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INTRACELLULAR COAGULATION INHIBITS THE EXTRACTION OF PROTEINS FROM *PROCHLORON*

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Abstract—Protein extraction from the prokaryotic alga *Prochloron* LP (isolated from the ascidian host *Lissoclinum patella*) was complicated by an irreversible loss of cell fragility in the isolated algae. Accompanying this phenomenon, which we term intracellular coagulation, was a redistribution of thylakoids around the cell periphery, a loss of photosynthetic O_2 production, and a drastic decrease in the extractability of cell proteins. Procedures are described for the successful preparation and transport of cell extracts yielding the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase as well as other soluble proteins.

INTRODUCTION

There is currently great interest in establishing the biochemical properties of *Prochloron* species, prokaryotic unicellular algae that live in symbiotic association with animal hosts, namely certain didemnid ascidians [1]. The phyletic origin of higher plant chloroplasts may be revealed by detailed analysis of this unique group of symbiotic algae which exhibit taxonomic affinities with both the blue–green algae (Cyanophyta) and the green algae (Chlorophyta). A new taxonomic class, Prochlorophyta, has been suggested for *Prochloron* [1, 2].

Biochemical analyses of *Prochloron* species have been hampered by a lack of fresh cell samples. Until recently no prochlorophyte had been successfully cultured in the laboratory, and thus all of the data on their biochemical properties are from field observations or from laboratory studies carried out on frozen or freeze-dried preparations of these algae. It has proved extraordinarily difficult to extract soluble proteins and active enzymes from such preparations, and a literature review of detectable enzymes in *Prochloron* reveals only one positive citation [3], while the activities of over twenty other enzymes could not be detected (R. Fall, unpublished observations). The recent success of Patterson and Withers [4] in culturing *Prochloron* DS (from *Diplosoma similis*) raises hope that fresh prochlorophyte samples will be available in the laboratory.

An underlying reason for the general failure to detect enzymatic activities in *Prochloron* preparations was revealed in a recent field trip to Palau, Western Caroline Islands, a location where *Prochloron* from six different host species can be easily collected. We discovered that enzyme extraction from *Prochloron* LP, from *Lissoclinum patella*, is complicated by an irreversible loss of cell fragility. This phenomenon, which we call 'intracellular coagulation', and its consequences for protein chemistry and enzyme studies of *Prochloron* are described here.

RESULTS

When viewed on a slide under a light microscope, freshly isolated suspensions of *Prochloron* LP could be seen to be relatively fragile. Application of a standard weight to the coverslip (see below) resulted in the rupture and lysis of virtually all the cells in the field of view. In contrast, when the algae were subjected to various stresses of pH, osmolarity or physical handling, the cells exhibited a dramatic loss of cell fragility. This intracellular coagulation, as viewed under the microscope, is seen as a condensation or aggregation of the thylakoids and congealing of the cell bodies to a rubbery consistency.§ When subjected to the standard weight pressure test these cell remnants do not readily rupture, and even if ruptured under high pressure (in a French press) they produce large cell fragments without appreciable release of cell protein. Thus coagulation appears to involve precipitation and/or aggregation of intracellular proteins. This change was found to be correlated with an irreversible loss of photosynthetic capacity, as measured with an oxygen electrode, and probably also with cell death.

Extensive coagulation was promoted by many treatments, including squeezing the algae into unbuffered sea water, cooling algal suspensions in buffered sea water to 4° or −15°, centrifuging algae into high-density cell pellets, or suspending them in hypertonic saline or half-strength sea water. Complete coagulation was produced on exposure of algal suspensions to 1% (v/v) toluene or 10% (v/v) dimethylsulfoxide or 100 mg/l. Triton X-100 in buffered sea water, treatments we hoped to use to permeabilize cells for *in situ* enzyme assays [5]. Cells could be maintained for short periods with minimal coagulation in Tris-buffered sea water or isotonic saline, and for longer periods (12–24 hr) in Bicine-buffered sea water.

§This sort of phenomenon may be common among prokaryotic cells when they die. Protoplasts of the blue–green alga *Chroococcus turgidus* exhibit a similar coagulation after treatment with chloroform (R. A. Lewin, personal observation).

Since our aim was to isolate and transport active enzyme preparations for subsequent laboratory analysis, we needed procedures for minimizing coagulation while maximizing protein extraction from the algae. One partially successful extraction procedure involved hypotonic lysis and immediate neutralization of acids released from the algae. Some typical results are shown in Table 1. Cells of *Prochloron* were rapidly but gently washed in buffered sea water, and then resuspended in various buffers of low ionic strength. After 5 and 120 min we determined the pH of the resulting lysates, and during the intervening period the extent of coagulation and of lysis was determined by the pressure test.

