ON THE MECHANICAL INSTABILITY OF RIBULOSE – 1,5-BISPHOSPHATE CARBOXYLASE

by

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The intrinsic mechanical instability of ribulose-1,5-bisphosphate carboxylase was analysed using highly purified enzyme from *Nicotiana sylvestris* and *N. tabacum*.

At concentrations of the enzyme above $0.125 \text{ mg}\cdot\text{ml}^{-1}$, mechanical agitation of the native protein in solution leads to the formation of insoluble fibrillar precipitates of high molecular weight. At concentrations above $2 \text{ mg}\cdot\text{ml}^{-1}$ of protein, fibrillar precipitate formation is reduced due to the protective effect of a stable protein foam. The fibrillar precipitate and the resulting supernatant contain both the large and small subunits of the enzyme in the same ratio as in the native protein. Mechanical instability affects the yields of ribulose-1,5-bisphosphate carboxylase obtained by different preparation procedures from leaves.

1. INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase (RuBP'case, Fraction I protein, EC. 4.1.1.39) is the major soluble protein in green leaves which fix carbon via the C_3 pathway of photosynthesis. Since at certain stages of leaf development RuBP'case can account for over 50% of the total soluble proteins in the leaf (8), this single

protein has been considered to be the most abundant protein on our planet (3). Utilization of this huge protein resource requires that isolation procedures be scaled up immensely from those presently in use in the laboratory (1), while retaining the high yields of the protein characteristic for these techniques.

Purified RuBP'case in solution often exhibits

precipitates when examined with strong backlighting, and normally these are removed by a centrifugation step (2). These precipitates are frequently referred to as »undissolved protein«.

In this paper the nature of these precipitates is examined and their formation studied. The yield of RuBP'case during isolation of the protein on a large scale can be greatly affected by the amount of this precipitate formed.

2. MATERIALS AND METHODS

2.1. Purification of RuBP'case

RuBP'case from *Nicotiana sylvestris* was prepared by direct crystallisation (2) and was a kind gift from Dr. K. CHEN & professor S. G. WILDMAN of The University of California, Los Angeles. RuBP'case from *Nicotiana tabacum* was purified by the Sepharose 6B method of STRØBÆK and GIBBONS (9).

2.2. Separation of subunits of RuBP'case

All samples were dissociated in 0.05 M-Tris/HCl pH 8.5 buffer containing 0.5% sodium dodecyl sulphate and 0.14 M- β -mercaptoethanol for 1 hour at 37°C. Crystalline RuBP'case and mechanically precipitated RuBP'case were dissociated directly. RuBP'case in solution was precipitated at 100°C for 60 min prior to subunit dissociation.

Subunit separation was performed on a 5.0 $\times$ 90 cm column of Sephadex G100 at room temperature with a flow rate of 90 ml·hour⁻¹. Fractions were continuously monitored at 280 nm with a LKB-Uvicord 2. The fractions containing large and small subunit were pooled and their absorbance at 280 nm was measured in a Jasco Uvidec 2 spectrophotometer.

2.3. Mechanical agitation

Crystalline RuBP'case was dissolved at the desired concentration in a buffer containing 0.025 M-Tris/HCl pH 7.6, 0.2 M-NaCl and 0.0005 M-EDTA. For agitation at 4°C, 4 ml aliquots in

a 10 ml Erlenmeyer flask were stirred with a magnetic stirrer bar (1.5 $\times$ 0.5 cm) at 600 rpm for 16 hours. The precipitate was isolated by centrifugation at 48,200 $\times$ g (maximum) for 45 min at 2°C in an SS34 rotor of a Sorvall RC-2B centrifuge.

Mechanical agitation in the experiment with different concentrations of RuBP'case presented in Figure 6 was carried out in stoppered 1 $\times$ 7 cm plastic tubes on a React-R-Shaker, model 1350 (Udy Analyser Company, Boulder, Colorado, U.S.A.) for 60 min at room temperature. The sample volume was 1 ml. The precipitate was isolated by centrifugation at 48,200 $\times$ g (maximum) for 45 min at 2°C in an SS34 rotor of a Sorvall RC-2B centrifuge.

Absorption of samples was measured at 280 nm before and after mechanical agitation in 1 cm cells on a Shimadzu MPS-5000 recording spectrophotometer.

2.4. Microscopy

Light microscopic examination of the aggregated RuBP'case was carried out on a Reichert Univar microscope using incident light objectives and interference contrast optics. For electron microscopy, samples of native and mechanically agitated RuBP'case were placed on 400 mesh carbon-coated formvar treated grids for 5 min. The protein was removed by suction and the grids stained with 2% w/v phosphotungstic acid (pH 7.0) for 2 min. After removal of the phosphotungstic acid and air drying the grids were examined in a Siemens Elmiskop 102 electron microscope at 80 KV.

