The Isolation of Rhabidosomes from the Blue-green Alga, *Spirulina*

By HELEN Y. Y. CHANG AND MARY M. ALLEN

*Department of Biological Sciences, Wellesley College,
Wellesley, Massachusetts 02181, U.S.A.*

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SUMMARY

Rhabidosomes, similar to those found in the flexibacterium *Saprospira*, have been isolated in the blue-green algal counterpart of *Saprospira*, *Spirulina*. They have been partially purified by renografin density gradients and shown to contain protein and a negligible amount of nucleic acid. Mitomycin C induction causes an increase in the number of rhabidosomes as well as an increase in the number of intact rhabidosome particles.

INTRODUCTION

Rod-shaped particles termed rhabidosomes were first observed and characterized by Lewin in autolysates of the flexibacterium *Saprospira grandis* (Correll & Lewin, 1964). These particles were demonstrated by negative staining under the electron microscope to be hollow cylinders 260 nm long, with or without a central wick-like cylinder (usually 300×15 nm) extending out of one end of the sheath. The wicks were constructed of six parallel elements while the sheath had a helical substructure with spiral subunits separated by 3.5 nm (Reichle & Lewin, 1968). Rhabidosomes, after isolation from actively growing cells lysed with chloroform in distilled water and purification by caesium chloride density-gradient centrifugation, were characterized by chemical analysis and u.v.-absorption spectroscopy (Reichle & Lewin, 1968). Intact particles had a sedimentation coefficient of 250 *S*, absorption peaks at 246 to 250 nm and 282 to 284 nm, and the usual amino acids. Although the presence of RNA was first reported in the rhabidosomes, later investigators proved that no or insignificant amounts of nucleic acid were present (Delk & Dekker, 1969; Price & Rottman, 1970).

How rhabidosomes originate in the cell is not clear, but there are indications that developing rhabidosomes are closely associated with the nuclear region (Lewin & Kiethe, 1965). Free rhabidosomes are abundant only in the cytoplasm of degenerated cells. The observation that strains which produce rhabidosomes are prone to lysis after the logarithmic phase of growth suggested that the particles are pathogens (Delk & Dekker, 1972). However, all attempts to demonstrate infectivity or toxicity in potential hosts have been unsuccessful.

Submicroscopic, rod-shaped particles, similar in appearance to rhabidosomes, have been found in a number of procaryotic microbes such as *Myxobacter* (Reichenbach, 1967), *Chondrococcus* (Pate, Johnson & Ordal, 1967), *Proteus* (van Iterson, Hoeniger & van Zanten, 1967), *Pseudomonas* (Yamamoto, 1967), and *Chromobacterium* (Rucinsky & Cota-Robles, 1973). Rhabidosomes are similar to a monocin described in *Listeria* (Bradley & Dewar, 1966), to the tail core of the contracted tail sheath of the bacteriophage T4 (Moody, 1967), and to the defective phage found in *Archangium violaceum* (Reichenbach, 1967).

The present investigation demonstrates, for the first time, rhabidosomes in a blue-green alga, *Spirulina*, an organism which is closely related to the colourless bacterium, *Saprospira*.

METHODS

Culture organism. The experimental procedures were carried out with a pure culture of *Spirulina* strain 6313 from the Berkeley culture collection, grown in liquid mineral no. 11 medium (Allen, 1968) in Erlenmeyer flasks on a rotary shaker or in stationary culture at room temperature, illuminated with cool white fluorescent lights. Cells were harvested two weeks after inoculation.

Isolation of rhabidosomes. Approximately 4 l of culture were harvested by low-speed centrifugation. The pellet was lysed by resuspension in 200 ml of chloroform. The mixture was stirred overnight at 4 °C, cleared of cell debris by low-speed centrifugation, and the pellet obtained was examined under the light microscope. If lysis was incomplete, as indicated by the presence of intact filaments and cells, the pellet was resuspended in distilled water and stirred vigorously at 4 °C, which resulted in immediate and complete cell lysis.

