Calcium Ion Induces Endwise Depolymerization of Bovine Brain Microtubules*

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Calcium ion induces rapid depolymerization of microtubules, and the mechanism of interaction of Ca²⁺ with cytoskeletal components may be metabolically significant. The sufficiency of an endwise depolymerization model for describing calcium-induced disassembly was demonstrated by three criteria: (a) correlation of the observed time course of disassembly with the initial polymer length distribution using a kinetic model described elsewhere (Kristofferson, D., Karr, T. L., and Purich, D. L. (1980) J. Biol. Chem. 255, 8567-8572); (b) observation that the initial rate of calcium-induced disassembly parallels the microtubule number concentration which is experimentally manipulated by mechanical shearing; and (c) determination of the average polymer length, microtubule number concentration, and extent of depolymerization at various levels of added calcium ion to achieve successive partial disassembly extents. The rate constant for protomer release and other aspects of the disassembly mechanisms are also presented. Critical concentration measurements at low calcium ion levels are also consistent with endwise interactions.

Weisenberg (1) was the first to recognize that in vitro self-assembly of brain microtubule protein requires a chelating agent to lower the uncomplexed calcium ion concentration. A number of other workers have subsequently demonstrated that calcium ion inhibits assembly and also induces rapid microtubule disassembly (2-4). In particular, Omlsted and Borisy (4) determined that calcium ion levels must exceed the tubulin dimer concentration to efficiently inhibit assembly, and that this divalent ion’s action is unrelated to sequestering GTP required for assembly. Solomon (5) has shown that calcium ion interacts with tubulin at a single high affinity site (K = 3 μM) and approximately 25 low affinity sites (K = 0.1 mM). However, at 0.1 M potassium chloride the high affinity site is not observable. As regards the action of calcium as an assembly inhibitor, it is interesting to note that Ca²⁺ affects the sedimentation properties of tubulin by causing aggregation (6). Interestingly, calmodulin has been observed to potentiate calcium ion depolymerization, and this calcium-regulatory protein has been localized in the microtubule network of the mitotic apparatus (7). Nonetheless, it is important to determine how calcium ions could destabilize the tubules. Two possible models exist: 1) calcium binds to the entire lattice structure and destabilizes the microtubule for dimer release along the tubule’s length or 2) calcium ion causes endwise depolymerization. This distinction is important for understanding the physiological effects of in vitro regulators of the state of microtubule assembly. Summers and Kirschner (8) have asserted that the endwise model is operant, but there is an obvious need for mechanistic characterization.

Recently, we developed a sound kinetic model for endwise depolymerization of linear protein polymers (9), which makes it possible to predict the shape of depolymerization progress curves (i.e. remaining polymer weight versus time). The method assumes that the rate of dimer release is independent of polymer length and requires polymer length distribution data. In a previous study (10), microtubule disassembly after rapid dilution to below the critical concentration was correlated to this endwise depolymerization model using length distribution data defined by electron microscopy. This approach yields useful information about the rate constant for dimer release and the mechanism of disassembly. We now extend this treatment to understand the dynamics and mechanism of calcium ion-induced disassembly of microtubules. This experimental approach and other evidence are presented herein to show that depolymerization occurs in an endwise fashion.

EXPERIMENTAL PROCEDURES

Materials

GTP and Mes¹ were obtained from Sigma. CaCl₂ was purchased from Fischer and all other chemicals used were reagent grade. The buffer used in this study contained 0.1 M Mes, 0.001 M MgSO₄, and KOH to adjust the pH to 6.8 (designated Mes/MgSO₄ buffer).

Methods

Protein Preparation—Microtubular protein was purified from bovine brains by the method of Karr et al. (11). Immediately prior to use, protein stored at -80°C in 3.5 M glycerol was thawed and sieved over a Sephadex G-25 column (1.6 × 30 cm) equilibrated with Mes/MgSO₄ buffer. The column effluent was monitored by absorbance at 280 nm and the peak of the absorbance was pooled, brought to 1 mM in GTP, and polymerized at 30°C for 30 min. Microtubules were harvested by centrifugation at 90,000 × g for 75 min. Microtubules were subsequently cold depolymerized at 4°C in Mes/MgSO₄ buffer and centrifuged for 10 min at 50,000 × g to remove any large aggregates.

Assembly/Disassembly Assays—Microtubular protein, prepared as described above, was diluted to the indicated final concentrations (see figure legends) and induced to assemble by raising the temperature to 30°C. Assembly was monitored in a thermally controlled chamber of a Cary 210 recording spectrophotometer equipped with a magnetic stirrer accessory. After the sample had achieved equilibrium as monitored by a stable plateau in the assembly curve, the sample

¹The abbreviation used is: Mes, 2-(N-morpholino)ethanesulfonic acid.

