The Kinesin-Related Protein MCAK Is an ATP-Tubulide Depolymerase that Forms an ATP-Hydrolyzing Complex at Microtubule Ends

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Summary

MCAK belongs to the Kin I subfamily of kinesin-related proteins, a unique group of motor proteins that are not motile but instead destabilize microtubules. We show that MCAK is an ATPase that catalytically depolymerizes microtubules by accelerating, 100-fold, the rate of dissociation of tubulin from microtubule ends. MCAK has one high-affinity binding site per protofilament end, which, when occupied, has both the depolymerase and ATPase activities. MCAK targets protofilament ends very rapidly (on-rate 54 μM⁻¹·s⁻¹), perhaps by diffusion along the microtubule lattice, and, once there, removes ~20 tubulin dimers at a rate of 1 s⁻¹. We propose that up to 14 MCAK dimers assemble at the end of a microtubule to form an ATP-hydrolyzing complex that processively depolymerizes the microtubule.

Introduction

The Kin I proteins are an unusual subfamily of kinesin-related proteins (Kim and Endow, 2000; Lawrence et al., 2002): in the presence of ATP they do not move along the surface of MTs (microtubules) like other motor proteins but instead bind to the ends of MTs and depolymerize them (Desai et al., 1999). The Kin I family includes mitotic centromere-associated kinesin (MCAK) from hamster (Wordeman and Mitchison, 1995), mKIF2 from mouse (Noda et al., 1995; Santama et al., 1998), XKCM1 from Xenopus (Walczak et al., 1996), and FKIF from fish (Bost-Usinger et al., 1997). Consistent with the depolymerization activity of Kin I kinesins observed in vitro, depletion or inhibition of XKCM1 increases the size of the mitotic spindle in egg extracts, presumably by increasing the length of the constituent MTs (Walczak et al., 1996; Tournebize et al., 2000). Also, inhibition of MCAK interferes with the poleward movement of chromosomes during anaphase A in CHO cells (Maney et al., 1998); again, this is consistent with MCAK depolymerizing MTs because chromosome movement during anaphase is associated with the shortening of the MTs that connect the chromosome (via the kinetochore at which MCAK is localized) to the pole. The finding that overexpression of MCAK causes loss of MTs in interphase as well as mitotic cells (Maney et al., 1998, 2001), together with the high levels of expression of FKIF2 and mKIF2 in nondividing cells in the retina and the brain, suggests that Kin I kinesins also regulate MT depolymerization outside of mitosis (Kinoshita et al., 2001).

The evidence that Kin I kinesins can depolymerize MTs in vitro is strong. Addition of purified, recombinant proteins to MTs that have been stabilized by taxol or the slowly hydrolyzed GTP analog GMP-CPP causes almost complete loss of polymer in the presence of ATP after 10–30 min (Desai et al., 1999; Maney et al., 2001; Niederstrasser et al., 2002; Moores et al., 2002). Furthermore, when MTs are incubated with Kin I kinesins in the presence of the nonhydrolyzable ATP analog AMP-PNP, electron micrographs reveal curled protofilament structures at their ends (GMP-CPP-stabilized MTs; Desai et al., 1999) or in solution (taxol-stabilized MTs; Moores et al., 2002). This again supports a depolymerizing activity because protofilament curls are intermediate structures in the depolymerization pathway (Mandelkow et al., 1991; Muller-Reichert et al., 1998).

In contrast to the evidence for depolymerization activity, the evidence that Kin I kinesins are motors that move along the MT surface is weak. An early report suggested that mKIF2 has directed motility (Noda et al., 1995), but we and others (Desai et al., 1999) have not been able to replicate this finding, and a critical examination of the experimental methods indicates that contamination by kinesin or another plus-end-directed motor cannot be ruled out.

With their unique functional and structural properties, Kin I kinesins may provide a window into the mechanism by which motor proteins in general regulate the depolymerization of MTs (Lombillo et al., 1995; Hunter and Wordeman, 2000). There are many open questions about how Kin I kinesins depolymerize MTs. How does a Kin I kinesin get to the end of a MT? Does it stay at the end of the MT while it removes tubulin dimers—is it processive? Or does it dissociate from the MT end with each tubulin dimer that it removes—is it nonprocessive? How is ATP hydrolysis coupled to the depolymerization reaction? Kin I proteins are classified as kinesins because they share the conserved motor domain that defines this superfamily of proteins. Because the motor domain of conventional kinesins is an ATPase, it is expected that Kin I kinesins are also ATPases. However, the ATPase activity of Kin I kinesins has not been measured, and there are reports that ATP hydrolysis is not necessary for the depolymerization activity (Moores et al., 2002). To address these questions, we have devel-
Results

Real-Time Microscopy Assay of MCAK-Mediated Microtubule Disassembly

The depolymerization activity of MCAK was quantified by comparing the rates of depolymerization of individual MTs in the presence and absence of purified MCAK using a microscopy assay. To circumvent the complexity introduced by MT dynamics, the switching of MTs between growing and shrinking phases (Mitchison and Kirschner, 1984), all the MTs used in this study were polymerized in the presence of the slowly hydrolyzed GTP analog GMP-CPP. Such MTs are very stable (Hyman et al., 1992; Caplow et al., 1994), and in the absence of MCAK they depolymerize at a negligible rate.

In the presence of a saturating concentration of MCAK (20 nM), MTs depolymerized at a rate of $0.880 \pm 0.015$ μm/min (mean ± SE, n = 8) (Figures 1A and 1B). In the absence of MCAK, MTs depolymerized at a rate of only $0.008 \pm 0.026$ μm/min (mean ± SE, n = 6) in agreement with previous measurements (0.003 to 0.02 μm/min; Hyman et al., 1992; Caplow et al., 1994) and with our turbidity results (0.013 ± 0.006 μm/min, mean ± SE, n = 8; see below). Thus, saturating MCAK accelerates the shrinkage rate of MTs 110-fold. In molecular terms, MCAK accelerates the rate at which tubulin dimers dissociate from the two ends of a MT from $0.23 \text{s}^{-1}$ to $26 \text{s}^{-1}$ (GMP-CPP MTs are composed of 14 protofilaments [Table 1] and therefore contain 1750 tubulin dimers per micron). By using the inhomogeneity of the fluorescence intensity along the length of the MT (the “speckles” of Waterman-Storer and Salmon, 1998) as reference points, we confirmed that MTs depolymerized from both ends. The rate of depolymerization at the faster end divided by that at the slower end was $1.56 \pm 0.18$ (mean ± SD, n = 5).

