

Kinetics and Subunit Interactions of Ribulose Bisphosphate Carboxylase-Oxygenase from the Cyanobacterium, *Synechococcus* sp.*

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Ribulose 1,5-bisphosphate carboxylase-oxygenase was purified to electrophoretic homogeneity from the unicellular, marine cyanobacterium, *Synechococcus* sp. It was composed of large (57,000 dalton) and small (12,000 dalton) subunits in a 1:1 stoichiometry. It was rapidly activated by Mg^{2+} plus HCO_3^- , even in the presence of ribulose 1,5-bisphosphate which had no effect on the extent of activation. Michaelis constants for CO_2 , O_2 , and ribulose 1,5-bisphosphate were 3- to 10-fold higher than for the enzyme from higher plants. Maximum specific activities of the carboxylase and oxygenase reactions were 2.9 and $0.34 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, respectively. After being three times precipitated at pH 5.4 and redissolved, 13% of the initial small subunit content remained, together with 41% of the original catalytic activity. Inactive small subunits remained in the supernatant after precipitation. Sucrose density gradient centrifugation and pore-gradient electrophoresis showed that the small subunit-depleted enzyme consisted of an oligomeric core of large subunits with varying numbers of small subunits attached. However, activity was not correlated with degree of saturation with small subunits. When isolated small subunits were added back to the small subunit-depleted enzyme, substantial re-formation of the native electrophoretic form occurred and catalytic activity increased 2-fold or more.

The enzyme D-ribulose 1,5-bisphosphate carboxylase-oxygenase (EC 4.1.1.39) is a key determinant of the capacity of organisms for the net assimilation of inorganic carbon, and has always been so throughout evolution. It catalyzes the initial reactions of both the photosynthetic carbon reduction cycle and its apparently wasteful appendage, the photosynthetic carbon oxidation cycle (Lorimer and Andrews, 1981). This is because the enzyme is bifunctional, catalyzing the oxygenation of Rbu- P_2 ¹ to produce 2-phosphoglycolate and 3-phosphoglycerate (Bowes *et al.*, 1971; Andrews *et al.*, 1973), as well as its carboxylation to produce two molecules of 3-phosphoglycerate. The presumed antiquity of cyanobacteria, their prokaryotic subcellular organization, and the possibility that they may be related to the endosymbiotic ancestor of chloroplasts (for a review, see Broda, 1977) suggest that study of cyanobacterial Rbu- P_2 carboxylase-oxygenase may provide information about evolutionary progress of the enzyme.

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¹ The abbreviations used are: Rbu- P_2 , D-ribulose 1,5-bisphosphate; L, large subunits; S, small subunit; SDS, sodium dodecyl sulfate; Bicine, N,N'-bis(2-hydroxyethyl)glycine.

Rbu- P_2 carboxylase-oxygenases from eukaryotes are large molecules (M_r = 500,000-550,000) composed of 8 large subunits, which bear the active site, and 8 small subunits of unknown function (Jensen and Bahr, 1977). However, studies of cyanobacterial Rbu- P_2 carboxylase-oxygenases have presented a confusing picture. Some authors have reported an L_8S_8 quaternary structure lacking small subunits (Tabita *et al.*, 1974; 1976), while others have detected the presence of small subunits and favored a eukaryote-like L_8S_8 structure (Takabe *et al.*, 1976; Stewart *et al.*, 1977; Codd and Stewart, 1977; Takabe, 1977; Tabita and Colletti, 1979). In one case, this disagreement occurred even when the same species, *Anabaena cylindrica*, was studied (Tabita *et al.*, 1976; Takabe, 1977). Codd and Stewart (1977) suggested that the small subunits might have been lost during a purification step used by Tabita *et al.*, (1976) which involved precipitation of the enzyme from crude extracts at pH 5.4. This controversy is further complicated by a recent report that Rbu- P_2 carboxylase-oxygenase from another cyanobacterium, *Aphanothece halophytica*, may have an L_4 structure (Codd *et al.*, 1979).

Early studies of kinetic properties of cyanobacterial Rbu- P_2 carboxylase-oxygenases (Takabe *et al.*, 1976; Codd and Stewart, 1977) were clouded by a lack of knowledge about the activation state of the enzymes (Lorimer *et al.*, 1976; 1977). This situation has been redressed recently by Badger (1980) for the enzyme from *Anabaena variabilis*; it resembled the higher plant enzyme in requiring a preliminary incubation with Mg^{2+} and CO_2 before becoming fully catalytically active, but showed much lower affinities for CO_2 and O_2 .

This paper reports the purification in high yield and characterization of a cyanobacterial Rbu- P_2 carboxylase-oxygenase from a previously unstudied source, the unicellular marine species, *Synechococcus* sp. (Strain RRIMP N1). Its subunit structure, its activation and kinetic properties, and the effect of isoelectric precipitation on its subunit composition were investigated. Particular scrutiny was directed toward the inference found in previous literature (see above) that isoelectric precipitation might remove the small subunits without grossly reducing catalytic activity, thus opening the way for a study of the role of the small subunit, which is presently a complete mystery.

MATERIALS AND METHODS²

² Portions of this paper (including "Materials and Methods," part of "Results," Figs. 1-3, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md 20014. Request Document No. 80M-2640, cite authors, and include a check for \$3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

RESULTS

Effects of Precipitation at pH 5.4—When the purified enzyme solution was adjusted to pH 5.4, a pH which is probably near to the enzyme's isoelectric point, the enzyme slowly precipitated over a 30-min period at 0 °C (Table II). SDS¹-gel electrophoresis of the redissolved precipitate showed a reduction of its small subunit content relative to the native enzyme. When the precipitation was repeated twice more, the reduction became very obvious, with only 13% of the native enzyme's small subunit content remaining (Fig. 4). Catalytic activity also was reduced progressively by the successive precipitations, but not to the same extent as small subunit content. Thrice-precipitated enzyme retained 41% of its original specific activity (34% if specific activity in terms of large subunit content only is considered) (Table II). Supernatant solutions, which were enriched in small subunits (Fig. 4), were inactive. Usually, the amount of small subunit protein recovered in the supernatants was less than that lost from the precipitates (Table II). Apparently some small subunits must have been lost from the system during the precipitation procedure, perhaps adsorbed on glassware from the dilute supernatant solutions.