The supernatant of the lysate was centrifuged at 15000 rev./min for 15 min at 4 °C. The pellet, consisting mainly of smaller elements of cell debris, was discarded. The supernatant was centrifuged in a no. 30 head in a Spinco model L ultracentrifuge at 27000 rev./min for 0.5 h. A small greenish pellet (crude pellet) which contained rhabidosomes and other cell fragments such as membranes, was resuspended in 0.1 M-phosphate buffer (pH 7) for 15 min at 4 °C before being layered on a 30 to 60 % (v/v) renografin gradient pre-chilled at 4 °C for 15 min according to the method of Tamin & Gilvarg (1966). Gradients were centrifuged at 23000 rev./min in a SW 25 rotor of a Spinco model L ultracentrifuge at 10 °C for 12 h. Bands were removed, diluted with 0.1 M-phosphate buffer (pH 7) and concentrated by centrifugation at 40000 rev./min for 30 min. Washed pellets were resuspended in a drop of buffer and examined under the electron microscope by means of negative-staining techniques. In some cases, the rhabidosome-rich pellet was treated with 3 % Triton X-100 for 3 h at 4 °C before layering on a second 30 to 60 % (v/v) renografin gradient.

Electron microscopy. Samples were stained using phosphotungstic acid (PTA) according to Huxley & Zubay (1960). Samples in 0.1 M-phosphate buffer (pH 7) were dropped on 200 mesh Formvar-coated copper grids and allowed to settle. PTA containing 0.4 % (w/v) sucrose was then placed on the grids for 30 s. Grids were examined, after drying, with a Siemens Elmiskop I electron microscope.

Chemical analyses. 1. Protein content: Samples of the rhabidosome-rich fraction were analysed by the Lowry-modification of the Folin reaction with bovine serum albumin (Sigma Chemical Co., St Louis, Missouri, U.S.A.) as standard. 2. RNA content: Samples were extracted in phenol as described by Correll & Lewin (1964), and RNA was analysed by the orcinol reaction. 3. Action of enzymes and solvents: Samples were treated with the following reagents for 24 h at 25 °C unless stated otherwise. (i) Organic solvents: 50 % (v/v) chloroform; 50 % (v/v) acetone. (ii) Detergents: 2 % (w/v) sodium dodecylsulphate (SDS); 1 % (w/v) sodium deoxycholate (SDC); 3 % (v/v) Triton X-100, for 3 h at 4 °C. (iii) Protein denaturant: 8 M-urea. (iv) Enzymes: pronase (1:100, w/w, ratio of enzyme to protein) at 37 °C (pH 7.2); 0.001 M-phosphate buffer; trypsin and chymotrypsin (1:50, w/w, ratio enzyme to protein); 0.001 M-tris buffer at 37 °C (pH 8.1); RNase, 0.1 mg/ml for 2 h at 30 °C; DNase, 0.1 mg/ml for 2 h at 30 °C. (v) Others: EDTA, 0.1 M; NaOH, 0.1 N at 100 °C and 1 N at 25 °C; HCl, 1 N.

Mitomycin induction. Mitomycin C (Sigma) was added to logarithmic-phase cultures to a

Fig. 1. Renografin density gradients. (a) Gradient layered with crude pellet. Band II is the rhapidosome-rich fraction. (b) Gradient layered with band II after treatment with Triton X-100.

final concentration of $0.5 \mu\text{g/ml}$. Cultures were centrifuged by low-speed centrifugation after 6 h and 24 h of treatment. Both pellet and supernatant were examined under the electron microscope.

RESULTS

Isolation of rhapidosomes

Fig. 1 shows two renografin density gradients with their resulting bands. Fig. 1a describes a gradient layered with the crude pellet. Below the upper blue band of phycocyanin was a dark green band of small membrane fragments (band I). About two-thirds down the tube there was a pale green band with a tinge of orange (band II) which was seen by electron microscopy to be rich in rhapidosomes. Immediately below this was a green band dotted with dark green aggregates (band III). The lowest band (band IV) was a green band of heavier membrane fragments. No pellet was found at the bottom of the tube. Rhapidosomes were found in all bands, but the number was much greater in band II, and the contaminating membranes were proportionately much less than in the other fractions.

Fig. 1b describes a gradient layered with band II which had been treated with Triton X-100 to destroy membrane contamination. Two bands were observed: a green band near the top of the tube and a pale orange band at approximately the same position as band II of Fig. 1b.

Electron microscopy

Negatively stained preparations of the rhapidosome-rich band (band II) from renografin gradients are shown in Fig. 2. Most of the rhapidosomes appear as sheaths without wicks. The central core stains darker, presumably owing to the penetration of the stain. The hollow cylinders are about 23 nm in diam with a core of about 7.5 nm. Although most of the rhapidosomes are seen with an empty central canal, thin filaments with a diameter that equals that of the central canal are usually seen near the rhapidosomes (Fig. 2b, insert). These wick-like structures are themselves hollow cylinders with a narrow central core of about 2.5 nm diam.