MCAK Is a Depolymerase

To measure the kinetic parameters associated with MCAK-mediated depolymerization, we used turbidity, the absorbance at 350 nm, to assay the amount of polymerized tubulin. Because MTs scatter light much more strongly than free tubulin dimers, the turbidity of a solution is expected to be proportional to the amount of polymer and independent of the length of the polymers, provided that their lengths are $>> \lambda/2\pi = 56$ nm ($\lambda$ is the wavelength of the light) (Berne, 1974). We confirmed that $\sim95\%$ of the MTs used in these experiments were longer than 600 nm before addition of MCAK by sedimentation in the black arrowhead. The small, slow increase in the turbidity is an artifact, perhaps due to the slow formation of non-MT oligomers.

(B) Length versus time traces of MTs depolymerized in the absence or presence of MCAK (20 nM). In the presence of MCAK, MTs depolymerized at a rate of $\sim1$ μm/min.

(C) Turbidity traces show the loss of 300 nM GMP-CPP-tubulin polymer over time in the absence (black) and presence (blue) of 2 nM MCAK. The addition of 5 mM CaCl$_2$ to the control reaction reduced the turbidity to the background level measured in the absence of MTs (dashed line) and caused complete depolymerization as confirmed by the spindown assay done at the time marked by the black arrowhead. The small, slow increase in the turbidity is an artifact, perhaps due to the slow formation of non-MT oligomers.

(D) The amount of tubulin polymer as a function of time. The addition of 4 nM MCAK caused a complete loss of polymer after 500 s (circles). The validity of turbidity as a measure of MT depolymerization was confirmed by the spindown assay (squares). The initial rate of depolymerization and the length distribution of the starting population of MTs (inset) were used to model the loss of polymer over time; the model curve (solid line) represents a MT shrinkage rate of 0.8 μm/min.
MCAK Has a High Affinity for Microtubule Ends

We used the turbidity assay to determine how much MCAK is needed to depolymerize MTs at the maximum rate. When increasing amounts of MCAK were added to a fixed concentration of MTs, the rate of depolymerization increased (Figure 2A), and at MCAK concentrations of \( \geq 2 \text{nM} \), depolymerization was complete within 600 s. To analyze these results, we plotted the initial rate of depolymerization against the MCAK concentration (Figure 2B). The initial rate was used to minimize the contribution from any possible repolymerization of tubulin dimers released during the reaction and also to minimize the effect of the turbidity artifact that occurs after 1000 s (Figure 1C). Because the concentration of MTs was known from the spindown assay, the initial rate of polymer loss could be expressed as the average shrinkage rate of individual MTs.

The initial rate of depolymerization depended on the MCAK concentration according to a sigmoidal curve. At low concentrations, the depolymerization rate increased approximately with the square of the MCAK concentration (Hill coefficient = 1.6, 1.9, and 2.6 for the three curves shown in Figure 2B). This indicates that there may be some cooperativity; for example, a few MCAKs per end may be required before any significant depolymerization occurs. At high MCAK concentrations, depolymerization increased to an extrapolated maximum shrinkage rate of \( 0.95 \pm 0.10 \text{ } \mu\text{m/min per MT} \), which corresponds to 28 tubulin dimers per second per MT. This is in close agreement with the depolymerization rate measured in the microscopy assay (Figure 1B).

The data shown in Figure 2A accord with the expectation that MCAK has its depolymerase activity at the ends of MTs, rather than on the lattice. If MCAK acted on the MT lattice then we would expect the depolymerization rate to saturate when the lattice tubulin was saturated, which would require 300 nM MCAK. Instead, depolymerization saturates at 4 nM MCAK. Furthermore, the half-maximal rate of depolymerization occurred at \( 1.6 \pm 0.2 \) nM MCAK, indicating that MCAK must have a high affinity for MT ends.

Table 1. Depolymerase Activity of MCAK

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum shrinkage rate per MT+</td>
<td></td>
<td>0.95 \pm 0.1 \mu m/min</td>
</tr>
<tr>
<td>Maximum tubulin off rate per MT+</td>
<td></td>
<td>28 \pm 3 s^{-1}</td>
</tr>
<tr>
<td>Protofilaments per MT+</td>
<td></td>
<td>14.0 \pm 0.2</td>
</tr>
<tr>
<td>Maximum MCAKs per protofilament end</td>
<td>N/(14 x 2)</td>
<td>0.86 \pm 0.14</td>
</tr>
<tr>
<td>Maximum tubulin off rate per protofilament end</td>
<td>( k_{cat} )</td>
<td>1.0 \pm 0.2 s^{-1}</td>
</tr>
<tr>
<td>Affinity for protofilament end</td>
<td>K</td>
<td>1.0 \pm 0.2 nM</td>
</tr>
<tr>
<td>Association rate to protofilament end</td>
<td>( k_{on} )</td>
<td>54 \pm 9 \mu M^{-1} s^{-1}</td>
</tr>
<tr>
<td>Dissociation rate from protofilament end</td>
<td>( k_{off} )</td>
<td>0.054 \pm 0.013 s^{-1}</td>
</tr>
</tbody>
</table>

Temperature: 23 \pm 1°C.

From the turbidity experiments.

From Hyman et al., 1995.

MCAK Has One High-Affinity Binding Site per Protofilament End

To estimate the number of MCAK binding sites per MT end and the binding affinity, we considered a model in which MCAK [M] binds to sites at the ends of MTs [P] where they form catalytically active complexes [MP] that remove tubulin dimers [Tb] from the end with a rate constant \( k_{cat} \). We write

\[
M + P \xrightleftharpoons[k_{off}]{k_{on}} MP
\]

\[
K = \frac{k_{on}}{k_{off}} = \frac{[MP]}{[P]}
\]

\[
\frac{d[Tb]}{dt} = k_{cat}[MP].
\]
when it dissociates. For example, MCAK could dissociate from the MT in a complex with a tubulin dimer or it could change its nucleotide state after binding to the MT. In what follows, and in the supplemental data at http://www.molecule.org/cgi/content/full/11/2/445/DC1 where the model is solved, we assume only that the association-dissociation reaction is at steady state (i.e., that the fraction of free and bound MCAKs does not change with time).

