Crystalline Ribulose 1,5-Bisphosphate Carboxylase-Oxygenase from Spinach

Abstract. *Spinach fraction I protein (ribulose 1,5-bisphosphate carboxylase-oxygenase, E.C. 4.1.1.39) was crystallized on both an analytical and a preparative scale by vapor diffusion with polyethylene glycol (molecular weight, 6000) used as the precipitant. The identity of the crystalline material with fraction I protein was shown by gel electrophoresis in the presence of sodium dodecyl sulfate and immunological properties. The carboxylase and oxygenase activities copurify during crystallization, and the crystalline enzyme lacks copper and iron.*

Fraction I protein (ribulose 1,5-bisphosphate carboxylase-oxygenase, E.C. 4.1.1.39) is an oligomeric enzyme, the most abundant protein in higher plant chloroplasts, which plays a vital role in both photosynthesis and photorespiration (*1, 2*). In photosynthesis, the carboxylase activity of fraction I protein catalyzes the addition of CO_2 to ribulose 1,5-bisphosphate (RuBP) to yield 2 moles of 3-D-phosphoglycerate (PGA). This and subsequent reactions in the Calvin cycle result in net photosynthetic CO_2 fixation. The enzyme is also responsible for the addition of O_2 to RuBP to give PGA and 2-phosphoglycolate, the major substrate for photorespiration, which results in a net loss of fixed carbon and reduced crop yields. The carboxylase and oxygenase reactions are competitive and may both share the same catalytic site (*3*). Fraction I protein has a molecular weight of approximately 550,000 and consists of eight large (55,000 daltons) and eight small (13,000 daltons) subunits (*4*). The large subunits are coded by chloroplast DNA (*5*) and contain the catalytic site (*6*), whereas the small subunits are coded by nuclear DNA (*7*) and possibly have a regulatory function (*6*).

Although it is generally accepted that spinach RuBP carboxylase-oxygenase requires Mg^{2+} to activate both enzymatic activities (*2*), the presence of a metal, such as copper or iron, as a prosthetic group is uncertain. Measurements of chromatographically purified spinach enzyme have yielded values ranging from 0.14 (*8*) to 1.0 g-atom of copper per mole of enzyme (*9*). In contrast, crystalline RuBP carboxylase-oxygenase preparations from tobacco have a copper content of less than 0.2 g-atom per mole of protein (*10*). However, a recent report (*11*) suggests that the carboxylase and oxygenase are really two different proteins and that only the oxygenase contains copper.

Even though it has been isolated from a variety of plants, fraction I protein readily crystallizes after low-salt dialysis of protein concentrates only from species of *Nicotiana* (*12, 13*). By low-salt dialysis and other methods, three crystal forms of tobacco fraction I protein have been obtained and subjected to structural analysis (*4*). Although there are numerous similarities in the chemical and physical properties of fraction I proteins from various photosynthetic organisms (*14*), this protein obtained from many other plant species has proved refractory to crystallization (*13, 15*).

The application of a vapor diffusion microcrystallization technique in our laboratory has yielded enzymatically active, crystalline fraction I protein from the leaves of a variety of plants (*16*), including spinach (*Spinacia oleracea* L. var. Viroflay). At present, the major body of knowledge regarding the carboxylase-oxygenase properties of fraction I protein has been derived from studies of the chromatographically purified spinach enzyme. The availability of pure crystalline spinach fraction I protein should spur additional biochemical investigations probing its enzymatic function as well as provide crystals suitable for structural studies by x-ray diffraction.

