total fraction. This method was chosen instead of measurements of protein concentration in each fraction, as many of the fractions contained BSA, added to the extraction buffer as a stabilizing agent.

The protein blots were incubated with a mixture containing a 1:5000 dilution of rabbit anti-form II RuBisCO, raised against a *Lingulodinium* RuBisCO cDNA expressed in *Escherichia coli*, and a 1:5000 dilution of rabbit anti-PCP, raised against PCP purified from *Lingulodinium* by a combination of column chromatography and preparative electrophoresis (Nassoury et al., 2001). Antibody binding was visualized using a peroxidase-linked goat antirabbit secondary antibody and chemiluminescence (Amersham).

Oxygen Evolution Measurements. Oxygen evolution rates were measured from whole cells and purified plastid preparations essentially as described (Dionisio-Sese et al., 2001). Briefly, whole cells were concentrated by centrifugation and resuspended at 10 µg/ml chlorophyll *a* in f/2 medium containing 5 mM $NaHCO_3$. Purified plastid preparations were measured at similar chlorophyll concentrations in plastid isolation buffer containing 5 mM $NaHCO_3$, and 2.5 mM potassium ferricyanide ($FeK_3(CN)_6$) and 1 mM methyl viologen were also added as artificial electron acceptors to facilitate sustained O_2 evolution. Measurements were made at 18°C under illumination from an IR-filtered 300-W quartz halogen bulb (Kondo) with intensities controlled by neutral density filters (Lee Colortran).

2D-PAGE. To prepare samples for 2D-PAGE, cells from 1 L of culture medium were broken in a minibead beater (BioSpec Products) with 500 µl 4%

aqueous 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and the proteins were precipitated overnight at −20°C by addition of 3 volumes of acetone. The precipitate was washed 6 to 8 times with water-acetone 1:3, dried under vacuum, and redissolved in the strip rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.02 M dithiothreitol, 0.5% pH 4–7 immobilized pH gradient [IPG] buffer; Amersham-Pharmacia Biotech) overnight at room temperature. The insoluble material was removed by centrifugation at 13000*g* for 30 minutes. Protein concentrations were measured using the Bio-Rad protein assay and BSA as standard. Typically, approximately 600 μg protein in 250 μl rehydration buffer was applied to an isoelectric focusing (IEF) strip (Amersham-Pharmacia Biotech) and left overnight at 20°C to rehydrate. The first dimension was run at 500 V for 1 hour, 1000 V for 1 hour and 8000 V for 6 hours (maximum current, 50 μA). After IEF the strip was equilibrated for 15 minutes in 10 ml SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue), 15 minutes with 10 mg ml^{-1} dithiothreitol, followed by a 15-minute equilibration in 10 ml SDS equilibration buffer with 250 mg iodoacetamide. The strips were then placed on top of a standard 10% polyacrylamide gel and electrophoresed at constant voltage. Following electrophoresis the gel was stained with Coomassie blue for 1 hour at 55°C, and destained overnight at room temperature.

Electron Microscopy. The isolated plastids were fixed separately with 2% glutaraldehyde and 2% OsO_4 in isolation buffer for 1 hour each. The fixation medium was removed by washing with saline buffered with 0.1 M phosphate (100 mM Na_2HPO_4/KH_2PO_4, pH 7.2, 150 mM NaCl) in a microfuge. The plastid pellets were dehydrated in a graded ethanol series, and embedded in either LR white or Epon. Ultrathin sections were viewed in a JEOL JEM-100S transmission electron microscope at 80 kV. All EM supplies were obtained from Ted Pella (Redding, Calif).

