Small Subunit Contacts in Ribulose-1,5-bisphosphate Carboxylase†

Harry Roy,* Anthony Valeri, Daniel H. Pope, Linda Rueckert, and Kris Ann Costa

ABSTRACT: The arrangement of subunits of ribulosebisphosphate carboxylase in solution has been studied by exposing the enzyme to the cross-linking agents tetranitromethane, dimethyl suberimidate, and dimethyl adipimidate, and the cleavable cross-linking agent, methyl 4-mercaptobutyrimidate, followed by gel electrophoresis in the presence of dodecyl sulfate. All these agents caused the formation of dimers of the enzyme's small subunit, independently of protein concentration. In addition, trimers and tetramers of small subunit were detected in the mercaptobutyrimidate-treated enzyme. The data show that small subunits are closely paired in the native enzyme and may be in layers of four, or a ring of eight.

The chloroplast protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39), consists of two types of subunits: the large subunit (55 000 daltons) comprises about 78% of the mass of the enzyme, while the small subunit comprises the remainder. Estimates of the molecular weight of the enzyme and of the small subunit vary considerably depending on species. However, it is widely believed that eight of each of the subunits are aggregated in most forms (Kung, 1976; Baker et al., 1975, 1977a,b).

The arrangement of these subunits in the native enzyme is unknown, although it appears likely that the x-ray and electron microscopic studies of crystals of tobacco ribulosebisphosphate carboxylase will ultimately provide this information (Baker et al., 1977b).

We are interested in both the structure and the assembly of this enzyme in vivo. To further our studies we conducted a literature search with the aid of computers. This failed to reveal any published work on reactions of the carboxylase with cross-linking agents. We report here the results of exposing pea ribulosebisphosphate carboxylase to four different cross-linking agents. Our data demonstrate that the small subunits are arranged at least in pairs, and probably in higher orders, in the native enzyme.

Experimental Methods

Isolation of Ribulosebisphosphate Carboxylase. Pea leaves (100 g) were homogenized at top speed for 30 s in 300 mL of 0.007 M mercaptoethanol, 0.05 M Tris–0.02 M ascorbic acid (pH 8.0) at 0–4 °C in a Waring Blendor, and the homogenate was filtered through four layers of cheesecloth. The filtrate was centrifuged at 30 000*g* for 10 min and the clear yellow supernatant was rendered 50% saturated in ammonium sulfate by addition of the solid salt; the pH was adjusted to 7.5 after the beginning of this step by dropwise addition of 3 N HCl. The mixture was centrifuged at 10 000*g* for 10 min, and the pellet was resuspended in 15 mL of 0.05 M Tris-HCl (pH 7.6) containing 0.007 M mercaptoethanol, applied to a Bio-Gel A5M column (2.5 × 50 cm), and eluted overnight with ca. 1 L of the same buffer. The carboxylase peak (the second major peak in the A_{278} profile) was identified by ring tests with antisera prepared against wheat or pea ribulose-1,5-bisphosphate carboxylase and by the characteristic pattern of bands produced in dodecyl sulfate–polyacrylamide gel electrophoresis. This material was reprecipitated with ammonium sulfate, dialyzed vs. 0.01 M Tris-HCl (pH 7.5), and applied to 65–20% (w/v) sucrose gradients in the same buffer and centrifuged at 120 000*g* for 17 h at 4 °C. The sucrose gradients were fractionated and monitored at 278 nm simultaneously. Over 80% of the material absorbing light at 278 nm sedimented in a single broad band. The material in this peak was subjected to a second sucrose gradient centrifugation, or was applied to a DEAE-Sephadex A50 column (1.5 × 15 cm) equilibrated with 0.01 M Tris-HCl (pH 7.5), 0.1 M NaCl at 4 °C, and eluted with a 500-mL linear gradient from 0.1 M to 0.6 M NaCl. The enzyme emerged in the region between 0.25 and 0.5 M NaCl. Regardless of the method used, the final preparation was 99% pure ribulosebisphosphate carboxylase, judging from staining patterns of nondenaturing polyacrylamide gel electrophoretograms (Figure 1A). With $NaDodSO_4$[1] gel electrophoresis on slabs, we occasionally found several extremely minor low molecular weight contaminants, none of which was present in sufficient concentration to interfere with straightforward analysis of the data.

In the experiments shown here, no contaminants were seen. Sometimes a band of lower mobility, which was identified as a large subunit dimer, was observed. This appeared to be the result of storing leaves in the freezer before grinding. In the experiments shown, that band was not seen in control gels.