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was induced to disassemble by the addition of calcium chloride. The magnetic stirrer was on during addition of calcium to ensure thorough mixing and was subsequently turned off 4 s after the addition.

Critical Concentration Measurements—The critical concentration for microtubule assembly in the presence of calcium ions was measured as follows. Microtubule protein prepared as described above, was pelleted by 150000 × g for 2 h in 30 mM MOPS buffer, pH 7.0, 200 mM KCl, and then rediluted into buffer containing 10 mM MgCl₂, 200 mM KCl, and 0.5 mM GTP to yield aliquots of varying protein concentrations but maintaining constant levels of GTP and CaCl₂. These samples were induced to assemble by raising the temperature to 30°C. As the assembly reaction was monitored in a Cary 210 recording spectrophotometer. One cuvette was brought to 30°C. After each addition of calcium as indicated in Table I. The calcium ion level was then increased to 10 μM. The sample was assembled to steady state in a 3-ml cuvette equipped with a magnetic stirrer. Data was entered by tracing along microtubules on an electron photomicrograph with the bombsite. The data is then processed and a Tektronix graphics terminal and hard copy unit produces the histograms (12).

Comparison of Theoretical and Experimental Curves—The experiments on the theoretical curve were used to calculate a theoretical curve according to the equations derived by Kristofferson et al. (9). A computer program based on a series first order mechanism is employed for this calculation. The theoretical and experimental depolymerization curves are matched on the same axes as described in Karr et al. (10). Briefly, the ratio of the initial turbidity to the turbidity at the steady state is used to calculate the half-life change for the disassembly reaction. The half-life change is then increased to 100 μM to convert units on the x axis (polymer weight axes) of the two curves. The half-life for the experimental curve is calculated as described elsewhere (12). The solid line in Fig. 1 represents the observed kinetics of disassembly.

Shearing Experiments—Two samples of microtubule protein were independently assembled to steady state as monitored by turbidity on a Cary 210 spectrophotometer. One of the samples was sheared by three rapid passes through a 22-gauge hypodermic needle. Aliquots were taken for electron microscopic length distribution analysis and then the samples were depolymerized by making each 2 ml in calcium chloride. As determined by the method of Lowry et al. (14), the protein concentration for the sheared and unsheared samples was 1.05 and 1.18 mg/ml, respectively. A 3-ml cuvette equipped with a magnetic stirrer was employed in both cases to ensure rapid mixing of the calcium with the sample.

Partial Depolymerization Experiments—A 0.89 mg/ml tubulin sample was assembled to steady state in a 3-ml cuvette equipped with a magnetic stirrer. The sample was depolymerized by successive addition of calcium as indicated in Table I. After each partial depolymerization, the turbidity was allowed to stabilize at a new plateau before further addition of calcium. The change in the apparent absorbance at 350 nm for these plateau values are noted in Table I.

RESULTS AND DISCUSSION

Correlation of Calcium-induced Disassembly Kinetics with a Linear Endwise Depolymerization Model—Microtubules are capable of undergoing rapid disassembly upon dilution as was shown in an earlier report (13). The rate constant for polymer loss was approximately 0.02 s⁻¹, and we realized that the rapidity of the Ca²⁺-induced depolymerization, as shown in Fig. 1, was qualitatively similar to dilution-induced disassembly and therefore might follow an endwise disassembly model. In a recent report (10), we demonstrated that the time course of microtubule depolymerization upon rapid dilution can be analyzed on the basis of a kinetic model with the following assumptions: (a) microtubule disassembly only proceeds from the tubule ends in a series first order fashion; (b) the rate constant for microtubule depolymerization depended on the rate of calcium ion concentration, and the zero time value was established by the turbidity value prior to disassembly. The solid line is the progress curve calculated on the basis of the linear depolymerization kinetic model. Inset, distribution of microtubule polymer lengths prior to calcium-ion induced disassembly. Lengths were determined by a microcomputerized digitizer as described under "Experimental Procedures." The average polymer length was 8.9 μm. The sample size was 734.
sents the results of this rate analysis, and it demonstrated that the kinetic model is suitable over the greatest fraction of the time course. A rate constant of 860/s was determined for protomer\textsuperscript{2} release from the microtubule ends, almost eight times that determined by the dilution induced disassembly technique. As discussed elsewhere (9), this rate constant possibly reflects the rate of protomer loss from several helix-start sites on the end of the tubule; thus, independent methods must eventually be used to establish the microscopic rate constant for each site. Nonetheless, the results of Fig. 1 supports the idea that calcium ion provokes endwise disassembly.