Results

The plastids in *Lingulodinium* are highly reticulate in electron micrographs of whole cells, as plastid profiles often branch out and merge with one another (Schmitter, 1971) (Fig. 1, A). It is thus possible that what are often observed as small discrete plastids in fact represent sections cutting through an end of a more reticulate structure. This makes purification of the organelles problematic, as considerable care must be used to reduce breakage of the organelles when the cell wall is disrupted. The standard protocols developed for purification of plastids from higher plants (protocol 1) (Schuler and Zielinski, 1989) produced preparations containing discrete pigmented bodies when examined using the light microscope (data not shown). When examined by transmission electron microscopy (Fig. 1, B), these preparations contained plastids surrounded by multiple membranes and containing dark and folded thylakoid membranes inside a light gray stroma (Fig. 1, C). The thylakoids must fold if long cylinders become spheres when released from the cell, although we note the spherical plastids in the purified samples appear smaller than the plastids observed in the intact cells. Thus it appears likely that many of the plastids have broken and resealed during cell lysis or purification.

Electron microscopy clearly shows that thylakoid membranes are purified by this procedure. In agreement with this, we found that roughly half the total cellular pigment (estimated by chlorophyll absorbance at 680 nm) was retained in these preparations (data not shown). However, when these plastid preparations were tested with antibodies against our marker proteins PCP and RuBisCO, the plastid preparations (Fig. 1, D, lane 4) contained less PCP than the supernatant that did not enter the Percoll gradient (lane 3). More importantly, no RuBisCO was detected in the purified plastid preparations (Fig. 1, D, lane 4). These observations are consistent with a breakage of the plastid membranes and complete loss of soluble stromal contents. They also suggest partial breakage of the thylakoid membranes and partial loss of the lumenal contents. These observations thus underscore the importance of assessing organelle integrity during purification.

The protein profile of the purified plastids after 2D-PAGE is much simpler than the profile in whole cells (Fig. 1, E, F). There is no indication of cytoplasmic contamination as judged by the presence of luciferin binding protein (LBP; box at 72 kDa). However, these analyses also confirm the absence of RuBisCO (box at 55 kDa) noted by the immunologic assays. In terms of the complexity of the protein pattern observed, this analysis superficially resembles that of pea thylakoid lumenal proteins (Peltier et al., 2000). Taken together, our results suggest that this protocol purifies thylakoids away from both cytoplasmic and chloroplast stroma proteins, and that some of the thylakoid lumen proteins have been lost.

Several previous studies have shown that plastid ultrastructure varies over the course of a day (Rensing et al., 1980; Herman and Sweeney, 1975). More importantly, these morphologic changes correspond

