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Structure and transformation of chitin synthetase particles (chitosomes) during microfibril synthesis *in vitro*

(fungal wall/*Mucor rouxii*/chitin biosynthesis/ultrastructure)

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ABSTRACT **The fine structure of isolated chitin synthetase (UDP-2-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamidodeoxyglucosyltransferase; EC 2.4.1.16) particles (chitosomes) from *Mucor rouxii* and the elaboration of chitin microfibrils were studied by electron microscopy. Chitosomes are spheroidal, but often polymorphic, structures, mostly 40–70 nm in diameter. Their appearance after negative staining varies. Some reveal internal granular structure enclosed by a shell measuring 6–12 nm thick; others do not show internal structure but have a pronounced depression of the external surface. In thin sections, isolated chitosomes appear as microvesicular structures with a tripartite shell 6.5–7.0 nm thick. Morphologically similar structures can be seen in intact cells of *M. rouxii*. Isolated chitosomes undergo a seemingly irreversible series of transformations when substrate and activators are added. The internal structure changes, and a coiled microfibril (fibroid) appears inside the chitosome. The shell of the chitosome is opened or shed, and an extended microfibril arises from the fibroid particle. During prolonged incubation, the fibroid coils become less common and extended microfibrils appear thicker. We regard the chitosome as the cytoplasmic container and conveyor of chitin synthetase *en route* to its destination at the cell surface. Isolated chitosomes are well suited for integrated ultrastructural-biochemical studies of microfibril biogenesis *in vitro*.**

Chitin microfibrils can be assembled *in vitro* by chitin synthetase (UDP-2-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamidodeoxyglucosyltransferase; EC 2.4.1.16) preparations isolated from the yeast form of the fungus *Mucor rouxii* (1, 2). Electron microscopy of shadow cast specimens showed that the fibrils were formed from particles ("granules") smaller than 100 nm in diameter (2). These particles, which we now call *chitosomes*, contain a chitin synthetase complex capable of forming a microfibril by collective synthesis of the polysaccharide chains. The biochemical properties of chitosomal chitin synthetase will be reported elsewhere.¶ An understanding of the underlying molecular mechanism of microfibril biogenesis requires knowledge of the detailed fine structure of the enzyme complex and the way in which microfibrils are assembled. Here, we show the first evidence of the morphological complexity of chitosomes as well as the striking transformations they undergo during the course of microfibril elaboration.

MATERIALS AND METHODS

Isolation and Purification of Chitin Synthetase. *Mucor rouxii* strain IM-80 was grown in liquid medium (0.3% yeast extract, 1% peptone, 2% glucose, pH 4.5) at 28° for 13 hr in a N_2:CO_2 (30%:70%) atmosphere (2). Cells were harvested, mixed with 50 mM KH_2PO_4–NaOH buffer, pH 6.5, and 10 mM $MgCl_2$, and broken in a Braun model MSK cell homogenizer (2). All subsequent manipulations were done in this buffer. After cell walls were removed at 1000 × *g* for 5 min, the cell-free extracts were centrifuged at 54,000 × *g* (R_{av}) for 45 min. The supernatant was subjected to gel filtration in Bio-Gel A-5m, then treated with ribonuclease to remove ribosomes, and centrifuged as described (2). The resulting supernatant was concentrated to 3 ml with an Amicon model 202 cell equipped with an XM-300 filter, then applied to a 5–20% linear sucrose gradient (36 ml), and centrifuged in a Beckman SW-27 rotor at 81,500 × *g* (R_{av}) for 3 hr. The gradients were fractionated (2), and fractions with highest chitin synthetase activity were pooled. Chitin synthetase was assayed as described (2) except that rennilase, an acid protease from *Mucor miehei* (a gift from Novo Enzyme Corp., Mamaroneck, N.Y.) (0.83 mg/ml), was substituted for the acid protease from *Rhizopus chinensis* (2). Samples for electron microscopy either were not incubated or were activated with rennilase and incubated with 1.5 mM UDP-GlcNAc, 25 mM GlcNAc, and 10 mM $MgCl_2$ in 50 mM KH_2PO_4–NaOH buffer, pH 6.5. Most incubations were at 22–24°; some were at 0–3° to slow down chitin synthesis (Figs. 13–16, 19, 20, and 23).