Cells resuspended in four volumes of a hypotonic solution with low buffering capacity, such as 10 mM Tris–Cl at pH 8.0, became acidic, with the pH dropping to as low as 5.5. The bright green colour of the cell suspension changed to brown as acids released from ascidian host cells converted the algal chlorophylls to phaeophytins [1]. In hypotonic bicarbonate solutions the cells lysed but the pH remained around pH 8 and the cells remained green.

The success of hypotonic lysis depended on rapid and gentle treatment of the *Prochloron* cells before the lytic step. For example, the cells in densely packed cell pellets were poorly lysed when resuspended in hypotonic solutions, and little acid was released (Table 1). This was evidently due to coagulation at the high cell density in such pellets. We also tried pretreating the algae with lysozyme in an attempt to increase the degree of lysis, but even a 6-hr treatment with lysozyme (2 mg/ml) in buffered sea water yielded no increase in lysis over a control incubation (Table 1).

Cell extracts could also be successfully prepared by passage of suspensions in an isotonic buffer solution through a French press. Extracts of this type, as well as hypotonic lysis extracts, were prepared to evaluate the release of algal proteins and enzymes. Since attempts to assay these cellular components in the field were hampered, cell-free extracts were adjusted to 90% saturation with ammonium sulfate, and then transported on ice and assayed (in the U.S.A.) *ca* 12–25 days after collection. Two other types of *Prochloron* preparations were also transported for subsequent laboratory analysis: freeze-dried cells and acetone-powder preparations of whole cells.

Table 2, summarizing the results of experiments performed on various types of *Prochloron* preparations, indicates the type of pretreatment of the algae, the mode of cell breakage, the total extractable protein obtained (expressed as mg protein per ml packed cell) and the activity of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) in each preparation.

Cells subjected to hypotonic lysis released more protein at room temperature than at 4°. Cooling the cells to 4° caused much coagulation, and freezing them, with or without subsequent drying (lyophilizing), led to complete coagulation, and then very little lysis or protein release could be obtained (Table 2). Cells could also be ruptured by passage through a French press: at room temperature the yield of released protein was comparable to that obtained by hypotonic lysis at 28°, but at 4° less protein was released. When freeze-dried cells were rehydrated and passed through a French press the resulting homogenate contained mainly fragments of coagulated cell protein and debris, but little soluble protein (Table 2). Acetone-powder preparations yielded the highest level of extractable protein. It was not easy to dry the acetone powders in the humid Palau environment, and much of the protein may have been denatured, and thus precipitated, during subsequent laboratory extraction.

On our return from the field we analysed the *Prochloron* extracts for a variety of enzymes, focussing on dehydrogenases known to be present in blue–green algae [6]: G6PD, 6PGD, isocitrate and malate dehydrogenases. Only the first two could be definitely detected. These activities were therefore assayed in all of the transported samples: the results are summarized in Table 2. The level

Table 1. Lysis of *Prochloron* in hypotonic media.

Pretreatment of algae	Lysis solution	pH of suspension after 5 min	pH of suspension after 120 min	% Lysis
(A) Control (gentle washing and immediate lysis)	TN	7.5	5.5	60
	TNE	6.1	5.5	55
	TBE	7.1	6.9	45
	BE	8.1	8.4	55
(B) Packed cells	TN	7.8	7.8	< 10
	BE	8.5	8.4	< 10
(C) Stirring for 6 hr	TN	—	6.2	45
(D) Stirring for 6 hr with lysozyme	TN	—	6.4	45

Cells were collected and washed in Tris-buffered sea water (TBSW) as discussed in the Experimental. All operations in Palau were carried out in room light at 28°. Before lysis some cells were (B) packed by centrifugation and the cell pellet was allowed to sit for 15 min after resuspension in lysis buffer, (C) stirred on a reciprocating shaker in TBSW for 6 hr, or (D) stirred as in (C) but with the addition of 2 mg/ml lysozyme. Just before lysis, cells were centrifuged for 90 sec at *ca* 50 *g*, and the cell pellet (~ 0.5 ml) was immediately resuspended in 4.5 ml of lysis solution. Lysis solutions included: TN: 0.01 M Tris–Cl, 0.01 M NaCl, pH 8.0; TNE: TN plus 0.01 M EDTA, pH 8.0; TBE: 0.01 M Tris–Cl, 0.01 M $NaHCO_3$, 0.01 M EDTA, pH 8.0; BE, 0.01 M EDTA in 2.2% (w/v) $NaHCO_3$. %Lysis was measured after 10 min as described in Experimental.