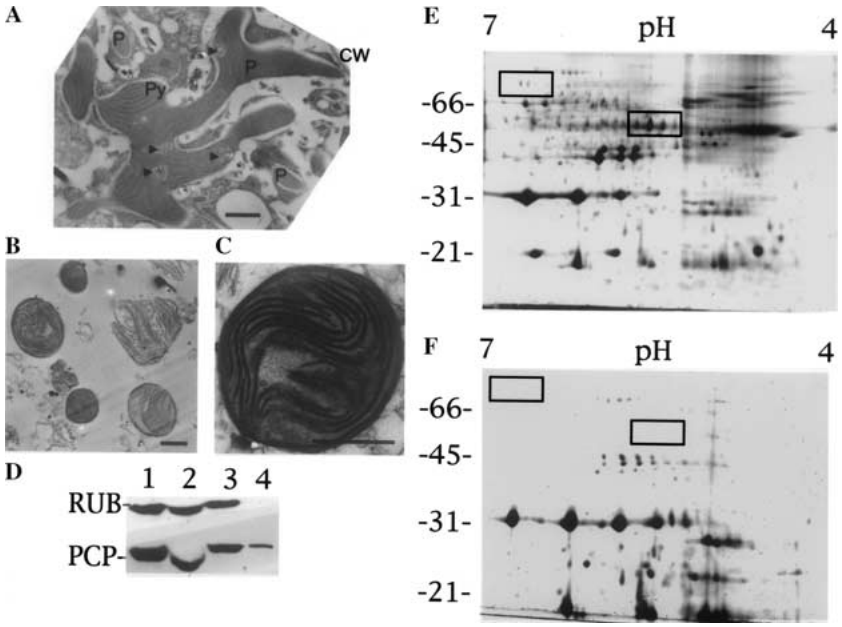

Fig. 1. Analysis of plastids prepared by protocol 1. **A**: Transmission electron micrograph of *Lingulodinium*. One large highly reticulate and several small individual plastids (P) can be seen. The pyrenoid (Py), characterized by more widely spaced thylakoid stacks, is near the center of the cell away from the cell wall (CW). Arrowheads indicate plastid DNA with a fibrous structure. **B,C**: Representative TEM of purified plastids. Pyrenoids are not present in any of the purified organelles, consistent with a lack of RuBisCO. **D**: Representative Western blot using 0.2% of the sample from lane 1, total protein extract; lane 2, crude plastid preparation; lane 3, sample above the Percoll; and lane 4, plastid band in Percoll using a mixture of anti-RuBisCO and anti-PCP. Total protein load cannot be measured owing to the presence of BSA in the buffers. **E:** Representative 2-dimensional electrophoresis of total *Lingulodinium* extract (0.6 mg). F. Representative 2D-PAGE of purified plastid protein (~0.6 mg). Molecular mass markers (kDa) are shown at left, and pH extremes (linear gradient) are at the top of each gel. The boxed areas in each 2D-PAGE gel indicate the position of LBP (molecular weight, 72 kDa) and RuBisCO (molecular weight, 55 kDa). Scale bars: 1 μm.

to movements of RuBisCO, which becomes sequestered in small regions of the chloroplast, called pyrenoids, during the early day when photosynthesis rates are high (Nassoury et al., 2001). Pyrenoids are readily identifiable in TEM images of whole cells by their more widely spaced thylakoid stacks (Fig. 1, A), but have never been observed in any of the purified organelles, consistent with the lack of RuBisCO in the preparations. We thought it possible that the loss of RuBisCO reflected a particularly sensitive time during the daily cycle of plastid structure and function. To test this, we also prepared plastids from early night-phase cells, which do not contain pyrenoids and show RuBisCO distributed over the entire plastid (Nassoury et al., 2001). We found no difference in marker protein retention of plastids prepared from cells taken at several different times throughout the Light-dark cycle (data not shown). These results indicate that RuBisCO retention is unaffected by the chloroplast ultrastructure at the time of extraction.

The ''stirred plastid'' method (protocol 2) produced organelles that did not appear spherical. Instead, these preparations contained irregularly shaped organelles as well as considerable quantities of broken plastids and isolated thylakoid membranes (Fig. 2, A). These plastids also contained roughly half the cellular pigments as measured by absorbance at 680 nm (data not shown). The plastid preparations contained roughly half the total PCP, as judged by the similar signals found for the purified plastids (Fig. 2, B, lane 3) and the fraction that did not enter the Percoll (lane 2). Unlike the standard method, however, these preparations also retained a small and variable fraction of the stromal marker RuBisCO (Fig. 2, B). The protein profile after 2D-PAGE can be seen to contain a greater number of proteins than observed using the standard protocol (compare Fig. 2, C, with Fig. 1, F), suggesting that lumenal integrity is more highly preserved. These 2-dimensional gels also confirm the presence of small amounts of RuBisCO and the absence of the cytoplasmic marker LBP. We conclude from these data that the stirred plastid protocol produced relatively clean preparations enriched primarily in both lumenal and membrane-bound thylakoid proteins.

To test if these plastids retained function after isolation, chlorophyll-specific O_2 evolution rates