3. RESULTS

The primary observations leading to this investigation were made whilst purifying RuBP'case from a variety of plants, including *Hordeum vulgare*, *Nicotiana tabacum*, *N. sylvestris*, *Spinacia oleracea*, and a number of different *Oenothera* spp. (5). During RuBP'case ultrafiltration in stirred pressure

Figure 1. Photomicrograph of RuBP'case aggregates in a mechanically agitated solution of enzyme. The small aggregates and larger fibrillar structures were visualized using interference contrast optics. 1a 200 x, 1b 290 x.

Figure 2. Electron micrograph of a negatively stained preparation of RuBP'case from a mechanically agitated solution. 82,300 x.

Figure 3. As in Figure 2 but solution not agitated. 82,300 x.

Figure 4. Electron micrograph of a negatively stained preparation of RuBP'case from a mechanically agitated solution. 281,000 x.

Figure 5. As in Figure 4 but solution not agitated. 281,000 x.

Table I**Effect of stirring RuBP'case from *N.tabacum*.**

Sample	A 280	Protein mg·ml ⁻¹	% loss
Control	1.58	1.12	0
Stirred	0.82	0.57	51.3

cells or hollow fibre ultrafiltration units where the concentrated protein stream was permitted to agitate the protein in the reservoir, small particulate precipitates were often seen in the solution. The same precipitation phenomenon was observed when pooling fractions containing RuBP'case. The form of these precipitates was variable but they looked like cellulose fibres.

During a series of experiments on isolation of RuBP'case from *Nicotiana* it was observed that decreasing the mechanical disturbance during the isolation procedure enhanced the final yield of protein. To study this phenomenon more closely a sample containing 1.12 mg·ml⁻¹ of RuBP'case purified from *N.tabacum* was subjected to 16 hours of stirring at 4°C, as described under Methods (2.3.). The control was allowed to stand in the presence of a magnetic stirrer bar for the same period of time and at the same temperature. Both samples were centrifuged and the amount of RuBP'case in the supernatant determined. As seen in Table I about 50% of the RuBP'case had precipitated out in the stirred solution.

Prior to centrifugation a small sample was taken from the stirred RuBP'case and examined by both light and electron microscopy. The aggregated RuBP'case formed fibrils with a diameter of 2.9 μ and variable length, as well as smaller particles (Figure 1).

Fibrils of the size seen in Figure 1 were too big for electron microscopic analysis in negative stained preparations, but smaller fibrils were observed in the preparations and one of these is depicted in Figure 2. Figure 3 illustrates the absence of such fibrils in an unstirred solution of RuBP'case. The prominent fibril seen in Figure 2 is approximately 1/50 of the diameter of the smallest fibril seen in the light microscope. The fibrils appear as aggregates of individual 100 Å diameter RuBP'case molecules (Figure 4).

In order to examine the effect of concentration of RuBP'case on the mechanical stability of the enzyme in solution a series of dilutions of the protein was prepared. The stock solution contained 8.25 mg·ml⁻¹ RuBP'case purified from *N.sylvestris* by direct crystallisation. The diluted samples were shaken at room temperature for 1 hour as described under Methods (2.3.). The results are shown in Figure 6. At concentrations of above 0.25 mg·ml⁻¹ the loss of protein by mechanical disturbance was greater than 90%.

At concentrations above 2 mg·ml⁻¹ a stable foam plug formed in the sample tube and protected the RuBP'case in the solution from precipitation during shaking reducing the loss of protein to less than 20% (data not shown). The degree of disturbance in this system was noticeably greater than in the magnetic stirrer experiment.

In order to determine the composition of the fibrillar precipitate in mechanically disturbed RuBP'case solutions, a solution of *N.sylvestris* RuBP'case containing 1.65 mg·ml⁻¹ was subjected to magnetic stirring for 16 hours at 4°C as described under Methods (2.3.). After centrifugation both the supernatant and the precipitate (amounting to 57% of the starting material) were subjected to subunit analysis. As a control crystalline *N.sylvestris* protein was also dissociated and analysed. The resulting subunit distribution is shown in Table II.

It is concluded that the mechanically precipitated protein is identical in subunit composition to both the native protein and the protein remaining in the supernatant.

This strongly indicates that during mechanical disturbance aggregation occurs between native RuBP'case molecules and not as a result of a dissociation of subunits and subsequent separate aggregation of either large subunits or small subunits.

