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Ribulose Bisphosphate Carboxylase from Three Chlorophyll *c*-Containing Algae[1]

PHYSICAL AND IMMUNOLOGICAL CHARACTERIZATIONS

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ABSTRACT

Distinctive properties are identified in the molecular structure of ribulose, 1,5-bisphosphate carboxylase/oxygenase (RuBPCase) in chlorophyll *c*-containing algae (*i.e.*, chromophytes). Using purified enzyme from *Cryptomonas* sp., *Coccolithophora* sp., and *Cylindrotheca fusiformis*, we have determined that the RuBPCase holoenzyme of each species has a molecular weight, subunit composition, and isoelectric points of its subunits similar to the purified enzymes from pea and *Chlamydomonas reinhardtii*. The large subunits from chromophytes exhibit microheterogeneity in their isoelectric points, whereas two to four well-resolved isoelectric variants of the small subunit were observed in each RuBPCase preparation. In spite of the high degree of similarity in terms of physical properties, both the small and large RuBPCase subunits of the chromophytes are structurally different from those of chlorophytes; immunological studies demonstrate that RuBPCase subunits of these two groups have few antigenic determinants in common.

Ribulose 1,5-bisphosphate carboxylase/oxygenase is a bifunctional enzyme which initiates the photosynthetic reduction of CO_2 and the first step of the photorespiratory pathway. Present in all autotrophs, RuBPCase[3] is probably the most abundant enzyme in nature. In vascular plants, green algae, and most bacteria, the RuBPCase holoenzyme is a high mol wt complex (~550 kD) composed of eight copies each of large (~55 kD) and small (~15 kD) subunits. The large subunit (LS) contains the sites for catalysis and CO_2/Mg^{2+} activation of the enzyme. The function of the small subunit (SS) is unknown, although it appears to be important for regulating enzyme activity (19). Unlike most RuBPCase species, SS is not essential for enzyme activity in the purple nonsulfur photosynthetic bacteria where RuBPCase is a homomultimer of LS. One nonsulfur photosynthetic bacterium, *Rhodopseudomonas*, is unique in possessing both a homomultimer LS (form II) and a more typical heteromultimer (form I) of RuBPCase (6, 19).

Little is known concerning the extent to which structural modifications of RuBPCase can affect the enzyme's catalytic properties. In an extreme case, the two forms of *Rhodopseudomonas* RuBPCase differ in their enzymic properties (21) but it has not been determined if the differences in RuBPCase in *Rhodopseudomonas* are due to the absence of SS in the form II enzyme or to the known structural differences in LS in the form I and II enzymes (19, 21). In plants, variations in RuBPCase have been positively correlated with the presence of CO_2 concentrating mechanisms (22, 29); species with carbonic anhydrase or those with C_4 photosynthesis have RuBPCase with higher specific *in vitro* activity (turnover number), but decreased CO_2 affinity ($K_m[CO_2]$), than species without the ability to concentrate CO_2 (29). The structural modifications in RuBPCase which accompany changes in catalytic properties are not known. Although the structural properties of RuBPCase from a variety of autotrophs have been extensively examined, RuBPCase of Chl *c*-containing algae (*i.e.* chromophytes) has not been well characterized. Studies of one chromophyte, the diatom *Cylindrotheca fusiformis*, indicate that the RuBPCase holoenzyme has the same mol wt and subunit composition as plant RuBPCase (7, 23), but its enzymic activity may be regulated differently than that from higher plants. Metabolites such as malate, aspartate and phosphoenolpyruvate appear to be effectors of the activity of diatom, but not spinach, RuBPCase.

The purpose of this study was to assess the degree of physical and structural similarity of RuBPCase from higher plants, green algae and Chl *c*-containing algae. We have found that RuBPCase of chromophytes has structural characteristics that are not shared with RuBPCase of other organisms. The data indicate that these RuBPCase species warrant further studies on their catalytic properties.

MATERIALS AND METHODS

Culture Conditions. All algae were grown in continuous light (250 $\mu E/m^2 \cdot s$). *Chlamydomonas reinhardtii* (137, mt-) from the *Chlamydomonas* Genetics Center, Duke University, was grown in Tris-acetate phosphate (TAP) medium as previously described (23). *Euglena gracilis* strain Z was grown photoheterotrophically in malate-glutamate medium (27). *Anabaena* sp. and *Isochrysis* sp. were obtained from Carolina Biological Supply and grown in Alga-gro (Carolina Biological Supply). *Synechococcus* sp. (SYN,