Analytical Gel Electrophoresis. 1. Nondenaturing gel electrophoresis was performed essentially by the method of Ornstein & Davis (Davis, 1964).

2. Electrophoretic analysis of dodecyl sulfate treated material was carried out as follows: Samples of protein were mixed with equal volumes of a solution containing 0.02 M Na_2CO_3, 5% (w/v) mercaptoethanol, 2.3% (w/v) sodium dodecyl sulfate (Chua & Bennoun, 1975) and heated 1 min at 90 °C. These samples were applied, as indicated in figure legends, to sample wells in a Studier-type (Studier, 1973) apparatus (Bob Dillingham, Aquebogue Machine Shop, Aquebogue, Long Island, N.Y.). In the experiment shown in Figure 1B, a gradient polyacrylamide gel with a Tris–borate buffer system was employed (Chua & Bennoun, 1975). Usually, however, the Tris–glycine stacking system of Ornstein and Davis as modified for dodecyl sulfate gel electrophoresis (O'Farrel, 1975) was employed. Gels were stained by the method of Weber & Os-

† From the Biology Department, Rensselaer Polytechnic Institute, Troy, New York 12181. *Received May 9, 1977.* Supported by National Institutes of Health Grant No. GM 23353 to H. Roy.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; TNM, tetranitromethane; MBI, methyl-4-mercaptobutyrimidate; RuBPCase, ribulose-1,5-bisphosphate carboxylase; RuBP, ribulose 1,5-bisphosphate.

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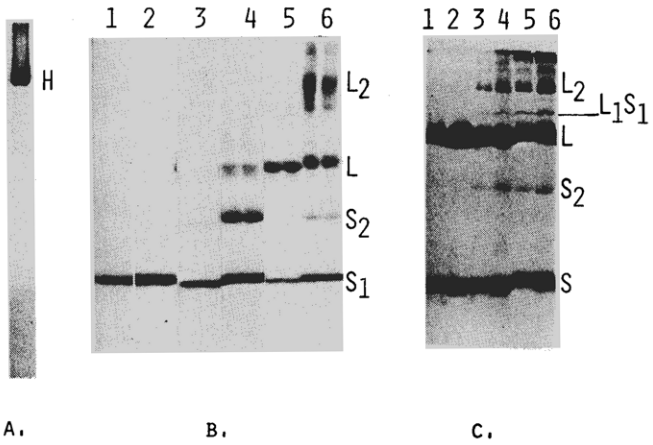

FIGURE 1: Analysis of RuBP carboxylase by electrophoresis. (A) Thirty micrograms of purified ribulosebisphosphate carboxylase was electrophoresed in the *absence* of sodium dodecyl sulfate (2.5% polyacrylamide stacking gel, 6% polyacrylamide running gel) and stained by the method of Weber & Osborn (1969). No contaminants were visible in the original gel. H = holoenzyme. (B) Two to 15 micrograms of proteins, previously incubated at 0.5 mg protein/mL with or without 6 mg dimethyladipimidate/mL at 0 °C for 1 h at pH 8.5 in 0.2 M triethanolamine were dialyzed vs. 0.05 M Na_2CO_3, 1% $NaDodSO_4$, pH 6.1 for 2 h, denatured in the $NaDodSO_4$ cocktail described under Experimental Methods, and electrophoresed in duplicate in a gradient polyacrylamide gel by the method of Chua & Bennoun. (Tracks 1) Cyctochrome *c*, no cross-linker; (tracks 2) cytochrome *c* treated with cross-linker; (tracks 3) hemoglobin, no cross-linker; (tracks 4) hemoglobin + cross-linker; (tracks 5) RuBP carboxylase, no cross-linker; (tracks 6) RuBP carboxylase, + cross-linker. L_i and S_i = large and small subunits in ith aggregation state. (C) Proteins were incubated with tetranitromethane or 3.7 mg/mL dimethyl suberimidate as described in Experimental Methods, at room temperature or 4 °C, respectively, for 1 h, denatured as described, and *equal volumes* subjected to discontinuous $NaDodSO_4$ slab gel electrophoresis with a "running" gel of 11% polyacrylamide. Thirty microliters of solution was applied to each sample well. Odd numbered wells: 1 mg/mL RuBPCase during cross-linking. Even numbered wells: 2 mg/mL enzyme during cross-linking. (1, 2) Untreated; (3, 4) tetranitromethane treated; (5, 6) dimethyl suberimidate treated. L_1S_1 and L_1S_2 are best seen in well number 6, because more protein is applied in that sample well than in well 5. No contaminants were seen in the untreated preparation.

born (1969) and destained in ethanol:acetic acid:H_2O (1:1:8).