TABLE II

Effect of precipitation at pH 5.4 on subunit structure of *Synechococcus Rbu-P₂ carboxylase-oxygenase*

Purified enzyme (1.6 mg·ml⁻¹ in 50 mM K phosphate buffer, pH 7.6, containing 1 mM EDTA) was precipitated by addition of a one-fifth volume of 1.43% (v/v) acetic acid at 0 °C. Turbidity was faint at first, but increased progressively over a 30-min period. After 150 min, the suspension was centrifuged at 27,000 × g for 20 min at 4 °C and the supernatant decanted as completely as possible. The firm white pellet was dissolved in a volume of the above phosphate/EDTA buffer equivalent to the original volume of native enzyme solution. The pH of the supernatant was 5.45. This procedure was repeated twice more, each time using the redissolved pellet from the previous step. All three redissolved pellets (P₁, P₂, and P₃) and supernatants (S₁, S₂, and S₃), together with the native enzyme, were assayed for protein and Rbu-P₂ carboxylase activity (standard assay under N₂) and subjected to SDS-gel electrophoresis (see "Materials and Methods"). After staining, the gels were scanned (Fig. 4) and the areas under the large and small subunit peaks measured. The ratio, S/L, of these areas was converted to the molar ratio of subunits by dividing by the ratio between the molecular weights of the two subunits as determined from Fig. 1. To indicate the reproducibility of this method of determining subunit ratios, data for duplicate determinations with different gels are reported for native enzyme and P₁. Protein concentrations of supernatants were corrected for dilution incurred by addition of acetic acid solution. Specific carboxylase activities are shown in terms of total protein (L + S) and in terms of large subunit protein (L only). The percentage of specific activities compared to the native enzyme is given in parentheses.

Sample	Protein mg·ml ⁻¹	Ratio of subunits, S/L		Specific carboxylase activity	
		Ratio of peak areas from gel scans	Molar ratio	Total protein L + S	L only
Native enzyme	1.62	0.202	0.99	1.97 (100)	2.38 (100)
		0.216			
		mean 0.209			
P ₁	1.46	0.116	0.52	1.52 (77)	1.69 (71)
		0.103			
		mean 0.110			
P ₂	1.15	0.069	0.33	0.95 (48)	1.02 (43)
P ₃	1.00	0.028	0.13	0.80 (41)	0.82 (34)
S ₁	0.038	ND ^a	ND	<0.02 (<1)	NA ^b
S ₂	0.056	4.17	19.8	<0.02 (<1)	NA
S ₃	0.030	1.27	6.04	<0.02 (<1)	NA

^a ND, not determined.

^b NA, not applicable.

Lack of an obligate relationship between catalytic activity and small subunit content was further shown by linear sucrose density gradient centrifugation of the native and small subunit-depleted enzymes according to the procedure of Martin

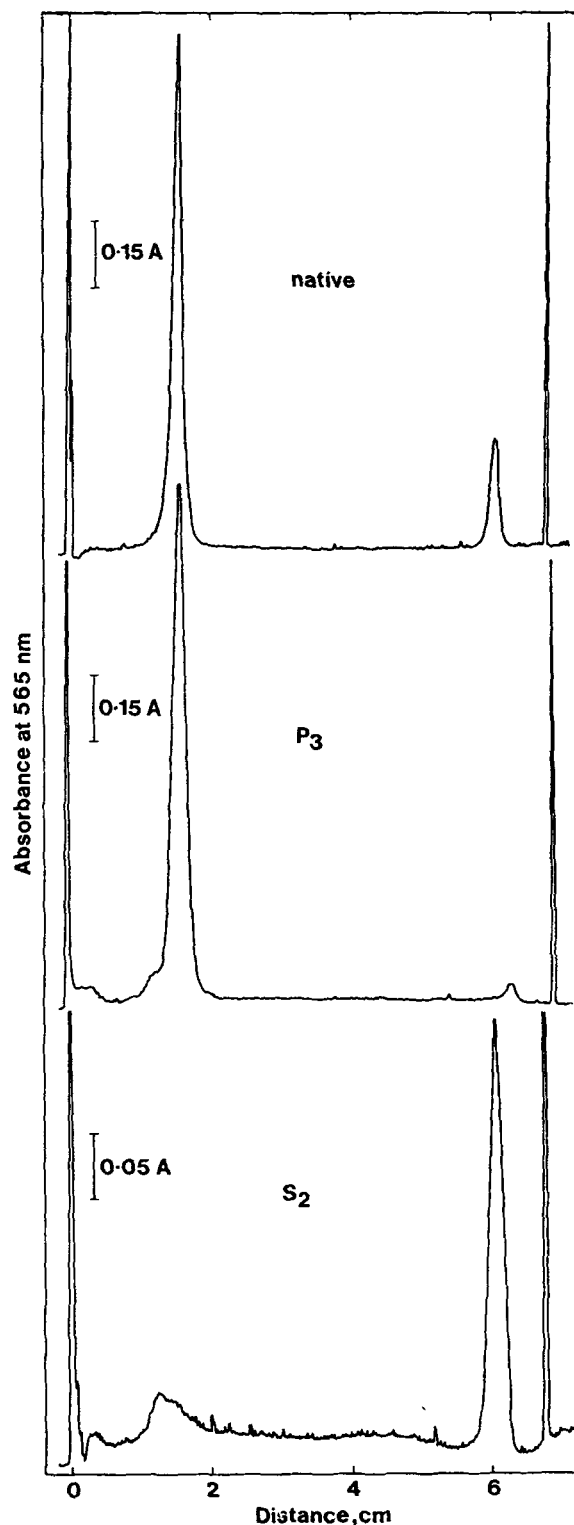


FIG. 4. Absorbance scans of stained gels after SDS electrophoresis of native *Synechococcus Rbu-P₂ carboxylase-oxygenase* and two of the fractions produced by precipitation of the enzyme at pH 5.4. Top, native enzyme (8 µg); middle, thrice-precipitated and redissolved enzyme (P₃, 5 µg); bottom, supernatant from second precipitation (S₂, 3 µg). Further details are given in the legend to Table II. The sharp spikes at 0 cm and approximately 6.7 cm are caused by the top of the gel and the dye front marker, respectively.