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[3] Abbreviations: RuBPCase, ribulose 1,5-bisphosphate carboxylase; LS, large subunit of RuBPCase; SS, small subunit of RuBPCase; αG-LS, antibody against the RuBPCase LS from the green alga *Chlamydomonas*; αE-LS, antibody against RuBPCase LS from *Euglena*; αD-LS, antibody against RuBPCase LS from the diatom *Cylindrotheca fusiformis*; αG-SS, antibody against RuBPCase SS from the green alga *Chlamydomonas*; pI, isoelectric point; M_r, molecular mass.

clone WH5701) was obtained from the Bigelow Culture Collection and grown in f/2 with natural seawater (10). *Rhodopseudomonas sphaeroides* was grown photoheterotrophically in a malate-glutamate medium (14). *Cryptomonas* sp. was grown in freshwater f/2 (10). Artificial sea water medium (23) was used for *Cylindrotheca fusiformis* and *Coccolithophora* sp. The dinoflagellates, *Amphidinium* sp. (Carolina Biological Supply) and *Zooanthus* sp. and the brown alga *Ectocarpus* sp. (Carolina Biological Supply) were grown in GPM medium (15).

Pea seedlings (Little Marvel, Carolina Biological Supply) were grown for 10 to 14 d in a greenhouse in vermiculite and pine bark.

Physical Characterization of Purified RuBPCase. RuBPCase was partially purified from *C. fusiformis*, *C. reinhardtii*, *Cryptomonas* sp., and *Coccolithophora* sp. Approximately 4 L of exponentially growing cells were collected and broken in a French pressure cell at 6,000 to 14,000 p.s.i. The buffers and conditions used for extraction and subsequent isolation were as described by Mishkind and Schmidt (17). Protease inhibitors (1 mM phenylmethyl-sulfonylfluoride, 5 mM ϵ-aminocaproic acid, and 1 mM benzamidine) were included in the extraction and gradient buffers to minimize proteolysis. Briefly, RuBPCase was obtained from the soluble fraction of whole cell extracts by $(NH_4)_2SO_4$ (30–60% saturation) precipitation. The precipitated protein was resuspended to 40 A_{280} units/ml and further purified by loading 20 A_{280} units on 10 to 30% linear sucrose gradients prepared with 25 mM Tris-HCl, pH 7.5, 200 mM NaCl. After centrifugation (284,000*g*, 18 h, 4°C), 18 fractions were collected and an aliquot of each was added to gel sample buffer (final concentrations: 60 mM Tris-HCl, pH 8.6, 100 mM DTT, 5 mM ϵ-aminocaproic acid, 1 mM benzamidine, 15% sucrose (w/v), 2% (w/v) lithium dodecyl sulfate) before heating at 100°C for 1 min. The sedimentation characteristics of RuBPCase was assessed by electrophoresis of gradient fractions at 4°C in gels composed of a 10 to 20% polyacrylamide gradient prepared as previously described (17) and stained with Coomassie blue R-250.

RuBPCase was purified from pea essentially as described above except that the leaves were homogenized with a Polytron (Brinkman) and the extract filtered through four layers of Miracloth. Chloroplasts were pelleted at 3,000*g* and then lysed hypotonically. After thylakoids were pelleted at 48,000*g*, RuBPCase was precipitated from the soluble fraction with $(NH_4)_2SO_4$ and 20 A_{280} units loaded onto sucrose gradients.

The isoelectric points of the RuBPCase subunits were determined with purified RuBPCase from fractions 6 to 8 of the sucrose gradients (see Fig. 1). These fractions were combined, solubilized (without heating) in gel sample buffer, precipitated in an excess of 80% (v/v) acetone, and applied to isoelectric focusing gels as described previously (17). The pH gradient in the gel after electrophoresis was measured in 25 mM KCl with a pH gradient scanner (Hoeffer Scientific Instruments). The second dimension 10 to 20% polyacrylamide gradient gels were silver-stained according to the procedure of either Morrissey *et al.* (20) or Wray *et al.* (32).

Production and Characterization of Antibodies against RuBPCase. Purified RuBPCase was heat denatured and subjected to preparative gel electrophoresis. Gels were stained with Coomassie blue and individual protein bands corresponding to either LS or SS were excised and used as a source of antigen for antibody production in rabbits. The schedules and procedures for injection, bleeding and purification of IgG fractions have been described (24); the specificity of antibodies against *Chlamydomonas* RuBPCase subunits also has been described (24). IgG preparations were used in all cases except for the *Euglena* antibody where whole serum was used.

The antibodies used in this study were all generated using denatured proteins. Thus, only sequential determinants, and not conformational determinants (3), should be recognized. The immunological assays we used (see below) were also performed with denatured proteins.