The solubility properties of native RuBP'case have been closely studied by KAWASHIMA and AYABE (6). Their work demonstrated that the protein could be rendered insoluble by either lowering the NaCl concentration or by addition of divalent metal cations, under conditions where the enzyme's substrate ribulose-1,5-bisphosphate (RuBP) was lacking. In all cases

Table II

Subunit composition of mechanically disturbed RuBP'case from *N.sylvestris*.

	Large subunit		Small subunit	
	A 280 total	%	A 280 total	%
Native protein	7.64	63.2	4.44	36.8
Mechanically agitated precipitate	1.82	64.2	1.01	35.8
Mechanically agitated supernatant	0.76	66.4	0.38	33.6

the metal ion - or low salt - induced precipitates could be resolubilised by incubation with 1 mM-RuBP at 40°C for 10 min. Similarly the precipitates induced by Ca^{2+} , Mg^{2+} , Mn^{2+} , Co^{2+} and Ni^{2+} ion treatment

Figure 6. The effect of increasing concentration of RuBP'case on the mechanical stability of the enzyme in solution. Samples of RuBP'case were subjected to mechanical agitation for 1 hour and the aggregated material removed by centrifugation (see Methods 2.3.).

could be resolubilised in 0.2 M-NaCl in 0.025 M-Tris/HCl buffer (pH 7.4).

In order to determine to what extent the mechanical precipitates could be prevented or resolubilised by RuBP or high salt concentration, aliquots of dissolved RuBP'case in 0.2 M-NaCl/Tris buffer (0.025 M, pH 7.8) were subjected to stirring in the presence or absence of 1 mM-RuBP for a period of 22 hours at 4°C (Methods 2.3.). The results are seen in Table III and reveal that RuBP offered only slight protection against mechanical disturbance of RuBP'case by stirring. The solubility of aggregated material after incubation with 1 mM-RuBP for 10 min at 40°C was similar for both the protein aggregated in the presence and in the absence of RuBP during stirring. The material was furthermore only slightly soluble in 1 M-NaCl.

These results indicate that the nature of the intra-molecular reactions causing fibril formation are not identical to those which occur during crystallisation or amorphous precipitate formation as described by KAWASHIMA and AYABE (6).

Table III

Effect of RuBP on the mechanical stability of RuBP'case from *N.sylvestris*.

	A 280	Loss on stirring		Solubility of precipitate	
		Protein mg·ml ⁻¹	% loss	% 1 M-NaCl	% 1 mM -RuBP
Control	1.20	0.84	0	—	—
+ RuBP 1 mM	0.16	0.11	87	25	77
- RuBP	0.04	0.03	97	22	72

4. DISCUSSION

The loss of RuBP'case by mechanical disturbance provides an explanation for previously observed unaccounted losses. It is well established both in this laboratory and others (10) that the direct crystallisation technique for tobacco RuBP'case yields in the order of 2-5 mg per gram fresh weight of leaves. When, however, one attempts to scale up this process, it is necessary to use larger columns with higher flow rates, continuous centrifugation, and other methods of ultrafiltration than the unstirred collodion bag utilised in the direct crystallisation technique. This gives greatly reduced yields of RuBP'case as estimated by extrapolation from the small-scale direct crystallisation technique.

During continuous centrifugation of solutions containing RuBP'case, the high shearing forces on entry to and exit from a rapidly rotating rotor are sufficient to aggregate the protein and thus remove it from solution. Similarly the high liquid velocities during column chromatography and ultrafiltration can also cause sufficient mechanical disturbance for aggregation of RuBP'case. Prudent design of flow patterns, collection vessels etc. can partly remedy this problem.

The mechanical agitation techniques described in this paper under Methods (2.3.) are naturally extreme as are the losses of RuBP'case observed. On the other hand during normal laboratory practice, losses of RuBP'case can become a major factor in the final yield of protein if care is not taken to avoid all mechanical disturbance during redissolving of ammonium sulphate pellets, redissolving of crystals etc.

Bundles of fibrils were previously observed inside the chloroplast especially under conditions of stress (4, 7). GUNNING et al. (4) examined these fibrils by negative staining using a homogenate prepared from *Avena* leaves. They found the fibrils to consist of linearly aggregated particles with a size and morphology similar to isolated RuBP'case and suggested that the fibrils consist of RuBP'case molecules. Further electron microscopical studies will aid in determining if the arrangement of the molecules in the fibrils produced by mechanical

agitation is the same as that in the fibrils formed inside the chloroplasts.

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