Cross-linking Reactions. 1. Tetranitromethane was mixed 1:9 (v/v) with H_2O and agitated for 1 min. The aqueous phase was then diluted 50-fold into a solution of the enzyme in 0.02 M sodium phosphate, 0.05 M triethanolamine·HCl (pH 8.0). The reaction was allowed to proceed for 1 h at room temperature. Samples were denatured and electrophoresed as described.

2. Imidodiesters were prepared in stock solutions of 0.2 M triethanolamine·HCl (pH 8.5) and diluted with the enzyme in 0.05 M triethanolamine·HCl (pH 8.0) at 4 °C. After 1 h, samples were denatured and electrophoresed.

3. The reaction with 0.01 M methyl 4-mercaptobutyrimidate was conducted essentially as described by Traut et al. (1973) at 4 °C for 20 min in 0.05 M triethanolamine·HCl (pH 8.0) containing 3% (w/v) mercaptoethanol. The enzyme was then dialyzed against 0.05 M triethanolamine·HCl (pH 8.0) in the cold for 1–2 h, adjusted to 1 mg of protein/mL or less, and allowed to react 30 min at room temperature in the presence of 0.04 M H_2O_2. The cross-linked enzyme was then dialyzed to remove H_2O_2, rendered 0.5% (w/v) in sodium dodecyl sulfate, heated at 90 °C for 1 min, lyophilized, reconstituted with a minimal volume of H_2O, reheated, and electrophoresed as described above using the Tris–glycine slab gel system in the presence of 0.1% sodium dodecyl sulfate.

For cleaving the cross-linked species, the strip of polyacrylamide containing the first dimension electrophoresed cross-linked proteins was cut out and equilibrated with 10% (w/v) glycerol, 5% (w/v) β-mercaptoethanol, 2.3% (w/v) sodium dodecyl sulfate, 0.0625 M Tris-HCl (pH 6.8) for 1 h. (The mercaptoethanol is the cleaving agent.) The strip was placed in the slot of the slab gel apparatus on top of a stacking gel (4.75% acrylamide, 0.1% sodium dodecyl sulfate, 0.125 M Tris-HCl (pH 6.8)), which had been cast on top of a gradient polyacrylamide gel (8.1–12.5% acrylamide in 0.1% $NaDodSO_4$, 0.375 M Tris-HCl (pH 8.8)). The spacers used to cast this second gel were 0.3-mm thicker than the standard spacers, in order to allow the strip to fit in the slot easily. A few drops of 0.4% (w/v) bromophenol blue were added to the upper tank buffer (O'Farrel, 1975), and electrophoresis was conducted at 20 mA until the potential reached 150 to 170 V. Then the apparatus was turned off, set to run at constant voltage of 150 to 170, and the electrophoresis was completed when the tracker dye reached a point 1 cm above the bottom of the gradient gel.

Results

Ribulosebisphosphate carboxylase behaves as a single hydrodynamic species in aqueous buffer systems, including the discontinuous polyacrylamide gel electrophoresis system (Davis, 1964) (Figure 1A). Upon denaturation with sodium dodecyl sulfate, the protein disaggregates into large (55 000 daltons) and small (12 800 daltons) (e.g., Roy et al., 1976) subunits, with approximately a 3:1 mass ratio (Figure 1B, track 5; Figure 1C, tracks 1, 2).

Treatment of the enzyme with tetranitromethane, over a range of enzyme concentrations from 0.5 to 10 mg/mL, causes the formation of an additional set of major bands detectable in sodium dodecyl sulfate gel electrophoresis (Figure 1C, tracks 3, 4). The most easily identifiable of these is a dimer of small subunits (S_2) located between large and small subunit monomer bands. Several other bands appear trailing the large subunit, including a putative large-subunit–small subunit dimer (L_1S_1) and higher order aggregates which are tentatively assigned L_1S_2 and L_2. The patterns observed are completely independent of the protein concentration during cross-linking.