and Ames (1961) (Fig. 5). The small subunit-depleted enzyme sedimented more slowly than the native enzyme. Furthermore, the 280-nm absorbance peak for the small subunit-depleted enzyme was much broader, suggesting that it did not represent a homogeneous population of molecules. This heterogeneity was presumably the result of partial saturation of an oligomeric large subunit core with varying numbers of small subunits. However, the carboxylase activity profile of the small subunit-depleted enzyme on the sucrose gradient coincided exactly with the 280-nm absorbance profile (Fig. 5). This constancy of specific activity across the heterogeneous peak suggests that all species were approximately equally active regardless of their degree of saturation with small subunits. When referred to catalase as standard (data not shown) the $s_{20,w}^{0.725}$ values (Martin and Ames, 1961) for the native and small subunit-depleted enzymes were 17.6 and 15.8, respectively. This corresponds roughly to an average molecular weight difference from peak to peak of 70,000 (Martin and Ames, 1961).

Heterogeneity of the small subunit-depleted enzyme was confirmed by pore-gradient gel electrophoresis under non-denaturing conditions (Fig. 6, lanes 3 and 7). At least three clearly separated bands were obvious, spanning a region corresponding to a molecular weight difference of approximately 50,000. Traces of aggregated protein which barely entered the gel were also noticeable.

Reconstitution—When the small subunit-depleted enzyme was mixed at 0 °C with a supernatant fraction containing isolated small subunits in proportions designed to recreate the subunit ratio of the native enzyme, a substantial portion of the activity lost during the original separation was regained (Table III). Activity after reconstitution was double or more than that before. Reactivation could have occurred either during the incubation period at 0 °C or during the 15-minute preincubation at 25 °C which was part of the standard assay procedure. The picture is complicated by the observation that, unlike the native enzyme, small subunit-depleted enzyme lost activity slowly during storage at 4 °C (compare Tables II and

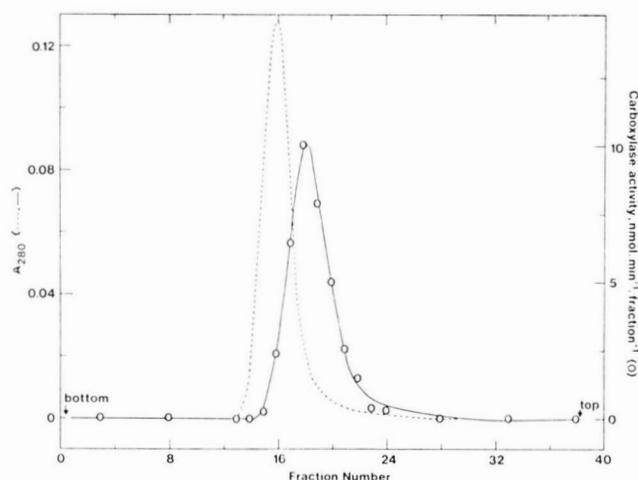


FIG. 5. Linear sucrose density gradient centrifugation (see "Materials and Methods") of native *Synechococcus* Rbu-P₂ carboxylase-oxygenase and after three times repeated acid precipitation and re-solution as described in the legend to Table II. The UV absorbance monitor traces were digitized and replotted (as dashed and solid lines for the native and acid-precipitated preparations, respectively) so that the areas under the peaks are equal. This was achieved by dividing the native enzyme trace by 1.8, which was approximately the ratio between the amounts of protein applied to the two gradients. Sample volume was 100 μ l and fraction volume was 110 μ l. Carboxylase activities of the fractions of the gradient for the acid-precipitated enzyme are shown also (○).

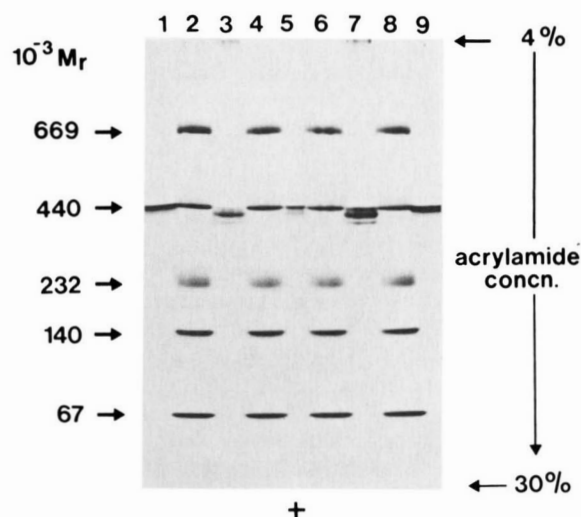


FIG. 6. Pore-gradient gel electrophoresis of *Synechococcus* Rbu-P₂ carboxylase-oxygenase. See "Materials and Methods" for details of the method and legends to Tables II and III for details of the samples. Lanes 2, 4, 6, and 8, standard proteins (from top: thyroglobulin, ferritin, catalase, lactate dehydrogenase, bovine serum albumin; molecular weights as indicated); lanes 1 and 9, native enzyme (8 and 16 μ g); lanes 3 and 7, thrice-precipitated and redissolved enzyme (P₃, 5 and 10 μ g); lane 5, 3 μ g P₃ plus 0.8 μ g neutralized S₂ mixed together and stored at 0 °C for 30 min before application. P₃ and S₂ fractions had been prepared 21 days previously.

