THE EMBO JOURNAL

Manuscript EMBO-2011-77119

The kinesin-13 MCAK has an unconventional ATPase cycle adapted for microtubule depolymerization

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Review timeline:

Submission date:	26 January 2011
Editorial Decision:	02 March 2011
Revision received:	30 June 2011
Editorial Decision:	13 July 2011
Accepted:	18 July 2011

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 02 March 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three reviewers express interest in your work, but referee 3 in particular raises a number of serious technical concerns that call in to question the conclusions drawn. His/her comments are explicit, and I will not go into detail here, but most notably, he/she argues that your kinetic experiments are not conducted under single turnover conditions, and that therefore you can not conclusively demonstrate the rate limiting step for the MCAK cycle.

Given the interest expressed by the referees, we would like to invite a revision of your manuscript. However, I would stress that addressing the concerns of referee 3 in particular will be essential for an eventual positive outcome here. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension. If you have any questions or comments about this revision, please don't hesitate to get in touch.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

In this manuscript the authors carry out the first extensive kinetic analysis of the ATPase cycle for a member of the Kinesin-13 family, MCAK. They find that the Kinesin-13 MCAK has an ATPase cycle distinct from motile kinesins in that the rate-limiting step is ATP cleavage rather than ADP and Pi release. From their studies they propose a model wherein ATP bound MCAK interacts with the MT lattice, which stimulates hydrolysis putting MCAK into a diffusible ADP-Pi state, which facilitates MT end targeting. Upon end binding nucleotide exchange of ADP for ATP is stimulated, and this ATP binding can result in the removal of a tubulin dimer. ATP hydrolysis of MCAK is stimulated on tubulin dimer resulting in the dissociation of tubulin and MCAK-ADP + Pi. This unique catalytic cycle is ideally suited for the depolymerization activity of MCAK. Overall the studies are very well done and should be of interest to the large number of scientists interested in motor proteins. There are several points that the authors should need to address before publication.

1. For ATP binding in Fig 2, other kinesins show a linear increase in rate followed by a plateau that follows an exponential/hyperbolic model. The authors tested up to 20 μM, which may be insufficient to see this effect. More points should be assayed to make the extrapolation described on page 6 more accurate.

2. The authors attribute the fast phase of the biphasic stop-flow traces in Fig 3 and 5 to association or dissociation of ADP or ATP. Isn't it possible that this fast phase reflects direct nucleotide exchange and not (for example) ADP -MCAK to apo-MCAK to ATP-MCAK? What is the rate associated with the fast phase in Fig 3?

3. The authors say in the discussion "Because the proposed tubulin binding site on MCAK is distant from the nucleotide-binding pocket (Ogawa et al, 2004), this stabilization likely occurs via an allosteric mechanism rather than a direct interaction of residues from tubulin with the nucleotide-binding site." Isn't this true for all kinesins - ATP hydrolysis in the nucleotide pocket is stimulated by MT binding a distance from the pocket? This should be reworded so as not to suggest that this is a unique feature of the Kinesin-13 family members.

4. In the introduction, please clarify the conformational change that you're referring upon ATP binding to kinesin - changes in switches or neck linker docking

5. Moore & Wordeman (2004) also show that MCAK ATPase activity is coupled to MT ends. They should be cited along with Desai et al, Hunter et al, and Wagenbach et al. in intro and in discussion.

6. Waganbach et al also identify unique aspects of the MCAK catalytic cycle. This should be emphasized more in the discussion.

7. Stop flow signals are not a smooth line as seen in the figures. Please show the raw data with the best fit line.

8. Please be consistent within the manuscript and figures with how you report your values in terms of significant digits and standard deviation/error. It got very confusing to try and match numbers in the text to the table because the text often had SD where the table is SEM.

9. Incorporation of Scheme 1 into the model would be helpful so that appropriate comparisons can

be made.

10. Overall the paper is fairly wordy. The text could be cut by 10-20% without a loss of clarity.

11. Organizational considerations
a. Combine Figure 1A, C, D and Figure 2. Figure 1B better suited in supplemental
b. Supplemental Fig 2 is more suited as a figure in the body of the paper because it is new work and represents steps in the cycle being determined
c. Combine figure 3 and figure 4

Referee #2

This is excellent work that establishes that MCAK, a depolymerase kinesin, has a kinetic cycle that differs radically from that of translocase kinesins, in that (a) ATP cleavage is suppressed until the enzyme binds to microtubules and (b) microtubule-activation of ADP release is partially suppressed until the enzyme encounters a microtubule tip. These findings very substantially advance our understanding of the catastrophase mechanism of MCAK, and for this reason I certainly think this belongs in EMBO J.

My suggestions are for improvements:

[1] The manuscript is written in didactic style that carefully assembles each component of the argument. I appreciated this, but on the other hand I think to lecture the reader in very basic kinetics is a bad idea. Fine to put this in but please move to supplementary, and use the freed space to move back some vital material that is currently in supplementary - for example the kinetic comparison of 1-headed and 2-headed MCAK.

[2] After lattice binding stimulated hydrolysis, what happens to Pi? I would like to know about the ADP.Pi state - my reading is that Pi release is assumed fast in the presence and absence of microtubules, which differs considerably from previous models from this lab, yet is not discussed - we need to see discussion of this, if only to make it clear that the possible suppression of Pi release from lattice-bound MCAK remains a moot point. Figure 7 is noncommital.

[3] Acknowledgement needs to be made of the prior work of Taylor, who identified a Mant signal associated with ATP cleavage by kinesin-1 (Ma & Taylor Biochem 1995, JBC 1997). The present work establishes that for MCAK, this signal-generating change is indeed coupled to hydrolysis.

