When ribulose bisphosphate carboxylase-oxygenase from *Synechococcus* (strain RRIMP N1) was precipitated under mildly acidic conditions, most of its small subunits remained in solution. The precipitated enzyme readily redissolved at neutral pH and remained as an octamer of large subunits with some small subunits still attached. Optimum pH for this separation was 5.3 and disulfide-reducing reagents were not necessary. The fraction of small subunits removed by a single precipitation increased with increasing NaCl concentration. Catalytic activity of small subunit-depleted enzyme was linearly proportional to the fraction of small subunits remaining, while the carboxylase:oxygenase activity ratio and the affinity for CO\(_2\) remained constant. When isolated small subunits were added back to depleted enzyme preparations at neutral pH, reassociation occurred with return of catalytic activity. Under the usual assay conditions at pH 7.7, the binding constant of the small subunits was estimated to be about 10\(^{-7}\) M. The small subunits also bound avidly to surfaces. However, loss of small subunits from the enzyme during the course of purification was minimal. The results are consistent with a simple model in which only those large subunits which have a small subunit bound to them are catalytically competent. Thus, an essential, even if indirect, role for the small subunits in catalysis is indicated.

Rbb-P\(_2\) carboxylase-oxygenase (EC 4.1.1.39) is the bifunctional enzyme which catalyzes the initial CO\(_2\)-fixing reaction of photosynthesis as well as the initial O\(_2\)-fixing reaction of photorepiration. In many prokaryotes and all eukaryotes the N,N'-bis(2-hydroxyethyl)glycine; by the payment of page charges. This article must therefore he hereby contributed to be about 10\(^{-7}\) M. The small subunits also bound avidly to surfaces. However, loss of small subunits from the enzyme during the course of purification was minimal. The results are consistent with a simple model in which only those large subunits which have a small subunit bound to them are catalytically competent. Thus, an essential, even if indirect, role for the small subunits in catalysis is indicated.

Enzyme Purification—*Synechococcus* Rbb-P\(_2\) carboxylase was purified either as described previously (Andrews and Abel, 1981) or by the following modification of that procedure. The 25-50% saturated (NH\(_4\))\(_2\)SO\(_4\) pellet was prepared as before and redissolved in 250 mM K phosphate buffer, pH 7.6, containing 1 mM EDTA and 10 mM diethiothreitol, dialysed against the same buffer with 1 mM diethiothreitol using an Amicon XM-300 membrane, and applied directly to the DEAE-Sephacel column. The phycobiliproteins were not retained by the column under these conditions. *Synechococcus* enzyme were studied quantitatively using improved methods for measuring the L and S content of enzyme preparations. Results support the view that S is just as essential for catalysis as L.

**EXPERIMENTAL PROCEDURES**

Subunit Quantitation—A reliable method for measuring the L and S content of enzyme preparations was essential for these studies. Conventional SDS-gel electrophoresis followed by densitometry of Coomassie blue-stained bands frequently underestimated S, particularly when present in the gels at low levels, despite fixation of the bands with 10% (w/v) trichloroacetic acid before staining. Scanning of fixed but unstained gels at 280 nm improved reproducibility but diminished sensitivity. A rapid, sensitive, and quantitative procedure was developed using HP gel filtration. An LKB Ultragel TSK-G 3000SW (7.5 \times 600 mm) column was used, equilibrated with 20 mM Na phosphate buffer, pH 6.5, containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol and diluted 10-fold immediately prior to injection. Column flow rate was 1 ml-min\(^{-1}\) with an inlet pressure of 2.8 megapascals. The eluate was monitored at 210 or 280 nm. The peaks of L and S were well separated and their areas were linearly related to the amount of sample applied. The peak area ratio, S/L, of the purified enzyme was similar to the two wavelengths (0.25 and 0.28 at 210 and 280 nm, respectively) and close to the ratio between the subunit molecular weights. Therefore, calibration was effective assuming that the mass extinction coefficients of L and S are equal. While this may introduce a slight systematic error, the data will be internally consistent. The same procedure was used to estimate subunit molecular weights. The plot of log M\(_s\), versus retention time for several standard proteins was linear and yielded M\(_s\) estimates (60,300 for L and 14,600 for S) which were similar to those obtained by SDS-gel electrophoresis (Andrews and Abel, 1981).