TABLE III

Reactivation of *Synechococcus* Rbu-P₂ carboxylase on remixing of fractions enriched in large and small subunits

Fractions enriched in large subunits (P₃) and small subunits (S₂) were prepared by repeated precipitation at pH 5.4 (see Table II) and stored at 4 °C for the times indicated in column 2. S₂ was neutralized (pH 7.2) by addition of a 1/30 volume of 1 N NaOH. P₃ and S₂ were mixed in the proportions shown, which were designed to recreate the subunit ratio of the native enzyme. After storage at 0 °C for the times indicated in column 3, the mixture was assayed for carboxylase activity in air-saturated solution using the standard assay procedure, including the usual 15-min preincubation with Mg²⁺ and HCO₃⁻ at 25 °C (see "Materials and Methods"). Large subunit content of the various fractions was determined from the subunit ratios given in Table II.

Sample	Time since preparation days	Time between mixing and assay min	Carboxylase activity nmol·min ⁻¹	Specific activity in terms of large subunit content μ mol·min ⁻¹ ·mg ⁻¹
Native enzyme				1.97
P ₃ (5 μ g)	22		2.28	0.47
S ₂ (1.1 μ g)	22		<0.02	
P ₃ (5 μ g) + S ₂ (1.1 μ g)	14	60	5.92	1.17
P ₃ (5 μ g) + S ₂ (1.1 μ g)	14	82	5.95	1.17
P ₃ (5 μ g) + S ₂ (1.1 μ g)	14	115	6.14	1.21
P ₃ (5 μ g) + S ₂ (1.1 μ g)	22	82	4.55	0.90
P ₃ (5 μ g) + S ₂ (1.1 μ g)	22	116	4.47	0.88

III; note that assays for Table II were performed under N₂ and those for Table III under air). Whereas, for the native enzyme, the difference was only that expected between O₂-free assays and those in air (see later), the small subunit-depleted enzyme lost about 30% of its activity during 22 days storage. This may explain the observed reduction in degree of reactivation achieved by reconstitution experiments as the small subunit-depleted enzyme aged (Table III).

Reconstitution of enzymatic activity was accompanied by substantial reformation of the native electrophoretic form of the enzyme, leaving only a trace of the predominant middle band and none of the smallest molecular weight band of the small subunit-depleted enzyme (Fig. 6, lane 5).

DISCUSSION

Although Rbu-P₂ carboxylase-oxygenase represents a much smaller proportion of total cellular protein in *Synechococcus* and other cyanobacteria than it does in leaves of higher plants, it nevertheless appears to be the predominant large protein in extracts. The efficacy of the zonal ultracentrifuge purification step stems from this. The DEAE-Sephacel step was also efficient because the enzyme was unusually tightly bound. Either step by itself was capable of achieving substantial purification, and in combination they provided a very reliable procedure which yielded 30–50% of the crude extract's carboxylase activity (data not shown). The product was homogeneous, had a high specific activity, and was stable in dilute solution at 4 °C.

Synechococcus Rbu-P₂ carboxylase-oxygenase is composed of both large ($M_r = 57,000$) and small ($M_r = 12,000$) subunits (Fig. 1) in a 1:1 molar ratio (Table II). The number of L-S pairs comprising the native enzyme molecule is less certain. Comparison of its mobility with those of standard proteins on pore-gradient electrophoresis gels showed that it was apparently slightly smaller than ferritin, i.e. $M_r \approx 430,000$. This seems more in keeping with a hexameric (Lawlis *et al.*, 1979; Taylor and Dow, 1980) than an octameric structure but, since shape plays such an important part in the pore exclusion process, further studies with other techniques for molecular weight estimation are required. These will be the subject of a separate communication.

Two aspects of the Mg²⁺- plus HCO₃⁻-induced activation of *Synechococcus* Rbu-P₂ carboxylase-oxygenase are noteworthy (Fig. 2). First, the rate of activation was rapid, being at least as fast as that of the higher plant enzyme under optimum conditions. Furthermore, this rapid activation occurred in the presence of Rbu-P₂, whereas the higher plant enzyme activated only very slowly when Rbu-P₂ was present (Andrews *et al.*, 1975; Lorimer *et al.*, 1976; Laing and Christeller, 1976; Gibson and Tabita, 1979). The *Synechococcus* enzyme's activation rate was much faster than that reported by Christeller and Laing (1978) for the *Rhodospirillum rubrum* enzyme. However, faster rates of activation of the *R. rubrum* enzyme have been reported more recently (Gibson and Tabita, 1979; Whitman *et al.*, 1979). Second, the extent of activation under given conditions was unaffected by the presence of Rbu-P₂. This again contrasts with the deleterious effect of Rbu-P₂ on activation of the higher plant enzyme and is also unlike the *R. rubrum* enzyme, where Rbu-P₂ increased the extent of activation (Christeller and Laing, 1978). The data indicate that the inactive form of the *Synechococcus* enzyme may be unable to bind Rbu-P₂.

Kinetic data obtained for both carboxylase and oxygenase activities of the *Synechococcus* enzyme (Table I) confirm and extend those of Badger (1980) for the *A. variabilis* enzyme. The $K_m(\text{CO}_2)$, about 240 μM , was an order of magnitude greater than that of the fully activated, higher plant enzyme (Jensen and Bahr, 1977) and resembled those of the Rbu-P₂ carboxylases from a variety of other prokaryotes.³ The affinity

of the *Synechococcus* enzyme for O₂, as indicated by its similar $K_m(\text{O}_2)$ -oxygenase and $K_i(\text{O}_2)$ -carboxylase, was also about 4-fold weaker than that of the higher plant enzyme (Badger and Andrews, 1974). The maximum velocities of both carboxylase and oxygenase reactions, as well as the ratio between them of approximately 9, agreed closely with those of Badger (1980). It seems that cyanobacterial Rbu-P₂ carboxylase-oxygenases, and perhaps those of prokaryotes in general, may have weaker affinities for their gaseous substrates, but higher carboxylase activities and greater carboxylase/oxygenase maximal activity ratios than the higher plant enzyme. While the $K_m(\text{Rbu-P}_2)$ values for both reactions of the *Synechococcus* enzyme were 3–6-fold greater than the similar parameters for the spinach enzyme, the two enzymes were similar in having a lower $K_m(\text{Rbu-P}_2)$ in the oxygenase reaction than in the carboxylase reaction (Badger *et al.*, 1980).