We purified spinach fraction I protein from 2-month-old plants by homogenizing leaves in high-salt buffer [buffer A (*12*)] for 1 minute at 4°C with a Waring Blendor (we used 2 ml of buffer per gram fresh weight of leaf tissue). The homogenate was filtered through a layer of Miracloth and two layers of cheesecloth. The filtrate was centrifuged (105,000*g*, 60 minutes), and the supernatant passed through a Sephadex G-25 column (diameter, 15 cm; height, 35 cm) at room temperature which had been equilibrated with a low-salt buffer [buffer B (*12*)]. The

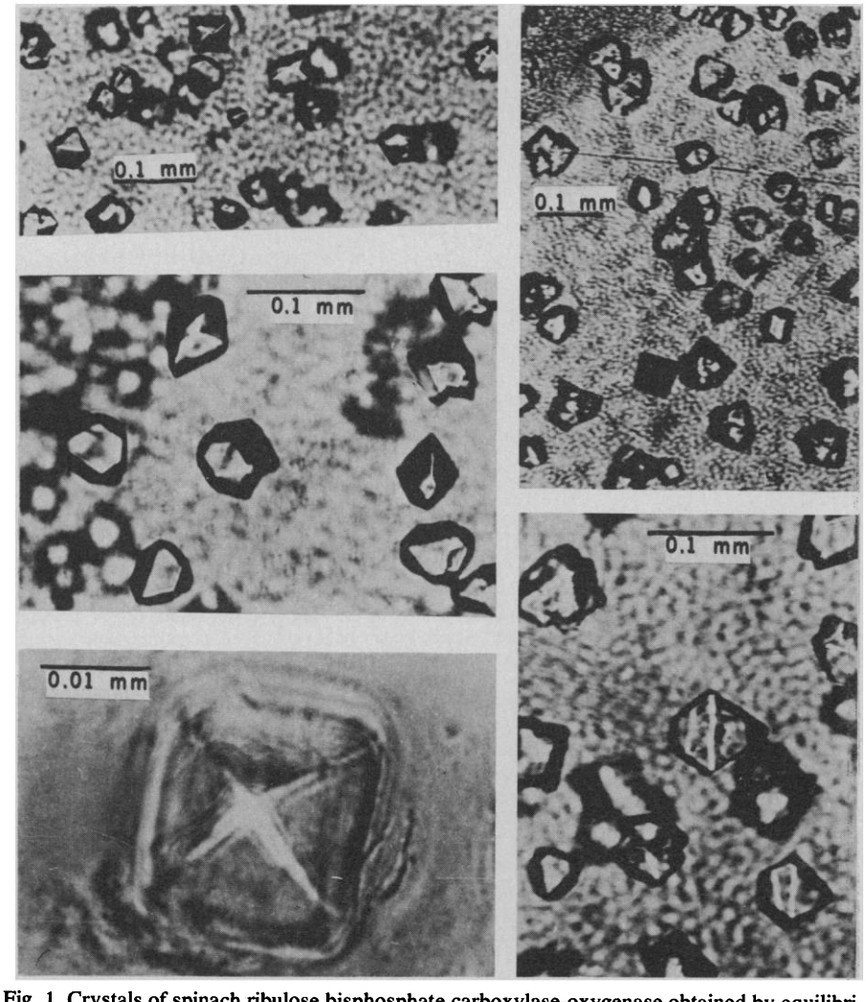

Fig. 1. Crystals of spinach ribulose bisphosphate carboxylase-oxygenase obtained by equilibrium vapor diffusion with 8 percent PEG 6000 used as the precipitant.

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protein was collected from the G-25 eluate as a precipitate obtained between 30 and 50 percent saturation with $(NH_4)_2SO_4$. The protein (100 to 500 mg) was dissolved in a minimum volume of buffer B (*12*) and then applied to a sucrose gradient (5 to 35 percent) containing buffer B. Centrifugation was carried out in a zonal rotor (Beckman Ti-14) for 16 hours at 4°C. The homogeneous 18*S* fraction I protein peak was collected and the protein precipitated with $(NH_4)_2SO_4$ (50 percent saturation). The precipitate was again dissolved in buffer B and dialyzed against 50 m*M* potassium phosphate buffer (*p*H 7.2) at 4°C for at least 24 hours.

Table 1. Enzymatic activities, copper content, and iron content of RuBP carboxylase-oxygenase from spinach.