According to the model, the half-maximal depolymerization rate occurs when the total concentration of MCAK (free and bound) is

\[ M_{1/2} = \frac{1}{2} P_0 + K = \frac{1}{2} N \cdot [M] + K \]  

(see the supplemental data at http://www.molecule.org/cgi/content/full/11/2/445/DC1). \( P_0 \) is the total number of binding sites (free and occupied), \([M]\) is the MT concentration, and \( N \) is the number of MCAK binding sites per MT. This equation makes intuitive sense. If the affinity is very high \( (K \sim 0) \), then the half-maximal rate occurs when there is enough MCAK to fill half the sites. Conversely, if the affinity is very low \( (K >> P_0) \), then the half-maximal depolymerization rate occurs when the MCAK concentration is equal to the constant \( K \).

Depolymerization data were obtained at two other MT concentrations: 0.033 and 0.50 nM (Figure 2B). The half-maximal depolymerization rates occurred at 1.8 ± 0.2 nM and 8.1 ± 0.9 nM MCAK, respectively. Fitting Equation 2 to a plot of the MCAK concentration required for half-maximal depolymerization versus the MT concentration (Figure 2B, inset) gave a correlation coefficient of \( r = 0.93 \) when the data were weighted by their uncertainties and yields \( K = 1.0 \pm 0.2 \) nM and \( N = 24 \pm 4 \). The latter number corresponds to 12 ± 2 binding sites per MT end. Because the MTs used in this study have 14 protofilaments, the number of binding sites corresponds to approximately one MCAK per protofilament end \((12 \div 14)\). Assuming one MCAK per protofilament end, the maximum catalytic activity of MCAK, \( k_{cat} \), is the maximum rate at which one MCAK dimer can remove one tubulin dimer from the end of a MT, \( 1 \text{ s}^{-1} (=28 \text{ tubulin dimers per MT per second} \div 28 \text{ protofilament ends per MT}; \text{Table 1}) \). The low level of cooperativity—as few as two MCAKs at an end are sufficient for depolymerization—indicates that MCAK can probably switch between protofilaments.

From this analysis, we also conclude that the affinity of MCAK for the tubulin dimers in the lattice must be very low. If MCAK had even a modest affinity for the surface of a MT, then the lattice would act as a sink because the tubulin dimers in the lattice outnumber those at the ends \( \sim 100:1 \). Thus the dissociation constant for tubulin in the lattice must be \( \geq 100 \) nM \((100 \times 1 \text{ nM})\).

**MCAK Finds Microtubule Ends Very Rapidly**

To measure how quickly MCAK targets MT ends, we used a stopped flow apparatus to measure the association rate

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Figure 2. Kinetics of MCAK-Mediated Microtubule Depolymerization

(A) Turbidity traces show the loss of 300 nM GMP-CPP-tubulin polymer (0.086 nM MTs) over time in the presence of various amounts of MCAK.

(B) Initial rates of depolymerization measured by turbidity or light scattering as a function of MCAK concentration at three different MT concentrations (\( \square \), 0.033 nM; \( \bullet \), 0.086 nM from the data in [A]; and \( \square \), 0.50 nM). The solid curves correspond to the Hill equation

\[ f(x) = \frac{V_{max} \cdot x^n}{K_{diss} + x^n} \]

where \( V_{max} \) is the maximum value, \( K_{diss} \) is the value at which a half-maximum value is obtained, and \( n \) is the Hill coefficient \((n = 1.6, \square; 2.6, \bullet; 1.9, \square)\). The open circles (○) correspond to the depolymerization rate measured in the microscopy assay (MT concentration = 0.01 nM). The inset graph shows the MCAK concentration required for a half-maximal depolymerization rate plotted against the MT number concentration. The line is the fit to Equation 2 and corresponds to there being 24 binding sites per MT, \( K = 1 \) nM.

(C) The initial rate of depolymerization measured by stopped flow. MCAK (at various concentrations) and MTs (800 nM GMP-CPP-tubulin polymer) were rapidly mixed, and the amount of polymer was assayed by the intensity of scattered light. The black curves correspond to the processive model (see the supplemental data at http://www.molecule.org/cgi/content/full/11/2/445/DC1).
constant $k_{on}$. If the total MCAK concentration ($M_0$) is in excess of the binding constant ($i.e., K$) and the total concentration of binding sites ($P_0$) then the delay, $\Delta t$, between the addition of MCAK and the attainment of the steady-state depolymerization rate is

$$\Delta t \approx \frac{k_{on} \cdot (M_0 - P_0)}{M_0} \quad M_0 >> K \quad (3)$$

(see the supplemental data at http://www.molecule.org/cgi/content/full/11/2/445/DC1). To measure the delay, MCAK and ATP in one syringe were rapidly mixed with MTs in the other, and the depolymerization was monitored by the intensity of light scattered 90° in the mixing chamber. The onset of depolymerization is very rapid, with delays of only a few seconds at the highest MCAK concentration (Figure 3C). The delay was measured by extrapolating back the steady-state depolymerization to where it intercepts the initial polymer concentration. Six measurements of the delay at MCAK concentrations $\approx 5$ nM obtained in three independent experiments gave a second order association rate of MCAK binding to protofilament ends of $k_{on} = 54 \pm 9 \times 10^{6} \text{ M}^{-1} \text{s}^{-1}$ (mean ± SE).

The Catalytic Activity of MCAK Requires ATP Hydrolysis

The experiments described so far were all performed in the presence of ATP. However, the hydrolysis of ATP is not required for MCAK to disassemble GMP-CPP-stabilized MTs. When 315 nM tubulin polymer was mixed with 150 nM MCAK in the presence of 2.5 mM of the nonhydrolyzable ATP analog AMP-PNP, there was 60% disassembly after 10 min and 70% disassembly after 50 min. Given that in the absence of MCAK, GMP-CPP MTs depolymerize <10% after 10 min (Figure 2A), we can positively conclude that MCAK accelerates depolymerization, as opposed to inhibiting polymerization. This confirms the finding of Moores et al. (2002) showing that the motor domain of the Kin I from P. falciparum can disassemble taxol-stabilized MTs in the presence of AMP-PNP.

ATP hydrolysis, however, is necessary for catalytic depolymerization. When 315 nM tubulin polymer was mixed with 25 nM MCAK in the absence of ATP, there was <10% disassembly after 10 min, as measured by the spindown assay. By contrast, 20 nM MCAK was sufficient to completely depolymerize 800 nM GMP-CPP-tubulin polymer within 1 min in the presence of 2 mM ATP as determined by turbidity assays (data not shown).