From studies with the two variants of *Rhodopseudomonas sphaeroides* Rbu-P₂ carboxylase-oxygenase, which either possess or lack small subunits, Gibson and Tabita (1979) suggested that presence of small subunits (i) causes an accelerated rate of Mg²⁺- plus HCO₃⁻-induced activation; (ii) inhibits this activation when Rbu-P₂ is present; and (iii) causes a high affinity for CO₂. Present data concerning activation and kinetics of the *Synechococcus* enzyme are in accord with (i) but opposed to (ii) and (iii).

Removal of small subunits from *Synechococcus* Rbu-P₂ carboxylase-oxygenase by repeated isoelectric precipitation at pH 5.4 is particularly interesting. However, unless cyanobacterial Rbu-P₂ carboxylase-oxygenases differ with respect to the ease with which small subunits are lost, it seems an unlikely explanation for the inability of some previous authors to detect small subunits in preparations which had been subjected to this procedure (Tabita *et al.*, 1976; Codd and Stewart, 1977). These authors precipitated the enzyme only once, which according to present data removes only about half of the small subunits (Table II). Furthermore, Tabita *et al.* (1974) did not observe small subunits even when the precipitation step was omitted. Although a single precipitation resulted in only partial separation of large and small subunits, repetition produced a preparation greatly depleted in small subunits (13% remaining after 3 precipitations, Table II). This preparation apparently consisted of an oligomeric core of large subunits, similar to that of the native enzyme, with varying degrees of saturation with small subunits (see "Results"). It retained 41% of the native enzyme's catalytic activity, but this activity was not preferentially associated with any particular molecular species, apparently being borne equally by all large subunit cores regardless of their degree of saturation with small subunits (Fig. 5). Although the small subunit-depleted preparation lost activity slowly with storage at 4 °C, re-addition of sufficient small subunits to restore the native L:S ratio of 1:1 resulted in a doubling, or more, in activity (Table III) accompanied by a substantial re-formation of the native configuration (Fig. 6).

Akazawa and co-workers dissociated the small subunits from the oligomeric large subunit core of Rbu-P₂ carboxylase-oxygenases from *Chromatium* (Akazawa *et al.*, 1972; Takabe and Akazawa, 1973; 1975) and spinach (Nishimura *et al.*, 1973; 1974; Kobayashi *et al.*, 1979) by treatment at alkaline pH and, in the case of spinach, with *p*-chloromercuribenzoate. The

³ It is difficult to compare $K_m(\text{CO}_2)$ values between laboratories because of the lack of standardization in the method of computing CO₂ concentrations from HCO₃⁻ concentrations. The main uncertainty surrounds the choice of the appropriate value for the pK' of the CO₂ \leftrightarrow HCO₃⁻ interconversion. Most workers appear to have used the value for distilled water solution (6.35 at 25 °C, Edsall and

Wyman, 1958), although this is often not stated. We have followed this convention. However, Edsall and Wyman's value for 0.1 M NaCl solution (6.12 at 25 °C) is probably more appropriate to the composition of assay solutions commonly used. If this value is adopted, the CO₂ concentrations, and therefore the $K_m(\text{CO}_2)$ values, are reduced by approximately 40%.

percentage of native activity retained by these preparations (10–15%) was lower than that of the small subunit-depleted *Synechococcus* enzyme (41%) and their specific activities were much lower. Furthermore, the alkaline procedures tended to disrupt the integrity of the core and to promote aggregation. No such disruption was apparent with the small subunit-depleted *Synechococcus* enzyme and it had only a slight tendency to aggregate (Figs. 5 and 6). Most importantly, and in marked contrast with the alkali-dissociated preparations, recombination of the large and small subunit preparations from *Synechococcus*, resulting in at least a doubling of catalytic activity, could be demonstrated even after several weeks of separate storage.

At first sight, the observation that the specific activities of the various species present in the small subunit-depleted preparation were independent of their L:S ratio (Fig. 5) seems difficult to reconcile with the reconstitution data. The difficulty may be overcome by postulating that any degree of undersaturation with small subunits, even the loss of only one per molecule, forces the large subunit core to adopt a different, less active conformation. The native, high activity conformation may be regained only when sufficient small subunits are rebound to recreate fully the native L:S ratio. We are thus led to hypothesize that the small subunit of Rbu-P₂ carboxylase-oxygenase, while not itself bearing the active site, nevertheless has a role in catalysis. Its binding to the oligomeric large subunit core in a 1:1 stoichiometry with large subunits must result in a more active conformation. Whether or not this binding also changes substrate affinities, or the Mg²⁺- plus CO₂-induced activation process, (Kobayashi *et al.*, 1979) are questions requiring further research. In view of the obvious disadvantages in requiring two polypeptides to express fully a single (albeit bi-functional) catalytic activity, might it not be correct to regard those Rbu-P₂ carboxylase-oxygenases (*e.g.* from *R. rubrum*, etc.) which are able to attain the necessary high activity conformation in the absence of small subunits (presumably by changes in the primary sequence of the large subunit) as the more advanced?

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SUPPLEMENTARY MATERIAL

KINETICS AND SUBUNIT INTERACTIONS OF RIBULOSE BISPHOSPHATE
CARBOXYLASE-OXYGENASE FROM THE CYANOBACTERIUM,
SYNECHOCOCCUS sp.