The major bands observed with tetranitromethane are also observed when the enzyme is treated with dimethyladipimidate (Figure 1B, track 6) or dimethyl suberimidate (Figure 1C, tracks 5, 6). In these cases, the relative amounts of cross-linking differ, with a higher proportion of the large subunits being cross-linked. All of the imidodiesters, however, produce a dimer of small subunit with electrophoretic mobility identical with that produced by tetranitromethane (Figure 1). We tested whether trace contaminants or intermolecular cross-linking might be responsible for appearance of some bands with the imidodiesters. In Figure 1C, more concentrated samples were run, for example, in track 6, which shows the putative L_1S_1 and L_1S_2 species most clearly. The *relative* concentration of these and all other species, however, was independent of the concentration of enzyme during cross-linking.

Controls showed that, under the conditions employed with the imidodiesters, a monomeric species, cytochrome *c*, does not exhibit cross-links (Figure 1B, tracks 1, 2), while the oligomeric species, hemoglobin, shows four distinct bands (Figure 1B, tracks 3, 4). Because hemoglobin has an anomalous free mobility in $NaDodSO_4$, it was *not* used to calibrate the gels, however. The assignment of molecular weights to various bands produced by treatment with cross-linkers was performed instead by two other methods.

Method I. It has been shown that, in discontinuous dodecyl

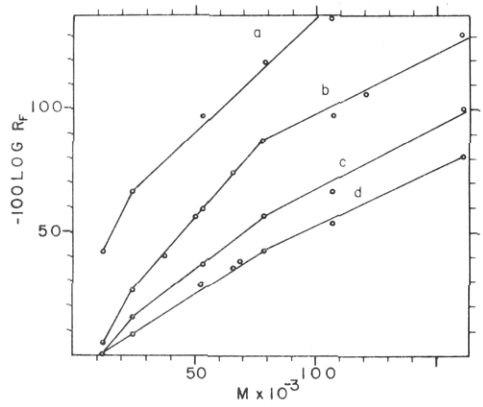

FIGURE 2: Assignment of molecular weights to oligomers of RuBPCase. RuBPCase, treated with cross-linkers as described in Experimental Methods, was subjected to disc electrophoresis with running gels of various concentrations of polyacrylamide. The $-\log R_f$'s of bands determined were plotted vs. assumed molecular weights. (a) Dimethyl suberimidate, 15% polyacrylamide; (b) cleavable cross-linker, methyl-4-mercaptobutyrimidate, 9.9% polyacrylamide; (c) dimethyl suberimidate, 9% polyacrylamide; (d) dimethyl suberimidate, 7.5% polyacrylamide. Assumptions about molecular weights were based on known values for large and small subunit, relative staining intensities and mobilities, and, in the case of the cleavable cross-linker, the recovery of monomeric small subunit upon second dimension electrophoresis from putative dimers, trimers, and tetramers of small subunit as seen in Figure 3.

