ATP-induced Gelation–Contraction of Microtubules Assembled In Vitro

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ABSTRACT We report here an ATP-dependent formation and contraction, or syneresis, of microtubule gels using microtubule proteins prepared from calf brains. Gel contraction is typically observable 15–30 min after ATP addition to microtubules assembled to steady state, and is complete after ~60 min, at which time the gel volume is reduced by as much as 75%. In contracted gels, microtubule bundles and aster-like structures are observable. Gelation–contraction requires only microtubule proteins present after purification by three cycles of assembly and disassembly.

Cytoplasmic microtubules are involved in various motile phenomena and in the control of cell shape, but little is known about the mechanism of microtubule function in the cell. In flagella, sliding of microtubules relative to one another is responsible for motility (25), but whether such a process is involved in cytoplasmic motility is not known (2, 30). Cytoplasmic microtubules are often organized into parallel arrays, or associated with microtubule-organizing centers, but the significance of such structures in either motility or cell shape is not understood. It obviously would be helpful to be able to form similar structures in vitro, and to induce in them some type of motile activity. Microtubules do undergo a type of internal motility in which subunits add at one end and gradually “treadmill” to the other end (15); however it is unclear if this phenomenon has any relationship to in vivo processes (11).

Cross-bridging of cytoplasmic microtubules in vitro has been obtained using ciliary dynein (7), a neuronal protein from squid (18), the enzyme glyceraldehyde 3-phosphate dehydrogenase (12), the spectrin-like protein “fodrin” (9), and the microtubule-stabilizing drug taxol (29), but these reactions have not been related to physiological processes and have not produced motile phenomena. Microtubules that have been cross-bridged with either dynein or glyceraldehyde 3-phosphate dehydrogenase or in the presence of taxol are sensitive to ATP, but the response to ATP in each case is the breaking of cross-bridges, not motility as such. We now report the ATP-dependent contraction, or syneresis, of microtubule gels formed in vitro using microtubule proteins prepared from calf brain by three cycles of assembly and disassembly. (Note that the terms gelation and contraction are used only to describe the observed phenomenon, and are not intended to imply any particular physiological or biochemical process.)

MATERIALS AND METHODS

Microtubule protein was isolated by slight modification of previously described methods (32), but we do not presently know if this procedure is essential to obtain a preparation competent to undergo gelation–contraction. Whole calf brains, processed within 2 h of slaughter, were homogenized in a Waring blender in 0.1 M 2-(N-morpholino)ethane sulfonic acid (MES) buffer at pH 6.6 (this buffer and pH were used throughout) containing 0.1% mercaptoethanol, 0.1 mM GTP, and 25% glycerol, using two brains in 500 ml of medium. The homogenate was spun in a Beckman T115 zonal (Beckman Instruments, Inc., Palo Alto, CA) rotor at 30,000 rpm for 60 min at 4°C. The supernatant was recovered and brought to 2 mM EGTA, 1 mM MgCl2, and 0.5 mM GTP, and 33 ml of additional glycerol was added per 100 ml of supernatant. The supernatant was then incubated at 37°C for 30 min to induce assembly, and the microtubules were collected in a Beckman T114 zonal rotor spun at 45,000 rpm for 45 min at 25°C. The microtubule pellet was resuspended in 115 ml of MES buffer with 0.2 mM GTP and incubated at 0°C for 30 min, and glycerol was added to make a final volume of 150 ml (25% glycerol). The protein was stored overnight at −20°C and centrifuged at 40,000 rpm for 20 min at 4°C in a Beckman TI60 rotor. The supernatant received 1 mM EGTA, 0.5 mM MgCl2, and 0.2 mM GTP, and a second cycle of polymerization and centrifugation was performed (using centrifugations of 40,000 rpm for 30 min in a TI60 rotor). The microtubule protein was stored overnight at −70°C in MES buffer, 0.1 mM GTP plus 25% glycerol. A third cycle of polymerization and centrifugation (using spins of 40,000 rpm for 30 min in a type-65 rotor) was performed to prepare the protein for each experiment. The third cycle pellet was resuspended in MES buffer and 1 mM dithiothreitol and then was spun through at least 20 vol of packed G-25 Sephadex (19) in MES buffer plus dithiothreitol, to remove most remaining traces of glycerol.