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MATERIALS AND METHODS

Materials. The unicellular marine cyanobacterium, *Synechococcus* sp. Nāgell (Chroococcales, Cyanophyta) (strain RRIMP N1) was kindly supplied in axenic culture by Dr. L. J. Borowitzka. Tetrasodium Rbu-P₂ and standard proteins for SDS gel electrophoresis were obtained from Sigma Chemical Co., St. Louis, Mo. NaH¹⁴CO₃ (60 Ci.mol⁻¹) came from New England Nuclear, Boston, Mass. Pore-gradient acrylamide gels and high molecular weight standard proteins were supplied by Pharmacia, Uppsala, Sweden.

Growth of *Synechococcus*. The cyanobacterium was grown to late log phase in 60 l batch cultures in a nutrient-enriched, 0.2 µm filter-sterilized, seawater medium based on the *14*C medium of Guillard and Rhyther (1962). Cultures were magnetically stirred and gassed with air, or 1% (v/v) CO₂ in air, passed through a 0.45 µm filter. Temperature was 30–33° and light was provided by a bank of cool white fluorescent lights (60–120 W.m⁻² at the surface of the culture vessels). Microscopic examination revealed negligible bacterial contamination. Cultures were harvested with once by continuous flow centrifuge (1500xg, 12 l.h⁻¹) and the cells washed once by centrifugation with 0.25–0.5 M Bicline[®]/NaOH buffer, pH 7.6, and stored at –20°.

Purification of Rbu-P₂ carboxylase-oxygenase. All procedures were carried out at 0–4°. Forty to 50 g of wet packed cells were thawed and suspended in 4 vol. of 0.25 M Bicline/NaOH buffer, pH 7.6, containing 5 mM EDTA, 10 mM dithiothreitol and 1 mM phenyl methyl sulfonyl fluoride. The suspension was passed once through a prechilled French pressure cell at 140 MPa, which broke most of the cells. The homogenate was centrifuged (31,000xg, 60 min) and the pellet discarded. The intensely blue supernatant was fractionated with saturated (NH₄)₂SO₄ solution (pH 7, 4°) to yield the fraction precipitating between 25 and 50% saturation. This fraction was dissolved in sufficient (40–60 ml) 50 mM K phosphate buffer, pH 7.6, containing 1 mM EDTA and 1 mM dithiothreitol (PED buffer) to ensure that the solution was less dense than 13% (w/w) sucrose in the same buffer and then centrifuged at 140,000xg for 60 min to remove a small pellet. The blue supernatant was applied via the core line to a 525 ml linear sucrose gradient (13–31% (w/w) in PED buffer) in a Beckman Ti14 zonal ultracentrifuge rotor followed by a 30 ml PED buffer overlay. After centrifugation at 48,000 rpm for 17 h, the gradient was unloaded from the core line by pumping 36% (w/w) sucrose solution in the rim line. Fifteen ml fractions were collected. Rbu-P₂ carboxylase activity sedimented with a 280 nm-absorbing peak which was well separated from the slower-sedimenting blue phycobiliproteins. Active fractions were pooled and applied directly to a 1.5 x 5.8 cm column of DEAE-Sephacel (Pharmacia) equilibrated with PED buffer. The column was washed with 2 column vol. of PED buffer and then eluted with a 400 ml linear gradient of 0.05–0.75 M K phosphate buffer, pH 7.6, containing 1 mM EDTA and 1 mM dithiothreitol. Ten ml fractions were collected. Rbu-P₂ carboxylase-oxygenase emerged as a symmetrical peak of both activity and 280 nm absorbance at a phosphate concentration of about 0.3 M. Active fractions were pooled and concentrated to approximately 1.5 mg protein. ml⁻¹ using an Amicon Model 52 ultrafiltration cell with a YM-30 membrane (Amicon Corporation, Lexington, Mass.) and then dialyzed against at least 5 vol. of 50 mM K phosphate buffer, pH 7.6, containing 1 mM EDTA. This enzyme solution was stored at 4° and required centrifugation (27,000xg, 30 min) from time to time to remove faint cloudiness, presumably the result of protein aggregation.

Carboxylase assay. For the standard assay the ¹⁴C fixation method of Lorimer et al. (1977) was used at 25°, except that the buffer was changed to 50 mM Bicline/NaOH, pH 8.3, and the H¹⁴CO₃⁻ (7.7 Bq.mmol⁻¹) concentration increased to 50 mM. The gaseous phase was either air or N₂. The enzyme was activated by preincubation in the complete assay solution, minus Rbu-P₂, for at least 15 min at 25° before starting the reaction by addition of Rbu-P₂. Because of the high K_{0.5} (Table 1), this standard assay procedure measured only about 70% of the true maximum velocity. Higher HCO₃⁻ concentrations were undesirable because of substrate inhibition (see "Results"). For kinetic analyses, the enzyme was preactivated by dialfiltration at 23° against 50 mM Bicline/NaOH buffer, pH 8.3, containing 20 mM MgCl₂ and 25 mM NaHCO₃ using an Amicon model 50C ultrafiltration cell with a YM-30 membrane. Assay solutions (minus H¹⁴CO₃⁻ and enzyme) were sealed in septum-capped vials and purged with N₂. H¹⁴CO₃⁻ was then added, followed by activated enzyme to start the reaction. Allowance was made for carryover of HCO₃⁻ with the enzyme solution.

Oxygenase assay. The polarographic method of Lorimer et al. (1977) was used with a Hansatech (King's Lynn, Norfolk, England) O₂ electrode modified for high sensitivity as described by Badger et al. (1980). Assay volumes were 0.5 or 1.0 ml, buffer was 50 mM Bicline/NaOH, pH 8.3, containing 20 mM MgCl₂, and the enzyme was preactivated by dialfiltration against Bicline/MgCl₂/NaHCO₃ buffer as described above.

Electrophoresis. Electrophoresis of the enzyme dissociated into its constituent subunits in the presence of SDS and 2-mercaptoethanol was carried out according to Weber et al. (1972) with cylindrical 10% (w/v) polyacrylamide gels. After electrophoresis, the position of the tracking dye (bromophenol blue) was marked by inserting a piece of fine wire. The protein bands were fixed with 10% (w/v) trichloroacetic acid for at least 2 h and then stained with Coomassie Blue R-250 and diffusion destained according to Weber et al. (1972). Stained gels were scanned at 565 nm with a Gilford spectrophotometer adapted for gel scanning.

