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RuBisCo activase is present in the pyrenoid of green algae

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Summary. RuBisCo activase catalyzes the activation and maintains the activated state of the photosynthetic enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo, EC 4.1.1.39). We employed antisera prepared against the RuBisCo holoenzyme purified from tobacco and RuBisCo activase isolated from spinach to determine the localization of these proteins in leaves of C_3-type higher plants and green algae. In leaves of *Vicia faba*, both RuBisCo activase and RuBisCo are distributed throughout the chloroplast stroma. In contrast, RuBisCo activase and RuBisCo are predominantly localized to the pyrenoid in the green algae *Chlamydomonas reinhardtii* and *Coleochaete scutata*. The co-immunolocalization of RuBisCo activase and RuBisCo to the pyrenoid in these two green algal species suggests that pyrenoid-localized RuBisCo is catalytically competent. We conclude that the pyrenoid functions as a unique metabolic compartment of the chloroplast in which the reactions of the photosynthetic carbon reduction pathway are initiated.

Keywords: Green algae; Immunocytochemistry; Pyrenoid; RuBisCo; RuBisCo activase.

Abbreviations: RuBisCo ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP ribulose 1,5-bisphosphate; Pipes 1,4-piperazinediethanesulfonic acid; BSA bovine serum albumin; PBS phosphate-buffered saline; TBS Tris-buffered saline; IgG immunoglobulin G; C_i inorganic carbon.

Introduction

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo, EC 4.1.1.39) catalyzes the carboxylation of ribulose 1,5-bisphosphate (RuBP) in the initial reaction of the photosynthetic carbon reduction pathway. In higher plants, immunocytochemical investigations have shown that RuBisCo is localized in the chloroplast stroma (e.g., Vaughn 1987, Rother et al. 1988). The chloroplasts of a number of algal and hornwort species, however, are characterized by the presence of proteinaceous inclusion bodies known as pyrenoids. Moreover, immunocytochemical analyses have shown that in pyrenoid-containing algae and hornworts, RuBisCo is predominantly localized in this region (Table 1). Many of these reports, however, indicate that a portion of the RuBisCo in pyrenoid-containing organisms is also located in the stroma. In non-pyrenoid-containing algae and hornworts, RuBisCo is distributed throughout the chloroplast stroma (Lacoste-Royal and Gibbs 1985, Ekman et al. 1989, Vaughn et al. 1990). Because RuBisCo is commonly regarded as being a stromal enzyme, the observation that RuBisCo may be partitioned between the pyrenoid and stroma has led to speculation that the pyrenoid may be a storage region containing inactive RuBisCo. Moreover, due to the inability of RuBisCo antiserum to differentiate between active and inactive forms of the enzyme, we have been unable to assess the activity state of pyrenoid-localized RuBisCo.

Recently, a soluble chloroplast protein, RuBisCo activase, has been implicated as being responsible for catalyzing the activation and maintaining the activated state of RuBisCo (Salvucci et al. 1985). The mechanism by which activase catalyzes activation of RuBisCo is not well understood; however, it likely involves ATP hydrolysis (Streusand and Portis 1987) and protein-protein interactions in order to promote enzyme carbamylation (Werneke et al. 1988 a). In the present investigation, we have employed antiserum prepared against a mixture of the 41 and 45 kDa polypeptides of spinach RuBisCo activase in order to determine the

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Table 1. Immunocytochemical investigations of RuBisCo localization in pyrenoid-containing species

Class	Species	Technique[a]	Reference
Rhodophyceae	*Porphyridium cruentum*	G	McKay and Gibbs (1990)
		G	Mustardy et al. (1990)
Cryptophyceae	*Chroomonas* sp.	G	McKay and Gibbs (1991)
Bacillariophyceae	*Phaeodactylum tricornutum*	G	McKay and Gibbs (1991)
Dinophyceae	*Symbiodinium kawagutii*	G	Blank and Trench (1988)
Euglenophyceae	*Euglena gracilis*	F	Kiss et al. (1986)
		G	Osafune et al. (1989, 1990)
Chlorophyceae	*Bryopsis maxima*	E, F	Kajikawa et al. (1988)
	Chlamydomonas reinhardtii	F	Vladimirova et al. (1982)
		G	Lacoste-Royal and Gibbs (1985, 1987)
		G	this report
	Chlorella pyrenoidosa	G	McKay and Gibbs (1989)
	Chlorella vulgaris	G	Nisius and Ruppel (1987)
	Coleochaete scutata	G	this report
	Dunaliella salina	F	Vladimirova et al. (1982)
Anthocerotae	various species	G	Vaughn et al. (1990)