Electron Microscopy. For negative staining, droplets of the sample were placed on carbon-coated formvar films on 300-mesh copper grids. The grids were floated, sample side down, on 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.1, for 1–2 min, and then floated on distilled water for a few seconds to remove glutaraldehyde, buffer, and residual sucrose. Finally, 2.5% aqueous uranyl acetate was added to each grid and withdrawn with filter paper.

For thin sectioning, chitin synthetase samples were mixed, in the dark at 0°, with an equal volume of fixative containing 1.5% glutaraldehyde, 1% OsO_4, and 0.1 M sodium cacodylate, pH 7.1 [modified from the procedure of Franke *et al.* (3)]. The resulting suspensions were centrifuged at 196,000 × *g* for 1 hr; then the pellets were washed 8 to 10 times with cold buffer and postfixed with fresh 2% OsO_4 in cacodylate buffer at 0° for 1 hr. The fixed pellets were then washed with distilled water, soaked in 2% aqueous uranyl acetate at room temperature for 4–8 hr, washed again in water, and embedded in 2% water agar for ease of handling during subsequent steps. Specimens were dehydrated in a graded series of acetone and embedded in Epon. Intact cells were prepared likewise, but without the 196,000 × *g* centrifugation. Thin sections with gray to pale gold interference colors were stained for 10 min with lead citrate.

Specimens were examined and photographed with Philips EM 200 and EM 300 electron microscopes at 60 kV or with a Hitachi HU-12 at 75 kV. Negatively stained catalase crystals and a waffle-type diffraction grating replica (463 nm spacing) were used as magnification and size standards.

RESULTS

Negatively stained preparations of purified chitin synthetase contain a heterogeneous population of particles. Two types of