Pore-gradient electrophoresis was conducted using a vertical slab gel apparatus and 4–30% (w/v) acrylamide gradient gels (Pharmacia). The electrophoresis buffer was 90 mM Tris/80 mM boric acid, pH 8.4, containing 2.5 mM EDTA. After electrophoresis at 125 V for at least 16 h at 9°, the gels were fixed, stained and destained as for the SDS gels.

Analytical sucrose density gradient centrifugation. The constant sedimentation rate procedure of Martin and Ames (1961) was modified to suit the Beckman SW60Ti rotor. Linear sucrose gradients (3.9 ml, 7–20% (w/w) sucrose in 50 mM K phosphate buffer, pH 7.6, containing 1 mM EDTA, were prepared in polyallomer tubes using a multichannel peristaltic pump. One hundred µl samples (containing 100–160 µg protein) in the above buffer were layered on top of separate gradients. After centrifugation at 60,000 rpm for 130 min at 20°, the gradients were fractionated by pumping from the bottom of the gradient via a narrow-bore glass tube. The effluent was monitored for absorbance at 280 nm with an LKB Uvicord S monitor and 110 µl fractions were collected. The very small volume of the fractionation system (<0.3 ml) ensured minimal peak broadening.

Protein determination. The dye-binding method of Bradford (1976) was used with bovine gamma globulin as standard. According to this procedure, a solution of the purified enzyme with an absorbance at 280 nm of unity had a protein concentration of 1.47 mg.ml⁻¹.

RESULTS

Purification. The purification procedure described reliably produced homogeneous enzyme preparations (Fig. 1) with maximum specific carboxylase activities of about 3 µmol. min⁻¹.mg⁻¹ at 25° (Table I) and a ratio of absorbances at 280 and 260 nm of 1.8. The preparations clearly were composed of both large and small subunits with molecular weights of 57,000 and 12,000, respectively (Fig. 1), in a molar ratio of 1:1 (Table II). No heterogeneity of either subunit band was observed, even at protein loadings as low as 8 µg (Fig. 4). Electrophoresis of the undissociated enzyme (Fig. 6, lanes 1 and 9) showed predominantly one band. A very faint additional band of marginally smaller molecular size, which was difficult to photograph, was sometimes resolved from the predominant band at low protein loadings. This faint band may represent a small fraction of enzyme which lacked some of its small subunits (see later). The initial stages of the purification procedure, up to the zonal centrifugation step, must be accomplished with dispatch. Otherwise, despite the inclusion of the proteinase inhibitor phenyl methyl sulfonyl fluoride in the extraction medium, preparations were obtained which had several additional bands on SDS electrophoresis gels, lying between the large and small subunit bands. These bands were presumably derived from the large subunit by proteinase action. However, the catalytic activities of such preparations were not reduced and they were still homogeneous as judged by the non-denaturing, pore-gradient, electrophoretic procedure (data not shown).

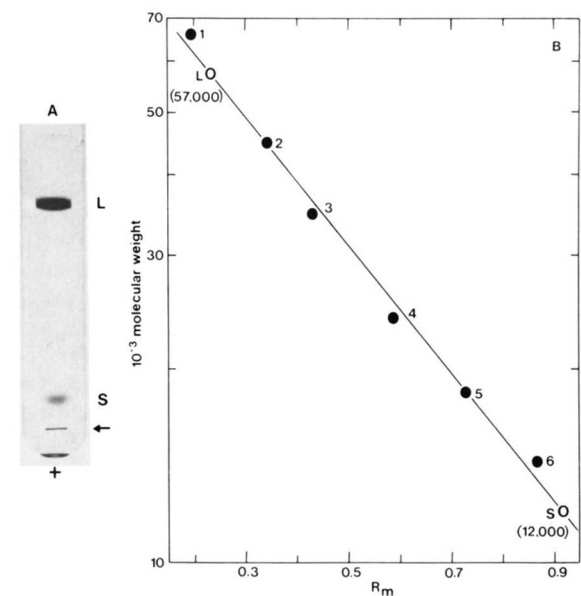


Figure 1 Polyacrylamide gel electrophoresis of purified *Synechococcus* Rbu-P₂ carboxylase-oxygenase dissociated in the presence of SDS and 2-mercaptoethanol (see "Materials and Methods"). A - Photograph of a gel which was loaded with 23 µg of enzyme. The arrow indicates the position of the tracking dye. B - Plot for determination of the molecular weights of the large (L) and small (S) subunits. The standard proteins used were: 1, bovine serum albumin (66,000); 2, ovalbumin (45,000); 3, pepsin (34,700); 4, trypsinogen (24,000); 5, α-lactoglobulin (18,400); 6, lysozyme (14,100).

Stability. When stored at 2–4° in 50 mM K phosphate buffer, pH 7.6, containing 1 mM EDTA, at a concentration of 1.5 mg protein. ml⁻¹, the purified enzyme lost little or no activity over a 5 month period. Furthermore, no changes in its electrophoretic or sedimentation properties were detected. The enzyme was less stable when 10 mM 2-mercaptoethanol was included in the storage buffer or when activated in 50 mM Bicine/NaOH buffer, pH 8.3, containing 20 mM MgCl₂ and 25 mM NaHCO₃. It was particularly unstable when stored at 19 mg.ml⁻¹ in 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid/NaOH buffer, pH 7.8, containing 1 mM EDTA and 5 mM dithiothreitol. Under these conditions 70% of the carboxylase activity was lost over a 3 week period at 4° and this was accompanied by massive aggregation to very high molecular weight material which barely entered the pore-gradient electrophoresis gels (data not shown).

