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

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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

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

labeled to approximately equal intensities. It is likely that this loss of functionality is due to the disruption of one or more of the outermost chloroplast membranes and the concomitant loss of regulatory macromolecules. Thus, the use of isolated plastids for the analysis of chromophytic chloroplast function is not technically advisable.

A second approach that has been successful in the identification of ctDNA coded proteins is to label cells in the presence of compounds that specifically inhibit protein synthesis on 70S or 80S ribosomes. Although this approach avoids the use of isolated plastids that may not be fully functional, it is not free of ambiguity. Protein synthesis inhibitors often affect other cellular processes (Galling, 1982; Reardon & Price, 1983), resulting in conflicting and/or erroneous conclusions about the coding site (either nuclear or chloroplast) of plastid proteins. However, inhibitor artifacts can be avoided if inhibitor concentration and exposure are minimized and if complementary pairs of inhibitors (one which inhibits 80S ribosomes and one which inhibits 70S ribosomes) are employed to detect nonspecific inhibitor activity. This experimental approach has allowed Chua & Gillham (1977) to demonstrate that 9 of 33 thylakoid membrane polypeptides are synthesized by the *Chlamydomonas* chloroplast. Inhibitors have also been effectively used to probe the coding site of specific chloroplast proteins in the *Euglena* (Schiff, 1971) and pea (Ellis & Hartley, 1971) systems. Many of the ctDNA-coded proteins identified by these studies have since been confirmed by in vitro protein synthesis or DNA mapping techniques (Whitfeld & Bottomley, 1982). Therefore, when suitable caution is exercised, the use of protein synthesis inhibitors to determine the site of chloroplast polypeptide synthesis can be an effective approach.

In this paper, we report upon the use of cycloheximide (CHI) and chloramphenicol (CAP) to investigate *Olisthodiscus* ctDNA coded polypeptides. These inhibitors block protein synthesis on 80S and 70S ribosomes, respectively. Four of the approximately 50 proteins observed to be coded on *Olisthodiscus* ctDNA have been identified. Similar to all chlorophytic plants investigated to date, the apoprotein of the P700–chlorophyll *a*–protein complex, the 32 000-dalton photogene, and the large subunit of RuBPCase are ctDNA coded in *Olisthodiscus*. Our evidence also indicates that the small subunit of RuBPCase is ctDNA coded in *Olisthodiscus*. This polypeptide is coded by a nuclear gene in all chlorophytes. The variation observed between chlorophytic and chromophytic plants in the coding sites for this important photosynthetic enzyme should provide a useful clue in the determination of evolutionary relatedness among major plant taxa.

MATERIALS AND METHODS

Cell Growth and Labeling. Olisthodiscus luteus Carter was grown synchronously in artificial seawater medium (McIntosh & Cattolico, 1978) with continuous but gentle agitation. Cultures were maintained on a 12 h light 12 h dark cycle and were stringently monitored for both bacterial and fungal contamination (Reith & Cattolico, 1985). Cell counts were made in a Model ZB1 Coulter cell counter. Cultures were sampled in the exponential phase of growth [$(1–5) \times 10^4$ cells/mL] between hours 4 and 6 of the light period. Cells were labeled by incubating 100 mL of culture with 25 μCi of NaH^{14}CO$_3$ (53 mCi/mmol) for 30 min under standard growth conditions (20 °C, 753 μE). The efficiency of bicarbonate incorporation was increased by minimizing gas exchange during the incubation period. Thus, labeling was performed in a sterile 125-mL Ehrlenmeyer flask that was not agitated. Protein synthesis inhibitors were added 5 min prior to the

addition of label. CHI and CAP were prepared as 10 mg/mL stocks in 95% ethanol. Final concentrations were 1 and 100 μg/mL of culture, respectively. Addition of ethanol at these concentrations had no effect on cell growth or labeling characteristics. At the end of the labeling period, cells were chilled on ice and collected by centrifugation at 1470g for 5 min.