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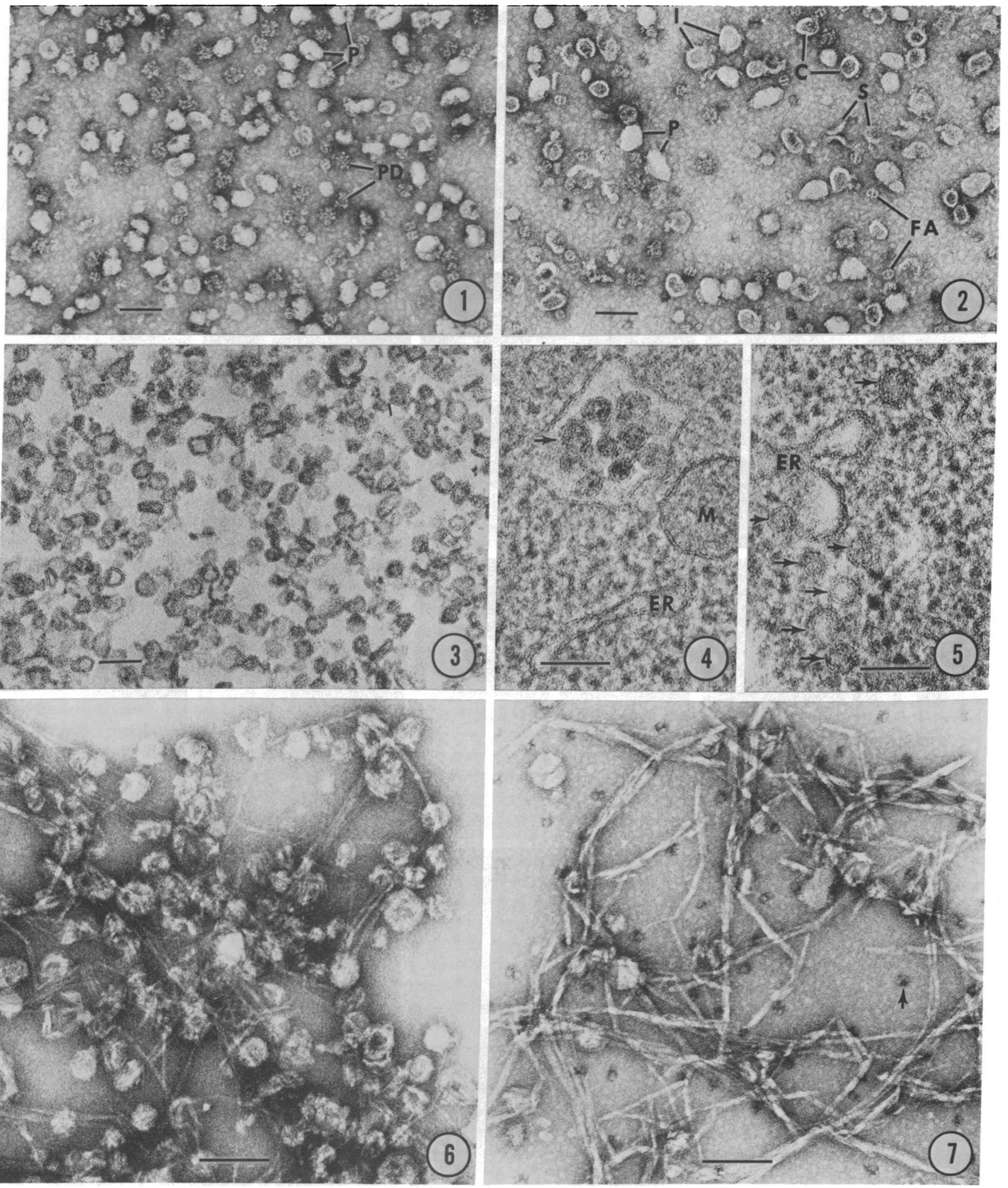
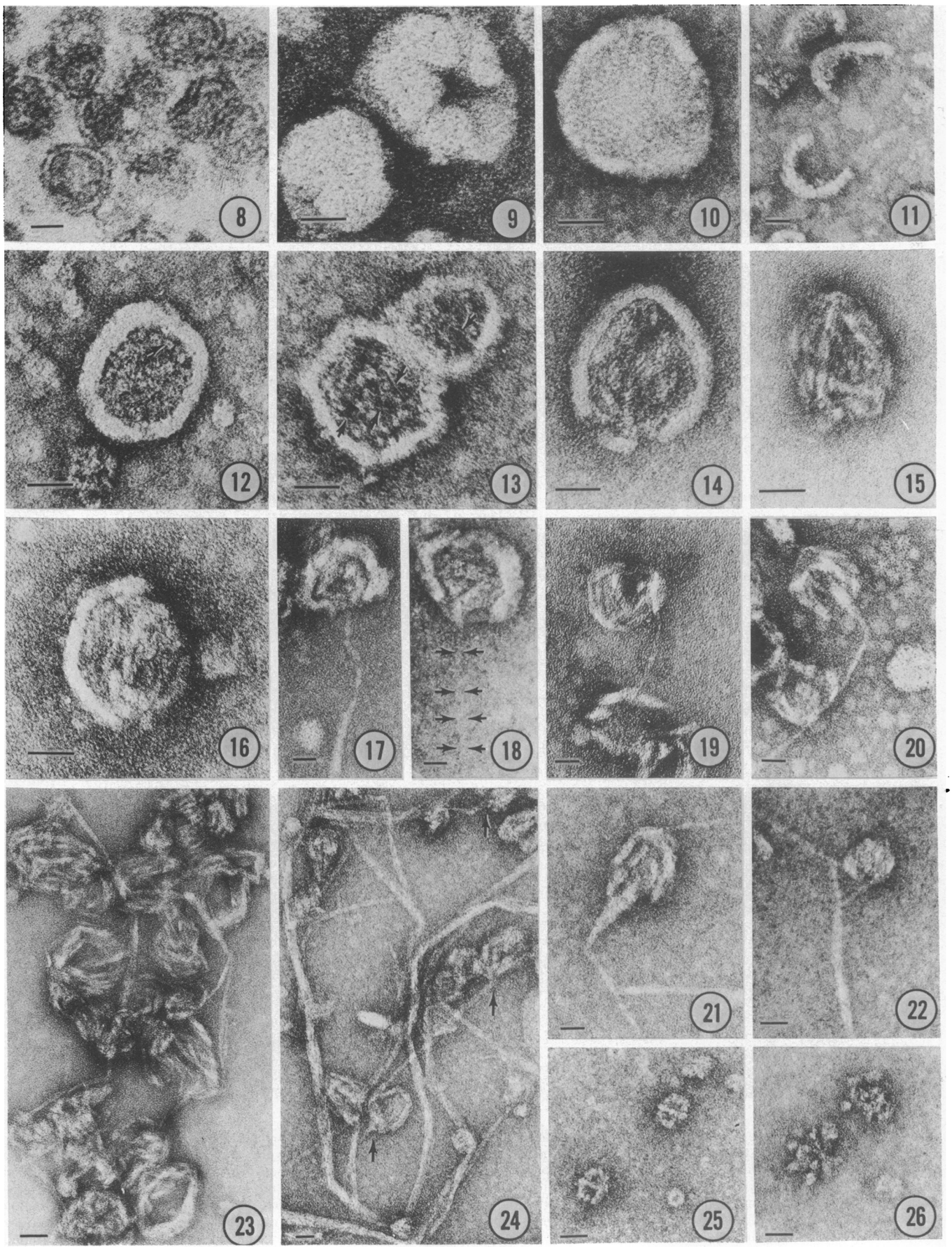
¶ J. Ruiz-Herrera, E. Lopez-Romero, and S. Bartnicki-Garcia, "Properties of chitin synthetase in isolated chitosomes from yeast cells of *Mucor rouxii*," submitted for publication.