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*The abbreviations used are: Rbb-P\(_2\), ribulose 1,5-bisphosphate; L, large subunit; S, small subunit; SDS, Na dodecyl sulfate; Bicine, N\(_2\)N' -bis(2-hydroxyethyl)glycine; HP, high performance.*
Other Methods—Protein content of purified enzyme preparations was measured spectrophotometrically using an ε_{280} of 12.6, determined by amino acid analysis (Andrews et al., 1981). Carboxylase and oxygenase activities were measured at 0.25 mM O₂ as previously described by Lorimer et al. (1977) and modified by Andrews and Abel (1981). The pH of the carboxylase assay buffer was altered to 7.7 and a gassing manifold-electrode vessel was used for the oxygenase assay. Previously described methods were used for SDS-gel electrophoresis and nondenaturing gel electrophoresis (Andrews and Abel, 1981).

RESULTS

Conditions Favoring Separation of L and S—As shown in previous studies (Andrews and Abel, 1981), a fraction of the small subunits of Synechococcus Rbu-P₅ carboxylase remained in solution when the enzyme was precipitated under mildly acidic conditions. A more detailed study of conditions favoring this separation of L and S was conducted with the aim of effecting as large a depletion of S as possible, consistent with good recoveries and minimal irreversible denaturation. The optimum pH for precipitation was 5.25 where a satisfactory compromise between these requirements was attained (Table I). Depletion of S was a little greater at pH 5.6 but precipitation was slow and incomplete, leading to poor recovery of L in the pellet. Furthermore, irreversible denaturation of the recovered protein was extensive, perhaps due to the long period at acidic pH required for precipitation to occur. Precipitation did not occur at pH 6 or above. Precipitation was more rapid at pH values below the optimum, being virtually instantaneous at pH 4.5. However, irreversible denaturation became progressively more serious as the pH was lowered and recovery of L declined because an increasing fraction of the precipitated protein did not redissolve when resuspended at pH 7.6.

At optimum pH, the fraction of S released during precipitation increased with increasing NaCl concentration (Fig. 1a). At high NaCl concentrations, two precipitations were sufficient to achieve a virtually inactive preparation, containing <8% of the native S content. Further precipitations were deleterious because of the cumulative effects of concomitant irreversible denaturation. Isolated S was retained in the supernatant after precipitation. Even under optimum conditions, it was usually accompanied by traces of undissociated enzyme, resulting in L protomer levels in supernatants which ranged from the barely detectable up to one-tenth (on a molar ratio basis) of the amount of S present. This contamination was least when the native enzyme was reduced with dithiothreitol, which was then removed by diafiltration, immediately prior to precipitation. The presence of dithiothreitol during the precipitation itself did not increase the fraction of S released (Table I). Isolated S was difficult to manipulate because it has a considerable affinity for surfaces, including those of glass and ultrafiltration membranes. Correlation of Catalytic Activities with S Content—Carboxylase activities of enzyme variably depleted in S (by precipitation over a range of NaCl concentrations) were found to be exactly proportional to S content (Fig. 1b). This indicates that S, as well as L, probably is required absolutely for activity.

**Table I**

| pH  | [Dithiothreitol] mM | [NaCl] mM | Recovery of L % | S:L | Activity/unit S µmol.min⁻¹.mg L⁻¹ |
|-----|-------------------|-----------|----------------|-----|----------------------------------|
| 6.00 | 0                 | 550       | 0.092 (11)     | 5.6 (42) |
| 5.50 | 0                 | 550       | 0.060 (23)     | 10.2 (76) |
| 5.04 | 0                 | 550       | 0.075 (30)     | 7.7 (59) |
| 4.49 | 0                 | 550       | 0.113 (45)     | 2.7 (20) |
| 5.22 | 0                 | 300       | 0.091 (36)     | 12.0 (90) |
| 5.22 | 10                | 300       | 0.099 (39)     | 11.3 (84) |

* No precipitation occurred.
* Precipitation occurred extremely slowly. In this case, centrifugations was carried out after 7 days at 4 °C.
* Precipitate only partially redissolved when resuspended in the pH 7.6 buffer.