Sample Preparation and Gel Electrophoresis. Labeled cells were separated into membrane and soluble fractions by resuspending the pelleted cells in 100 μL of solution A [0.1 M *N*-(2-hydroxyethyl)piperazine–*N'*-ethanesulfonic acid and 0.1 M dithiothreitol, pH 7.6 with KOH] (Chua, 1980) and homogenizing 5–6 times with a Dounce homogenizer. The samples were then centrifuged at 15000g for 5 min. The soluble fraction was removed and the membrane-containing pellet washed with 50 mM Tris(hydroxymethyl)aminomethane, pH 7.6, and resuspended in solution A. The composition of the sample buffer was adjusted as previously described (Reith & Cattolico, 1985) for either one- or two-dimensional gel electrophoresis. One-dimensional electrophoresis was performed on 10%–20% linear gradient polyacrylamide gels in the buffer system of Laemmli (1970). Two-dimensional gel electrophoresis was done according to O'Farrell et al. (1977) with pH 3–10 ampholines in the first dimension and a 10%–16% exponential acrylamide gradient in the second. Fluorography of gels was done as described previously (Reith & Cattolico, 1985).

Antibody Preparation and Detection of Antigens. Antibodies were raised against purified RuBPCase (S. Newman and R. A. Cattolico, unpublished results) in white New Zealand rabbits by standard techniques (Garney et al., 1977). IgG was purified from the antisera by passage over a CM Affi-Gel Blue column (Bio-Rad) followed by precipitation with 45% (NH$_4$)$_2$SO$_4$. The precipitate was desalted by passage over Sephadex G-50 and concentrated in an Amicon ultrafiltration unit. Antigens were detected essentially according to Towbin et al. (1979). Labeled proteins were separated by SDS gel electrophoresis and electrophoretically transferred to nitrocellulose. The nitrocellulose was then blocked, incubated with antibody, and washed. Following incubation with horseradish peroxidase conjugated goat anti-rabbit antibody (Cappell Laboratories) and washing, antigens were visualized by staining for peroxidase activity with 4-chloro-1-naphthol. Parallel lanes were prepared for fluorography as noted above.

RESULTS

Specificity of Inhibitor Action. Initial in vivo labeling experiments were designed to investigate appropriate CHI concentrations and labeling times that are necessary for the exclusive expression of ctDNA gene products. *Olisthodiscus* cells were labeled for 30 min in the presence of CHI, which ranged in concentration from 0.01 to 500 μg/mL of culture. At inhibitor concentrations below 0.1 μg/mL, increasing amounts of total cellular proteins are detectable by gel electrophoresis and fluorography, indicating that these levels of CHI only partially inhibit protein synthesis on cytoplasmic ribosomes. However, concentrations of CHI above 0.1 μg/mL resulted in no difference in the pattern of labeled proteins (data not shown). Variations in the length of the Na^{14}CO$_3$ labeling period (10 min to 3 h in the presence of 1 μg/mL CHI) had no effect on the pattern of labeled protein products. A CHI concentration of 1 μg/mL and a Na^{14}CO$_3$ labeling period of 1 h were chosen for all further experiments.