FIGS. 1–7. *Figs. 1 and 2.* Negatively stained samples of isolated, unincubated chitin synthetase preparations. Proctoid chitosomes (P), cycloid chitosomes (C), intermediate forms (I), fragments of chitosome shells (S), particles resembling pyruvate dehydrogenase (PD), fatty acid synthetase particles (FA). *Fig. 3.* Thin section of fixed and embedded pellet of unincubated chitin synthetase. *Figs. 4 and 5.* Thin sections of intact yeast cells of *M. rouxii* showing microvesicles morphologically similar to isolated chitosomes inside a multivesicular body (arrow, Fig. 4) or free in the cytoplasm (arrows, Fig. 5). Endoplasmic reticulum (ER), microbody (M). *Fig. 6.* Cluster of chitin microfibrils after 5-min incubation of isolated chitin synthetase, substrate, and activators. Numerous fibroid particles are associated with the extended microfibrils. *Fig. 7.* Cluster of chitin microfibrils after 3-hr of incubation. Fibrils are thicker than in Fig. 3 and have only a few associated particles. Dark spots in the background (arrow) are aberrations of the carbon support film. Magnification bars equal 100 nm in all figures on this plate.



FIGS. 8–26. *(Legend appears at bottom of the following page.)*

spheroidal particles (chitosomes) predominate in the unincubated samples, and most of these are 40–70 nm in diameter (Figs. 1 and 2). The most prevalent particles have a "proctoid" appearance and reveal little internal structure by negative staining (Figs. 1, 2, and 9). Apparently, uranyl acetate does not penetrate into these particles. The proctoid chitosomes are highly polymorphic, sometimes flattened, sometimes with an angular profile, and often with radial folds or clefts leading to a depression in the surface of the particle (Fig. 9). The other abundant particle type is similar to the proctoid form in size and outline, but stain accumulation within these particles reveals some internal structure and a shell approximately 6–12 nm thick (Figs. 2 and 12). The shell gives these particles a "cycloid" appearance. Internally, the cycloid chitosomes exhibit either a diffuse grainy texture (not shown) or discrete "microgranules" (Figs. 2 and 12). Fragments of chitosome shells are also found among the intact chitosomes (Figs. 2 and 11). Some of the fragments retain adhering material, presumably from the interior of the chitosomes. Some chitosomes are intermediate between the proctoid and cycloid forms, with varying degrees of internal stain accumulation (Figs. 2 and 10). Gradient fractions with the highest chitin synthetase specific activity have the highest proportion of proctoid chitosomes relative to other particle types.

Thin sections of pelleted chitosomes reveal particles (Fig. 3) in the same size range as those seen by negative staining. The particles are bounded by a shell, only 6.5–7 nm thick in section. The shell appears tripartite, with two electron-opaque bands separated by an electron-lucent layer (Figs. 3 and 8). In intact yeast cells of *M. rouxii*, the only structures that appear as morphological correlates of the isolated chitosomes are microvesicles approximately 35–50 nm in diameter within multivesicular bodies (Fig. 4) and microvesicles free in the cytoplasm (Fig. 5).

Other structures found in the chitin synthetase preparations include: (*i*) particles 28 × 25 nm (Figs. 2 and 25), similar in appearance to the fatty acid synthetase complex from *Saccharomyces cerevisiae* (4), and (*ii*) particle aggregates, 35–45 nm overall diameter (Figs. 1 and 26), resembling pyruvate dehydrogenase complexes (5). The biochemical identity of these particles will be reported elsewhere.

After substrate and activators are added to the chitin synthetase suspensions, clusters of extended microfibrils are found among the chitosomes. Although microfibrils can be seen within 30 sec after an incubation begins, they are not conspicuous or common in negatively stained preparations until after about 5 min of incubation at 22–24°. During the first 15 min, the clusters of microfibrils have many associated particles (Fig. 6). However, these particles do not have the proctoid and cycloid appearances prevalent in the unincubated preparations. Instead, they consist of one or more fibrils repeatedly bent at more or less sharp angles (e.g., Figs. 6, 15, 19, and 23). Most of these "fibroid" structures are roughly spherical to ellipsoid, about 35–60 nm in diameter. They vary from a simple loop to seemingly continuous spirals with several loops of similar dimensions, or even clews of loops of different widths running in different directions, giving the appearance of delicate fibrillar baskets (Figs. 15–24). The loops have the appearance and insolubility properties (1 M HCl, 1 M NaOH) of chitin fibrils. Most of the fibroid particles associated with a cluster of microfibrils lack a shell, but some retain remnants of a shell, similar to the shells of cycloid chitosomes. Both cycloid and proctoid chitosomes, morphologically unchanged, remain in the incubated samples, but they are not specifically associated with microfibrils. With increasing time of incubation, the relative proportion of fibroid particles to extended microfibrils within the clusters declines, and by 3–11 hr, clusters of microfibrils have few or no associated fibroid particles (Fig. 7).