MCAK Has a Microtubule-Stimulated ATPase Activity

Given that ATP hydrolysis is necessary for MCAK’s enzyme activity, we measured the rate at which MCAK hydrolyzes ATP to its products ADP and inorganic phosphate (P). Following the addition of 2 nM MCAK to 300 nM of GMP-CPP-tubulin polymer in 250 μM ATP, we observed a fast initial rate of P release (~6 nM·s⁻¹) of duration ~1000 s, followed a slower steady-state rate of P release (~0.13 nM·s⁻¹) (Figure 3A). The time course of the initial phase closely matched the time course of depolymerization (Figure 3B); when appropriately scaled, the amount of phosphate released from ATP (large squares) could be superimposed on the amount of tubulin released from MTs. A spindown assay confirmed that depolymerization was >90% complete after 600 s and >99% complete after 3000 s. Because the initial phase depended on there being MTs present, we believe that it corresponds to MT-stimulated ATPase activity. This is further supported by the observation that when the concentration of polymerized tubulin was increased, the ATPase rate also increased (Figure 3C).
MCAK Has an ATPase Activity that Is Stimulated by Free Tubulin Dimers

We propose that the second, slower phase of P_i release seen in Figure 3A is due to an ATPase activity stimulated by free tubulin dimers. This is based on the following observations. (1) There are no MTs present during the slow phase, so the ATPase cannot be due to lattice or ends. (2) The slow phase is not due to MCAK’s basal ATPase activity. In the absence of any tubulin, the ATPase is 0.014 ± 0.003 s⁻¹ per MCAK (mean ± SE, n = 2), too low to account for the slow phase (Figure 3A). (3) In the presence of 300 nM GDP-CPP tubulin (maintained as free dimer by the addition of 5 mM CaCl₂) and 25 nM MCAK, the ATPase rate was 0.052 ± 0.004 s⁻¹ per MCAK (mean ± SE, n = 2), similar to the slow phase of the ATPase seen in depolymerization reactions after the loss of 300 nM GDP-CPP polymer was complete (0.06 s⁻¹ per MCAK, Figure 3A). (4) We measured the ATPase rate of MCAK over a wide range of GDP-tubulin concentrations (Figure 3D). The maximum GDP-tubulin-stimulated ATPase rate was 0.15 ± 0.01 s⁻¹ per MCAK, with a half-maximum ATPase at 490 ± 150 nM tubulin. The ATPase at 300 nM GDP-tubulin (0.056 s⁻¹ per MCAK) was similar to that observed during the slow phase (Figure 3A).

These results (Table 2) show that MCAK has an ATPase activity that is stimulated by free tubulin. This is another property that distinguishes Kin I kinesins from other members of the kinesin family. Interestingly, the tubulin-activated ATPase does not depend on whether the tubulin dimer has GDP or GMP-CPP in its active site (compare [3] and [4]).

MCAK Has an End-Stimulated ATPase Activity

The ATPase activity of MCAK could be stimulated by the MT lattice or it could be stimulated by the MT ends. Or it could be stimulated by both. In order to distinguish between these possibilities, we did two additional sets of experiments.

First, we measured the ATPase rates at various MCAK concentrations but at a fixed tubulin polymer concentration. If the ATPase is stimulated by MT ends, then the specific ATPase activity (ATPase rate per MCAK) should fall when the MCAK concentration exceeds the concentration of end binding sites. If the ATPase is stimulated by the MT lattice, then the specific ATPase activity should increase until the MCAK concentration exceeds the concentration of lattice tubulin.

We found that as the MCAK concentration was increased, both the initial and steady-state rates of P_i release increased (Figure 4A). However, the MT-stimulated ATPase rate (the initial rate of P_i release divided by the MCAK concentration) had a maximum of ~3 s⁻¹ per MCAK at ~3 nM MCAK and then decreased to ~1 s⁻¹ per MCAK at higher MCAK concentrations (Figure 4B). We interpret the ATPase rate at the higher MCAK concentrations as being due to a small lattice-stimulated ATPase (see below). However, the peak at ~3 nM MCAK cannot be accounted for by a lattice-stimulated ATPase because it occurs at an MCAK concentration that is much less than the concentration of lattice tubulin (300 nM). Instead, we attribute this predominant component of the ATPase to an end-stimulated activity. Because the peak occurs at an MCAK concentration similar to that of protofilament ends (2.4 nM = MT concentration × 14 protofilaments per MT × 2 ends per protofilament), the end-stimulated ATPase is consistent with there being one high-affinity binding site per protofilament end which, when filled with MCAK, has the ATPase activity.

In the second experiment, we increased the concentration of MT ends, without changing the total concentration of tubulin polymer, by shearing the MT solution. If MCAK has an end-stimulated ATPase, the sheared MTs should lead to a higher ATPase rate than the un-sheared MTs. The result accorded with this expectation (Figure 4C). In the presence of unsheared GMP-CPP MTs, the initial hydrolysis rate was 1.38 ± 0.18 s⁻¹ (mean ± SE, n = 4) per MCAK; after shearing, the initial rate increased to 2.92 ± 0.22 s⁻¹ (mean ± SE, n = 4) per MCAK. Electron microscopy showed that shearing increased the concentration of MT ends approximately 3-fold from 0.22 nM to 0.65 nM (Figure 4C, inset). The ~2-fold increase in the ATPase is somewhat larger than expected given the MCAK concentration was 2.5 nM; if K_i = 1 nM for the high-affinity sites, then a 43% increase is expected. However, we do not consider this as being an important discrepancy because the exact time course of the hydrolysis curve depends on the length distribution of MTs which is different for the unsheared and sheared solutions. Furthermore, shearing may create more ragged ends that contain more MCAK binding sites.

Together, these experiments show that MCAK has an end-stimulated ATPase activity. However, they do not exclude a lattice-stimulated activity.

MCAK Hydrolyzes Multiple ATPs for Each Tubulin Dimer Removed from the Polymer

Surprisingly, we found that MCAK hydrolyzes ~5 ATP for each tubulin dimer that it removes from a GMP-CPP MT. A stoichiometry of five is evident from the scaling in Figure 3B that was necessary to superimpose the P_i and tubulin dimer release curves. It can also be obtained by dividing the initial rate of phosphate release (during which time there is little tubulin released and therefore little dimer-activated ATPase) by the initial rate of tubulin release: 6 nM·s⁻¹ divided by 1.2 nM·s⁻¹ equals 5 ATP hydrolyzed per tubulin dimer released (Figure 3B). The stoichiometry can also be estimated by simply dividing
Depolymerase and ATPase Activities of MCAK

Figure 4. The Predominant ATPase Activity of MCAK Is Stimulated by Microtubule Ends

(A) Time course of Pi release in reactions with 300 nM GMP-CPP-tubulin polymer (0.086 nM MTs) at various MCAK concentrations. The curves (dotted lines) correspond to a model in which the Pi rise is due to the sum of a MT-stimulated ATPase (which decays as the MTs depolymerize) and a tubulin-stimulated ATPase (which rises and then plateaus as the tubulin is released).