The specificity of CHI for inhibiting only cytoplasmically synthesized proteins was investigated by comparing the patterns of radioactive proteins from cells labeled in the presence
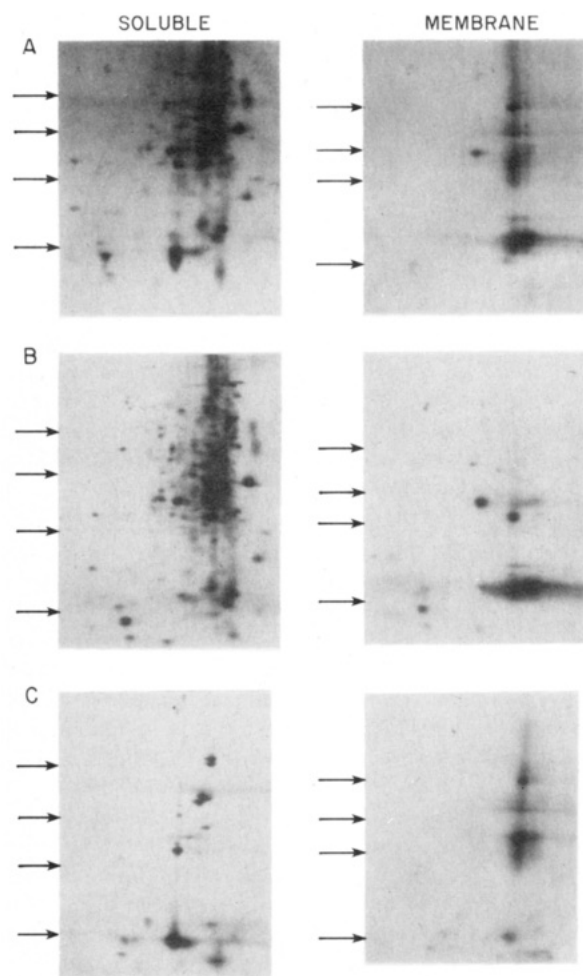
FIGURE 1: Specificity of protein synthesis inhibitors. Cells were labeled in the presence of (a) no inhibitor, (b) 100 μg/mL CAP, or (c) 1 μg/mL CHI and separated into soluble and membrane fractions. The autoradiographs obtained following two-dimensional gel electrophoresis are shown. Arrows indicate the molecular weight markers bovine serum albumin (68 000), ovalbumin (45 000), carbonic anhydrase (29 000), and myoglobin (17 000).

Table I: Effect of Proteinase K and Inhibitor Treatment on Protein Synthesis[a]

treatment	% incorporation
proteinase K[b]	2.8
+CAP	62.2
+CHI	39.1
+CAP, +CHI	3.7

[a] Cells were labeled with $NaH^{14}CO_3$ as described (Materials and Methods) for 30 min. Aliquots (0.05 mL) were processed to determine TCA-precipitable incorporation. The control for the inhibitor treatments contained 6645 cpm while the proteinase K treatment control contained 7822 cpm. [b] Cells were labeled with $NaH^{14}CO_3$ for 30 min and then incubated with proteinase K (10 mg/mL) for 1 h at 37 °C in the dark.
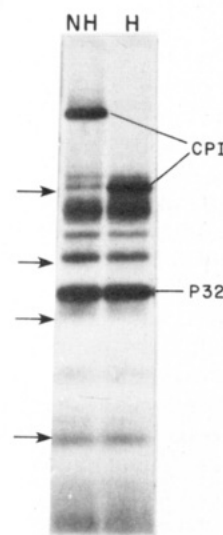

FIGURE 2: Identification of ctDNA-coded membrane proteins. Autoradiography of nonheated (NH) and heated (H) membrane samples from cells labeled in the presence of CHI. The apoprotein of CP1 (CP1) and the 32 000-dalton PS II protein (P32) are indicatd. Arrows indicate molecular weight as described in Figure 1.

of either CHI (1.0 μg/mL) or CAP (100 μg/mL) or no inhibitor. The results (Figure 1) demonstrate that all proteins labeled in the control gels are present in either the +CHI or +CAP gels and that there is no overlap between the two populations (CHI vs. CAP) of peptides. It is important to note that labeling of cells in the presence of a second pair of protein synthesis inhibitors, anisomycin (50 μM) or erythromycin (100 μg/mL) (Gillham et al., 1978), produces the same pattern of labeled proteins as CHI or CAP, respectively. Thus for *Olisthodiscus*, labeling cells in the presence of CHI appears to accurately reflect the synthesis of chloroplast DNA coded proteins.