Preparation of Whole Cell Extracts. Algae were collected by centrifugation (50–300 μl packed cell volume), resuspended in approximately two volumes of cold distilled H_2O, and then immediately extracted with an equal volume of 20% (w/v) cold TCA. The cells were sonicated (5–10 pulses, output = 1; Heat Systems-Ultrasonics, Inc.) and further extracted in 10% (w/v) TCA for approximately 5 h. The precipitated material was collected by centrifugation (13,000*g*, 2 min), resuspended in distilled H_2O (100–200 μl), and sonicated as described above. Acetone was added to a final concentration of 90%. The precipitated protein was collected by centrifugation (13,000*g*, 2 min), and the pellet resuspended in 3 to 5 volumes of gel sample buffer (see above). Before PAGE, the samples were sonicated and boiled for 1 min to denature the proteins.

Detection of Cross-Reacting Polypeptides. The cross-reactivity of the antibodies with proteins from either purified RuBPCase or from the whole cell extracts of the various algae was determined by the protein blot procedure. A complete description of the buffers and protocols is presented elsewhere (18). Briefly, samples were subjected to PAGE as described above and proteins electrophoretically transferred to nitrocellulose. The nitrocellulose was exposed sequentially to antibodies against the RuBPCase subunits (10–15 μg IgG per ml; 20–30 ml final volume) and goat anti-rabbit IgG antibodies (1:2000 dilution; Sigma) conjugated with alkaline phosphatase. The colorless substrate 5-bromo-4-chloro-3-indolyl phosphate produced an insoluble blue color after enzymic cleavage of phosphate.

In assays of whole cell extracts, the cross-reactions were ranked on a scale of zero (no cross-reaction) to four (see Table II). Positive cross-reactions were listed in Table II when the protein band recognized by the antibody was near the known relative mobility (M_r) of RuBPCase subunits; with few exceptions our antibodies cross-reacted only with proteins of the expected M_r. The notable exceptions were reactions between the diatom antibody and high mol wt proteins from whole cell preparations of cyanobacteria, *Chlamydomonas* and *Euglena*. These proteins had a M_r ($\simeq$60 kD) greater than the expected M_r of LS and higher than the proteins recognized by antibody to LS from *Chlamydomonas* and *Euglena*. The biological significance of these cross-reactions is unclear, but nonspecific cross-reactions are not uncommon with the protein blot procedure (18). The 'second' antibody has also been noted to bind nonspecifically to protein bands (M Mansfield, personal communication) (18); this potential problem was determined not be the cause of nonspecific binding in our experiments.

RESULTS

Physical Characteristics of RuBPCase. The first goal of this study was to compare physical properties of RuBPCase isolated from the Chl *c*-containing algae (*i.e.* chromophytes) with RuBPCase isolated from a green plant (pea). PAGE of aliquots from 10 to 30% sucrose gradients showed that the chromophyte RuBPCase sedimented similarly to that of pea RuBPCase (Fig. 1). Therefore, the sedimentation coefficients of pea and chromophyte RuBPCase are nearly the same (18 S). These results, indicating that RuBPCase holoenzyme from chromophytes has a similar molecular size as the holoenzyme from higher plants ($\simeq$550 kD), are in agreement with those of Estep *et al.* (7) who found RuBPCase activities from the diatom *Cylindrothaca* and spinach comigrate during nondenaturing PAGE. Estep *et al.* (7) and Plumley (23) also showed that *Cylindrotheca* RuBPCase has two classes of subunits, similar in molecular size to the subunits isolated from higher plant RuBPCase. We obtained similar results with *Cryptomonas* and *Coccolithophora* (Fig. 1; Table I).