Rates of Calcium Depolymerization of Sheared and Unsheared Microtubules—To further test the endwise model, we examined the depolymerization rate dependence on microtubule number concentration. As shown by a number of investigators (2, 10, 14), mechanical shearing of microtubules can be effected by rapid passage through a 22-gauge hypodermic needle. In a typical experiment, we assembled tubules to their stable plateau turbidity value and subsequently sheared the microtubule sample. Electron microscopy demonstrated that the average lengths of sheared and unsheared samples were 3.5 and 9.4 \mu m, respectively. This corresponded to a microtubule number concentration ratio of 2.36 when corrected for the protein concentrations of each sample. Observed rates of disassembly for the two samples upon calcium addition were 5 \times 10^{-3} and 2.2 \times 10^{-3} A units/s for the sheared and unsheared samples; thus the ratio of initial rates (sheared/unsheared) was 2.27. These data represent a significant demonstration that calcium-induced disassembly rates may be correlated with the initial number of polymer ends to within a 4% error.

Changes in the Microtubule Number Concentration and Average Length Attending Partial Depolymerization by Calcium Ion—The above experiments rather conclusively demonstrate that endwise depolymerization results from calcium ion addition. Nonetheless, we developed an additional qualitative criterion for probing this point. Added calcium ion could induce total random disintegration of microtubules. If so, the average length would not change if the probability of disintegration is independent of polymer length. Conversely, if endwise depolymerization were the mechanism, then the number concentration and average length should progressivly drop. To test this notion, we incrementally increased the calcium ions levels in a sample of assembled microtubules and permitted the turbidity to reach a new stable value after each addition. The electron microscopy data obtained at each plateau is shown in Table I, and the results are clearly at odds with a random disintegration model. These results are in qualitative agreement with endwise depolymerization at the levels of calcium ion used in these experiments.

Microtubules Demonstrate Critical Concentration Behavior in the Presence of Calcium Ion—In our studies of calcium ion effects, we noted that microtubule assembly will proceed at low levels of calcium (5 to 250 \mu M), in agreement with experiments reported by Marcum et al. (7). We therefore undertook a more detailed study of the assembly reaction in the presence of Ca\textsuperscript{2+}. We employed the theory of protein polymer condensation-equilibrium as developed by Oosawa and Kasai (15). An agreement between this theory and experiment implies that the protomer subunits are in equilibrium with the polymeric form of the protein. As discussed by Oosawa and Kasai (15), a linear plot of polymer weight versus total protein weight indicates an equilibrium between the protomer and polymer. The results shown in Fig. 2 demonstrate a strong linear relationship between the polymer and total protein present. The line represents a least-squares regression fit through the data points with a correlation coefficient of 0.999. The intercept represents the so-called critical concentration which for this experiment was 0.18 mg/ml. This value is approximately 2-fold higher than the critical concentration for microtubule protein derived by method of Karr et al. (11), and this reflects lower stability of the ends toward complexation with unbound tubulin.

CONCLUSION

The findings presented in this report suggest that calcium ion induces microtubule depolymerization in an endwise manner. In terms of regulation of microtubule assembly and disassembly, this mechanism may afford cells a means to halt microtubule-associated processes without complete disassembly of the tubules to form a large unpolymerized tubulin pool. At high calcium or in the presence of calmodulin (7), the extent of depolymerization can be total. For example, using electron microscopy, we have found no evidence for any remaining tubules after the experiments illustrated in Fig. 1. It is also interesting to note that calcium ion does not block microtubule assembly in vitro at levels substantially below 1 mM (Ref. 7 and this report), but the rates of assembly are somewhat slower. For example, in the experiment shown in Fig. 2, the assembly rates for the control samples containing no calcium were approximately 2-fold faster (data not shown). Fig. 2 also demonstrates that microtubule assembly in relatively low levels of calcium behaves identically to assembly in the absence of calcium with regards to the critical concentration. We interpret this behavior to indicate that calcium ions are regulating both the assembly and disassembly rates in a manner consistent with the proposed condensation-equilibrium model (15). It will be fascinating to examine the effects of calcium ion on the opposite-end assembly/disassembly process which is sometimes called microtubule treadmilling (16), and we also believe that the mechanism of disassembly in the presence of both calcium and calmodulin should be explored to determine the kinetic pathway in the presence of this important regulatory protein.

\textsuperscript{2}The term protomer is used here to avoid confusion with the tubulin subunit structure. The tubulin heterodimer is considered the protomeric unit involved in assembly.
Finally, we must stress that the present experiments do not reveal whether tubulin protomers are desorbed from microtubule ends or larger aggregates are released upon calcium ion addition. This will be an important goal of further dynamic measurements of the interactions of protomers with microtubules. At this point, it is nevertheless satisfying to observe that the process does occur endwise.

REFERENCES