In these experiments, a potential labeling artifact stems from the use of the precursor [14C]bicarbonate. This choice is dictated by the observation (Reith & Cattolico, 1985a) that neither radioactive amino acids nor sulfate are incorporated at significant levels by *Olisthodiscus luteus*. Since [14C]bicarbonate is ultimately a precursor for virtually all cellular macromolecules, it is possible that some proteins may become indirectly labeled through the addition of polysaccharide or oligosaccharide moieties. Two observations give evidence that this potential problem is minimal. First, incubation of labeled protein extracts with an excess of proteinase K (Table I) results in the solubilization of greater than 97% of the TCA-precipitable counts. Second, in the presence of both CHI and CAP, all de novo protein synthesis is blocked, but glycosylation of

proteins should be unaffected. Under these conditions (Table I) incorporation into TCA-precipatable counts is reduced to less than 4% of the control. These results, as well as the previously observed lack of overlap between +CHI and +CAP labeled proteins, indicate that in *Olisthodiscus* [14C]bicarbonate is primarily incorporated into protein through de novo synthesis.

Identification of ctDNA-Coded Proteins. As seen in Figure 1, approximately 50 chloroplast proteins are labeled in vivo when cells are labeled in the presence of CHI. Of these polypeptides 35 are soluble and 15 are membrane localized. A number of ctDNA-coded proteins can be easily identified on the basis of their molecular abundance or labeling pattern. Among the *Olisthodiscus* membrane proteins labeled in the presence of CHI, two have been identified.

The apoprotein of CP1 can be readily detected by comparing heated vs. nonheated samples of membrane proteins. In unheated samples, chlorophyll remains associated with the apoprotein, and this complex runs as a green band in polyacrylamide gels. Heating in the presence of SDS dissociates the complex, and the apoprotein thus runs at a lower molecular weight. This technique has been used to identify the apoprotein of CP1 in spinach (Zielinski & Price, 1980) and *Acetabularia* (Green, 1980). In both systems, the 68 000-dalton apoprotein is synthesized in isolated chloroplasts. When heated and nonheated samples of in vivo labeled *Olisthodiscus* membrane proteins are compared, the data seen in Figure 2 are obtained. A high molecular weight protein in the non-
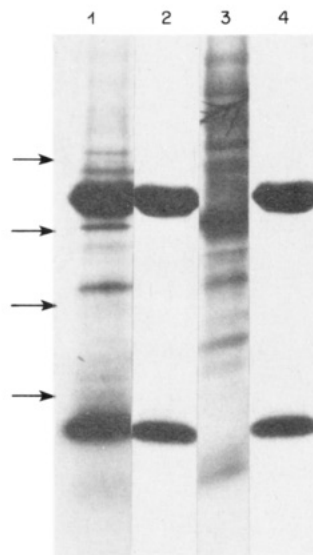
FIGURE 3: Identification of ctDNA-coded soluble proteins. Soluble proteins from cells labeled in the presence of either CHI (lanes 1 and 2) or CAP (lanes 3 and 4) were separated by SDS gel electrophoresis and either prepared for fluorography (lanes 1 and 3) or electrophoretically transferred to nitrocellulose and incubated with antibody to *Olisthodiscus* RuBPCase (lanes 2 and 4). Arrows indicate molecular weight markers as described in Figure 1.

heated lane that comigrates with a green band (the chlorophyll–apoprotein complex) migrates with a molecular weight of approximately 68 000 after heating. Thus, the apoprotein of CP1 also appears to be a ctDNA-coded protein in *Olisthodiscus*.

The second readily identifiable membrane protein is a 32 000-dalton PS II protein, which is known to bind several herbicides. This protein is abundantly labeled in isolated chloroplasts (Whitfeld & Bottomly, 1983) yet is not detectable by staining. A labeled protein having these characteristics can be seen Figure 2, indicating that this protein is also a ctDNA-coded protein in *Olisthodiscus*.

