Microtubular Spherulites: Development and Growth in Solutions of Bacteriochlorophyll Protein

Abstract. *Macromolecules of the water-soluble complex of bacteriochlorophyll protein can assemble in vitro to form fully developed spherulites composed of radiating microtubules. The changes that occur in the pattern of long-range order during the growth of these structures are typical of the development of the spherulitic crystal habit in numerous other materials of nonbiological origin.*

The properties of the conical paracrystalline aggregates of bacteriochlorophyll (Bchl) protein (*1*) suggest that these structures represent the spherulitic crystal habit of the protein. Although the spherulitic crystal habit is common to many compounds, from inorganic salts to high polymers, it has rarely been observed among proteins. Only one other protein, carboxy peptidase, has been reported to form typical, fully assembled spherulites in vitro (*2*). Typical spherulites were absent in our preparations (*1*), and the prevailing conical domains of radiating Bchl protein microtubules were often contiguous. These conditions suggest that Bchl protein spherulites growing at high nucleation density are arrested at a conical stage of assembly by exhaustion of protein in the mother liquor. Low nucleation density, then, should permit the development of more complete spherulites. Confirmation of implied features of spherulite development requires observation of undisturbed material under conditions appropriate for growth. I now report that Bchl protein forms fully developed radial spherulites and that the development of these spherulites from origin to maturity is almost identical to that of spherulites formed by nonproteins.

Bacteriochlorophyll protein spherulites (Fig. 1) are derived from sheaflike bundles of crystals from the ends of which fibrils (microtubules) extend and fan out to form the conical domains. These domains, by continuous extension and thickening through branching or intercalation, finally occupy a quasi-spherical space. With the exception of some terminal differences this is the same pattern of spherulite formation reported for high polymers (*3*), organic monomers (*4*), and inorganic salts in a convection-free environment (*5*). The pattern of forces which governs the long-range order of these widely different crystallites may have new implications for the interpretation of the organization of proteins in organelles of living systems.

Bacteriochlorophyll protein is a water-soluble complex with a molecular weight of 152,000; it contains 20 molecules of bacteriochlorophyll a (*6*). The regular hexagonal crystal habit of the protein belongs to the space group $P6_3$ with a hexamolecular unit cell which has the following dimensions along the crystal axes: $a = b = 195 \pm 1$, $c = 94.4 \pm 0.5$ Å (*7*). The crystal lattice is composed of hexagonally arranged macromolecules surrounding channels running parallel to the long axis (c axis) of the crystal (*7*, *8*). Crystals exhibit a weak birefringence (*9*), and the polarization color is orange. Maximum extinction is parallel to the c axis. The weakly polarized absorption spectrum shows that polarized light is preferentially absorbed parallel to the c axis at 809 nm [D (dichroic ratio) = 1.30] and perpendicular to the c axis at 603 nm ($D = 1/1.21$). This dichroism (*9*) indicates that the binding sites for Bchl are oriented.

The optical anisotropy of Bchl protein spherulites (*1*) is similar in order and sign to that of the regular crystals. Polarized absorption with respect to the axes of the microtubules is more positive at 809 nm ($D = 1.50$) and less negative at 603 nm ($D = 1/1.09$). The polarization color is blue, and maximum extinction is parallel to the microtubule axes. The microtubules which comprise Bchl protein spherulites are more or less mutually parallel and randomly spaced. Cross sections show a six-pointed star-shaped array of unresolved electron-dense elements; this array is larger in diameter than the hexagonal array in the crystal lattice (*1*). Longitudinal periodicity of macromolecules in Bchl protein microtubules can be resolved, and recent electron-microscopic observations on both crystal habits (*8*) suggest that the Bchl protein molecular shape is a noncompact sphere 81.5 Å in diameter.

Spherulites were grown for microscopic observation in a multiplicity of small volumes formed by the fusion of