The length of rhapidosomes varied considerably, ranging from short particles of 100 nm to long ones of 400 nm. The fact that short rhapidosomes were broken fragments of long

Fig. 2. Negatively stained preparations of the rhabdosome-rich band from renografin gradients (band II). Bar markers = 100 nm.

ones is illustrated in Fig. 3*a*. Although most rhabdosomes had regular ends, some appear to have appendages at one end (Fig. 3*b, d*) that may be structures for attachment. Rhabdosomes were usually found in close association with membrane fragments in band II; the main contaminants in the rhabdosome-rich fraction were the small membrane fragments of the photosynthetic membrane system. In some preparations the wicks are seen within the central core of the sheath, sometimes protruding from one end (Fig. 3*c*). In other preparations the wicks partially fill the core (Fig. 3*d*) or completely fill the core (Fig. 3*c*).

Fig. 3. Negatively stained rhapidosomes. (a) Fragmented rhapidosomes. Note end appendages in (b) and (d), and wicks in (c) and (d). Bar marker (a) = 100 nm; bar markers (b), (c), (d) = 50 nm.

RNA and protein content

The rhapidosome-rich fraction contained 220 μg protein/mg dry wt. RNA determination showed the fraction to contain 18 μg RNA/mg dry wt, giving an RNA to protein ratio of 0.08.

Action of various chemical reagents

When the rhapidosome-rich fraction was treated with RNase, DNase, chloroform, acetone, SDS, SDC, EDTA, Triton X-100 or urea, the integrity of the rhapidosomes was preserved. Prolonged treatment (24 h) with acid, alkali, or the proteolytic enzymes pronase, trypsin, and chymotrypsin resulted in less well-defined structure, but in all instances, intact rhapidosomes were observed.

Fig. 4. Negatively stained preparations of mitomycin-lysed cells showing peripherally situated rhabidosomes distinguishable from the parallel folds of the photosynthetic and mesosomal membranes.

Mitomycin induction

After 6 h treatment with mitomycin C most of the cells were partially lysed. Peripherally situated rhabidosomes were seen lying within the cells. They appeared randomly oriented with no apparent attachment to any cell organelle and were easily distinguishable from the parallel folds of the photosynthetic and mesosomal membranes (Fig. 4).

After 24 h treatment most of the cells were lysed, releasing the rhabidosomes to the supernatant which had a much larger number of rhabidosomes than had supernatants from

Fig. 5. Negatively stained rhapidosomes from mitomycin-treated cells. Note intact forms with the central core protruding from the sheath.

untreated cultures. A larger number of rhapidosomes present in supernatants from mitomycin C-treated cells were also in the intact form with the central core protruding from the sheath (Fig. 5).

DISCUSSION

Rhapidosomes, particles morphologically similar to those found in the bacterium *Saprospira grandis*, were found in and isolated from lysed cultures of *Spirulina*, the blue-green structural counterpart of *S. grandis*, hence strengthening the close relationship between these two classes of organisms.

The main difficulty in the purification of the rhapidosomes from *Spirulina* was contamination by membrane fragments. In *Saprospira*, which is free of photosynthetic membranes, the rhapidosomes can be successfully purified by a CsCl gradient (Reichle & Lewin, 1968). For *Spirulina*, a similar gradient preparation gave no clean separation of rhapidosomes. Similarly, Ficoll and sucrose gradients, of various concentrations, gave banding patterns more related to membrane fragment size than to concentration of rhapidosomes. Even in

the greatly enriched rhabidosome fraction from the renografin gradient (band II), small fragments of membrane were still present. Neither SDS nor EDTA treatment allowed further purification of *Spirulina* rhabidosomes. EDTA promoted membrane aggregation, trapping the rhabidosomes among the meshwork of membrane aggregates. Treatment of band II with Triton X-100, followed by layering on a renografin gradient, produced the best purification of rhabidosomes.

Negatively stained preparations show rhabidosomes from *Spirulina* to consist of two structures, both hollow cylinders. The core is about 350 nm in length and about 7.5 nm in diameter with an axial channel about 3.0 nm wide. The sheath varies in length and is about 24.0 nm in diameter with a channel about 8.0 nm wide. These dimensions agree with those reported by previous workers for *Saprospira* (Lewin, 1963; Reichle & Lewin, 1968). In agreement with Delk & Dekker's (1972) observation, few intact rhabidosomes were present when compared to free cores and sheaths. In *Saprospira*, twelve curved projections around the periphery of the sheath in cross-sectional views, and parallel markings at an angle of 15° to the axis of the sheath in lateral views were observed. These observations strongly support the suggestion that the sheath is made up of a helical array of subunits (Delk & Dekker, 1972). In our preparations, we were unable to find cross-sectional views although some samples were sonicated for 10 min to produce fragments that would rest vertically on the grid.