Carboxylase activity* ($\frac{\mu mole\ CO_2}{mg\ protein\ min}$)	Oxygenase activity† ($\frac{\mu mole\ O_2}{mg\ protein\ min}$)	Carboxylase/ oxygenase	Copper‡ ($\frac{g\text{-}atom}{mole\ protein}$)	Iron§ ($\frac{g\text{-}atom}{mole\ protein}$)
	Before crystallization			
0.85	0.08	10.6	0.05	0.22
	Once crystallized			
0.94	0.08	11.8	0.05	0.15
	Twice crystallized			
0.86	0.08	10.8		

*The zonal centrifuge-purified enzyme and washed crystalline enzyme were dissolved in buffer B (*12*) at 0.75 to 1.5 mg/ml, and solutions were heat-activated for 20 minutes at 50°C. The enzyme (38 to 75 μg) was incubated for 4 minutes at 25°C in 500 μl of the assay mixture (25 m*M* Hepes, *p*H 8.0, 20 m*M* $MgCl_2$, 2.5 m*M* dithiothreitol, and 20 m*M* [^{14}C]$NaHCO_3$; specific activity, 0.5 Ci/mole). Then RuBP was added (0.51 m*M* final concentration), the reaction was terminated after 30 seconds with 100 μl of 2*N* HCl, and the reaction mixture was evaporated to dryness. An additional 500 μl of 2*N* HCl was added to the residue, and the resulting solution was again evaporated to dryness to completely remove free $^{14}CO_2$. Finally, the $^{14}CO_2$-fixation was measured as acid-stable radioactivity in the residue by scintillation counting. An absorbance of 1.0 at 280 nm equals 0.61 mg of protein per milliliter. †Crystalline enzyme was dissolved in buffer B (*12*) and dialyzed against activation medium (*19*) for 12 hours. Zonal centrifuge-purified enzyme was dialyzed at 4°C against activation medium for 12 hours. Enzyme solutions were heat-activated for 20 minutes at 50°C prior to assay. We determined the oxygenase activity polarographically (*19*) at 25°C after adding 0.5 mg of enzyme to 1.0 ml of CO_2-free assay medium (25 m*M* Bicine, *p*H 8.2, 5 m*M* $MgCl_2$, 20 m*M* RuBP). ‡The copper content of 10-mg samples was determined by atomic absorption spectrophotometry. All glassware was acid-washed. Measurements were made in the absence and presence of known amounts of added copper. The copper content of these and other samples of crystalline and noncrystalline protein ranged from 0.02 to 0.125 g-atom per mole of protein (assuming a molecular weight of 5.5×10^5 for fraction 1 protein). §The iron content was determined by the same method as for copper but without acid-washed glassware.