The most distinctive ctDNA-coded soluble protein is the large subunit of RuBPCase. This enzyme can represent as much as 20%–80% of the total cellular protein of plants cells (Wildman, 1982). Consequently, the large subunit, with a molecular weight of about 55 000, is easily detectable by both staining and fluorography. This protein has been shown to be coded on ctDNA by both in vivo and in vitro labeling procedures in a wide variety of plants (Whitfeld & bottomly, 1983). As expected, a protein with these characteristics is found in *Olisthodiscus* (Figure 3).

An unexpected observation was the appearance of a second highly labeled soluble protein with a molecular weight of about 15 000. Both the molecular weight and the intensity of labeling suggest that this protein might be the small subunit of RuBPCase, a peptide that has been unequivocally demonstrated to be a nuclear DNA coded protein in higher plants and *Chlamydomonas* (Whitfeld & Bottomly, 1983). The identity of the 15 000-dalton polypeptide was investigated by preparing antibodies to the *Olisthodiscus* RuBPCase holoenzyme. As seen in Figure 3 (lanes 2 and 4), the RuBPCase antibody binds exclusively to two proteins of approximately 55 000 and 15 000 daltons, which comigrate with the two highly labeled bands detected in soluble extracts from cells labeled in the presence of CHI (Figure 3, lane 1). This observation indicates that these two proteins are the LS and SS of RuBPCase. The 55 000- and 15 000-dalton proteins are conspicuously absent in fluorographs of soluble extracts from cells labeled in the presence of CAP (Figure 3, lane 3).

Identical data are obtained when the inhibitor set erythromycin and anisomycin is used. This observation demonstrates that the small subunit of RuBPCase is ctDNA encoded in *Olisthodiscus*.

DISCUSSION

Olisthodiscus ctDNA coded polypeptide production has been investigated by labeling cells in the presence of specific protein synthesis inhibitors. The chloroplast proteins that are labeled in vivo by this technique form a distinct subset of the total proteins synthesized by the cell. Our data demonstrates that the inhibitor pair CHI and anisomycin or CAP and erythromycin allows the identification of cytoplasmic and chloroplast protein populations, respectively. Thus, these selective inhibitors can serve as effective probes in determining the nuclear or chloroplast DNA coding sites of *Olisthodiscus* proteins.

When this experimental approach was used, it was shown by two-dimensional gel electrophoretic analysis that approximately 50 proteins are synthesized by the *Olisthodiscus* chloroplast. Fifteen of these proteins are membrane polypeptides, a number similar to that reported in several studies using either isolated chloroplasts or in vivo labeling techniques. Twelve ctDNA-coded membrane proteins have been reported for pea (Ellis, 1977), 15 for spinach (Zielinski & Price, 1980), and nine for *Chlamydomonas* (Chua & Gillham, 1977). This reasonable agreement between previous reports in which other experimental systems were used and the data from the *Olisthodiscus* systems suggest that in all plant taxa plastid DNA codes only for a limited number of membrane proteins.

Our results demonstrate that labeling Olisthodiscus cells in the presence of CHI more accurately reflects the in vivo protein synthetic patterns of the plastid than does the labeling of isolated chloroplasts. Isolated chloroplasts synthesize (Reith & Cattolico, 1985) 100 proteins vs. the 50 produced by whole cells incubated in the presence of CHI. A similar discrepancy in chloroplast protein number was noted by Ellis (1981), who reported that significantly more soluble proteins are labeled in isolated chloroplasts than are seen when intact pea plants are pulse labeled in the presence of CHI.

A possible explanation for this difference is that premature termination of translation occurs in isolated chloroplasts. The presence of a large number of incomplete polypeptides would then result in an inflated estimate of total plastid proteins produced. To analyze this possibility, labeled polypeptides that had been synthesized by isolated *Olisthodiscus* chloroplasts were examined by the *S. aureus* protease digestion method of Bordier & Crettol-Jarvinen (1979). Data from this preliminary experiment demonstrated that only a few proteins with similar digestion products occurred. Thus, it appears that incomplete polypeptide products do not account for the increased protein complement observed when *Olisthodiscus* plastids labeled in vitro vs. in vivo are compared.