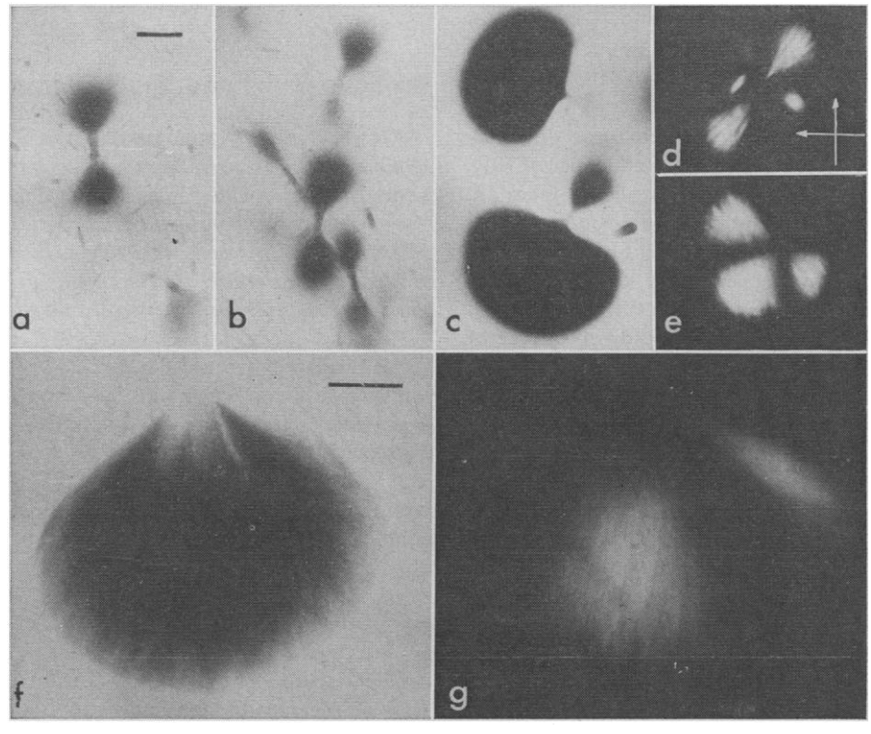

Fig. 1. Typical spherulites of Bchl protein in various stages of growth. (a) Early extension phase (scale indicates 50 μm); (b) same at higher nucleation density; (c) rounding up phase; (d and e) birefringence of early bilobate spherulite (d) and nearly mature spherulite (e) (arrows indicate the E vectors of light transmitted by the polarizer and analyzer); (f and g) mature, conical, single spherulite mounted for observation with a × 90 N.A. 1.4 apochromat (scale line indicates 10 μm); microtubular texture is indicated in this optical section by absorption at 603 nm in (f) and in (g) by birefringence in white light. (Note the small regular crystals in a, b, and c.)

two small droplets of equal size (5 to 25 μl), one consisting of Bchl protein (8 to 10 g/liter), 1*M* NaCl, and 0.01*M* phosphate buffer (*p*H 7.8) and the other of 13 percent $(NH_4)_2SO_4$ solution weight per volume (*10*). The droplets of each pair, juxtaposed in acrylic depression slides, were united by a long, narrow liquid bridge drawn from the droplet containing the Bchl protein. The preparations, when completed, were covered, sealed, and stored in the refrigerator (~5°C). Spherulites which appeared after a period of several hours to several days apparently formed at sites favorable to their nucleation in the numerous gradients occurring during and after droplet union.

Typical Bchl protein spherulites in various stages of growth are shown in Fig. 1. In the early stage (Fig. 1, a and b) dissimilarity in the length of the extending microtubules gives a fuzzy appearance to the outer edge of conical lobes formed by their growth; the sheaflike structure from which they originate appears to be twinned or symmetrically expanded in two directions. However, bilobate, twinned spherulites do not always occur; the single-lobed, conical form (half structure) is also present. The next stage in the growth of the Bchl protein spherulites involves the rounding up and enlargement of one of the conical lobes. During this process, bundles of microtubules and the originating sheaflike structure gradually disappear until only one lobe is left (Fig. 1c). The edges of these larger lobes are less fuzzy and indicate a more uniform length of the radiating microtubules. The final stage in the development of Bchl protein spherulites is reached when sharp resolution of the outer surface of the lobe (Fig. 1f) indicates that the shorter microtubules have grown in length to fill in the spaces between the longer ones. The microtubular texture can be nearly resolved by absorption and birefringence microscopy (Fig. 1, f and g). The weak birefringence and low density of tubule packing in Bchl protein spherulites are indicated by the polarization color, which remains blue (first order) even in the larger, mature forms.

The final stages of spherulite development in Bchl protein differ from that in most other materials. In most spherulites complete rounding involves the expansion of both fibrillar lobes until they meet and fill a spherical space. This often leaves two fibril radiation centers which, when the originating sheaf structure is incorporated, become cavities (*3*, *5*). The predominance, in bilobate spherulites, of one lobe developing at the expense of the other, as well as the competence to originate single-lobed structures, appears to be unique to proteins. These characteristics of Bchl protein may be linked with the different long-range order effects involved in an assemblage composed of large single molecules as opposed to those involved with atoms or small molecules. The occurrence of microfibrillar spherulites of fraction 1 protein in the chloroplast stroma of *Avena* and *Phaseolus* (*11*) indicates that under certain intracellular conditions this crystal habit can assemble even in the presence of other macromolecules.