**Fig. 1. Effect of NaCl concentration during precipitation on S content (a) and relationship between S content and carboxylase activity (b).** Precipitation was carried out at pH 5.2 and a range of NaCl concentrations as described for Table I and the redissolved enzyme was assayed for subunit content and carboxylase activity. In one experiment (Δ) precipitation was allowed to proceed for 55 min, the pellet was dissolved in 10 mM Na phosphate, 1 mM EDTA, pH 7.6, and the SDS-HF gel filtration to measure subunit content was monitored at 210 nm. In another (○), precipitation proceeded for 90 min, the pellet was dissolved in 30 mM Bicine-NaOH, 1 mM EDTA, pH 7.6, and 280 nm detection was used. The S:L peak area ratios and carboxylase activities (µmol.min⁻¹.mg L⁻¹) of the untreated enzyme were, respectively, 0.25 and 3.3 (Δ) and 0.27 and 2.7 (○) for the two experiments. Surface adsorption was minimized by assaying for carboxylase activity at high enzyme concentrations (180 mM L protomers).
Effect of S content on carboxylase:oxygenase activity ratio

S content was depleted to varying degrees by precipitating the native enzyme [by the appropriate NaCl concentrations as described in the legend of Table I, with other pertinent details, as well as identifying the resultant estimates of V and plotted as the ordinate. The together with other pertinent details, as well as identifying the resultant estimates of V and plotted as the ordinate.

Data set
| S content of Ls preparation | Ls proton concentration | V | Ks |
|-----------------------------|-------------------------|---|----|
| (a) Albumin absent           |                         |   |    |
| - - -                        | 15                      | 42 | 3.4 ± 0.3 | 65 ± 22 |
| O - O                       | 6                       | 178 | 3.2 ± 0.3 | 20 ± 19 |
| O - O                       | 100                     | 51 | 4.3 ± 0.4 | 81 ± 23 |
| O - O                       | 100                     | 201 | 3.9 ± 0.05 | 7.9 ± 2.1 |
| (b) Albumin present         |                         |   |    |
| - - -                        | 7                       | 10 | 4.2 ± 0.1 | 2.4 ± 0.58 |
| O - O                       | 7                       | 100 | 4.6 ± 0.1 | 0.83 ± 1.1 |
| O - O                       | 100                     | 18 | 4.3 ± 0.2 | 0.72 ± 0.75 |

Extrapolation of the regression line in Fig. 1b to the S content of unprecipitated enzyme results in an activity about 12% below that of unprecipitated enzyme. This is consistent with the data of Table I which show that a small amount of irreversible denaturation accompanies precipitation even at optimum pH.

Oxygenase and carboxylase activities were affected similarly by depletion of S so that the carboxylase:oxygenase activity ratio remained constant, within experimental error, for preparations with different S/L subunit ratios (Table II). The affinity of the enzyme for CO2 in the carboxylase reaction was not changed significantly by depletion of S. The Ks (CO2) of a preparation with about 50% of the native enzyme's S content was 96 μM compared to 115 μM for the untreated enzyme.

Reconstitution—Earlier studies (Andrews and Abel, 1981) showed that S-depleted enzyme exists as Ls with varying degrees of saturation with S. Depleted species were distinguishable from the native enzyme by their electrophoretic mobility and sedimentation coefficient. When remixed with isolated S, reconstitution occurred to a form electrophoretically indistinguishable from the native enzyme. This was accompanied by a return of catalytic activity. These observations have been extended by the present studies. The reconstitution process was quantitated by varying the concentrations of L and S, and their ratio, on remixing. When a fixed quantity of highly S-depleted enzyme was mixed with increasing quantities of isolated S, activity increased with increasing S concentration until saturation was reached (Fig. 2a). The reassociation process was too rapid to be detected under normal assay conditions. No preincubation of L-S mixtures prior to assay was necessary beyond the usual period required for activation by CO2 and Mg2+ (minimum 2 min). However, S-depleted enzyme deteriorated with storage. After 24 days at 4°C, activity after full saturation with S was reduced by 40% although the shape of the reconstitution curve was unaltered, indicating that the binding constant was similar (data not shown). Nondenaturing gradient-gel electrophoresis of aged preparations revealed multiple bands both higher and lower in molecular weight than the predominant native enzyme band (not shown). Therefore, both disruption and aggregation of the Ls core must be involved in the deterioration of unprecipitated enzyme.