(B) The initial rate of Pi release as a function of MCAK concentration. The data are fit to a model in which there are 3 nM of high-affinity binding sites ($K_{H} = 1$ nM), which hydrolyze ATP at a rate of 8 s$^{-1}$, and a low-affinity binding site with a hydrolysis rate of 0.7 s$^{-1}$. The high-affinity binding has a Hill coefficient of 2, indicating cooperativity.

(C) End-stimulated ATPase activity. A comparison of initial ATPase rates during depolymerization of unsheared (red circles) versus sheared (green triangles) GMP-CPP MTs. Both reactions contained 630 nM tubulin polymer and 2.5 nM MCAK. EM analysis of MT lengths before and after shearing (inset) showed that shearing decreased the average lengths of the MTs from 3.1 to 1.2 μm.

(D) Stoichiometry of ATP hydrolyzed per tubulin dimer released as a function of MCAK concentration. The stoichiometry was a minimum of approximately five when the number of MCAKs per MT end was ≤15 MCAK dimers (dashed lines). Reactions contained 300 nM GMP-CPP-tubulin polymer and were performed in BRB80 supplemented with 75 mM KCl, except for the reaction at 25 nM MCAK (square) which was supplemented with 150 mM KCl.

The total number of P$_i$ released (during the depolymerization phase) by the total number of tubulin dimers removed. Because the latter estimate is independent of the MCAK concentration, the stoichiometry is a robust measurement.

The requirement of five ATPs to remove one tubulin dimer was not expected. To make sure that this high stoichiometry was not due to the particular conditions used in Figure 3A, we measured the amount of ATP hydrolyzed during the depolymerization of a fixed amount of GMP-CPP-tubulin polymer over a wide range of MCAK concentrations (Figure 4D). Quantitative spin-downs performed during steady-state hydrolysis confirmed that depolymerization was complete in all reactions (≤5% polymer remaining after 3000 s) except at the lowest MCAK concentration (at 1 nM, 25% of the polymer remained at 3000 s). At the highest concentration of MCAK tested (25 nM), the stoichiometry during the depolymerization phase was 30 ATP hydrolyzed per tubulin dimer removed (Figure 4D). However, at lower MCAK concentrations the stoichiometry remained constant at five (Figure 4D).

The stoichiometry of five is not due to additional rounds of ATP hydrolysis associated with removing
GMP-CPP dimers that reassociated with the MTs after their initial removal. When such reassociation was blocked by adding 20 μM GDP, a condition in which polymerization is strongly inhibited because GDP displaces GMP-CPP from the free dimer (Caplow et al., 1994), a high stoichiometry was still observed (data not shown).

Combining the maximum tubulin dimer dissociation rate of 1 s⁻¹ per MCAK with the stoichiometry of five ATPs hydrolyzed per tubulin removed, we deduce a maximum GMP-CPP-MT stimulated ATPase rate of 5 s⁻¹ per MCAK (Table 2). This is similar to the maximum ATPase rate of 3.4 s⁻¹ estimated from the data in Figure 3C. Because the former estimate of the maximum ATPase rate is independent of the MCAK concentration, the agreement with the latter estimate confirms that the MCAK concentration was correctly measured.

Effect of Ionic Strength on the ATPase and Depolymerase Activities of MCAK

In addition to an end-stimulated ATPase activity, MCAK has an additional MT-stimulated ATPase that we attribute to stimulation by the MT lattice. This ATPase is observed when the MCAK concentration is in excess of the concentration of end binding sites (Figures 4A and 4B). When the lattice-stimulated ATPase is high, the stoichiometry is high (>5) showing that this ATPase is not tightly coupled to depolymerization (Figure 4D). The lattice-stimulated ATPase rate can be estimated from the data in Figures 4A and 4B; at 25 nM MCAK the ATPase rate is 1.5 s⁻¹ and if the component due to the end-stimulated ATPase is subtracted, we obtain a value of 1 s⁻¹ per MCAK for the lattice-stimulated ATPase.

Adding salt decreased the lattice-stimulated ATPase. In buffer that is optimal for depolymerase activity (BRB80 plus 75 mM KCl), 25 nM MCAK has a large lattice-stimulated ATPase (Figure 4A) and the stoichiometry is equal to 30 (Figure 4D). However, when the salt was increased (BRB80 plus 150 mM KCl), the amplitude of the initial phase of phosphate release decreased to a level that was indistinguishable from the corresponding curve measured at 2.5 mM MCAK. As a consequence, the stoichiometry decreased from 30 to 6 (Figure 4D) indicating that the additional 75 mM KCl almost completely abolished the lattice-stimulated ATPase. Conversely, decreasing the amount of added salt (BRB80 plus 6 mM KCl) increased the lattice-stimulated ATPase even further, and a stoichiometry of 60 was obtained (data not shown). Very high levels of added salt (BRB80 plus 225 mM KCl) inhibited both the ATPase and the rate of depolymerization (data not shown).

Interaction of MCAK with the Microtubule Lattice

The effects of salt suggested that MCAK interacts electrostatically with the MT lattice and that this interaction modulates both the ATPase and depolymerase activities. We therefore looked for a direct interaction of MCAK with the MT lattice in motility assays. We found that MTs bound to MCAK-coated surfaces in the presence of ATP and underwent a small back-and-forth motion without apparent directionality (Figure 5A). The mean-square displacement increased in proportion to time, consistent with the movement being due to a randomly directed process (Figure 5B). This type of motion has been observed for flagellar dynein (Vale et al., 1989) and for the kinesin-related proteins Ncd (Chandra et al., 1993) and KIF1A (Okada and Hirokawa, 1999), and has been called one-dimensional diffusion. It is one-dimensional in the sense that the diffusion coefficient along the axis of the MT \(D_{\parallel}\) is larger than the diffusion coefficient perpendicular to the axis \(D_{\perp}\); the MT in Figure 5B had \(D_{\parallel} = 7000 \text{ nm}^2 \cdot \text{s}^{-1}\) and \(D_{\perp} = 20 \text{ nm}^2 \cdot \text{s}^{-1}\). The axial diffusion coefficient corresponds to a step between tubulin
dimers every ~5 ms (= (8 nm)^2/2D). Note that if a MT
were constrained to diffuse in a plane parallel to a sur-
face the axial diffusion coefficient would only be twice
the orthogonal diffusion coefficient (Howard, 2001). The
finding that the ratio \(D_x/D_\perp > 2\) indicates that MCAK
constrains the perpendicular motion more than the par-
allel motion. One-dimensional diffusion was also seen
in the presence of ADP but never in the absence of
nucleotides.