Careful examination of incubated samples reveals a variety of particles intermediate between the proctoid-cycloid chitosomes and the fibroid forms. Some cycloid chitosomes contain exceedingly fine fibrils measuring only 1–2 nm in diameter (Fig. 13), whereas others contain more robust fibrous configurations (Fig. 14) resembling those in the fibroid particles with extended microfibrils (Figs. 6 and 19–21). In most of the fibroid chitosomes, the shells have opened up and exposed their fibrillar contents. Occasionally, chitosomes of this type have an extended microfibril emanating from the coiled fibrous material in the shell (Figs. 17, 18, and 24). Fibroid particles lacking a shell can also be seen with (Figs. 19–22) and without (Fig. 15) a continuous extended microfibril. Images of isolated single particles with an extended microfibril are relatively scarce because of the tendency of fibroid particles and microfibrils to form aggregates (Figs. 6, 7, 23, and 24). At a given time in an incubated preparation, only a small proportion of the chitosomes are observed in intermediate structural stages such as those illustrated in Figs. 13–18. The chances of finding these intermediate forms are increased in samples incubated at 0–3°.

Chitin microfibrils are ribbon-shaped, kinked, and of various dimensions (Figs. 6, 7, 23, and 24). Thick fibrils are composed of thinner fibrils. Fine fibrils are seen merging into thicker ones (Figs. 7 and 24), and occasionally a microfibril arising from a fibroid particle can be seen as a lateral tributary to another microfibril (Figs. 22 and 24). The microfibrils synthesized during short (5 min) incubations (Fig. 6) are much thinner on the average than those observed after long (3–11 hr) incubations (Fig. 7).

DISCUSSION

This study has revealed some new aspects of chitin synthetase and the formation of chitin microfibrils *in vitro*. It is now evident that the isolated particles (chitosomes) containing the enzyme are relatively complex structures which may be re-

FIGS. 8–26 (*on preceding page*). *Fig. 8.* Isolated, unincubated chitosomes in thin section. Note the tripartite shell bounding the particles. *Fig. 9.* Isolated proctoid chitosomes (this and all subsequent figures are of negatively stained samples). *Fig. 10.* Chitosome, intermediate between proctoid and cycloid forms, shows faint stain accumulation within the particle. *Fig. 11.* Fragments of chitosome shells. *Fig. 12.* Cycloid chitosome with internal microgranules (arrow). *Fig. 13.* Incubated cycloid chitosomes with microgranules and fine fibrous elements (arrows) in the interior. *Fig. 14.* Incubated chitosome with internal fibroid material. *Fig. 15.* Fibroid particle consisting of coiled and bent fibrillar material, but without a shell. *Fig. 16.* Chitosome consisting of fibroid contents with only a remnant of the cycloid shell remaining. *Figs. 17 and 18.* Chitosomes with opened shells and extended chitin fibrils (arrows in Fig. 18) emanating from the fibroid contents. *Figs. 19–22.* Extended chitin microfibrils continuous with fibroid particles that lack shells. The small fibril continuous with the fibroid in Fig. 22 is a lateral tributary to a larger fibril. *Fig. 23.* Small cluster of extended chitin microfibrils and large fibroid particles produced during a low-temperature (0–3°) incubation of chitosomes with substrate and activators. *Fig. 24.* Chitin fibrils from a chitosome preparation incubated at 22–24°. The fibrils are kinked and ribbon-shaped, with slender fibrils associated to form larger fibrils. Three of the fibroid particles show extended fibrils emanating from them (arrows). *Figs. 25 and 26.* Enzyme complexes as contaminants of the isolated chitin synthetase preparations. Fig. 25, two fatty acid synthetase particles; Fig. 26, two particles resembling pyruvate dehydrogenase complexes. Magnification bars equal 20 nm in all figures on this plate.

garded as small organelles. The chitosomes exhibit structural detail that could not have been realized by the metal shadowing technique used previously (2). Moreover, the chitosomes are transformed during the process of fibrillogenesis. The diversity of particle morphology reflects both an inherent polymorphism among chitosomes as well as pleomorphism as chitosomes go through progressive transformations during incubation.