Activation. Like all Rbu-P₂ carboxylase-oxygenases so far studied, that from *Synechococcus* was activated by Mg²⁺ and HCO₃⁻ (CO₂) and was inactive in their absence. The rate of activation depended on HCO₃⁻ concentration but was generally quite rapid, even in the presence of Rbu-P₂. The time required for full activation ranged from about 2 min at 0.8 mM HCO₃⁻ down to a few seconds at 41 mM HCO₃⁻ (Fig. 2). The final extent of activation reached at a particular HCO₃⁻ concentration was the same regardless of whether or not Rbu-P₂ was present during activation.

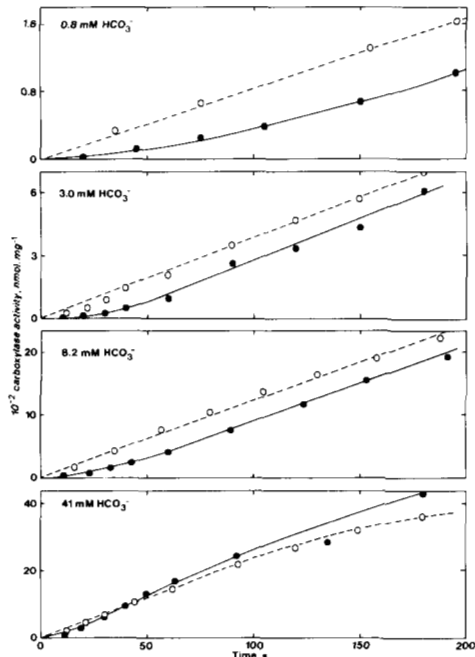


Figure 2 Activation of *Synechococcus* Rbu-P₂ carboxylase in the presence of 20 mM MgCl₂ and a range of HCO₃⁻ concentrations. O₂-free assay solutions of 5 ml total volume contained 50 mM Bicine/NaOH, pH 8.3, 20 mM MgCl₂, 0.5 mM Rbu-P₂, 75 to 600 μg enzyme and the stated concentrations of NaH¹⁴CO₃ (7.2 μg.mmol⁻¹). Since the enzyme was dissolved in a solution free of HCO₃⁻ and Mg²⁺ (50 mM K phosphate, pH 7.6, 1 mM EDTA), it was initially inactive. 0-----0, enzyme was preincubated in the assay solution (lacking Rbu-P₂) for 15 minutes before beginning the reaction by adding Rbu-P₂; O-----□, reaction was started by adding non-preincubated enzyme to the otherwise complete assay solution. At the indicated times, 0.5 ml samples were withdrawn and added to 1 ml of 20% (v/v) formic acid. After drying, acid-stable ¹⁴C was measured.

Kinetics. Apparent Michaelis constants and maximum velocities for both substrates in both carboxylase and oxygenase reactions were determined for two different purified enzyme preparations (Table I). Substrate inhibition by HCO₃⁻ at concentrations exceeding 51 mM was observed (data not shown) but otherwise all Lineweaver-Burk plots were linear. Relatively large standard errors in the kinetic parameters for oxygenase activity were observed when O₂ was the variable substrate. This occurred because the K_m(O₂) was equivalent to a pO₂ of nearly 1 atmosphere. Therefore data for O₂ concentrations above the K_m could not be obtained. As expected, O₂ was a competitive inhibitor of the carboxylase with respect to HCO₃⁻ (Fig. 3). The slope replot was linear (not shown), giving a K_i(O₂) of 1.3 mM, which was not very different from the K_m(O₂) of the oxygenase reaction.

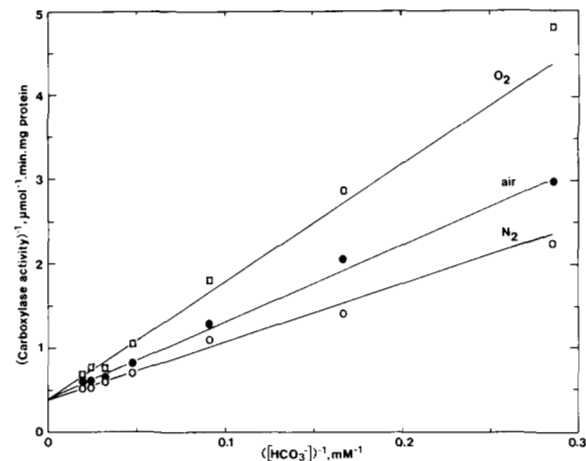


Figure 3 Lineweaver-Burk plots of carboxylase activity versus HCO₃⁻ concentration at varying O₂ concentrations. Assays were performed in septum-capped 1 ml vials as described in "Materials and Methods". The assay solutions were equilibrated with CO₂-free N₂ (○), air (●), or O₂ (□). The lines represent the best fit to the data points according to the statistical method of Wilkinson (1961).

TABLE I.
Summary of kinetic properties of *Synechococcus* Rbu-P₂ carboxylase-oxygenase.

Reaction	Variable substrate	Substrate concentrations, μM			K _m , μM	V, μmol.min ⁻¹ .mg ⁻¹
		Rbu-P ₂	HCO ₃ ⁻ (CO ₂)	O ₂		
Carboxylase	HCO ₃ ⁻ (CO ₂)	236	3500 (29) - 5100 (480)	0	26700 ± 4700 (250 ± 44)	3.23 ± 0.28
		311	3500 (144) - 5100 (645)	0	18300 ± 2300 (232 ± 29)	2.63 ± 0.11
	Rbu-P ₂	2-471	25100 (235)	0	41.8 ± 3.9	1.60 ± 0.04
		3-700	25100 (254)	0	47.7 ± 5.1	2.05 ± 0.06
Oxygenase	O ₂	221	1170 (11.5)	203-1000	1010 ± 162	0.370 ± 0.034
		366	935 (11.5)	180-1100	984 ± 369	0.304 ± 0.064
	Rbu-P ₂	3-220	1170 (11.5)	250	29.6 ± 2.7	0.0909 ± 0.0030
		4-360	935 (11.5)	250	32.3 ± 1.8	0.0600 ± 0.0010