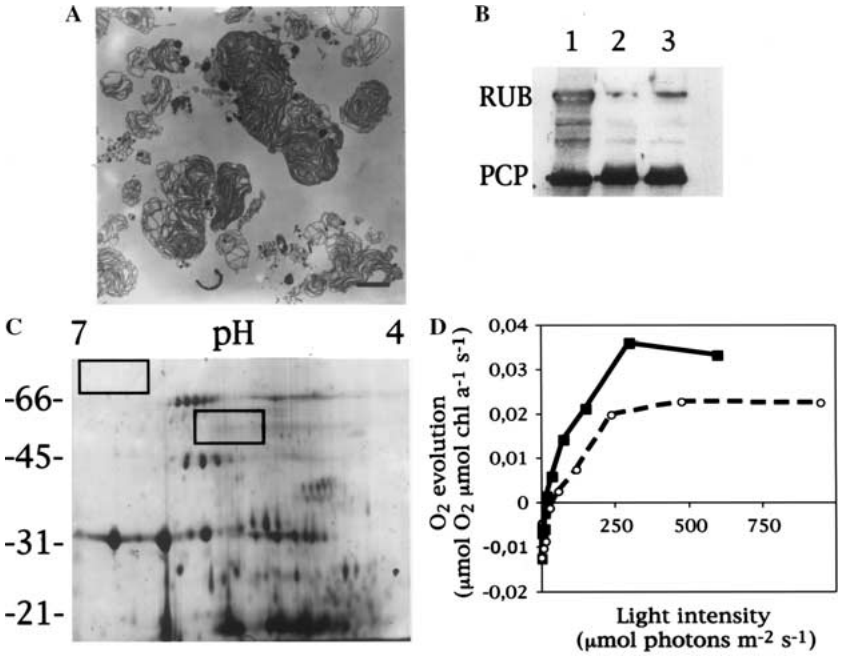

Fig. 2. Analysis of plastids prepared by protocol 2. **A**: TEM of purified plastids. Scale bars: 1 µm. **B**: Western blot using 0.2% of the sample from lane 1, crude plastid preparation; lane 2, sample above the Percoll; and lane 3, plastid band in Percoll using a mixture of anti-RuBisCO and anti-PCP. **C**: 2D-PAGE of purified plastid protein (~0.6 mg). Molecular mass markers (kDa) are shown at left, and pH extremes (linear gradient) at the top of the gel, and the positions of LBP and RuBisCO are boxed. The group of 6–7 protein isoforms at 66 kDa is BSA from the extraction buffer and not a true plastid component. **D**: Net photosynthetic O_2 evolution rates from *Lingulodinium* whole cells (closed squares) and purified plastids (open circles) measured over a range of light intensities.

were measured under a range of light intensities and compared with O_2 evolution rates in intact cells. The artificial electron acceptors ferricyanide and methyl viologen were added to replace the electron sinks normally maintained by stromal proteins in whole cells. The facilitated O_2 evolution rates from the plastid preparations were nearly as high (>80%) as the rates measured from whole cells (Fig. 2, D), showing that the photosynthetic complexes were indeed functionally active in the purified fractions. In control experiments we observed no major difference in addition of the artificial electron acceptors to whole cells (Table 1). However, plastids were photosynthetically competent only in the presence of artificial electron acceptors (Table 1), consistent with our observation that the stromal proteins mediating CO_2-dependent electron transport were absent from the prepared plastids.

To attempt to increase the yield of intact plastids, we also tested the addition of cellulase to degrade the cell wall. However, the optimal temperature for the cellulase is 40°C, and this heat shock produced effects on the ultrastructure of the intact cell that we interpret as consistent with denaturated protein (Fig. 3, A); indeed, the cells did not remain viable after a heat shock. Plastids purified following a heat shock (protocol 3) appeared to be the least pure and least intact. As viewed by electron microscopy, the plastids were primarily spherical but only approximately 50% were intact, and the preparations also contained considerable non-membrane-bound material (Fig. 3, B). Thus the non-membrane-bound material observed in the purified plastids might represent denatured protein released from the organelles as they rupture. Interestingly, immunologic analyses showed that the purified organelles retained the majority of the stromal marker RuBisCO (Fig. 3, C, lane 4), although considerable PCP had still been lost. Quantitative analyses of these immunoblots clearly showed this selective loss of PCP (Table 2), which we interpret to reflect a greater stability and increased solubility of PCP at the increased temperature of the heat shock. As in all previous protocols, there were no differences between day and night-phase plastids, showing that plastid ultrastructure had no effect on the extraction protocol. Finally, the presence of RuBisCO was confirmed on 2-dimensional gels (Fig. 3, D; box at 55 kDa). The protein profiles obtained by this protocol were the most complex of all the methods tested, and yet remained free from detectable levels of the cytoplasmic marker LBP (box at 72 kDa). These data suggest that the heat shock protocol is most

Table 1. Maximum Gross Chlorophyll-Specific O_2 Evolution Rates Relative to Whole Cells with Added CO_2[a]

Fraction	*Added CO_2 (%)*	*Added MV + $FeK_3(CN)_6$ (%)*
Whole cells	100	89
Plastids	0.5	87

[a]CO_2 was supplied as 5 mM $NaHCO_3$; MV and ferricyanide concentrations were as described in "Materials and Methods."