Rhabidosomes from *Spirulina* and *Saprospira* are similar in their remarkable stability. Delk & Dekker (1972) reported *Saprospira* rhabidosomes remained intact after prolonged treatment with a large number of reagents including the reagents used in the present study. Density values obtained in gradient studies are also similar. Delk & Dekker (1972) reported density values of 1.32 g/cm³ for cores, 1.295 g/cm³ for intact rhabidosomes, and 1.285 g/cm³ for sheaths. A value of 1.288 g/cm³ was determined in our study for a preparation composed mostly of hollow sheaths.

Information concerning the *in situ* location of the rhabidosomes would help in our understanding of the biological role of rhabidosomes. In partially lysed whole mounts of *Saprospira*, Correll & Lewin (1964) found large numbers of randomly oriented rhabidosomes. Similar observations were made on crude lysates of *Spirulina*. In thin sections, Lewin observed that developing rhabidosomes seemed to be mainly associated with the nuclear region (Lewin & Kiethe, 1965). Thin sections of *Spirulina* do not show the presence of rhabidosomes (Allen, 1972), probably because of the abundant photosynthetic and mesosomal membrane systems within the cells.

The abundance of rhabidosomes in mature or dying cultures and their general resemblance to the tail core and contracted sheath of several bacteriophages have led several investigators to comment on the possible viral role of rhabidosomes. Delk & Dekker (1972) compared the dimensions of a number of phage-related particles and suggested that the almost constant diameter for the core (7.5 to 9.0 nm) and sheath (20.0 to 28.0 nm) strongly linked rhabidosomes to this class of particles. Their amino acid composition and stability to denaturing conditions are also similar to those of phage-related structures (Delk & Dekker, 1972). Reichenbach (1967) regards rhabidosome-like particles (consisting of a core, a contractile sheath, and a basal plate with spikes and fibres) in *Archangium violaceum*, a myxobacterium, as degenerate bacteriophage because he could induce increases in the yield of the phage with mitomycin C. Only the tail of this defective phage is formed; phage heads were never observed. Similarly, Clark-Walker (1969) reported that in *Spirillum itersonii*, mitomycin C and u.v. light induced the formation of microcysts which contained phage tail parts, rhabidosomes, and a granular substance not seen in normal cells. Again,

complete virus particles were not formed. Clark-Walker (1969) suggested that rhapidosomes may be products of a defective phage genome, i.e. a form of polysheath arising by a self-assembly of excess sheath subunits.

The results of our induction experiments indicate that mitomycin C causes the prompt lysis of *Spirulina* and an increase in the number of rhapidosomes in the cells, suggesting that the rhapidosomes of *Spirulina* may be defective phages.

Since no proof of virulence of rhapidosomes has been demonstrated, there is the alternative hypothesis that they are integral components of normal cells. Their strong resemblance to the microtubules of ubiquitous distribution might suggest that they fulfil a general structural or functional role such as motility. Each procaryotic microbe in which structures similar to rhapidosomes have been demonstrated has some degree of motility, be it a gliding apochlorotic cyanophyte, a flexibacterium, or the contractile tail of a phage. Pate *et al.* (1967) have further suggested that the rhapidosomes in *Chondrococcus columnaris* may be derived from the disintegration of the mesosomal membrane. So far, *Spirulina* is the only blue-green alga found to have mesosomes (Allen, 1972). All of these hypotheses for the role of rhapidosomes are consistent with the data presented.

It can be concluded that *Spirulina* contains rhapidosomes which are similar in structural and chemical properties to those formed in *Saprospira*. Lewin (1962) considers *Saprospira* to be an alga assigned within the division Cyanophyta next to the genus *Spirulina*, while Stanier *et al.* (1971) consider blue-green algae to be bacteria. Both are helical, multicellular, spiral organisms; *Saprospira* is apochlorotic and heterotrophic, while *Spirulina* is photo-autotrophic, containing chlorophyll *a* and phycobiliprotein. The results of this study describe another shared structural component, further evidence that the organisms are closely related.

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