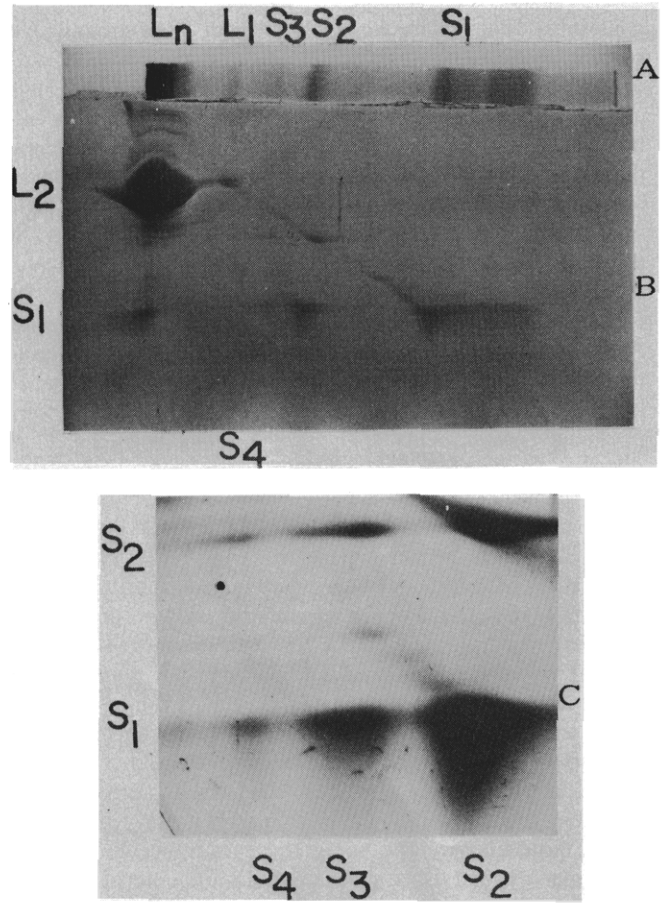

FIGURE 3: Two-dimensional electrophoresis of mercaptobutyrimidate-treated ribulosebisphosphate carboxylase. Enzyme, 5.7 mg, was dissolved in 0.5 mL of 3% mercaptoethanol, 0.05 M triethanolamine·HCl (pH 8.0) and dialyzed vs. 200 mL of the same buffer for 2 h at 4 °C. The retentate was mixed with 0.1-volume of 0.1 M mercaptobutyrimidate in 0.05 M triethanolamine·HCl (pH 8.0) and incubated 20 min on ice. This solution was then dialyzed at 4 °C vs. 0.05 M triethanolamine·HCl (pH 8.0) for 2 h to remove mercaptoethanol, adjusted to a protein concentration of 0.5 mg/mL, and 5 mL was mixed with 25 μL of 30% H_2O_2. After 30 min at room temperature, this solution was dialyzed 2 h at 4 °C, then made 0.5% (w/v) in sodium dodecyl sulfate, heated at 90 °C 1 min, and lyophilized. This material was dissolved in 0.2 mL of distilled water, re-heated, and applied to a discontinuous $NaDodSO_4$–polyacrylamide slab gel as described in Experimental Methods. Upper figure (A) shows the Coomassie blue staining pattern of a narrow strip cut out at the edge of the sample-containing region of the resulting slab gel. Middle figure (B) shows the pattern of spots produced when a 2-cm strip, cut out and equilibrated with mercaptoethanol and heated to cleave the cross-links as described in Experimental Methods, was electrophoresed in a second, thicker, gradient (7.5–12.5%) polyacrylamide slab using the same ionic conditions as for the first. Bottom figure (C) is contrast enhanced print of the central region of the gel shown above it, detailing more clearly the small subunit monomers and dimers derived from S_4, S_3, and S_2. First dimension electrophoresis left to right; second dimension top to bottom. Symbols as in Figure 1. Proteins released by cleavage are located below the diagonal joining the common origin with the small subunit monomer (S_1).

sulfate–polyacrylamide gel electrophoresis, standard proteins show a characteristic relationship when the negative logarithm of their relative mobilities is plotted against molecular weight (Neville, 1971). Since the large and small subunit show a three-to-one mass ratio, which is reflected in overall staining intensity, and since they differ markedly in molecular weight, it is possible to assign molecular weights systematically to the cross-linked bands. Thus, the small subunit and large subunit dimers are easily identified. The heterologous L_1S_1 and L_1S_2 species are less certain. After tentatively assigning molecular weights to all these bands on the basis of order and staining intensity, the $-\log R_f$ of each protein was plotted vs. calculated molecular weight as described by Neville (1971), who refers to this type of treatment as a Ferguson plot. The plots in Figure 2 show that, in the range between 20 000 and 80 000 daltons, the tentatively identified bands fall on a straight line in gels of various concentrations of polyacrylamide. Above 80 000 daltons the line changes slope as shown originally by Neville (1971). Below 20 000 daltons, the data are erratic for standard proteins as well as for small subunit. Thus, the partially intuitive assignment of molecular weights to these aggregates agrees with their positions on the Ferguson plot.

Method II. We employed methyl 4-mercaptobutyrimidate followed by H_2O_2-induced cross-linking. Figure 3 shows the first dimension analysis (after cross-linking) and the second dimension analysis (after reductive cleaving) of the MBI-cross-linked enzyme. In the first dimension (top), much of the protein entering the gel is of very low mobility suggesting a high degree of cross-linking; monomeric large subunits are seen, and small subunit dimer and monomer are seen. The monomer band is smeared, due to heavy loading of the gel, which reduces the usual effectiveness of the discontinuous buffer "stacking" system. Species present in lower concentration exhibit sharper bands. Note material in the region between the small subunit dimer and the large subunit monomer (labeled S_3). When the unstained first dimension gel strip was soaked in mercaptoethanol, a substantial proportion of the disulfide cross-links were reduced, so that in second dimension electrophoresis, monomers of small subunit were generated from spots originally containing monomers (S_1), dimers (S_2), trimers (S_3), and tetramers (S_4). (The tetramers are the only small subunit species observed in the second dimension which are not also visible in the first dimension. The presence of the S_4 spot was verified by densitometric tracing of the gel (not shown).) Large subunits and small subunit monomers and dimers were also recovered from the originally very high molecular weight material at the top of the first dimension gel. It is obvious from the patterns that the cleavage of cross-linked species by mercaptoethanol treatment was incomplete; for example, dimers of small subunit were recovered from the spots originally