The presence of an array of intermediate particle types in incubated preparations of chitin synthetase leads to the interpretation of an *in vitro* developmental sequence beginning with the proctoid chitosome and ending with an extended chitin microfibril. We propose that incubation of the purified enzyme with substrate and activators initiates the following irreversible sequence of events: (*i*) The proctoid chitosomes are transformed into cycloid forms. However, it is possible that the proctoid and cycloid forms are not two separate developmental stages but are differently stained forms of the same particle type. (*ii*) Cycloid chitosomes undergo a gradual internal conversion leading to the formation of a fibroid structure. The minute (1–2 nm in diameter) fibrils and the microgranules found inside some of these chitosomes may represent minimum assemblages of chitin chains and their assembling units, respectively.‖ (*iii*) The coiled fibroid structure is formed within the chitosome before an extended microfibril is evident. (*iv*) Once the chitosome shell is opened or shed, a slender microfibril arises from the fibroid particle. In any cluster of microfibrils and fibroid particles (e.g., Figs. 6, 23, and 24), most of the fibroid particles lack a shell, indicating that the ultimate stages in fibrillogenesis can occur without participation of the shell. (*v*) The chitin microfibrils formed after prolonged incubation are composed of slender fibrils in lateral association and have very few fibroid particles associated with them.

The following observations, taken collectively, support the overall scheme described above and establish developmental links from chitin microfibrils back to proctoid chitosomes: (*a*) fibroid particles and extended microfibrils appear only after incubation of chitosomes with substrate and activators; (*b*) extended chitin microfibrils are continuous with fibroid particles; (*c*) fibroid particles occur with and without cycloid shells; (*d*) forms intermediate between cycloid and proctoid chitosomes occur among the chitosome population; and (*e*) electron microscopy and enzyme assays of fractions along the sucrose gradient show that chitin synthetase activity is correlated with chitosomes, most of which are proctoid (in preparation).

The tripartite image of the chitosome shell in section resembles the image of a biological membrane, but whether this shell is a lipoprotein is currently under investigation. The detail of the shell is very subtle, and earlier failure to detect its tripartite organization (2) can be attributed to differences in obtaining the samples and preparing them for electron microscopy. Membranes such as the plasma membrane, tonoplast, and secretory vesicles are considerably thicker (8–9 nm) than the chitosome shells (6.5–7 nm) in thin sections. The only *in situ* structures that resemble the isolated chitosomes are the microvesicles shown in Figs. 4 and 5. The minute amounts of phospholipid found in chitin synthetase preparations obtained from cells grown in either [^{14}C]choline or inorganic [^{32}P] phosphate indicate that isolated chitosomes are not produced artificially by fragmentation of any of the major membrane components of the cells (unpublished data).

It is likely that the chitosome is the container and conveyor of chitin synthetase *en route* to its destination at the cell surface. A detailed model for the operation of chitosomes awaits further chemical and structural characterization as well as a determination of their origin and fate *in vivo*. A comparison of *M. rouxii* chitosomes with the chitin synthesizing complexes described for other fungi (6, 7) is outside the scope of this report. However, we have recently confirmed the existence of chitosomes and fibrillar structures shown here in chitin synthetase preparations isolated from *Allomyces macrogynus, Neurospora crassa, Saccharomyces cerevisiae,* and *Agaricus bisporus* (in preparation).

Aspects of the biosynthesis of cell wall polysaccharides in other systems have heretofore been investigated either biochemically (e.g., refs. 8–10) or cytologically (11, 12), but without the opportunity to follow structural and biochemical transformations together *in vitro*. In the chitosome, we have found a new and distinctive biosynthetic structure capable of undergoing transformation in the process of synthesizing a microfibril. It can be isolated and will operate *in vitro*. Thus, the process of fibrillogenesis can be monitored biochemically and observed directly by electron microscopy. The chitosomes have provided an exceptional opportunity for integrated ultrastructural-biochemical investigation of microfibril biogenesis.

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‖ Many synthesizing units must be present within a single chitosome to form a microfibril. Even a small microfibril, 4 × 8 nm in cross section, would contain at least 64 individual chitin chains.