[a] *E* Immunoenzymatic; *F* immunofluorescence; *G* immunogold

localization of activase in leaves of C_3-type higher plants and in green algae. Since RuBisCo activase catalyzes the activation of RuBisCo in vivo, its immunolocalization should indicate the location of functional RuBisCo in the chloroplast.

Material and methods

Plant material

Seeds of *Vicia faba* L. (cv. Longpod) were sown in potting mix (3:1:1, peat:perlite:vermiculite) and watered daily. Plants were maintained in a controlled environment chamber (continuous light, 250 µmol photons $m^{-2}s^{-1}$, 20 °C). *Chlamydomonas reinhardtii* Dangeard was obtained from the Culture Collection of Algae at the University of Texas at Austin (UTEX 90) and grown in batch culture in a modified Bold's minimal medium. Cultures were maintained at 25 °C and were continuously agitated by use of a rotary shaker. Light was provided by cool-white fluorescent lamps at a photon fluence rate of 40 µmol·m^{-2}·s^{-1} in 12 h light − 12 h dark cycles. *Coleochaete scutata* de Brébisson was obtained from Carolina Biological Supply Co. (Burlington, NC, U.S.A.) and was used for experimental work immediately upon receipt.

Immuno-electron microscopy

Plants of *Vicia faba* were harvested 2 months after planting and small pieces (1 mm^2) of fully expanded terminal leaflets were cut directly into ice cold fixative (3% glutaraldehyde in 0.05 M 1,4-piperazinediethanesulfonic acid (Pipes), pH 7.4). The leaf pieces were fixed for 2 h, following which they were washed with buffer and dehydrated through a graded ethanol series to 100% ethanol. Leaf tissue was embedded in Lowicryl K4M (Polysciences, Warrington, PA, U.S.A.) and tissue blocks were polymerized under ultraviolet light at 4 °C for 24 h in a commercially-obtained light box (Ladd Industries Inc., Burlington, VT, U.S.A.).

Logarithmic-phase cells of *Chlamydomonas reinhardtii* were harvested at hour 4 of the light period. All pellets were fixed at 4 °C for 90 min in a solution containing 1% glutaraldehyde in 0.05 M Pipes, pH 7.4. The pellets were washed with cold buffer and the cells dehydrated through a graded ethanol series and embedded in Lowicryl K4M (J. B. EM Services, Montreal, P. Q., Canada) as described in Lacoste-Royal and Gibbs (1985).

Small pieces (1 mm^2) of *Coleochaete scutata* thallus were cut directly into cold 3% glutaraldehyde in 0.05 M Pipes buffer, pH 7.4, and fixed in the same solution at 4 °C for 2 h. The specimens were washed in cold 0.1 M cacodylate buffer, pH 7.2, and then post-fixed in 2% OsO_4 in 0.1 M cacodylate, pH 7.2, for 2 h at 4 °C. The specimens were washed in de-ionized water and then dehydrated through a graded ethanol series to 100% ethanol, following which they were embedded in LR White soft grade resin (Fullam Inc., Latham, NY, U.S.A.).

Pale gold-coloured sections of the tissue were cut with a diamond knife and collected on formvar-coated nickel grids. For immunolabelling, grids were placed section-side down on drops of the following solutions: 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), 30 min; antiserum, 1 h; 1% BSA in PBS, 4 drops during 10 min; colloidal gold reagent diluted 1:25, 20 min; PBS, 4 drops during 10 min; de-ionized water rinse. In addition, immunolabelling of sections of *Chlamydomonas* and *Coleochaete* was preceded by incubation on a drop of 12% sodium *m*-periodate for 10 or 30 min followed by a de-ionized water rinse in order to restore antigenicity. Mouse antiserum against a preparation containing both the 41 and 45 kDa polypeptides of spinach RuBisCo activase was kindly provided by J. M. Werneke and W. L. Ogren (United States Department of Agriculture, Agricultural Research Service, Urbana, IL, U.S.A.) and was used at dilutions of 1:80 to 1:160. The preparation of this antiserum has been described previously (Salvucci et al. 1987). Rabbit antiserum against RuBisCo was prepared from purified tobacco holoenzyme and obtained commercially (Cappel Laboratories, Cochranville, PA, U.S.A.) and was employed at a dilution of 1:400. Goat anti-mouse-gold (15 nm particles; EY Laboratories,