Table 2. Subcellular Fractionation of RuBisCO, PCP, and Plastid Pigments After Heat Shock[a]

	Day-phase cells (%)			*Night-phase cells (%)*		
Fraction	OD_{680}	*RuBisCO*	*PCP*	OD_{680}	*RuBisCO*	*PCP*
Crude	100	100	100	100	100	100
Clarified	80	90	96	77	102	83
Supernatant	1	4	53	1	6	42
Plastids	43	86	69	40	78	57

[a]Aliquots of each fraction (0.2% total volume) were analyzed for absorption at 680 nm, and response to anti-RuBisCO and anti-PCP on Westerns blots. Western blots were quantitated by densitometric scans and compared with the values observed in crude extracts. The data are representative of 4 different experiments.

likely to produce plastids containing the full gamut of stromal and thylakoid proteins. The caveat, as based on electron microscopic examination, is that nonplastid impurities might still be present. These preparations appeared to contain high levels of denatured protein, precluding analysis of the level of contaminants using marker enzyme activity. At present we lack the immunologic tools to adequately determine the level of contaminants by Western blot analysis.

Discussion

We describe here the purification of chloroplast fractions from the dinoflagellate *Lingulodinium polyedrum*. The plastids from this organism appear to be more sensitive to rupture than those from higher plants, as normal protocols for the purification of plant plastids produce dinoflagellate plastids completely lacking the stromal marker enzyme RuBisCO (Fig. 1, D). In part, the fragility of the organelles might be related to problems of osmotic strength, as we observed that higher than usual concentrations of sucrose or other osmolytes were beneficial (compare protocols 1 and 2, for example). However, a more important issue may be the highly reticulate nature of the chloroplasts observed in electron micrographs (Fig. 1, A). Clearly purification of these structures is expected be difficult, and it remains to be seen if other dinoflagellates will prove to be more tractable. Our preliminary experiments, which have tested the stirred plastid protocol for purification of plastids from the dinoflagellate *Amphidinium carterae*, suggest that plastid fragility may be common in the dinoflagellates, as these plastids showed no noticeable improvement in RuBisCO retention over what was observed with the *Lingulodinium* plastids.

The complete lack of RuBisCO in the plastids purified by traditional methods was surprising, and underscores the importance of methods that can be used to monitor organelle integrity. For example, plastids prepared using protocol 1 evolve oxygen (data not shown), leaving the erroneous impression that the organelles have been isolated intact. It is only when the retention of the stromal enzyme RuBisCO