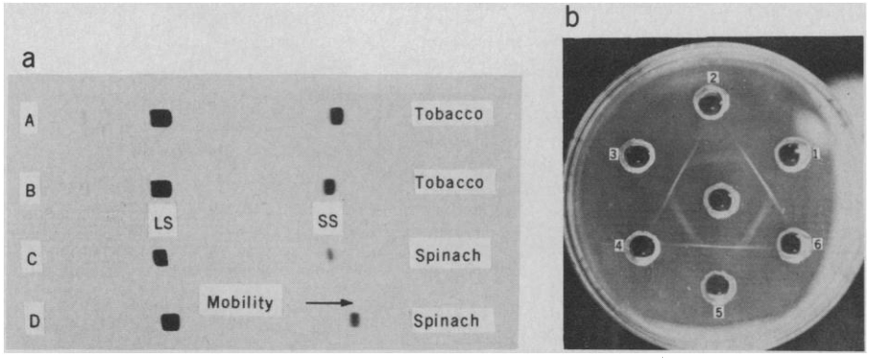

Fig. 2. Spinach fraction I protein crystals obtained by the large-scale method were transferred to test tubes and centrifuged (1085*g*, 5 minutes). The supernatant was decanted and the crystals were resuspended in 15 percent PEG 6000. After two successive washes, no protein was detected in the supernatant above the pellet of crystals. The washed (and structurally intact) crystals were dissolved in buffer B. The protein was then subjected to gel electrophoresis in the presence of SDS and Ouchterlony double diffusion analysis. (a) Polypeptide patterns of spinach and tobacco fraction I proteins resolved by SDS polyacrylamide gel electrophoresis. Tobacco fraction I protein was crystallized as described in (*12*). Electrophoresis was performed by the method of Weber and Osborn (*18*). Gels contained 7.5 percent acrylamide and 0.1 percent SDS; *LS*, large subunit; *SS*, small subunit. Polypeptide patterns: *A*, 20 μg of tobacco fraction I protein crystals, which had been equilibrated with 8 percent PEG 6000; *B*, 10 μg of tobacco fraction I protein crystals, no PEG 6000 added; *C*, 20 μg of spinach fraction I protein crystals, obtained in 8 percent PEG 6000, 50 m*M* phosphate; and *D*, 20 μg of spinach fraction I protein after purification by zonal centrifugation but prior to crystallization. (b) Ouchterlony double diffusion analysis of crystalline fraction I proteins from spinach and tobacco. The center well contains antibody to tobacco fraction I protein. Wells 1, 3, and 5 contain 20 μg of tobacco fraction I protein crystals; well 2 contains 20 μg of spinach fraction I protein after purification by zonal centrifugation; wells 4 and 6 contain 20 and 30 μg of spinach fraction I protein crystals, respectively. Spinach fraction I protein, before and after crystallization, gave a reaction of partial identity to tobacco fraction I protein.

Small amounts of spinach fraction I protein crystals were first obtained by equilibrium vapor diffusion (*17*), with polyethylene glycol (PEG) used as the precipitant. We mixed 50 μl of protein (10 mg/ml) in 50 m*M* phosphate buffer at *p*H 7.2 with an equal volume of 8 percent PEG (molecular weight, 6000) in a spot plate depression. These plates were supported in a sealed container above a reservoir of 8 percent PEG 6000. When the containers were left undisturbed at 4°C for approximately 2 weeks, crystals (Fig. 1) were observed in the solutions in each depression containing protein, whereas control solutions lacking protein were devoid of crystals or any other particles. The crystals completely disintegrated within several hours of the addition of a small amount of pronase; this result demonstrated that the crystals were indeed proteinaceous.

We then modified this microcrystallization technique, using a nonequilibrium vapor diffusion protocol to provide a practical, large-scale preparative procedure. The small sealed containers were replaced with desiccators, the depression spot plates with petri dishes, and the reservoir PEG solution with silica-gel desiccant. We mixed 3 ml each of protein solution (10 mg/ml) and 8 percent PEG 6000 in the petri dish, which was then supported in a sealed, evacuated desiccator containing a layer of silica gel on the bottom. The desiccator was left undisturbed at room temperature for 8 to 12 hours, at which time crystals were observed. These crystals were smaller and more irregularly shaped than those grown by equilibrium vapor diffusion; however, a high yield of crystals (≥ 80 percent) could be recovered from such preparations. Crystals obtained in this manner are morphologically stable for at least 2 months.

Depending on the method of preparation, the colorless, transparent crystals ranged in size from 5 to 50 μm (largest dimension). The predominant crystal habit observed in the spinach preparations appears to be a tetragonal bipyramid (Fig. 1), which has symmetry properties similar to those of the three crystal forms that have been obtained with tobacco fraction I protein in other laboratories (*4*). Indeed, some of the crystals shown in Fig. 1 are similar to the pseudorhombic dodecahedral form III crystals of tobacco which have been suitable for x-ray diffraction analysis (*4*).

The crystalline product is identical

with spinach fraction I protein. Gel electrophoresis [in the presence of sodium dodecyl sulfate (SDS)] of crystalline tobacco fraction I protein and spinach fraction I protein before and after crystallization reveal indistinguishable patterns (Fig. 2a) with only the two expected bands, corresponding to the large and small subunits. In addition, the protein crystals from spinach had identical immunological properties (Fig. 2b) to that of spinach fraction I protein before crystallization. Finally, the carboxylase and oxygenase activities of freshly prepared, dissolved protein crystals were comparable to those of noncrystalline spinach fraction I protein purified by zonal centrifugation (Table 1). The enzymatic activities were identical after the first and second crystallizations; thus the homogeneous enzyme was purified to constant specific activity.