San Mateo, CA, U.S.A.) was used to detect anti-activase whereas protein A-gold (15 nm particles; EY Laboratories) was employed to detect anti-RuBisCo on tissue sections. Antisera and colloidal gold reagents were diluted in 1% BSA in PBS. Immunolabelled sections were post-stained for 4 min with 2% aqueous uranyl acetate and for 1 min with lead citrate (Reynolds 1963) prior to observing in either a Philips EM 410 or Zeiss EM 10CR electron microscope at an operating voltage of 60 kV.

In control experiments, the antiserum was replaced by PBS prior to colloidal gold reagent incubation. In addition, one other RuBisCo activase antiserum (also prepared against spinach activase and kindly provided by M. E. Salvucci, USDA-ARS, Lexington, KY, U.S.A.) was employed and results obtained using this antibody were similar to those provided by anti-spinach RuBisCo activase (data not shown).

Quantitative evaluation

The density of labelling over various cell compartments was obtained by determining the number of gold particles per square micrometer of compartment sectioned. Area determinations were made using a Zeiss (New York, NY, U.S.A.) MOP-3 digital analyzer.

Antiserum specificity

A *Chlamydomonas* crude protein extract was obtained by sonicating (Sonifier Cell Disruptor, Model W 140D; Heat Systems Ultrasonics Inc., Plainview, NY, U.S.A.) freshly harvested cells resuspended in ice-cold extraction buffer (100 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). The protein extract was clarified by centrifugation, following which proteins from the soluble fraction were separated essentially as described by Laemmli (1970) on 12.5% sodium dodecyl sulfate-containing polyacrylamide gels. Separated polypeptides were transferred electrophoretically to nitrocellulose filters (0.45 µM; Bio-Rad Laboratories Ltd., Mississauga, Ont., Canada) at room temperature for 2 h at 60 V in 25 mM Tris, 192 mM glycine and 20% methanol. Filters containing transferred polypeptides were blocked with 2% BSA in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl) at 4 °C overnight and were then incubated at room temperature in the following solutions: anti-RuBisCo activase diluted 1:500, 30 min; PBS containing 0.05% Tween 20 (PBST), 3 × 10 min; goat anti-mouse IgG-horseradish peroxidase (heavy and light chains; BIO/CAN Scientific Inc., Toronto, Ont., Canada) diluted 1:1000, 30 min; PBST, 3 × 10 min; TBS, 10 min. Immunoreactive bands were visualized by incubating blots in a solution containing the chromogen 4-chloro-1 naphthol (Sigma Chemical Co., St. Louis, MO, U.S.A.). Primary and secondary antisera were diluted in PBST containing 1% BSA.

Protein concentration was determined with the Bio-Rad protein assay kit using BSA as a standard and following the manufacturer's instructions.

Results

Western immunoblotting

Antiserum prepared against a mixture of spinach RuBisCo activase 41 and 45 kDa polypeptides was used to probe a Western blot of a soluble protein extract from *Chlamydomonas reinhardtii* (Fig. 1). The antibody mixture cross-reacts with only a single polypeptide of about 42 to 43 kDa, an observation in accord with recent findings by Roesler and Ogren (1990). In addition, Salvucci et al. (1987) have demonstrated that

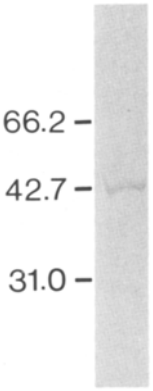

Fig. 1. Immunoblot of *Chlamydomonas reinhardtii* soluble protein extract (5 µg total protein). Transferred polypeptides were probed with anti-RuBisCo activase as described in the Material and methods. Positions of molecular mass standards (in kDa) are shown. The antiserum cross-reacted with only a single polypeptide band of about 42–43 kDa