Table 2. Recovery of protein, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from *Prochloron* LP

Pretreatment of algae	Cell breakage	Extracted protein (mg/ml packed cells)	Glucose 6-phosphate dehydrogenase (units/ml packed cells)	6-Phosphogluconate dehydrogenase (units/ml packed cells)
(A) TBSW, 28°	Hypotonic lysis	5.8	110	81
(B) TBSW, 4°	Hypotonic lysis	0.8	14	n.d.
(C) TBSW, frozen	Hypotonic lysis	0.2	n.d.	n.d.
(D) TBSW, freeze-dried	Hypotonic lysis	0.1	n.d.	n.d.
(E) TBSW, 28°	French press (28°)	4.6	50	67
(F) TBSW, 4°	French press (4°)	3.6	29	n.d.
(G) TBSW, freeze-dried	French press (4°)	0.2	n.d.	n.d.
(H) TBSW, acetone powder				
Preparation 1	—	6.1	3012	n.d.
Preparation 2	—	8.4	1736	n.d.

Cells were collected and washed in Tris-buffered sea water (TBSW) as described in the text, and pretreated before cell breakage as indicated. Hypotonic lysis was carried out as in Table 1 with TBE solution. When the French press was used, the cell pellet was resuspended in an isotonic buffer (0.1 M Tris–Cl, 0.3 M NaCl, pH 8.0) just before breakage. After cell breakage the lysates and homogenates were centrifuged at *ca* 1000 *g* for 10 min and the resulting supernatants were quickly adjusted to pH 8 with aliquots of 1 M Tris. Some samples, including preparations (A)–(F), were transported as ammonium sulfate suspensions, where the solid salt was added to give 90% saturation at 25°; just before assay these samples were dialysed (3 hr, 3 buffer changes) against 40 mM NaCl, 10 mM Tris–Cl, 0.1 mM dithiothreitol, 0.1 mM EDTA, pH 7.4, at 4°. Preparation (G) was a 5% (w/v) suspension of freeze-dried cells in the above buffer, passed through a French press at 16 000 p.s.i. at 4°, and centrifuged at 1000 *g* for 10 min; the 1000 *g* supernatant was assayed. The preparations shown in (H) were 5% (w/v) suspensions, stirred for 30 min at 4°, then centrifuged and assayed as above.

n.d. = Not detectable (in this case our assays would detect 0.1 units/ml).

of G6PD activity was generally proportional to the total extractable protein. An exception was the extract obtained from acetone-powder preparations, where G6PD activity (total units per ml packed cells) was the highest of any preparation, being 16- to 27-fold higher than that of the next best preparation, a hypotonic lysis extract (Table 2). 6PGD activity was not detectable in any cell-free extract derived from cooled, frozen, freeze-dried, or acetone-treated *Prochloron* cells.

The specific activities (units of nmol/min per mg protein) of these two enzymes ranged from 8 to 494 for G6PD and 9 to 15 for 6PGD. The G6PD values were comparable to those we obtained for a few selected blue–green and green algae: *Synechocystis* sp. (strain 27178), 21; *Synechocystis* sp. (strain 29108), 14; *Chlorella salina* (strain 1809), 203; and *Dunaliella salina*, 146.

Another observation may bear on the underlying chemistry associated with intracellular coagulation. We could not detect any low MW thiols in cell extracts prepared in 80% (v/v) methanol or ethanol. When mixed with Ellman's reagent [7, 8] the extracts produced no colour change detectable by eye. Control tests with a *Nostoc* sp. (collected in Palau) treated in the same way exhibited the usual positive reaction for cell thiols. These results suggested that in these *Prochloron* preparations cellular thiols were completely oxidized. Subsequent analysis for thiols in rehydrated cells using derivatization with monobromobimane [8], followed by high-performance liquid chromatography, indicated the absence of water-soluble thiols common to blue–green and green algae (R. Fahey, University of California, San Diego, personal communication).

DISCUSSION

The apparent fragility of these *Prochloron* cells was unexpected, since they have a cell wall like that of Gram-negative bacteria [1]. Frozen preparations after thawing are refractory to breakage even at high shear pressures. A dramatic loss in cellular fragility, as observed in the light microscope, is accompanied by a loss of photosynthetic O_2 production, a redistribution of thylakoids around the cell periphery, and an insolubilization of cellular enzymes and other proteins. The cell contents seem to coagulate. Intracellular coagulation is triggered by many treatments, including hypotonic shock, hypertonic shock, acidification of the medium, high cell densities, and cooling to 4° and below. It is now clear that special cell handling techniques must be developed if the coagulation is to be prevented: the development of such techniques may shed light on the chemical reactions involved.