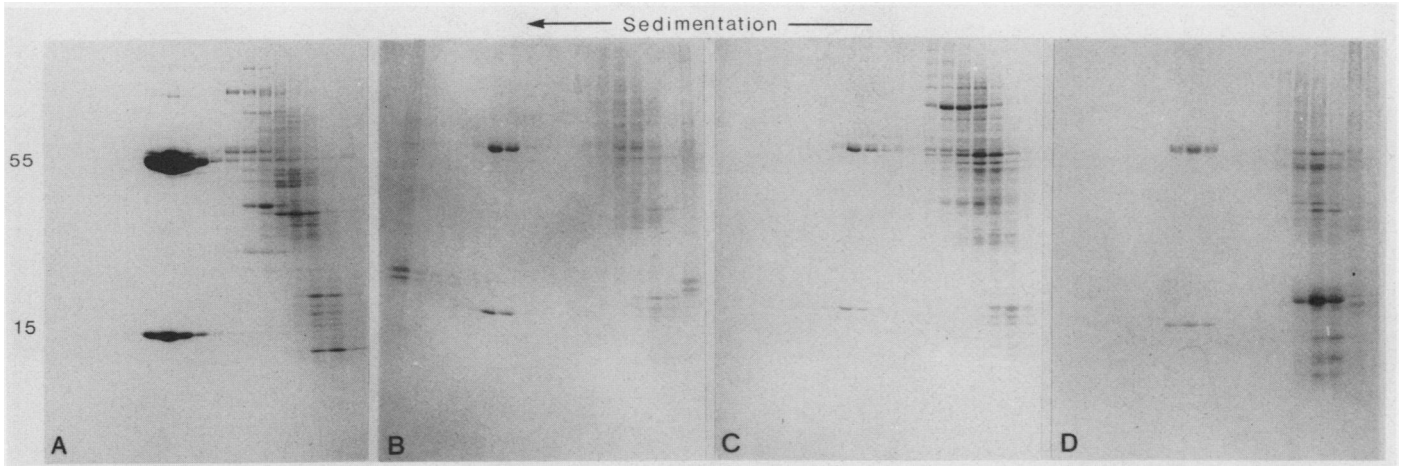

FIG. 1. RuBPCase analysis by PAGE of sucrose gradients fractions. Soluble protein (20 A_{280} units) from whole cell extracts of the algae or from purified pea chloroplasts, were loaded on 10 to 30% sucrose gradients. After centrifugation, 18 aliquots were removed from each gradient and prepared for SDS-PAGE. The bottom of the gradient is on the left. The gel was stained with Coomassie blue R-250 before photography. The migration of LS and SS at 55 and 15 kD are indicated. A, Pea; B, *C. fusiformis*; C, *Coccolithophora* sp.; D, *Cryptomonas* sp.

Table I. *Summary of Physical Characteristics of RuBPCase from Various Algae*

Species	Sedimentation Coefficient	Large Subunit		Small Subunit	
		Mol wt[b]	pI[c]	Mol wt[b]	pI[c]
	S	*kD*		*kD*	
Anabaena		52.7			
Synechococcus		52.7			
Chlamydomonas	18	53.9		14.8	
Euglena		52.7			
Pea	18	53.4	7.0	12.7	7.5, 7.1 7.8, 6.9[d]
Cylindrotheca	≃18[a]	52.3	6.6	13.8	5.5, 5.8
Ectocarpus		54.6			
Isochrysis		57.0			
Cryptomonas	≃18	53.8	6.8	15.1	7.6, 7.3, 7.8
Amphidinium		57.0			
Zooanthus		54.6			
Coccolithophora	≃18	52.8	7.1	15.5	7.6, 7.8, 7.2

[a] Sedimentation coefficient was estimated by co-sedimentation with RuBPCase isolated from pea in 10 to 30% sucrose gradients. [b] The apparent mol wt of the subunits of RuBPCase was estimated by SDS-PAGE with 10 to 20% acrylamide gels using the mol wt markers shown in Figure 2. [c] Isoelectric point was measured in denaturing isoelectric focusing gels. Proteins were focused toward the cathode. [d] Isoelectric variants listed in order of decreasing abundance. The second dimension gels were stained with silver nitrate (20).

Thus, the molecular size and subunit composition of RuBPCase of at least three chromophytes are quite similar to those of vascular plant RuBPCase.

In Figure 1, the yield of RuBPCase from the chromophytes appears to be substantially lower than for pea but this is not due to an inherently low level of RuBPCase in chromophytes. The data for the different species in Figure 1 are not directly comparable since the soluble protein fraction from pea was derived from isolated chloroplasts while those from chromophytes represent whole cell extracts. Analysis of whole cell extracts of pea and the algae examined in this study indicate that the vascular plant does not invest a significantly larger proportion of its total leaf tissue protein in RuBPCase (not shown). Furthermore, we have found that the levels of RuBPCase are rather similar in all algae examined.

Immunological Cross-Reactivity. To compare in more detail the structure of RuBPCase isolated from chromophytes and well-studied sources such as *Chlamydomonas* and pea, their immunological relatedness was examined by the protein blot procedure. Antibodies against the LS from the green alga *Chlamydomonas* (αG-LS) and *Euglena* (αE-LS) recognize only the LS in purified preparations of RuBPCase from pea and *Chlamydomonas* (Fig. 2, B and C) but not the LS from the chromophytes (Fig. 2D). The αG-LS antibody also cross-reacts weakly with proteins with a relative mobility (M_r) greater than LS (Fig. 2B). The antibody against the diatom LS (αD-LS) recognizes the LS from *Cylindrotheca* and *Cryptomonas*, and to a lesser extent, the *Coccolithophora* LS. No cross-reaction can be observed between the αD-LS antibody and proteins from pea and *Chlamydomonas*. Therefore, chromophyte LS has antigenic determinants that are present in LS from three evolutionarily diverse Chl *c*-containing algae but are absent in green algae and higher plants. Comparative peptide mapping studies also show that the LS of the three chromophytes is considerably different from that of pea (not shown).