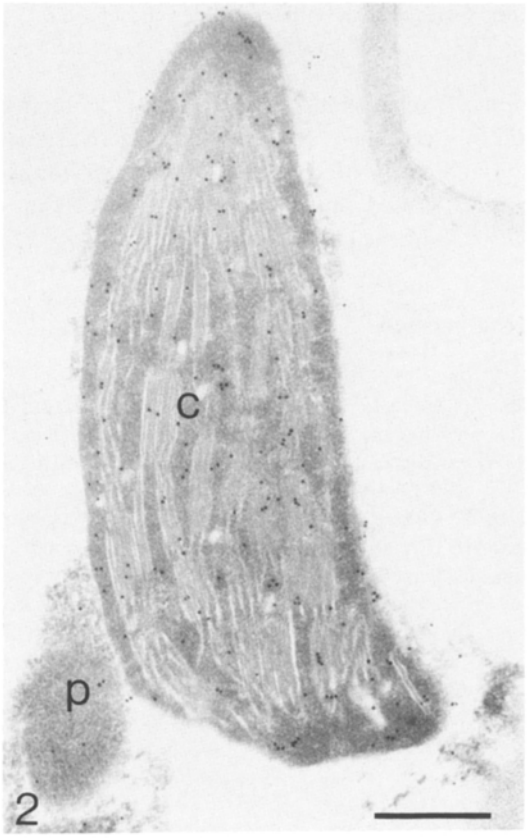

Fig. 2. Section through mesophyll cell of leaf of *Vicia faba* labelled by anti-RubisCo activase. Gold particles are distributed throughout the stromal region of a chloroplast (*c*). The cytoplasm and a peroxisome (*p*) are mainly unlabelled. × 30,500; bar: 0.5 µm