Discussion

Kin I Kinesins Have One High-Affinity Binding Site
per Protofilament End

Earlier work in vitro using fluorescence microscopy
showed that Kin I kinesins bind to the ends of MTs in
AMP-PNP (Desai et al., 1999; our unpublished data). The
present work extends these observations by measuring the
affinity and the number of binding sites per MT end
in ATP. The saturation of the depolymerization rate indi-
cates that the binding sites are high-affinity ones (\(K =
1\) nM in ATP) and that there are approximately 28 per
MT. The ATPase measurements provide additional sup-
port for this number of MCAK binding sites per MT;
the ATPase rate peaks when there is ~1 MCAK per
protofilament end (Figure 4B), and there is a constant
ratio of ATPs hydrolyzed per tubulin released provided
that the MCAK concentration does not exceed the con-
centration of protofilament ends (Figure 4D). If the MCAK
binding sites are evenly distributed between the two
ends of the MTs and between the protofilaments, then
there is one binding site at the end of each protofilament.
We propose that this site, when filled by MCAK, has
both the depolymerase and ATPase activities.

At physiological MCAK concentrations (10–100
nM \(> \cdot 1\) nM; L.W., unpublished data), the binding sites
will be occupied, and each MT end is expected to have a
complex of 12±14 MCAK dimers at its end (depending on
the number of protofilaments). The failure to detect
MCAK at the ends of cytoplasmic MTs (Wordeman and
Mitchison, 1995) might be explained by the lack of sensi-
tivity of conventional fluorescence microscopy. On the
other hand, MCAK has been localized to the spindle poles
and the midbodies in mitotic cells (Maney et al., 1998),
both of which contain high densities of MT ends.

Evidence that MCAK Is a Processive Enzyme

Knowledge of the on-rate (\(k_{on} = 0.054 \) nM\(^{-1}\)s\(^{-1}\)
measured by stopped flow) and the affinity (\(K = 1\) nM
measured from the half-saturation of the depolymeriza-
tion rate) allows us to estimate the off-rate, \(k_{off}\), of MCAK
from the protofilament end using Equation 1. This yields
\(k_{off} = 0.054 \pm 0.013\) s\(^{-1}\) (the 95% confidence range
is 0.027 to 0.10 s\(^{-1}\), when the errors are propagated
multiplicatively). Because the MCAK off-rate is one nine-
tenth the tubulin dimer off-rate from the protofilament
end (1 s\(^{-1}\)), we can rule out the possibility that MCAK
dissociates from the ends of the MT each time it removes
a tubulin dimer. Instead, MCAK removes on average 20
dimers before it dissociates from an end (>10 dimers
at the 97.5% confidence level). This implies that MCAK
is processive like conventional kinesin (Howard et al.,
1989).

Under physiological conditions, MTs can shorten at
rates up to 20 \(\mu\)m/min, much faster than the rates re-
ported here. But such rapid shortening is thought to be
triggered by loss of a stable cap of GTP-tubulin dimers,
which exposes the underlying unstable GDP-tubulin di-
mers (Mitchison and Kirschner, 1984; Walker et al.,
1988). Because MCAK can depolymerize a 1 \(\mu\)m long
GMP-CPP MT (analogous to a GTP cap) in 1 min,
MCAK’s depolymerase activity is high enough to ac-
count for the measured rates of catastrophe in cells (1–2
min\(^{-1}\); Tournebize et al., 2000; Kinoshita et al., 2001).
Furthermore, the high association rate of MCAK to pro-
to filament ends (Table 1) ensures that MCAK will reach
the cap quickly, within 0.2 to 2 s given the measured
on-rate and assuming a cellular MCAK concentration
of 10 to 100 nM.

Assuming an off-rate of 0.05 s\(^{-1}\), MCAK is expected
to remain bound to the end of a MT during the depoly-
merization of a GTP cap up to 160 nm in length. The
size of the GTP cap is not known; it is no larger than 1
\(\mu\)m (Walker et al., 1991) and could be as small as one
layer of GTP-tubulin dimers (Caplow and Shanks, 1996;
Drechsel and Kirschner, 1994). A shorter cap will take
less time to depolymerize, and if the GTP cap of a dy-
namic MT is only one or two dimers long, then it is not
clear what advantage there is to MCAK being processive
because, after uncapping a dynamic MT, MCAK may
be released from the end as the MT transits to rapid
shortening. However, it is possible that the high-affinity
interaction that manifests itself in our experiments as
processivity may serve a different function in cells. The
attachment of MCAK to the centromere is mediated via
nonmotor domains (Maney et al., 1998). Assuming that
the binding of MCAK to protofilament ends is mediated
by the motor domain, then the point of the multiple,
high-affinity interactions between the MCAK complex
and the MT end may be to create a link to the kinetocho-
there that is both dynamic and robust; i.e., the connection
remains intact even when tubulin dimers dissociate from
the end and when the whole structure is under tension
(Nicklas, 1988; Coue et al., 1991). One interpretation of
the similarity of the MCAK-mediated rate of depolymeri-
ization to the rate of chromosome movement during ana-
phase A (Skibbens et al., 1993) is that the MCAK complex
is regulating the MT shortening speed.

Rapid Targeting of MCAK to Microtubule Ends

The targeting of MCAK to the ends of MTs is extraordi-
narily rapid. The on-rate to protofilament ends, \(5 \times 10^6
\) M\(^{-1}\)s\(^{-1}\), far exceeds the fastest protein-protein interac-
tions. It is 20 times the on-rate for kinesin to the MT
lattice (Hackney, 1995), and it corresponds to an on-
rate of \(760 \times 10^6\) M\(^{-1}\)s\(^{-1}\) for MCAK binding to MT ends,
over 100 times the rate at which tubulin dimers bind to
MT ends during polymerization (2 to 5 \(\times 10^6\) M\(^{-1}\)s\(^{-1}\),
Drechsel et al., 1992; Caplow et al., 1994). This argues
strongly against a targeting mechanism based solely on
the direct diffusion of MCAK to MT ends. How, then,
does MCAK reach the ends so quickly?