The absence of immunological relatedness between the LS from chromophytes and the LS from pea and *Chlamydomonas* is unexpected. Previous studies with cyanobacteria and chlorophytes have demonstrated both sequence homology and antigenic relatedness for LS (1, 19). To determine whether the antibody we obtained against denatured LS from *Chlamydomonas* and *Euglena* would recognize LS from cyanobacteria, whole cell extracts of two cyanobacterial species were examined by the protein blot procedure. The αG-LS and αE-LS recognize the LS from *Synechococcus* while only the αG-LS binds to the LS from *Anabaena* (Table II). In contrast, αD-LS is not cross-reactive with LS from either cyanobacterium (Table II). Consistent with the immunoblots of purified RuBPCase (Fig. 2), αG-LS and αE-LS do not recognize proteins in whole cell extracts of any of the chromophytes tested whereas αD-LS recognizes the LS from all chromophytes (Table II). This analysis of whole cell extracts provides further evidence that LS of chromophytes resembles those of other eukaryotic phototrophs in having a M_r of ≃55,000 but is immunologically distinguishable from the LS of cyanobacteria, green algae, and higher plants.

Previous studies have indicated little immunological relatedness among SS from different plants species and between plants and green algae (8). These results are consistent with SS nucleotide sequence data which predict a low number of common

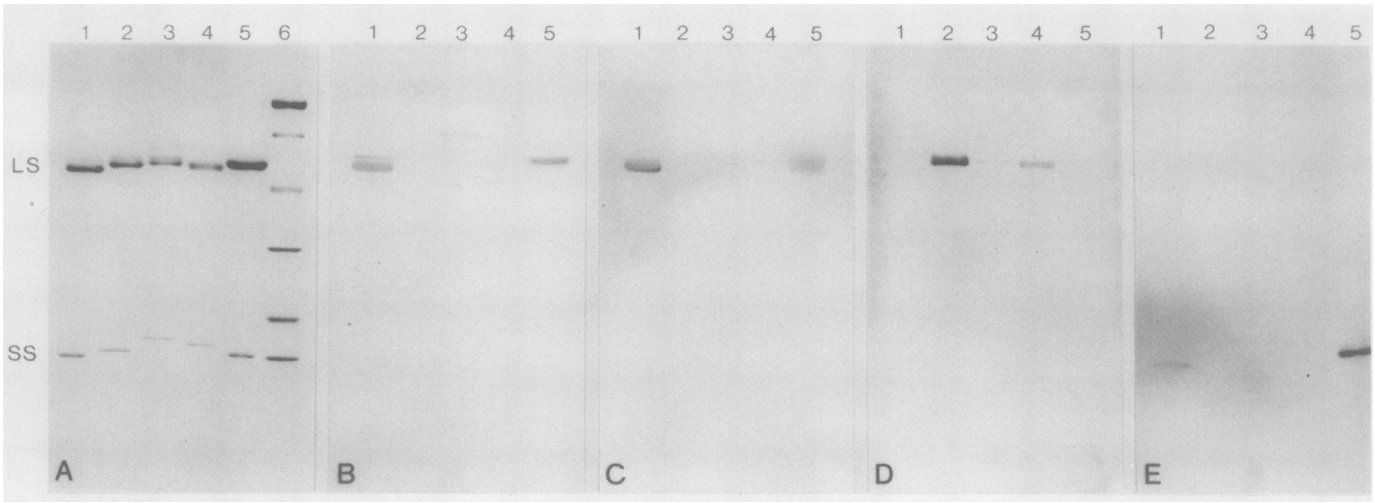

FIG. 2. Immunoblot analysis of purified RuBPCase from pea, *Chlamydomonas* and the three chromophytes. A, Coomassie stained profile of purified RuBPCase. Protein blots immunostained with: B, α *Chlamydomonas* RuBPCase LS; C, α *Euglena* RuBPCase LS; D, α *Cylindrotheca* RuBPCase LS; or E, α *Chlamydomonas* SS. Pea, *C. fusiformis*, *Coccolithophora* sp., *Cryptomonas* sp., and *C. reinhardtii* RuBPCase was applied respectively to lanes 1 to 5. Lane 6 in A contains molecular standards: phosphorylase *b* (92.5 kD), BSA (66.2 kD), ovalbumin (45 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD).

Table II. *Summary of Cross-Reaction of Four RuBPCase Antibodies with Whole Cell Extracts from Various Algae*

Cross-reactions were ranked qualitatively from no cross-reaction (−) to the strongest positive cross-reaction (++++).