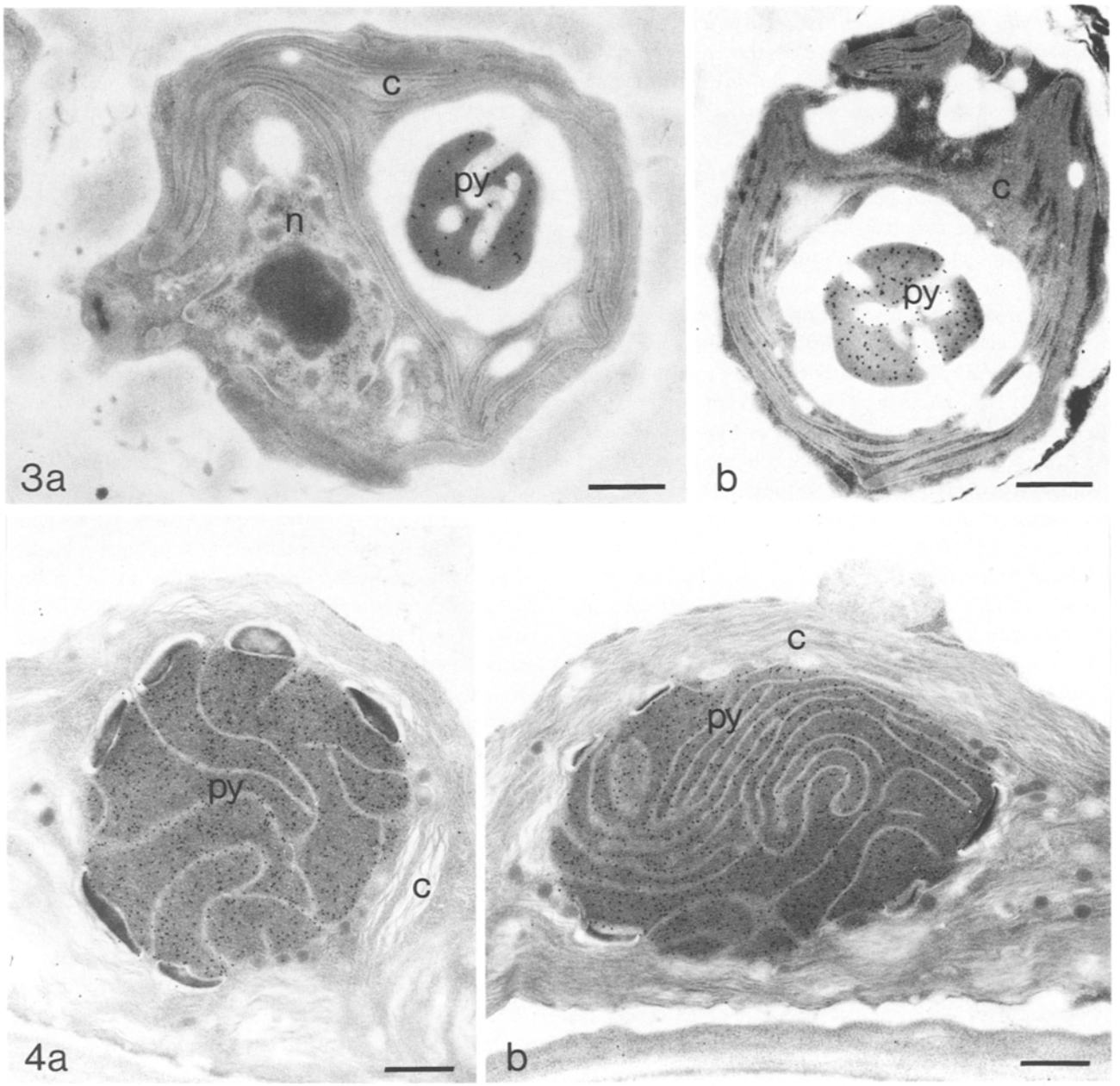

Fig. 3 a, b. Immunolocalization of RuBisCo activase and RuBisCo in logarithmic phase cells of *Chlamydomonas reinhardtii*. × 23,000; bars: 0.5 µm. **a** Anti-RuBisCo activase is concentrated over the pyrenoid (*py*) whereas the remainder of the chloroplast (*c*) is only lightly labelled. Few gold particles are evident over other cell compartments. *n* Nucleus. **b** Similarly, anti-RuBisCo is localized to the pyrenoid (*py*). Again, the remainder of the cell is relatively unlabelled

Fig. 4 a, b. Immunolocalization of RuBisCo activase and RuBisCo in *Coleochaete scutata*. × 21,000; bars: 0.5 µm. **a** Anti-RuBisCo activase is localized to the pyrenoid (*py*). The remainder of the chloroplast (*c*) contains only a few scattered gold particles. **b** The pyrenoid (*py*) is heavily labelled by anti-RuBisCo whereas the remainder of the chloroplast (*c*) is unlabelled

the spinach antiserum recognizes two polypeptides in the range of 41 to 47 kDa from leaves of a variety of other C_3-type higher plants. Specificity of the anti-RuBisCo used in this study has also been demonstrated previously (Vaughn 1987, Vaughn et al. 1990). These studies have shown that this antibody preparation specifically recognizes the large subunit of RuBisCo.

Immuno-electron microscopy

The intracellular localization of RuBisCo activase differs between C_3-type higher plants and green algae. We have employed antibodies to spinach RuBisCo activase to probe tissue sections of leaves of two higher plants (*Vicia faba* and pea) and two green algal species (*Chla-*

mydomonas reinhardtii and *Coleochaete scutata*). In all cases, the enzyme is restricted to the chloroplast. In *Vicia* (Fig. 2) and pea (data not shown), RuBisCo activase is restricted to the chloroplast stroma. Immunogold label over thylakoid grana stacks and cellular organelles is present at only low levels (Fig. 2). This pattern of immunolabelling by activase antiserum is identical to the observed intracellular localization of RuBisCo in C_3 plants (e.g., Vaughn 1987, Rother et al. 1988).

Figures 3 and 4 show the immunolocalization of RuBisCo activase and RuBisCo in both *Chlamydomonas* and *Coleochaete*. In both algae, immunolabelling by RuBisCo activase antiserum is confined mainly to the pyrenoid (Figs. 3 a and 4 a). For *Chlamydomonas*, a cross-section through an entire cell is provided from which it is evident that label over the chloroplast stroma and other cellular compartments is present only at low levels (Fig. 3 a). This observation is supported by results of a quantitative analysis of the density of labelling by anti-RuBisCo activase over different cell and plastid compartments in *Chlamydomonas*. Labelling density over the pyrenoid matrix (47.1 ± 2.7 particles/μm^2) is much higher than that over the chloroplast stroma (1.8 ± 0.2 particles/μm^2). Stromal labelling, however, is significantly higher than both cytoplasmic labelling (1.0 ± 0.2 particles/μm^2; one-tailed *t*-test, $P < 0.01$, n = 23) and nuclear labelling (0.7 ± 0.2 particles/μm^2; $P < 0.005$).