One obvious possibility is that MCAK finds the ends
of MTs by walking along the lattice. However, our ATPase
measurements provide positive evidence against MCAK
being a motor protein in the traditional sense. MCAK
must move an average distance of 1 μm from its initial binding site on the lattice to get to the end of a 2 μm long MT. If translocation occurred via directed motility, then MCAK would have to take 125 eight nanometer steps to reach the end of the MT, and if MCAK were like kinesin, which uses one ATP per step (Goy et al., 1999), this would cost 125 ATPs. While at the end, MCAK removes some 20 tubulin dimers before dissociating. If the coupling of hydrolysis to tubulin removal was 1:1, then a further 20 ATP would be consumed. The total cost would be 145 ATPs for 20 tubulin dimers or seven ATPs per tubulin dimer removed. Because the maximum dimer off-rate is 1 s⁻¹, the maximum ATPase rate would be ~7 s⁻¹ (assuming that it takes only a few seconds for the MCAK to motor to the end of the MT). This calculation shows that the stoichiometry and the ATP rate are not inconsistent with motor activity. Instead, it is the shearing experiment that provides the strong argument against MCAK being a motor. After shearing, the MTs are shorter and therefore it should take less, not more, ATP for a motor to reach the end. If MCAK were a motor, we would expect a 2.5-fold decrease in the total ATP hydrolyzed when the MTs are reduced in length from 3 to 1 μm; instead, the total Pᵢ released increases (Figure 4C).

MCAK May Target Microtubule Ends by Diffusion along the Microtubule Lattice

We propose that MCAK rapidly targets MT ends by using a two-stage mechanism: first MCAK binds to the MT lattice, and then it diffuses along the lattice before being captured at a protofilament end (Figure 6A). This proposal is based on three lines of evidence. (1) Direct microscopic observations show that MTs undergo one-dimensional diffusion on surfaces coated with MCAK. (2) MCAK has a weak electrostatic interaction with the MT lattice as evidenced by the salt-dependent lattice-stimulated ATPase. (3) Kin I kinesins interact with the glutamate-rich C-terminal region of tubulin known as the E-hook (Moores et al., 2002; Niederstrasser et al., 2002), perhaps via a positively charged domain near the motor domain (Ovechkina et al., 2002). A further argument in favor of a diffusional mechanism is that, because diffusion is not a directed process, it is equally effective in targeting the molecule to either end of the MT. Such a targeting mechanism is analogous to that used by some transcription factors (Berg et al., 1981) and restriction enzymes (Stanford et al., 2000) to find their target sites on DNA, in which case the diffusive interaction occurs via a weak electrostatic interaction between the protein and the filament (Spolar and Record, 1994).

The diffusion coefficient of 7000 nm²·s⁻¹ measured in Figure 5 can quantitatively account for the high on-rate. If MCAK remains weakly bound to the lattice for 1 s during a diffusive encounter with the MT, then it will cover an area of ~2800 nm² (assuming that MCAK can switch protofilaments), and so will visit ~70 tubulin dimers. This significantly increases the probability of finding a protofilament end. The acceleration of the targeting rate would be even greater if the single-molecule diffusion coefficient is greater than 7000 nm²·s⁻¹, which is likely because the MT in Figure 5 was held by several MCAK molecules.

The diffusive movement of MCAK between tubulin dimers on the MT lattice cannot be coupled to ATP hydrolysis. If it were, the ATPase rate would be much higher than observed because, on average, MCAK samples a new tubulin dimer every few milliseconds and this would lead to an ATPase rate of >100 s⁻¹. Instead, the small lattice-activated ATPase rate of 1 s⁻¹ may be linked to the dissociation of MCAK from the lattice which terminates the diffusive encounter.

A corollary of our lattice-diffusion model is that any-
thing that impedes the interaction of MCAK with the MT will decrease the affinity of MCAK for ends and will therefore inhibit MCAK depolymerase activity. This could explain the inhibition of depolymerization by high salt; if salt disrupts the electrostatic interaction between the motor and the lattice, it will reduce the time that MCAK spends in the diffusive stage and therefore lower the rate of end targeting. Likewise, if the E-hook of tubulin is necessary to maintain contact between MCAK and the MT lattice during diffusion, then its removal by proteolysis would inhibit end targeting and lead to inhibition of depolymerization (Moores et al., 2002; Niederstrasser et al., 2002).

Where Is the ATP Hydrolyzed?

Desai et al. (1999) proposed that ATP binds to a Kin I kinesin when it is at the end of the MT but that the actual hydrolysis and completion of the cycle takes place in solution after the Kin I-tubulin dimer complex has dissociated from the MT. However, the following observations indicate that the hydrolysis actually takes place at MT ends. First, MCAK dissociates from the ends of MTs at a rate of 0.05 s⁻¹. Because this is much slower than the maximum end-stimulated ATPase rate of 4 s⁻¹ (Table 2), MCAK hydrolyzes ~80 ATPs while at the end of the MT. By analogy to conventional kinesin, MCAK is chemically processive. And second, the high stoichiometry of five cannot be due to multiple ATP hydrolysis cycles taking place on the release tubulin dimer. If this were the case, we would expect that high concentrations of tubulin should maximally activate the ATPase. But, the maximum tubulin-activated ATPase rate is only 0.15 s⁻¹, much lower than the maximum MT-activated ATPase rate of 5 s⁻¹.

Though we do not agree with the Desai et al. (1999) model, our results are quite consistent with their finding that the Kin I kinesin, XKIF2, forms a stable complex with tubulin dimer in the presence of AMP-PNP (Desai et al., 1999). The fact that MCAK has a tubulin-stimulated ATPase implies that MCAK physically associates with tubulin dimers, and the Kᵦ of 0.5 μM tubulin is sufficiently low to account for their observations that were made in 10 μM tubulin. The failure of Desai et al. to detect a complex in the presence of ATP can be reconciled with our results if ATP hydrolysis and dissociation of the Kin I-tubulin complex is rapid (>0.15 s⁻¹).