containing dimers, trimers, or tetramers of small subunit. The pattern observed here is reproducible and was observed with carboxylase cross-linked at a protein concentration of 0.5 and at 0.17 mg/mL. In addition, it has been observed with carboxylase which was cross-linked, centrifuged on sucrose gradients, recovered from the 18S band, and then subjected to the same electrophoretic analysis. Of course, when mercaptoethanol is not used to cleave the cross-links, second dimension electrophoresis yields a diagonal pattern of spots, generates no new monomers, and therefore provides no additional information. Figure 2b shows a plot of the first dimension $-\log R_f$'s of the various complexes seen in Figure 3 vs. calculated molecular weight. The linearity of the plot over the range 20 kD to 80 kD supports the molecular weight (hence the compositional) assignments.

Discussion

The data presented here establish that small subunits in ribulosebisphosphate carboxylase are in molecular contact, or very nearly so, and may be arranged in groups of four.

Tetranitromethane treatment of the enzyme produces a band intermediate in mobility between the large and small subunit monomers. This band is produced in sufficient quantity that it cannot be derived from any extremely minor contaminants which may be present; furthermore, its appearance is accompanied by a decrease in the staining intensity of the small subunit monomer. The identity of the band as a dimer is supported by the position it occupies on a Ferguson plot of $-\log R_f$ vs. molecular weight, using accurate molecular weights determined by gel filtration in guanidinium chloride (Roy et al., 1976). Electrophoretically identical small subunit dimers are produced by the three other cross-linkers tested. Thus, the production of the dimer is not a unique chemical artifact. One of these cross-linkers, being cleavable, offered a further identification of the dimer, since, upon cleavage, small subunit monomer was demonstrated to be produced in the region of gel originally containing only the dimer. (The cleavage of dimer was not quantitative; although incomplete cleavage has been observed with other cleavable cross-linkers, it remains unexplained (Peretz & Elson, 1976). Perhaps it reflects incomplete penetration of mercaptoethanol into the first dimension gel.) The fact that tetranitromethane produces the small subunit dimer indicates that tyrosine residues on separate small subunits are within a few angstroms of each other (Martinson & McCarthy, 1975; Williams & Lowe, 1971). Hence, the small subunits are considered to be in close contact, at least in pairs. Higher order tetranitromethane-induced bands, specifically S_3 and S_4, were not resolved.

Three other bands produced by the cross-linkers can be identified rationally as L_1S_1, L_1S_2, and L_2. While this agrees with the Ferguson plots the resolution of our particular cleavable cross-linking gels in that molecular weight region is sufficient to confirm only one of these three identifications: L_1S_1 has been resolved in second dimension analyses (data not shown).

A more interesting result of the use of the cleavable cross-linker is the evidence for trimers and tetramers of small subunit. These are produced independently of changes in protein concentration during cross-linking and are not due to intermolecular cross-linking. Because they are present in trace amounts, very large quantities of enzyme must be applied to the gels. The large subunit monomer obscures the small subunit tetramer in a first dimension analysis. However, once cleaved, the small subunit trimers and tetramers each release dimers and monomers which can be easily recognized upon second dimension electrophoresis. Apparently any side reaction products are not cleaved; hence, they appear as components of a diagonal set of spots running from the origin toward the small subunit monomer. The appearance of the trimers and tetramers supports two ideas about the structure of RuBP carboxylase: (1) perhaps the small subunit pairs are themselves paired in a closely packed layer of four small subunits; or (2) more interestingly, the small subunit pairs might be arranged on four faces of a cube of eight large subunits in a belt-like fashion. The latter arrangement would predict the occurrence of pentamers, etc., which we have not detected but cannot rule out. The belt arrangement would account nicely for the eightfold symmetrical or circular faces of some carboxylase molecules in negatively stained electron micrographs (e.g., McFadden et al., 1975).

It should be pointed out that the dimeric character of small subunits at high pH has been questioned in the literature (Roy et al., 1976). We feel that, as found previously under mild conditions (Nishimura et al., 1973) or in the native enzyme, there is no question about the fact that small subunits are in at least a binary molecular complex with one another.

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