Species	Antibody Against			
	Diatom large	*Euglena* large	*Chlamydomonas*	
			Large	Small
Rhodopseudomonas	−	−	−	−
Anabaena	−	−	++++	−
Synechococcus	−	+	++++	++
Chlamydomonas	−	++++	++++	++++
Euglena	−	++++	++++	++++
Cylindrotheca	++++	−	−	−
Ectocarpus	++++	−	−	−
Isochrysis	++++	−	−	++++
Cryptomonas	+++	−	−	−
Amphidinium	++	−	−	++
Zooanthus	+++	−	−	−
Coccolithophora	++	−	−	−

antigenic determinants in these diverse algae (1, 19). However, the antibody against the *Chlamydomonas* SS (αG-SS) does recognize some determinant(s) in purified SS from pea (Fig. 2E) as well as the SS in whole cell extracts of *Euglena* and *Synechococcus* (Table II). These results and those of previous studies can be reconciled on the basis of methodological differences; in the protein blot procedure a single antigenic site can be detected whereas immunoprecipitation techniques used in earlier studies require at least three antigenic sites for detection of a positive cross-reaction (18). The cross-reaction of αG-SS with whole cell extracts of *Euglena* and *Synechococcus* also indicates the high degree of sensitivity afforded by the protein blot procedure.

The αG-SS antibody fails to recognize SS from purified RuBPCase fractions from any of the chromophytes examined (Fig. 2E). Similarly, αG-SS does not cross-react with proteins in whole cell extracts of most of the chromophytes (Table II). However, αG-SS did recognize a protein with an M_r of 13,000 to 20,000 in whole cell extracts of *Isochrysis* and *Amphidinium*. We employed several different procedures to minimize nonspecific binding of antibody to protein bands (18), but were not able to account for the results with *Isochrysis* or *Amphidinium* on the basis of procedural artifacts. Data for the crossreactivity of αG-SS with SS from the chromophyte *Ochromonas* also have been published (12) but these results need to be verified with purified RuBPCase from each species.

It is of interest that neither form of RuBPCase from the purple nonsulfur photosynthetic bacterium *Rhodopseudomonas* cross-reacts with any of the antibodies we have prepared to LS or SS (Table II). The unique structure and antigenicity of the form II RuBPCase (*i.e.* hexamer of LS) from these bacteria has been noted previously (21).

Isoelectric Variants of RuBPCase Small Subunits. The SS of RuBPCase in higher plants is nuclear encoded by a multigene family (4); nucleotide sequence variations in SS genes can be responsible, in part, for multiple isoelectric forms of RuBPCase SS (19). In contrast, the chloroplast encodes LS of a single isoelectric species. By subjecting partially purified RuBPCase to isoelectric focusing, we determined that LS from each chromophyte exhibits microheterogeneity but all have a pI that is similar, or only slightly more acidic, than the LS of pea (Fig. 3; Table I). Therefore, differences in antigenicity are not reflected in marked changes in the net charge of large subunits of chromophytes *versus* chlorophytes.

Similar to SS from higher plants and green algae, multiple isoelectric forms of SS from the three chromophytes are observed (Fig. 3); the pI of these variants from two of the chromophytes resemble those of green algae and higher plants while those of the diatom are considerably more acidic. We have observed that minor differences in the relative abundances of the SS variants depend on whether the second dimension gels are stained by the ammoniacal silver method (32) or by the silver nitrate method (20). In addition, slight differences in pI of the SS variants result if the samples are focused toward the anode instead of the cathode. Thus, the relative amounts and pI of the variants in Table I and Figure 3 must be considered as rough approximations.

Isoelectric variants of SS can arise from the loss of N-terminal methionine from some of the molecules (19). Also, carbamylation of primary amines can occur as a result of cyanate formation from urea, used as a denaturant in the isoelectric focusing