Although fully developed protein spherulites are rarely seen in vivo, the considerable order in the arrangement of the quaternary structured elements of proteins which comprise the paracrystalline microtubules, fibrils, lamellae, and so forth, of many organelles suggests that a spherulitic ordering may exist in the living cell. Many of these structures have been separated, disassembled, and then reconstituted in vitro; a few have been reassembled in crystal habits different from the originals, including one which resembles spherulitic crystals (*12*). In view of my observations, Bchl protein appears well qualified as a material for the study of the molecular forces involved in the assemblage of macromolecules to form functional organelles.

RODNEY A. OLSON
Laboratory of Physical Biology, NIAMD, National Institutes of Health, Bethesda, Maryland 20014

References and Notes

1. R. A. Olson, W. H. Jennings, C. H. Hanna, *Arch. Biochem. Biophys.* **130**, 140 (1969).
2. J. E. Coleman, B. J. Allan, B. L. Vallee, *Science* **131**, 350 (1960).
3. A. Keller and J. R. S. Waring, *J. Polymer Sci.* **28**, 447 (1955).
4. B. Popoff, *Latv. Farm. Zurn.* **1934**, 1 (1934).
5. H. W. Morse and J. D. H. Donnay, *Amer. Mineral.* **21**, 391 (1936).
6. J. P. Thornber and J. M. Olson, *Biochemistry* **7**, 2242 (1968).
7. J. M. Olson, D. F. Koenig, M. C. Ledbetter, *Arch. Biochem. Biophys.* **129**, 42 (1969).
8. L. W. Labaw and R. A. Olson, *J. Ultrastruct. Res.*, in press.
9. R. A. Olson, W. H. Jennings, J. M. Olson, *Arch. Biochem. Biophys.* **129**, 30 (1969).
10. Bchl protein was kindly provided by J. M. Olson, Brookhaven National Laboratory, Long Island, New York.
11. B. E. S. Gunning, M. W. Steer, M. P. Cochrane, *J. Cell Sci.* **3**, 445 (1968).
12. B. Poglazof, *Structure and Functions of Contractile Proteins* (Academic Press, New York, 1966), pp. 164–230; D. Abram and H. Koffler, *J. Mol. Biol.* **9**, 168 (1964); R. E. Stephens, *Quart. Rev. Biophys.* **1**, 377 (1969).

26 February 1970

Bronchograms and Tracheograms of Seals under Pressure

Abstract. *Radiograms of the upper portion of the respiratory system were obtained at pressures up to 31.6 atmospheres absolute in the Weddell seal,* Leptonychotes weddelli, *and the northern elephant seal,* Mirounga angustirostris. *The trachea was considerably compressed but not fully collapsed at the highest pressures. No measurable change in the size of the bronchioles and smaller bronchi was observed. Measurements of total lung volume obtained simultaneously showed that the seals consistently dived with a small volume.*

All explanations of the effects of pressure on marine mammals have been hypothetical and based on anatomical characteristics and inference. The subject has been reviewed recently (*1*); with the exception of heart rate (*2*) no physiological parameters have been directly measured, although Ridgway *et al.* (*3*) have measured expiratory gas composition, after diving, in a porpoise trained to dive to 300 m. We now describe some observations of the effects of pressure on the upper respiratory passageways of two species of seal. The Weddell seal, *Leptonychotes weddelli,* and the northern elephant seal, *Mirounga angustirostris,* were selected for this study for two reasons: (i) *Leptonychotes* is a deep diver (*4*), and general information that we have gathered on *Mirounga* indicates that it can endure great pressures as well; (ii) tracheal cartilage rings in *Leptonychotes* are highly modified which suggests that they are an adaptation to pressure (*1*, *5*), whereas the trachea of *Mirounga* is not so unusual. Consequently, we had subjects that could withstand high pressures, which in turn would enhance our chances of observing any gross changes in the bronchi and trachea. Comparison of changes in these two species might aid us in interpretation of the significance of certain anatomical configurations of the trachea and bronchi.

We used a 7-month-old female Weddell seal (109 kg) collected in the Antarctic in December 1968 and a male elephant seal (78 kg) estimated to be about 15 months old. The animals were anesthetized with halothane, and