Alternatively, if plastid expression is highly regulated by the nucleus, then chloroplast isolation could release ctDNA gene expression from this control, resulting in a relatively equal synthesis of all ctDNA-coded proteins. Heavily synthesized proteins such as the large subunit of RuBPCase might be produced in lower amounts in isolated plastids, while proteins synthesized at low levels or not at all would show an increase in expression. This uniformity in gene expression would allow the detection of substantially more peptides, and thus, the number of proteins synthesized by isolated chloroplasts may be a more accurate indicator of the total number of ctDNA-coded proteins, though not necessarily a true reflection of in vivo gene expression levels. Undoubtedly, until extensive

transcriptional mapping and/or DNA sequencing studies have been completed, the number of proteins encoded on ctDNA will remain uncertain.

The proteins identified as coded by the *Olisthodiscus* chloroplast include the apoprotein of CP1 and the photogene, plus both the large and small subunits of RuBPCase. Except for the small subunit of RuBPCase, the genes for each of these proteins have been previously shown to be located (Whitfeld & Bottomly, 1982) on the ctDNA of both algal and higher plant representatives of the Chlorophyta. Our studies present the first report that these genes are also encoded on the ctDNA of a plant within the chromophytic evolutionary line. This observation suggests that certain genes may be present on the ctDNA of all photosynthetic plants. In view of recent findings that the chloroplast genome appears to be evolving relatively slowly (Palmer & Zamir, 1982; Palmer et al., 1983), this result suggests that chloroplast DNA might be a repository in which vital photosynthetic genes are sequestered from evolutionary processes.

The observation that the small subunit of RuBPCase is a chloroplast-coded protein in *Olisthodiscus* was quite unexpected. However, recent reports suggest that this coding location may also occur in several other nongreen algal species. A number of laboratories (Heinhorst & Shively, 1983; K. Wassman, personal communication) have demonstrated that both large and small subunits of RuBPCase are coded on cyanelle DNA. Cyanelles are blue-green algal symbionts in which the genome has been reduced to approximately 115×10^6 daltons, a size very similar to that of most chloroplasts. For this reason, cyanelles have been regarded as an extant transition stage between blue-green algae and true plastids (Bohnert et al., 1982); Mucke et al., 1980). In addition, a recent report by Steinmuller et al. (1983) suggests that the small subunit of RuBPCase may also be a chloroplast-coded protein in red algae. These researchers found that poly(A^-) RNA from *Cyanidium caldarium* and *Porphyridium cruentum* programmed the synthesis of the RuBPCase small subunit in a rabbit reticulocyte translation system. Since most chloroplast mRNAs are recovered as poly(A^-) RNA (Wheeler & Hartley, 1975), these results indirectly suggest a plastid origin for the small subunit gene in red algae. Finally, we have recently demonstrated (Reith & Cattolico, 1985b) that a cloned *Olisthodiscus* ctDNA fragment, when expressed in a linked transcription/translation system, produces both RuBPCase large and small subunit proteins. These polypeptides have been positively identified by immunoprecipitation. It is an interesting possibility that all chromophytes and rhodophytes contain a ctDNA-coded RuBPCase small subunit. We are actively analyzing this possibility in our present research. Such a finding would suggest a common evolutionary origin for the chromophytes and rhodophytes, perhaps through a symbiotic event involving a blue-green algal cell. A second, different symbiosis possibly involving a *Prochloron*-like organism (Lewin & Withers, 1975) would have then produced the chloroplasts of green algae and higher chlorophytic plant species. Alternatively, transfer of the RuBPCase small subunit gene from the chloroplast to the nucleus may have occurred during the separation of chlorophytes from the chromophytes and rhodophytes. Further studies on the physical and genetic ctDNA maps of nongreen plant types are needed to provide a better understanding of chloroplast evolution.