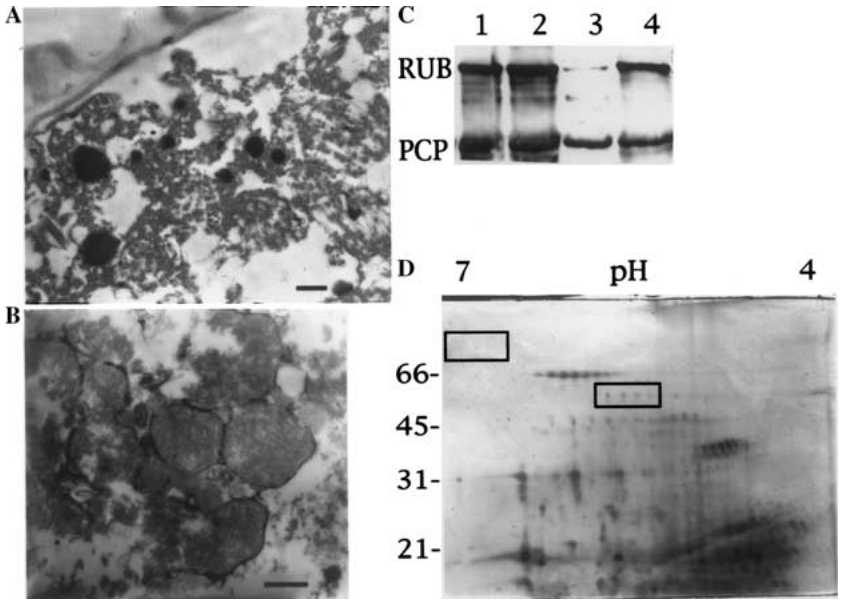

Fig. 3. Analysis of plastids prepared using protocol 3. **A:** TEM of *Lingulodinium* plastids in situ in heat-shocked cells. **B**: Representative TEM of purified plastids. **C**: Representative Western blot using 0.2% of the sample from lane 1, total protein extract; lane 2, crude plastid preparation; lane 3, sample above the Percoll; and lane 4, plastid band in Percoll using a mixture of anti-RuBisCO and anti-PCP. **D**: Representative 2D-PAGE of purified plastid protein (~0.6 mg). Molecular mass markers (kDa) are shown at left, and pH extremes (linear gradient) at the top of the gel. The positions of LBP and RuBisCO are boxed. The group of 6–7 protein isoforms at 66 kDa is BSA from the extraction buffer.

and the thylakoid protein PCP are directly monitored that the true situation is revealed. The method we have developed to assess marker protein retention simply compares the amount of immunoreactive protein in a sample representing 0.2% of each fraction. This differs from traditional biochemical measurements of organelle purity, in which specific activity (in terms of enzyme activity per unit protein) would be followed in successive stages of purification until no further increases could be observed. There are two advantages to our method in the present instance. First, the method does not require the proteins to be functional, and can be used to follow specific marker proteins through the purification process even though the sample has been heavily denatured (for example, as in protocol 3). Second, our method does not require general protein measurements, thus allowing inclusion of proteins such as BSA to the extraction buffers as a stabilizing agent.

Given the novelty of our assessment procedure, it was thought important to confirm our observations by determining the overall protein pattern by 2D-PAGE. We observed that the 2D-PAGE protein pattern was simplified in all plastid preparation protocols compared with the total cell extract. This, and in particular the lack of the cytoplasmic marker protein LBP, suggests that the plastids may have been purified away from other organelles and the bulk of the cytoplasmic components. However, the more important issue revealed by our studies is that of organelle integrity. The thylakoid membranes were clearly present in our preparations, as they were readily visualized by electron microscopy and pigments were retained in the purified plastid fractions. However, our more sensitive immunologic methods clearly demonstrated that the plastids had broken and resealed during the purification.

In general, we found that a higher osmotic strength medium was beneficial, as suggested by comparison of protocols 1 and 2. Furthermore, the addition of both KC1 and BSA appeared helpful, as also found during purification of plastids from higher plants (Jones, 1995). Thus these changes should be considered first if plastid purification protocols are being developed using other dinoflagellate species. The 3 different purification protocols described here reflect the full range of RuBisCO retention inside the purified plastids that we have observed. We believe that none of the procedures delivered intact organelles, presumably as a consequence of the plastid structure. However, the heat shock treatment that appears to have denatured most of the cellular proteins seems most promising for proteomic analyses. These preparations were the only ones in which plastid stromal contents co-purified with the plastid pigments. It is also possible that plastid DNA can be isolated from these preparations. However, it must be noted that the heat shock treatment precludes functional analyses of the plastids in vitro.

For researchers interested primarily in thylakoid proteins, such as the proteins encoded by plastid minicircles, our recommendation would be to employ the stirred plastid protocol described here. Examination of the electron micrographs and the protein patterns observed after 2D-PAGE suggests that this protocol should provide the most highly purified thylakoid preparations.