RuBisCo also appears to be confined to the pyrenoid in both *Chlamydomonas* and *Coleochaete* (Figs. 3 b and 4 b). In both green algae, the pyrenoid is heavily labelled by antiserum to the RuBisCo holoenzyme whereas other cellular compartments remain largely unlabelled. A pyrenoid localization of RuBisCo in green algae has been reported previously (e.g., Lacoste-Royal and Gibbs 1987, McKay and Gibbs 1989). Thus, as occurs with the two higher plant species surveyed, RuBisCo activase co-localizes with RuBisCo in green algae also. Control experiments were performed by replacing the antiserum with PBS, followed by either goat anti-mouse-gold (control for anti-activase) or protein A-gold (control for anti-RuBisCo). For each control, labelling densities were low (< 1 gold particle/μm^2) over each cellular compartment analyzed.

Discussion

RuBisCo activase has been detected in all higher plant species thus far surveyed (Salvucci et al. 1987). In addition, immunoblots have demonstrated its presence in the green alga *Chlamydomonas reinhardtii* (Salvucci et al. 1987, Roesler and Ogren 1990). In most higher plants, activase is comprised of two immunologically related polypeptides in the range of 40–47 kDa which appear to be derived from the same single-copy, nuclear-encoded gene (Werneke et al. 1988 b). Initial studies indicated that activase was synthesized in the cytoplasm as a single precursor polypeptide which was subsequently processed into mature activase polypeptides following transport into the chloroplast (Werneke et al. 1988 b). Recent findings, however, indicate that at least in spinach and *Arabidopsis*, RuBisCo activase polypeptides are generated by way of a conserved, alternative mRNA splicing mechanism (Werneke et al. 1989) which does not appear to exhibit developmental (Zielinski et al. 1989) or tissue-specific regulation (Werneke et al. 1989).

The exact nature of native RuBisCo activase remains unclear. The two higher plant activase polypeptides can be partially resolved under native conditions using ion-exchange chromatography (Werneke et al. 1988 a). This suggests that the functional holoenzyme is not necessarily heterogenous in composition. Moreover, analyses of individual activase polypeptides cloned and expressed in *Escherichia coli* indicate that the presence of both polypeptides is not requisite for RuBisCo activase activity (Werneke et al. 1988 a; also as reported in Portis 1990). This observation is further supported by the fact that *Chlamydomonas* contains only one activase polypeptide species (Roesler and Ogren 1990). The 41 kDa *Chlamydomonas* RuBisCo activase polypeptide is immunologically related to spinach RuBisCo activase and exhibits between 60–65% identity at the amino acid level with activase from both spinach and *Arabidopsis* (Roesler and Ogren 1990). Furthermore, in contrast to the higher plant gene, *Chlamydomonas* RuBisCo activase cDNA sequence analysis does not provide evidence for an alternative mRNA splicing mechanism (Roesler and Ogren 1990), thus supporting the observation of a single activase polypeptide species in this alga.

In the present investigation, we observed a co-localization of RuBisCo and RuBisCo activase in green algae and the leaves of C_3-type higher plants. In both *Vicia* (Fig. 2) and pea, activase is confined to the chloroplast stroma, an observation coinciding with a stromal location of RuBisCo as has been reported previously (e.g., Vaughn 1987, Rother et al. 1988). In green algae, RuBisCo is predominantly localized to the pyrenoid (e.g., Lacoste-Royal and Gibbs 1987, McKay and Gibbs 1989). We confirm this observation with