A Crossbridge Model for Kin I Kinesins

Our data suggest that MCAK interacts with the MT lattice and ends as shown in Figure 6A. We propose that these interactions are coupled to the ATP hydrolysis cycle according to the model shown in Figure 6B. First, we postulate that Kin I kinesins, like other kinesins, release ADP upon binding to the MT. Though there is no direct evidence for this, it is supported by the observation that MCAK binds uniformly to the MT lattice, and not preferentially at the ends, in the absence of nucleotides (Maney et al., 1998; Wordeman et al., 1999; our unpublished data). If MCAK diffused to ends in the ADP state and released its ADP at ends, then we would expect to see end binding in the absence of nucleotide.

We postulate that diffusion on the lattice occurs either in the ATP state or the ADP-Pᵦ state. The preferential binding of MCAK to MT ends in the presence of AMP-PNP (Desai et al., 1999; our unpublished data) means that Kin I kinesins bind weakly to the MT lattice in the ATP state (in which AMP-PNP traps the enzyme); otherwise, if the ATP state were a strongly bound state, then AMP-PNP-MCAK would uniformly decorate the lattice as is the case for conventional kinesin (Crevel et al., 1996). Weak binding in the ATP state is another feature that distinguishes MCAK from all other kinesins studied so far (Crevel et al., 1996). One possibility is that the electrostatic interaction between the E-hook and positive charges in the neck of the motor domain provides the tether during lattice diffusion (Ovechkina and Wordeman, 2002).

To complete the cycle, we postulate that the dissociation of the terminal tubulin dimer is triggered by either ATP hydrolysis or Pᵦ release. This is consistent with the observation that in the presence of AMP-PNP Kin I kinesins stabilize protofilament peels and rings (Desai et al., 1999; Moores et al., 2002; Niederstrasser et al., 2002) but do not remove individual tubulin dimers from the protofilament. The disassembly observed in AMP-PNP is therefore different from the true depolymerization that is observed in ATP. Thus, in the ATP state, Kin I kinesins bind to the bent conformation of tubulin and form a stable complex. Only after hydrolysis does the terminal tubulin dimer separate from the MT. One possibility is that binding in the ADP-Pᵦ state strains even more the link between the ultimate and penultimate tubulin dimers. After dislodging the terminal tubulin, the Kin I protein remains attached to the MT, perhaps through an electrostatic interaction. Alternatively, the tether could be the second head of a dimer, and Kin I kinesins may operate by a hand-over-hand mechanism analogous to conventional kinesin (Schief and Howard, 2001).

The simplest explanation for the stoichiometry of five ATPs hydrolyzed per GMP-CPP-tubulin removed is that MCAK slips; if it releases Pᵦ before the tubulin dimer has dissociated, then MCAK finds itself still bound to the MT in the ADP state. The ADP would be rapidly released, ATP would bind and be hydrolyzed, and a futile round of hydrolysis would have occurred. In this view, the low efficiency is due to a kinetic partitioning of the cycle; only 20% of the hydrolysis cycles are productive. MCAK is more efficient at removing taxol-bound dimers than GMP-CPP dimers; the minimum stoichiometry measured for taxol-stabilized MTs was only two (data not shown), as might be expected from the lower stability of taxol-MTs over GMP-CPP MTs. It is even possible that MCAK requires only one ATP to remove a GTP-dimer from the end of a dynamic MT.

Our proposed ATP hydrolysis cycle is meant to serve as a working model that illustrates how a hydrolysis scheme that drives a motor might be turned into one that drives a depolymerase.

Experimental Procedures

Protein Purification

Recombinant Histidine-tagged MCAK was expressed in SF9 cells (Pharmingen, San Diego, CA) and purified on a Ni-NTA-agarose column (Qiagen Inc., Chatsworth, CA) according to Maney et al. (1998). Because MCAK is a dimer (Maney et al., 1998), the concentration refers to dimers and was determined as half the concentration.
of nucleotide binding sites, which were measured radiometrically (Coy et al., 1999). Bovine brain tubulin was purified to 95% purity (Mickey and Howard, 1995). Aliquots in BRB80 buffer (80 mM potassium PIPES [pH 6.9], 1 mM EGTA, 1 mM MgCl2) were frozen in liquid nitrogen and stored at −80 °C. Tubulin concentrations, expressed in terms of 110 kDa tubulin heterodimers, were measured by absorbance at 276 nm in 6 M guanidine HCl using an extinction coefficient of 1.03 ml/mg/cm.

Microtubules

For microscopy assays, MTs were assembled in BRB80 plus 1 mM MgGMP-CPP from a mixture of 6 μM rhodamine-labeled tubulin (Hyman et al., 1991) and 18 μM unlabeled tubulin. For turbidity, stopped-flow, and ATPase assays, MTs were prepared by two cycles of assembly in GMP-CPP to deplete the GDP-tubulin dimers KCl, 1 mM DTT, 100–200 μM MgATP, and 10 mM ammonium molybdate. The phosphate concentration was determined by adding 1 ml organic reagent (cyclohexane, isobutyl alcohol, acetone, quench solution, mixed at ratio of 50:50:10:1), vortexing for 50 s, then allowing the phases to separate for 10 min at 0 °C. The light scattering signal (excitation and emission at 350 nm) was measured at 0.2–0.5 s intervals. Because MTs break during rapid mixing, we used the maximum initial depolymerization rate measured in the microscopy and turbidity assays (0.95 μm/min) to estimate the MT (and end) concentration.

ATPase Assays

ATPase rates were determined using two different methods to assay P, release over time. A malachite green assay (Coy et al., 1999) was used for experiments to determine ATPase activity stimulated by variable amounts of either GMP-CPP MTs or GDP-tubulin. A radiometric assay was used for all other experiments. Three or five microliters of the reaction mixture (BRB80 supplemented with 250 μM [γ-32P]ATP [NEN Life Sciences Products Inc., Boston, MA], 75 mM KCl, 1 mM DTT, 100–200 μM BSA, and a variable amount of MCAK or GMP-CPP MT) was quenched in 500 μl cold solution containing 1 M hydrochloric acid, 4% perchloric acid, 180 μM P2, and 10 mM ammonium molybdate. The phosphate concentration was determined by adding 1 ml organic reagent (cyclohexane, isobutyl alcohol, acetone, quench solution, mixed at ratio of 50:50:10:1), vortexing for 50 s, then allowing the phases to separate for 10 min on ice. After phase separation was complete, 63% or 65% of the organic phase containing radiolabeled P (complexed with molybdate) was recovered, mixed with 3 ml scintillation fluid, and read in a Beckman scintillation counter.

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