In summary, we present here the range of results that were obtained with respect to the purification of plastids from the dinoflagellate *Lingulodinium*. We found that traditional protocols yielded plastids from *Lingulodinium* that typically contained intact thylakoid stacks as judged from their morphology under the electron microscope. However, while retaining significant amounts of the thylakoid marker PCP, these preparations had lost all RuBisCO. The 2-dimensional electrophoretic patterns were roughly comparable to those observed with pea thylakoids. We conclude that this protocol purified essentially thylakoid membranes and that the plastids had broken and resealed during cell lysis or purification. This suggests that *Lingulodinium* plastids from this organism are remarkable for their fragility, in particular with regard to the level of RuBisCO retained in the purified fractions.

Nonetheless, we found several techniques that improved the purification when compared with the standard methods usually employed with higher plant plastid preparations. One modified version of the basic method was found to produce plastids that do retain small amounts of RuBisCO and appear reasonably pure as judged by the 2D-PAGE profile and electron microscopy. The most important improvements include an increase in the osmotic strength of the extraction buffer, and the addition of various stabilizing compounds to the extraction buffers. Finally, we report a protocol that retained almost all the RuBisCO, but electron micrographs of the purified fractions suggest it may not be as highly purified. In this case the application of a heat shock treatment to the algae in conjunction with a cellulase treatment was found to be the most important feature. These preparations, if used with caution, appear suitable for whole organelle proteomic studies and potentially genomic studies as well.