(a and b), there are data sets for both S-depleted (●, ▲, ■, ▲) and native (○, △, □, ◆) enzyme preparations. Except for the native enzyme in experiment b, there are two data sets, at different final concentrations of L, for each preparation. Table III lists these concentrations together with other pertinent details, as well as identifying the symbols used for each data set.

![Table II](link)

![Table III](link)

![Figure 2](link)
tion process. The native enzyme was quite stable under similar storage conditions.

Surface Adsorption Phenomena—Surprisingly, the activity of native enzyme preparations was stimulated by addition of S, especially when the former was present at low concentration (Fig. 2a). Direct assays of the native enzyme also gave progressively lower activities with decreasing enzyme concentration, with highly variable results being observed at low concentration (Fig. 3). When bovine serum albumin (0.1 mg·mL⁻¹) was included both the variability and the decline in activity at low concentrations were eliminated (Fig. 3). These observations are probably attributable to surface adsorption phenomena. Presumably albumin competes successfully with S for the binding sites on the surface of the glass assay vessel. In view of the already noted affinity of S for surfaces, it is likely that S was sequestered by surface-binding sites, thus depleting the enzyme of S and reducing its activity. However, the possibility that the holoenzyme was also bound to surface sites and denatured cannot be excluded. When binding experiments similar to those of Fig. 2a were repeated with serum albumin included in the solution, the data were consistent with much tighter binding between L and S, and additional S caused little or no stimulation of the activity of native enzyme, even when the latter was present at very low concentration (Fig. 2b).

Retention of S during Purification—In view of the apparently lower activity of S during purification must be included. However, present evidence is against this possibility. Firstly, the purified enzyme appeared to have a molar ratio of S:L close to unity. This is indicated by the similarity between the molecular weight ratio of the subunits (0.24) and their peak area ratios measured by SDS-HP gel filtration, monitored at both 210 and 280 nm (0.25 and 0.28, respectively; see under “Experimental Procedures”). Secondly, the purified enzyme appeared to be fully saturated with S since, when binding to surfaces was suppressed, additional S did not stimulate its activity (Fig. 2b). Thirdly, the final gel filtration step did not cause any detectable loss of S. The preparation was pure enough before this step to estimate its S:L ratio by SDS-HP gel filtration and the ratio, 0.27 at 280 nm, was insignificantly different from that of the final preparation.

DISCUSSION

Some limited information about the nature of the forces involved in maintenance of the quaternary structure of Synechococcus RuBP carboxylase may be gleaned from the conditions which promote dissociation of S from the L octamer. As expected, disulfide bridges are clearly not involved. The need for high NaCl concentrations suggests that ionic interactions may be important. This is also borne out by the efficacy of mildly acidic conditions where the subunits are probably approaching their pI points, thus reducing ionic interactions.

The simplest hypothesis consistent with the linear correlation of catalytic activity with S:L ratio (Fig. 1b) is that only those large subunits which have a small subunit bound to them are catalytically competent. This conflicts with previous conclusions that catalytic activity was not directly related to S content (Andrews and Abel, 1981). These conclusions were based on two lines of evidence. The first was data showing a nonstoichiometric relation between activity and degree of saturation with S (Andrews and Abel, 1981). These data must now be disregarded because they were based on SDS-gel electrophoresis of S content, which are now known to be unreliable (see under “Experimental Procedures”). The second line of evidence was based on the observation that, after centrifugation of an S-depleted preparation through a sucrose gradient, the peaks of catalytic activity and protein were coincident (Andrews and Abel, 1981). In the light of this data, this constancy of specific activity across the peak is difficult to reconcile with the presumption that there was a gradient in S:L ratio across the peak with the leading edge being richest in S. However, this presumption may not be correct if S partially dissociated from the faster-sedimenting S-rich complexes and reassociated with the slower-sedimenting S-depleted complexes during the course of the experiment. This would tend to even out the S:L ratio across the peak. Dissociation of S during sedimentation would have been promoted by the low enzyme concentrations used in the experiment in question.