For each experiment, unless otherwise noted, the third-cycle protein was polymerized by incubation at 37°C for 30 min in 0.5 mM GTP, 1 mM EGTA, 0.5 mM MgCl2. Protein concentrations were typically between 5 and 8 mg/ml. The solution was divided as needed, and the indicated additions were made. Experiments were generally performed in 1-ml polystyrene cuvettes to allow optimum visibility of the gels. Incubation was continued for at least 1 h after ATP addition, if gelation–contraction was observed, and for at least 3 h if gelation–contraction was not observed in any sample. The solutions were

1 Abbreviations used in this paper: MES, 2-(N-morpholino)ethane sulfonic acid.
RESULTS AND DISCUSSION

Approximately 30 min after ATP addition to steady-state microtubules, the turbid phase of the solution began to separate from the walls of the vessel and to either raise up from the bottom or separate from the meniscus (Fig. 1). A distinct gel was present at this time, as indicated by entrapment of bubbles and flow properties. The gel continued to decrease in volume for ~15–30 min after contraction, or syneresis, was first observable. The contracted gel often split, with half moving towards the meniscus and half settling to the bottom of the vessel (Fig. 1). Under less than optimum conditions, such as low protein concentration, a distinct gel may not form. Instead, the solution becomes flocculent and the turbid material settles slowly to the bottom of the vessel (not shown). The conditions for formation and the structure (e.g., the presence of aligned microtubules and aster-like structures) of the flocculent “precipitate” are similar to that of the contracting gel, and we consider them to reflect the same basic reaction.

The gels may contract to about ¼ the initial volume under optimum conditions. The volume and protein content of contracted gels were estimated by centrifugation in a graduated, narrow-bottom centrifuge tube in a table-top centrifuge. The amount of protein present in the gel ranged from 30 to 60% of the total protein present.

The contracted gels were temperature sensitive and were rapidly dispersed by incubation at 0°C. In most experiments a small amount (~10% of the initial volume) of insoluble material remained following cold incubation. Although the cold-stable material did not contain microtubules, the formation of the cold-stable aggregate appeared to correlate with gelation-contraction. Preliminary observations by timelapse video microscopy suggest that contraction involves the formation of microtubule focal centers which enlarge and move together, resulting in the formation of aster and spindle-like structures (visible in Fig. 2C). The cold-stable aggregate appears to be related to the microtubule focal centers.

Polarization microscopy of contracted gels indicated the presence of birefringent domains of various orientations (Fig. 2). No significant birefringence was observed before addition of ATP, at which time the solutions appeared uniformly grey (not shown). The pattern of birefringence was quite variable with respect to the size and anisotropy of birefringent domains. Although gel contraction was not detectable until ~30 min after ATP addition, birefringent domains were observable within 5 min. The birefringent structures formed early occurred the entire solution and eventually coalesced or contracted (Fig. 2C) at about the time that contraction was observed macroscopically.

Transmission electron microscopy by thin sectioning or negative staining with 0.5% uranyl acetate indicated that the gels contain microtubules aligned into roughly parallel arrays or bundles (Figs. 3 and 4). It is possible to observe apparent cross-bridges between microtubules, particularly in negatively stained preparations, but we do not know if these reflect real structures or are artifacts of the preparation (14). Considerable variation has been observed in the degree of alignment and in the distance between microtubules. It is likely, however, that the wider spacing observed in thin sections reflects the true distribution of microtubules, while the negatively stained images have been distorted by flattening of the sample. Besides microtubules, dense aggregates of material are often observed. These aggregates frequently appear to be associated with microtubules, resulting in the formation of aster-like structures (Fig. 3C).

ATP was found to be effective above 0.5 mM in inducing gelation-contraction. At higher ATP concentrations contraction was observed sooner, and the extent of contraction increased (Fig. 5). GTP added to steady-state microtubules (GTP was always present at the start of incubation) was occasionally observed to induce gelation-contraction, but it was less effective than ATP (Fig. 6). When tested at 1 mM, ITP and CTP were unable to induce gelation-contraction (Fig. 6). No gelation-contraction was observed in 1 mM AMP-PNP (5' guanylimidodiphosphate) or AMP-PCP (β,γ