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References

1. Asano CS, Okamoto OK, Colepicolo P (1998) Diurnal patterns of incorporation of gammaP32-ATP in isolated chloroplasts from *G. polyedra*. Biol Rhythm Res 29, 488–493
2. Barbrook AC, Howe CJ (2000) Minicircular plastid DNA in the dinoflagellate *Amphidinium operculatum*. Mol Gen Genet 263, 152–158
3. Barbrook AC, Symington H, Nisbet RE, Larkum A, Howe CJ (2001) Organisation and expression of the plastid genome of the dinoflagellate *Amphidinium operculatum*. Mol Genet Genomics 266, 632–638
4. Dionisio-Sese ML, Maruyama T, Miyachi S (2001) Photosynthesis of Prochloron as affected by environmental factors. Mar Biotechnol 3, 74–79
5. Fagan T, Hastings JW, Morse D (1998) The phylogeny of glyceraldehyde-3-phosphate dehydrogenase indicates lateral gene transfer from cryptomonads to dinoflagellates. J Mol Evol 47, 633–639
6. Fagan T, Morse D, Hastings JW (1999) Circadian synthesis of a nuclear encoded chloroplast glyceraldehyde-3-phosphate dehydrogenase in the dinoflagellate *Gonyaulax polyedra* is translationally controlled. Biochemistry 38, 7689–7695
7. Gibbs S (1981) The chloroplasts of some algal groups may have evolved from endosymbiotic eukaryotic algae. Ann NY Acad Sci 361, 193–208
8. Grossman AR, Bhaya D, Apt KE, Kehoe DM (1995) Light-harvesting complexes in oxygenic photosynthesis: diversity, control, and evolution. Annu Rev Genet 29, 231–288
9. Guillard RRL, Ryther JH (1962) Studies on marine planktonic diatoms: *Cyclotella nana* Hufstedt and *Denotula confervacea* (Cleve) Gran. Can J Microbiol 8, 229–239
10. Herman EM, Sweeney BM (1975) Circadian rhythm of chloroplast ultrastructure in *Gonyaulax polyedra*, concentric organization around a central cluster of ribosomes. J Ultrastruct Res 50, 347–354
11. Hiller R, Wrench P, Sharples F (1995) The light harvesting chlorophyll *a-c* binding protein of dinoflagellates: a putative polyprotein. FEBS Lett 363, 175–178
12. Hiller RG (2001) 'Empty' minicircles and petB/atpA and psbD/psbE (cytb559 alpha) genes in tandem in *Amphidinium carterae* plastid DNA. FEBS Lett 505, 449–452
13. Hofmann E, Wrench PM, Sharples FP, Hiller RG, Welte W, Diederichs K (1996) Structural basis of light harvesting by carotenoids: peridinin-chlorophyll-protein from *Amphidinium carterae*. Science 272, 1788–1791
14. Howe CJ, Barbrook AC, Koumandou VL, Nisbet RE, Symington HA, Wightman TF (2003) Evolution of the chloroplast genome. Philos Trans R Soc Lond B Biol Sci 358, 99–106; discussion 106–107
15. Jeffrey S, Seilicki M, Haxo F (1975) Chloroplast pigment patterns in dinoflagellates. J Phycol. 11, 374–384
16. Jones H (ed) (1995) *Plant Gene Transfer and Expression Protocols*. (Totowa, NJ: Humana Press)
17. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685
18. Le QH, Jovine R, Markovic P, Morse D (2001) PCP is not implicated in the photosynthesis rhythm of *Gonyaulax* despite circadian regulation of its translation. Biol Rhythm Res 32, 579–594
19. Le QH, Markovic P, Jovine R, Hastings JW, Morse D (1997) Sequence and genomic organization of the peridinin-chlorophyll-*a* rotein from Gonyaulax polyedra. Mol Gen Genet 255, 595–604
20. Morse D, Salois P, Markovic P, Hastings JW (1995) A nuclear encoded form II rubisco in dinoflagellates. Science 268, 1622–1624
21. Narang F, McIntosh L, Somerville C (1984) Nucleotide sequence of the ribulose bisphosphate carboxylase gene from *Rhodospirillum rubrum*. Mol Gen Genet 193, 220–224
22. Nassoury N, Fritz L, Morse D (2001) Circadian changes in ribulose-1,5-bisphosphate carboxylase/oxygenase distribution inside individual chloroplasts can account for the rhythm in dinoflagellate carbon fixation. Plant Cell 13, 923–934
23. Nassoury N, Cappadocia M, Morse D (2003) Plastid ultrastructure defines the protein import pathway in dinoflagellates. J Cell Sci 116, 2867–2874
24. Norris BJ, Miller DJ (1994) Nucleotide sequence of a cDNA clone encoding the precursor of the peridinin-chlorophyll-*a* binding protein from the dinoflagellate *Symbiodinium* sp. Plant Mol Biol 24, 673–677
25. Peltier JB, Friso G, Kalume DE, Roepstorff P, Nilsson F, Adamska I, van Wijk KJ (2000) Proteomics of the chloroplast: systematic identification and targeting analysis of lumenal and peripheral thylakoid proteins. Plant Cell 12, 319–341
26. Prezelin B, Haxo F (1976) Purification and characterization of peridinin-chlorophyll a-proteins from the marine dinoflagellates *Glenodinium* and *Gonyaulax*. Planta 130, 251–256
27. Rensing L, Taylor WR, Dunlap J, Hastings JW (1980) The effects of protein synthesis inhibitors on the *Gonyaulax* clock II: the effect of cycloheximide on ultrastructural parameters. J Comp Physiol 138, 9–18
28. Rowan R, Whitney SM, Fowler A, Yellowlees D (1996) Rubisco in marine symbiotic dinoflagellates: form II enzymes in eukaryotic oxygenic phototrophs encoded by a nuclear multigene family. Plant Cell 8, 539–553
29. Schmitter R (1971) The fine structure of *Gonyaulax polyedra*, a bioluminescent marine dinoflagellate. J Cell Sci 9, 147–173

30. Schuler M, Zielinski R (1989) *Methods in Plant Molecular Biology*. (San Diego, Calif: Academic Press)
31. Whitney S, Andrews T (1998) The CO_2/O_2 specificity of single-subunit ribulose-bisphosphate carboxylase from the dinoflagellate *Amphidinium carterae*. Aust J Plant Physiol. 25, 131–138
32. Zhang Z, Green BR, Cavalier-Smith T (1999) Single gene circles in dinoflagellate chloroplast genomes. Nature 400, 155–159
33. Zhang Z, Green BR, Cavalier-Smith T (2000) Phylogeny of ultra-rapidly evolving dinoflagellate chloroplast genes: a possible common origin for sporozoan and dinoflagellate plastids. J Mol Evol 51, 26–40
34. Zhang Z, Cavalier-Smith T, Green BR (2001) A family of selfish minicircular chromosomes with jumbled chloroplast gene fragments from a dinoflagellate. Mol Biol Evol 18, 1558–1565