both *Chlamydomonas* (Fig. 3 b) and *Coleochaete* (Fig. 4 b) and, in addition, we report the pyrenoid localization of RuBisCo activase in these two species. Moreover, Vaughn et al. (1990) have recently shown a pyrenoid localization of RuBisCo among several pyrenoid-containing hornworts and further studies from his lab indicate that activase is also predominantly localized to the pyrenoids of these hornwort species (K. Vaughn, unpubl. data). The observed co-localization of RuBisCo and RuBisCo activase in the present study was not unexpected. Interactions between the two proteins have been proposed in order to reconcile inconsistencies with previous ideas on RuBisCo activation. Foremost among these is the observation that spontaneous in vitro RuBisCo activation can be accomplished only in the presence of supraphysiological concentrations of CO_2 ($K_{activation}$ = 25–30 μM) and Mg^{++} (Miziorko and Lorimer 1983). The physiological concentration of CO_2 in the C_3 plant chloroplast, however, approaches only 10–12 μM, yet, RuBisCo extracted from leaves exposed to photosynthetic saturating light intensities is reported to be highly activated (Perchorowicz et al. 1981). Portis et al. (1986), however, reported that the addition of partially purified spinach RuBisCo activase to a reconstituted illuminated chloroplast system led to activation of RuBisCo with a $K_{activation}$ of about 4 μM CO_2. Thus, it seems that activase enables RuBisCo to achieve a high degree of enzyme activation in C_3 plants in vivo. Why, however, would activase be required for in vivo RuBisCo activation in green algae? It appears that its existence there might be superfluous in view of evidence that *Chlamydomonas* (Badger et al. 1978) and other chlorophytes (Aizawa and Miyachi 1986) possess an effective inorganic carbon (C_i) concentrating mechanism where C_i species are concentrated intracellularly to levels higher than in the surrounding medium. The high concentration of intrachloroplastic CO_2 as would result from the operation of this mechanism should be sufficient to ensure the spontaneous activation of RuBisCo. The presence of RuBisCo activase, however, might be anticipated if a major function of the enzyme was to catalyze the activation of RuBisCo when it is complexed to inhibitors. RuBP, in addition to acting as substrate for activated RuBisCo, is a potent inhibitor of the higher plant inactive enzyme form, preventing addition of the activator CO_2 and Mg^{++} (Jordan and Chollet 1983). The effectiveness with which RuBP inhibits RuBisCo activation among algae and photosynthetic prokaryotes, however, varies. Among the algae, RuBisCo isolated from the red alga *Griffithsia pacifica* and the chromophyte *Olisthodiscus luteus* is rather insensitive to inhibition by RuBP (Newman et al. 1989). Conversely, Jordan and Ogren (1983) report that RuBP strongly inhibits RuBisCo activation in *Euglena gracilis* and *Chlamydomonas reinhardtii*. It is not known whether RuBP is an inhibitor of RuBisCo in *Coleochaete*; however, given its position on the "bryrophytan" line of evolution towards higher plants (Graham 1985), it is reasonable to assume that is does. RuBP is present in millimolar concentrations in the chlorophyte *Chlorella pyrenoidosa* (Yokota and Cavin 1986). Thus, it appears that in green algae, RuBisCo activase might prevent RuBisCo deactivation by catalyzing the activation of the tight binding RuBisCo-RuBP complex or other potential RuBisCo-sugar phosphate complexes. In support of this, Roesler and Ogren (1990) have recently reported that purified spinach activase can promote the activation of *Chlamydomonas* RuBisCo in the presence of physiological concentrations of RuBP.

The observed pyrenoid localization of RuBisCo activase in green algae in the present investigation lends further support to the idea that the pyrenoid is an important metabolic compartment of the cell and not simply a protein storage region. Previous immunolabelling results from this lab (McKay and Gibbs 1989) strongly suggested that pyrenoid RuBisCo was functional in vivo; however, due to limitations of the technique of immunocytochemistry, the study was not able to provide a direct assessment of RuBisCo activation state. As a result, we were unable to ascertain directly whether or not the pyrenoid represented the site of initial CO_2 fixation. The immunolocalization of RuBisCo activase, however, provides for a more direct method to elucidate the function of pyrenoid localized RuBisCo. Since RuBisCo activation is catalyzed and regulated by activase in vivo (Salvucci 1989, Portis 1990), the presence of activase in the pyrenoid indicates that pyrenoid RuBisCo is functionally active.

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