FIGURE 1 Examples of contracted microtubule gels. The samples, 0.4 ml each, are in plastic cuvettes. All samples contained 1 mM GTP, and the indicated additions and were incubated at 37°C for 30 min at which time 1 mM ATP was added to all the solutions. The photograph was taken ~1 h after ATP additions. Note that the movement of the gels may vary with essentially identical extents of contraction. The additions were: cell 1, 1 mM ATP only; cell 3, 1% DMSO; cell 4, 1% DMSO and 10 µM taxol; cell 5, 0.1 mM cAMP, cell 6, 0.1 mM cGMP.

in the vessel (Fig. 1). Chemicals were all reagent grade. Water was distilled and then passed through a Barnstead Mixed Bed deionizer (Barnstead Co., Boston, MA). Protein concentration was determined by the Bradford method (1) using BSA standards. SDS acrylamide gel electrophoresis was done by the method of Laemmli (13). Nucleotides and DNase were all obtained from Sigma Chemical Co. (St. Louis, MO). Taxol was a gift from the National Institutes of Health.
FIGURE 2  Polarization micrographs of contracted microtubule gels. (A) A well-contracted gel showing large, distinct birefringence domains. (B) A contracted gel showing a more homogeneous appearance with small domains. (C) Birefringent structures formed when gelation-contraction was induced in a thin solution on a microscope slide. (D) Gelation-contraction in the presence of 0.1 mM taxol; birefringence domains are larger than normal. (A–D) × 640.

While much more remains to be learned about this phenomenon, several conclusion can be reached. First, the reaction is either the contraction or syneresis of a true microtubule-containing gel. Within the gel, microtubules are aligned to a significant degree. This is indicated by the birefringence of the gels and confirmed by electron microscopy. However, at this time we do not know whether microtubule alignment depends upon cross-bridges, is a result of physical interactions between rigid polymers (17, 26), or is related to the formation of apparent focal centers during contraction. Nor do we know...
whether contraction of the microtubule gel is a result of microtubule alignment, the "zipping" of microtubule bundles (28), or the sliding of microtubules, or the result of an as yet unknown process. There is ATPase activity in these preparations, but its relationship to contraction is unknown. ATP may also act as a substrate for a protein kinase (10, 24) which
FIGURE 3—Continued
The relationship of the phenomenon reported here to events in the living cell is, of course, unknown. However, the specificity for ATP is consistent with a physiological function for this reaction. Physiological significance is also indicated by the fact that ATP-dependent gelation-contraction requires

stimulates gelation-contraction, but the fact that cAMP sometimes inhibits gelation-contraction argues against this. However, more than one reaction is required for gelation-contraction to occur. Microtubules must first be formed, and inhibition by cAMP may reflect inhibition of microtubule assembly (10). The rather long lag time (30 min) between the addition of ATP and the start of an observable contraction also suggests that more than a single process is occurring.
only purified microtubule proteins, and as much as 60% of the total protein, and nearly all the microtubules, are incorporated into the contracted gel. Although the motility observed in vitro, contraction of a gel, has no obvious relationship to cellular motility as it occurs in nerve cells, this may reflect differences in the in vivo and in vitro environment of the microtubules (21). The reaction observed here may also reflect a developmental process, such as neurite extension. Results that indicate interactions between microtubules and actin (6), neurofilaments (8, 16, 20), and other microtubules (7, 9, 12, 18, 28) suggest the existence of complex interactions between cytoskeletal components in the living cell, consistent with ultrastructural observations (4, 8). The present observations may ultimately help resolve the role of such interactions.

We thank Moira Cioffi for her contributions to the electron microscopy, and Mortimer Labes and Joel Sheffield for their valuable assistance.

This work was supported by National Institutes of Health grant CA29985 to R. C. Weisenberg.

Received for publication 27 February 1984, and in revised form 21 May 1984.

Note Added in Proof: We have recently examined microtubule gelation-contraction by video-enhanced contrast microscopy in collaboration with Robert Day Allen (Dartmouth College) and Shinya Inoué (Marine Biological Laboratory, Woods Hole). We observed that gelation-contraction begins with the formation structures resembling mitotic spindle asters. These aster-like structures subsequently move towards one another at rates of 1-5 μm/min. In addition to the formation and movement of aster-like structures, we also observed linear particle movements. These are most often directed towards a single aster center, but are sometimes bi-directional, and particles may move back and forth between two connected asters. These results will be reported in a manuscript now in preparation.

REFERENCES