Registry No. RuBPCase, 9027-23-0.

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Changes in Protein Conformation and Stability Accompany Complex Formation between Human C1 Inhibitor and C1s̄[†]

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Received August 21, 1984

ABSTRACT: The fluorescence spectrum of C1 inhibitor (C1-Inh) in aqueous buffer has a maximum at 324 nm which shifts to 358 nm in 6.0 M guanidinium chloride (GdmCl), indicating that fluorescent tryptophans are buried in the native protein. When titrated with GdmCl, the fluorescence intensity, polarization, and emission maximum of C1-Inh and C1s̄ exhibited clear transitions which were more prominent than those of the enzyme–inhibitor complex. Two of the variables (intensity and emission maximum) suggest biphasic unfolding of C1-Inh. Differential absorption measurements and sodium iodide quenching of intrinsic fluorescence were consistent with a net increase in the exposure of tryptophans and tyrosines upon complex formation. This reaction, i.e., complex formation, was also accompanied by an increase in the ability to enhance the fluorescence of the hydrophobic probe 8-anilino-1-naphthalenesulfonate. Fluorescence assays of heat denaturation showed transitions at 40 and 52 °C for C1s̄ and at 60 °C for C1-Inh whereas there was no detectable melting transition for the complex. Similarly, differential scanning calorimetric measurements revealed transitions at 42, 52, and 62 °C for C1s̄ and one transition at 60 °C for C1-Inh, with no major transitions detectable for the complex. The ratio of the calorimetric enthalpy to the apparent van't Hoff enthalpy for thermal unfolding of C1-Inh was 1.6. Taken together, these results suggest that C1-Inh and C1s̄ are each composed of at least two independently unfolding domains and that complex formation, which involves conformational change, yields a protein substantially more stable than either component alone.

C1 inhibitor (C1-Inh),[1] the only circulating protease inhibitor known to react with the activated complement components C1r̄ and C1s̄ (Sim et al., 1979; Ziccardi, 1981), plays a crucial role in the control of the plasma complement cascade. This is illustrated by the sometimes severe medical symptoms displayed by individuals with inherited deficiencies of this protein (Donaldson & Evans, 1963). Native human C1-Inh is a single-chain glycoprotein with three disulfide bonds and an amino acid composition which is unremarkable except for its relatively low tryptophan and tyrosine contents (Haupt et al., 1970). Up to 35% of the inhibitor's 104 000 molecular weight is composed of sugar residues (Haupt et al., 1970; Harrison, 1983), making this one of the most heavily glycosylated plasma proteins. The limited information available

about C1-Inh three-dimensional structure comes from the circular dichroic spectrum which indicates that the C1-Inh secondary structure is composed of nearly equal proportions of α-helix, β-sheet, and aperiodic structure (Nilsson et al., 1983) and from electron microscopic visualization of the rotary-shadowed molecule which appears to consist of a globular domain 40 Å in diameter attached to the end of a rod-shaped domain, 330 Å long and 20 Å in diameter (Odermatt et al., 1981). In the past several years, it has been shown that C1-Inh reacts with C1r̄ and C1s̄ to form stoichiometric 1:1 complexes

[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; DSC, differential scanning calorimetry; P, polarization; Cbz-Lys-sBzl, N^{α}-carbobenzyloxy-L-lysine thiobenzyl ester; HPSEC, high-performance size-exclusion chromatography; SDS, sodium dodecyl sulfate; DTDP, 4,4'-dithiodipyridine; PBS, phosphate-buffered saline; C1-Inh, C1 inhibitor; GdmCl, guanidinium chloride; bis(ANS), 5,5'-bis[8-(phenylamino)-1-naphthalenesulfonate]; Tris, tris(hydroxymethyl)aminomethane; Me$_2$SO, dimethyl sulfoxide.