Our measurements (Table 1) show that neither the carboxylase nor the oxygenase activities, which copurify after repeated crystallizations, are associated with a protein containing tightly bound copper. Furthermore, the oxygenase activity is inseparable from the carboxylase by crystallization. Although iron could also function as a prosthetic group to activate molecular oxygen in the oxygenase reaction, only trace amounts of iron are present in the crystalline protein (Table 1). These results are in agreement with earlier measurements of spinach (*9*) and crystalline tobacco (*10*) fraction I protein.

The crystallization of RuBP carboxylase-oxygenase from spinach and other plant species (*16*) provides the experimental material necessary to carry out comparative structural, enzymatic, and evolutionary analyses. Such studies are germane to elucidating the biological significance of the counterproductive enzymatic activities of this bifunctional protein.

SARJIT JOHAL
Department of Nutrition and Food Science, University of Arizona, Tucson 85721

DON P. BOURQUE
Department of Biochemistry, University of Arizona

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Both Photons and Fluoride Ions Excite *Limulus* Ventral Photoreceptors

Abstract. *Illuminating* Limulus *ventral photoreceptors with dim light results in a response composed of discrete waves. In the dark, adding 10 mM sodium fluoride to the artificial seawater that bathes the photoreceptor includes discrete waves. The fluoride-induced waves are similar to those evoked by light. These findings suggest that fluoride induces a process similar or identical to visual excitation of the photoreceptor.*

Hecht, Schlaer, and Pirenne (*1*) suggested, on the basis of psychophysical experiments, that rod photoreceptors are excited by the absorption of a single quantum of light. Subsequently, Yeandle (*2*) recorded discrete waves from *Limulus* lateral eye photoreceptors that behaved statistically as if they were the result of the absorption of single photons. Over the ensuing years discrete waves have been recorded from a variety of invertebrate photoreceptors (*3*). Recently, light-induced discrete fluctuations in membrane current, presumably the result of the absorption of single photons, have been observed in vertebrate rods (*4*).

Discrete waves are typically observed when a receptor is stimulated with dim light. Photoreceptors respond to brighter light with a change in membrane potential that is graded with stimulus intensity. Dodge *et al.* (*5*) have suggested that the response of *Limulus* photoreceptors, for all stimulus intensities, is the result of a summation of these discrete waves. Furthermore, they proposed that a change in the size of discrete waves is the primary mechanism of light and dark adaptation. Thus, the mechanism of discrete wave

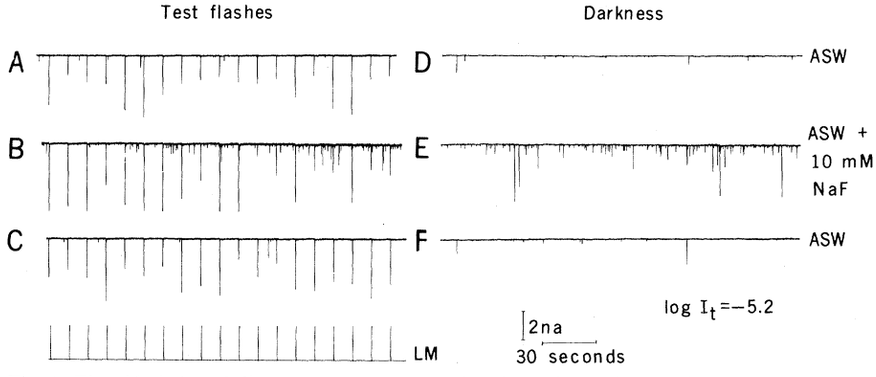

Fig. 1. Fluoride-induced discrete waves measured under voltage clamp. The cell was voltage-clamped to its resting dark potential throughout the experiment. Inward membrane current is shown as a downward deflection. I_t is the intensity of the 20-msec test flash, the occurrence of which is shown by the light monitor (*LM*).

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