Binding of S to S-depleted enzyme may be represented as follows.

\[ L_0 S_{n-1} + S \rightleftharpoons L_0 S_n \quad (n = 1-8) \]

If all binding sites act independently with similar binding constants, this model simplifies to

\[ L + S \rightleftharpoons LS. \]

In this case, the binding constant at each site, \( K_D \), is given by

\[ K_D = \frac{l \cdot s / ls}{(l - l)(s - ls) / ls} \]

where \( l, s \), and \( ls \) are the concentrations of unbound L and S and the LS complex, respectively, expressed in terms of protomers, and \( l \) and \( s \) are the total concentrations of L and S protomers. This gives rise to a quadratic expression for \( ls \) where the applicable root is

\[ ls = \left[ s_1 + l_1 + K_D - \sqrt{(s_1 + l_1 + K_D)^2 - 4 \cdot l_1 \cdot s_1}\right]/2. \]

If the LS complex is the only active species, the specific activity of a preparation per unit \( L \), \( V/L \), is given by

\[ V = ls \cdot V/ls. \]
where \( V \) is the specific activity per unit L after saturation with S. Therefore,

\[
v = \frac{V(s + l + K_D - [(s + l + K_D)^2 - 4 \cdot l \cdot s])^{1/2}}{(2 \cdot l)}
\]  

(1)

In the experiments reported in Fig. 2 and Table III, \( l \) was held constant and \( v \) measured as a function of \( s \). Each data set was fitted to Equation 1 by means of a nonlinear regression (PAR program of the BMDP package developed by the Health Sciences Computing Facility, University of California, Los Angeles) and the parameters \( V \) and \( K_D \) estimated (Table III). A convenient normalization of the data sets for different preparations and different \( l \) values was obtained by plotting the observed \( v \) divided by the estimated \( V \) of each data set (Fig. 2). While the estimates of \( V \) were reasonable, with small standard deviations, the estimates of \( K_D \) varied widely, ranging from 2 to 130 nM, and the standard deviations were large (Table IIIa and other data not shown). This variation was probably caused by surface binding phenomena with the apparent \( K_D \) values reflecting the affinity of S for surface sites as well as its affinity for L. When surface binding was suppressed with albumin, the \( K_D \) values were much lower (about \( 10^{-9} \) M) and the standard deviations much smaller (Table IIIb). The standard deviations in the presence of albumin were still large relative to the estimated \( K_D \) but this is to be expected since the \( K_D \) values are much lower than the lowest protomer concentrations which could be used. Therefore, the \( K_D \) estimates should be considered significant at the order of magnitude level only. However, it is clear that the small model embodied in Equation 1 provides a reasonable description of L-S interactions when surface binding is suppressed. The observed \( K_D \) value, measured at pH 7.7, is at least four orders of magnitude lower than the enzyme protomer concentrations commonly prevailing during the purification procedures, which were conducted at a similar pH. Therefore, very little dissociation of S is to be expected under these conditions and retention of S during purification is understandable.

A clear picture thus emerges from this study. The small subunits of Rbu-P₃ carboxylase are essential for catalytic activity. The data are consistent with a very simple model where only the complex between L and S is catalytically active. Both carboxylase and oxygenase activities are expressed by LS pairs present in the LₛSₛ complex regardless of the value of \( n \). The CO₂ affinity is also independent of \( n \). All active site-directed probes so far tested, as well as the CO₂ molecule which becomes bound during activation, react with amino acid residues of L (Lorimer, 1981). Therefore, at least part of the active site must reside on L. Since it appears that only the LS complex is active, two possible roles for S in catalysis may be inferred. The first possibility is that the active site occurs at a point of interaction between L and S with residues of both participating in catalysis; the second is that binding of S, at a site remote from the potential active site, induces a conformational change in L which promotes activity. Until an active site probe which labels S is found, the latter possibility must be favored. Regardless of which possibility is true, it is clear that the structure of S and the manner of its binding to L could have a profound effect on the kinetic properties of the active complex. The close similarity between the amino acid sequences of L in cyanobacterial and higher plant Rbu-P₃ carboxylases, inferred from the nucleotide sequences, has been emphasized by the recent studies of Reichelt and Delaney (1982). It is possible that the large differences in kinetic properties between cyanobacterial and higher plant enzymes (e.g. the poorer affinity for CO₂ and higher turnover number of the former (Badger, 1980; Andrews and Abel, 1981)) could be due to differences between the small subunits rather than the relatively few sequence differences between the large subunits.

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