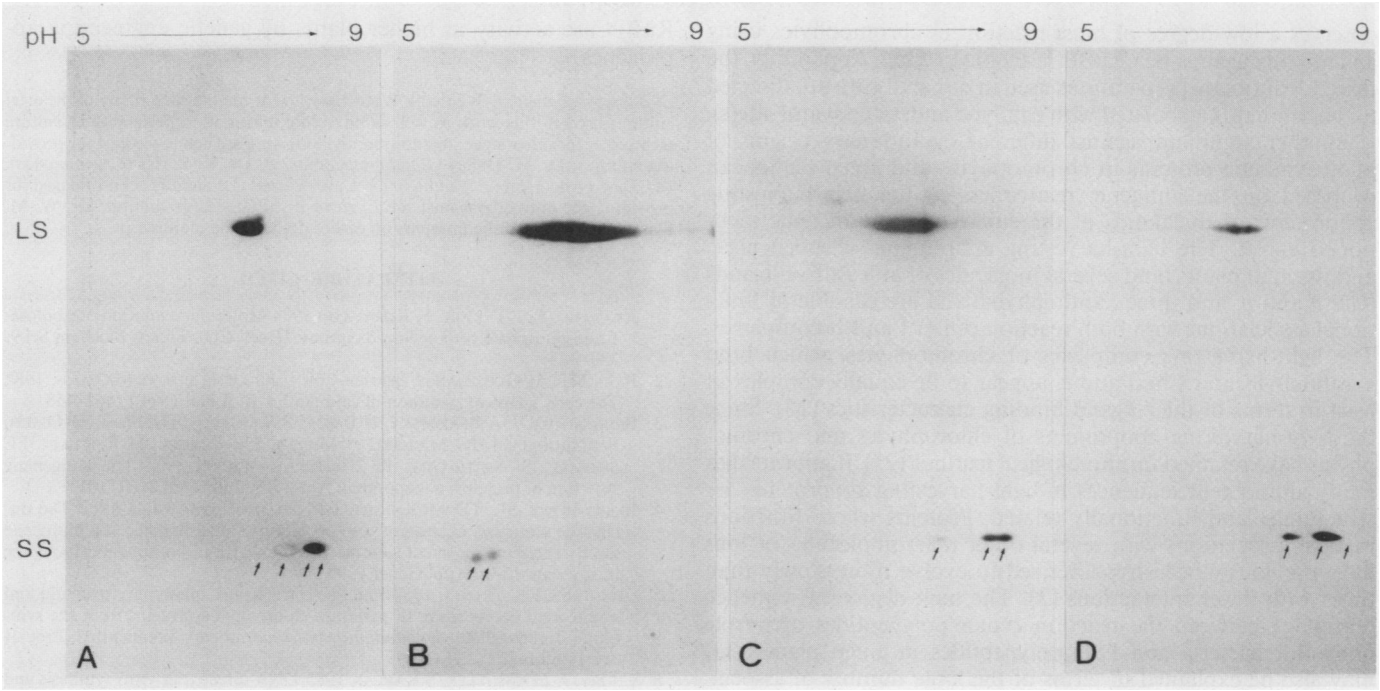

FIG. 3. Analysis of purified RuBPCase by two-dimensional polyacrylamide gel electrophoresis (isoelectric focusing first dimension, SDS-PAGE second dimension). The pH was determined with a gel scanner before the second dimensions were run. Proteins were visualized in the second dimension after staining with ammoniacal silver (32). A, Pea; B, *C. fusiformis*; C, *Coccolithophora* sp.; D, *Cryptomonas* sp. Arrows indicate positions of SS variants.

procedure. However, we include methylamine in our gel system to quench cyanate, minimizing artifacts of this sort. Other artifacts associated with ionic detergents and charged reducing agents employed for some isoelectric focusing procedures have also been documented but we do not believe these account for the charge variants of SS in this study. Identical results were obtained from experiment to experiment and our gel system duplicates previous findings of isoelectric variants of SS in pea and *Chlamydomonas* (19).

Differential post-translational modification can also account for the presence of isoelectric variants in two-dimensional gel systems. Examples of this for RuBPCase SS are N-terminal acetylation, detected in the SS precursor produced during *in vitro* translation of *Chlamydomonas* mRNA (28) and phosphorylation (16, 30). We cannot rule out the possibility that some of the mature SS from the chromophyte species are phoshorylated, acetylated or subject to other processing events.

DISCUSSION

RuBPCase from plants, green algae, and prokaryotes has been well-characterized biochemically and structurally (for recent reviews, see refs. 1 and 19). The similarity of structure of LS from green plants and prokaryotes was indicated first from immunological characterizations (8). Recent DNA sequencing studies show that the amino acid sequence homology is high (≃90%) among LS isolated from different plants and nearly as high when plant and cyanobacterial LS are compared (80–85%) (1). In contrast, both immunological and sequencing studies indicate that SS structures differ greatly (1, 19). Amino acid sequence homology among SS isolated from plants is 70 to 75% whereas the homology between plant and cyanobacterial SS is only 40% (1).

The high degree of structural similarity among the LS from cyanobacteria and green algae is consistent with the endosymbiont theory for the evolution of chloroplasts (9). In support of this hypothesis, several studies have demonstrated that the LS from cyanobacteria, green algae, and higher plants share a high degree of amino acid sequence homology and, as we also observe, are immunologically related. Moreover, our other observations (25) (FG Plumley, GW Schmidt, manuscript in preparation) indicate that chromophytes and green algae share antigenic determinants in a set of photosynthetic proteins generally considered to be unique to each line of algal evolution; the Chl *a/b* binding apoproteins of the light-harvesting complexes of *Chlamydomonas* have structural (*i.e.* immunological) counterparts in at least two of the chromophytes examined in this study. Thus, our observation that chromophyte LS and green algal LS do not possess antigenic determinants which are recognized by our three RuBPCase antibody preparations was surprising.