The mechanism of coagulation is unknown. Since the native algae are osmotically fragile it seems likely that they have mechanisms for maintaining turgor pressure similar to those of other cell types [9]. Possibly the cells, although prokaryotic, have subcellular structures analogous to the tonoplast of some eukaryotes, and the rupture and release of the vacuolar contents lead to coagulation. It is conceivable that the electron-transparent central bodies seen in electron micrographs of *Prochloron* [10] are related to such structures. Possibly the release of acids and/or phenolic compounds could explain the protein insolubilization noted in these studies. The release of oxidizable materials such as polyphenols could explain our inability to detect cellular thiols. Another possibility is that the isolated algae when illuminated may produce toxic levels of superoxide or hydrogen peroxide which are normally removed *in situ* by the symbiont and/or host, as suggested recently for a zooxanthella–sea anemone symbiosis [11]. Accumulation of an oxidant such as hydrogen peroxide could certainly result in thiol oxidation, and perhaps then a cross-linking of proteins by –S–S– linkages.

The demonstration of both G6PD and 6PGD activities in many of the *Prochloron* cell-free preparations, even after freezing and thawing, was satisfying, especially in light of our previous failures to detect enzymes in frozen or freeze-dried *Prochloron* cells (R. Fall, unpublished results). This limited success suggests that, for conservation of enzyme activities, future preparations of *Prochloron* should include hypotonic lysates and acetone powders, which may prove suitable for various enzyme assays. However, the variable recovery of 6PGD (Table 2) suggests that one should seek additional ways to conserve and transport the cells for subsequent protein chemistry studies.

With the recent report of partial success in culturing *Prochloron* [4], it may soon be possible to define under laboratory conditions the sequence of events that leads to intracellular coagulation. In addition, the chemical basis for acid release and for the (postulated) destruction of cell thiols could be further analysed, and the efficacy of protein stabilizers could be tested.

EXPERIMENTAL

Chemicals. Reagent-grade chemicals were used whenever possible. Lysozyme, glucose-6-phosphate, 6-phosphogluconate, NADP and Bicine (*N,N*-bis[2-hydroxyethyl]glycine) were obtained from Sigma.

Algae preparations. In February 1982, colonies of the ascidian *Lissoclinum patella* were collected at Palau, Western Caroline Islands, and transported in sea water to the Micronesian Mariculture Demonstration Center. The animals were cleaned and squeezed by hand into sea water buffered with 0.04 M Tris–Cl, pH 8.4. In a few experiments sea water buffered with 0.04 M Bicine, pH 8.4 (adjusted with KOH), was used. The algae were received in about an equal vol. of the buffered sea water to neutralize acids liberated by bruised ascidians [1]. The *Prochloron* cells were harvested by centrifugation at *ca* 50 *g* for 90 sec, quickly resuspended in buffered sea water, and washed twice by a similar procedure. Microscopic examination revealed that contamination by animal host cells or bacteria was negligible (much less than 1%). Some preparations were 'shell frozen' and then freeze-dried. Two preparations, each representing *ca* 8 ml of packed cells, were extracted with cold Me_2CO: washed algae resuspended in an equal vol. of buffered sea water were poured into stirred Me_2CO at $-15°$ and quickly collected on filter paper with suction; the residual Me_2CO was allowed to evaporate for 30 min, and the resulting powder was stored at $-15°$, except during shipment when it was maintained at 4°.

For comparative studies, other algae (pure cultures) were obtained as follows: *Synechocystis* strains 27178 and 29108 from the American Type Culture Collection were grown as described [12]; *Chlorella salina* 1809 from the Culture Collection of Algae University of Texas was grown in 'high yield' medium [13] supplemented with 10 g/l. NaCl; a *Dunaliella salina* strain obtained from Neil Burris (Solar Energy Research Institute) and its growth was previously described [14].

Cell-free preparations. Freshly washed *Prochloron* cells were ruptured by use of a French press or by hypotonic lysis as described in the text. Other algae harvested by centrifugation and washed with isotonic saline were disrupted (French press) in the dialysis buffer described in Table 2, and 1000 *g* supernatants assayed.

Cell viability and fragility. Cell viability was routinely monitored by measuring photosynthetic oxygen production with an oxygen electrode [15]. For a quantitative test of cell fragility the following procedure was developed. Living cells in 5 μl of buffered sea water were set on a slide under a coverslip and observed with a light microscope. Pressure was then applied to the coverslip; we used a 2 kg weight for 10 sec. Fresh, viable cells were completely ruptured by this procedure, whereas cells that had coagulated (e.g. cells exposed to pH < 6 or to low temperatures) were flattened but not ruptured. The percentage of fragile cells correlated with light-dependent O_2 production rates; non-fragile cells evolved no oxygen.

Assays. Protein was determined by a modified Lowry procedure in which sodium dodecyl sulfate was added to the alkali reagent [16], and absorption was measured at 550 nm to minimize interference by chlorophyll. Serum albumin was used as the standard. G6PD and 6PGD were assayed at pH 7.4. Extraction with 80% (v/v) alcohol and visual detection of algal thiols were attempted using Ellman's reagent [7].

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