RuBPCase from chromophytes has either diverged substantially during its molecular evolution or its evolutionary origin is different from that of cyanobacteria and green plants. The former hypothesis is more compelling and explicable because all RuBPCases appear to possess similar amino acid sequences which are essential for photosynthetic reduction of CO_2 (19). The catalytic site entails only a small portion of the LS polypeptide. In the remainder of the LS molecule, many amino acid substitutions could be "neutral" with respect to function but still cause major shifts in antigenicity. Single amino acid substitutions in another photosynthetic protein, ferredoxin, can drastically alter its antigenicity (31). In support of this line of reasoning, the amino acid sequence homology between LS from the purple nonsulfur bacterium *Rhodospirillum* and spinach is pronounced only in regions of the enzyme implicated as the catalytic and activator sites (11). The observations that an antibody preparation against *Chlamydomonas* LS cross-reacts with LS from two chromophytes, *Ochromonas* (12) and *Cryptomonas* (S Gibbs, personal communication), suggest that antibodies recognizing catalytic and/or activator site(s) have been obtained. Similarly, L. Rothschild (Brown University, personal communication) also

observes a low degree of cross-reaction in chromophytes using an antibody against pea RuBPCase holoenzyme. Apparently, the three LS antibody preparations used in our study are not directed to determinants associated with catalytic and/or activator site(s).

Another argument against different evolutionary origins of photosynthetic proteins in chromophytes and green plants can be based on the antigenic relatedness in the light-harvesting apoproteins of thylakoids of the eukaryotic phototrophs mentioned above. The light-harvesting apoproteins of green algae and vascular plants bind several molecules of at least five ligands (Chl *a* and *b*, and three xanthophylls) and are capable of functional associations with both reaction center I and II complexes. The light-harvesting complexes of chromophytes, which bind xanthophylls and Chl *a* and *c*, appear to be equally complex at least in terms of their ligand binding characteristics (13). Since the light-harvesting apoproteins of chlorophytes and chromophytes have retained immunological features (25), it appears that many amino acid sequences in light-harvesting apoproteins are structurally and functionally related. Proteins whose functions involve associations with several other macromolecules or ions have previously been hypothesized to evolve more slowly than those with fewer interactions (2). The high degree of sequence homology between the reaction center polypeptides of purple nonsulfur bacteria and PSII polypeptides in green plants (33) may also be explained in terms of the large number of associations necessary for function of polypeptides involved in the light reactions of photosynthesis. Therefore, if the proposed lineage 'purple nonsulfur photosynthetic bacteria → cyanobacteria → chloroplasts' is correct (1, 33), it is apparent that the rate of amino acid substitution in RuBPCase has been much higher than the substitution rate in photosynthetic reaction center and light-harvesting polypeptides.

The SS isoelectric variants in chromophytes resemble those of green algae and higher plants. However, it recently has been determined that SS in one chromophyte, *Olisthodiscus*, is encoded by the chloroplast genome (26). The small size of the chloroplast genome in Chrysophyceae (26) probably obviates the occurrence of large multigene families for SS in this group of Chl *c*-containing algae. Determination of the sites of synthesis of SS in other chromophytes, as well as other protein complexes from chromophytes which exhibit charge heterogeneity (5), might define further the phylogenetic trends of these algae. Such studies also could reveal modes of enzyme regulation that differ among algae and higher plants.

One recent observation could also explain the charge heterogeneity of SS in chromophytes. Lacoste-Royal and Gibbs (12) detected substantial quantities of SS in both the chloroplast and mitochondria of the chromophyte *Ochromonas* and have suggested mitochondrial DNA harbors a SS gene. If mitochondrial SS does not play a functional role in *Ochromonas*, the gene encoding it could have sustained a high rate of nucleotide substitution leading to charge heterogeneity in the mitochondrial SS relative to the chloroplast localized SS. Obviously, the immunocytochemical localizations of SS to mitochondria by Lacoste-Royal and Gibbs (12) should be examined in other chromophytes.

A better understanding of the extent to which chromophyte RuBPCase is conserved within the evolutionarily diverse Chl *c*-containing algae will undoubtedly require elucidation of the site(s) of subunit synthesis in various chromophytes as well as data on the amino acid sequences of the subunits. In addition, detailed analyses of RuBPCase enzymic properties in chromophytes are needed to assess if these structurally unique enzymes have altered carboxylase/oxygenase activity; if novel enzymic properties are observed, comparative analyses of the amino acid sequences of chromophyte RuBPCase with those of other plant species could help provide useful models for attempts to modify RuBPCase activity in higher plants by genetic engineering approaches.

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