[4] Nucleotide free MCAK is described, please indicate how stable the nucleotide free state of MCAK is in the absence of MTs. Most kinesins do not like having an empty active site - if MCAK is stable when empty, that is an interesting difference.

[5] Consider also acknowledging the work of Naber and colleagues, who detected a change in the kinesin-1 active site that they interpreted as a closure of the switch regions on microtubule binding, consistent with the acceleration of ATP cleavage by microtubule binding. In MCAK, it might be that this effect is exaggerated to the point that ATP cleavage in the absence of MTs is substantially inhibited.

[6] Regarding the coupling of depolymerisation to ATP turnover, consider referring to work from the Wordeman lab in which a hydrolysis-incompetent MCAK mutant can depolymerise (GMPCPP) microtubules, albeit very slowly (Wagenbach JCB 2008).

[7] MCAK is referred to throughout as a depolymerase, but it must be remembered that so far as we know it only depolymerises GMPCPP microtubules and GTP caps. With dynamic microtubules, MCAK is a catastrophase, destabilising caps and causing catastrophes. I am sure this is familar territory for MCAK specialists, but this reader at least was left wondering why catastrophase activity is not reported. Is it because with dynamic microtubules, free tubulin competes with microtubules for MCAK? Can the authors say something about this, based on their measurements of the activation of MCAK's ATPase by tubulin and by MTs?

[8] Regarding the scheme and comparison with other motors (figure 8) consider emphasizing that in all kinesins, K.ADP is weak and K.ATP is strong. I find this scheme a simplficiation too far, since for MCAK two steps, ATP cleavage and ADP release, are envisaged to be accelerated by the 'filament', but by different parts of the filament.

[9] Regarding the proposal that curved PFs are required for full activation of ADP release from MCAK, is it not also possible that separation of PFs is the key thing? The idea that the tips of GMPCPP microtubules have curved PFs is hard to swallow for me, considering that GMPCPP is supposed, like taxol, to straighten tubulin heterodimers. It is much easier for me to believe that MCAK cannot grip the lattice properly until neighbouring PFs are unzipped.

[10] On this same point, it has been shown that the loop2 of MCAK is crucial to get it to target to MT tips and destabilise tips. Could loop2 be a device for suppressing lattice-activation of ADP release?

Referee #3

The kinesin-13 MCAK has an unconventional ATPase cycle adapted for microtubule depolymerization.
Friel C.T. and Howard J. EMBO J

In this research article, Friel and Howard present a series of experiments designed to monitor the kinetics of several steps in the ATPase cycle of a kinesin-13, MCAK. Their aim for this study was to elucidate the rate-limiting step in the ATPase cycle and how the binding of unpolymerized tubulin dimer or microtubule polymer affects various kinetic steps in the overall cycle. The major point that was emphasized throughout the entire document was that the MCAK cycle was limited by ATP cleavage rather than product release, and that tubulin or microtubules accelerates this step in the cycle. A second significant point illustrates how ADP product release is the rate-limiting step in the tubulin- or MT-stimulated cycle, with the ends of MTs being necessary and sufficient to stimulate ADP release in the absence of MT depolymerization.

While the latter experiments are clear and conclusive, there are significant issues with the design of the kinetic experiments addressing the rate limiting step of the MCAK cycle in the absence of microtubules. Primary among these are that the kinetic experiments described as being done under single turnover conditions were not actually performed under single turnover conditions, which require enzyme concentrations to be significantly (normally 10x) over substrate concentrations, with substrate concentrations at least 2-3x over the Km. A second major concern is the lack of determination of the Km, ATP for MCAK in the absence of microtubules. Determination of this important parameter would allow the subsequent design of the kinetic experiments to produce unambiguous conclusions. Unfortunately, these issues make one of the main conclusions of the paper, that is, that ATP hydrolysis is rate limiting in the absence of MTs, unproven by the studies described.

In order to be suitable for publication, the authors need to repeat the experiments under true single-turnover conditions. However, this requires a great deal of protein and may not be achievable. In that case, they should utilize pre-steady state kinetic techniques to look for a burst of phosphate product formed during the first turnover event using acid quench methods that have been described for mysoins as well as other kinesins.

Following is a list of major and minor concerns:

Major Concerns:

1. The authors do not set the context in the introduction for what is already known about the ATPase cycle of kinesin-13 motors (e.g. Hertzer et al. 2006, which is not even cited). This is a critical component that is essential to placing the current work into the literature.

2. Basal ATPase of MCAK (pg. 4; paragraph 2) - the authors do not report the steady-state kinetics of ATP turnover by MCAK (no tubulin) as a function of ATP concentration. Hertzer et al. 2006 report the MT-stimulated steady state parameters for MCAK (kcat and Km,ATP), but no study that presents these kinetics in the absence of polymer. It is essential to know these values for the interpretation of the presteady-state kinetics of mantATP binding that are presented later in this manuscript.

3. MT-stimulated ATPase of MCAK (pg. 4; paragraph 2) - What was the average length of MTs used for these steady-state assays? It seems that there is a discrepancy between ATPase rates presented in this manuscript and it is not clear how the experiments were performed. It says in the "Methods" section that "MTs of average length of 2.1um....were used for all assays, unless otherwise stated". And in the second to last section of the "Results" section ("The MT end stimulates ADP dissociation from MCAK"; pg 11) says the ATPase rate for 2.1um MTs was 3.13 s-1. Based on Fig. S1C, the rate at 5.5 uM microtubules should be ~2.2 s-1. The authors need to clarify these internal inconsistencies.

4. Dissection of ATP turnover cycle (pg 5/6; paragraph 3) - These data suggest a two-step nucleotide binding mechanism (at a minimum) as the rates observed in Fig. 2B and S2B are far too slow to be diffusion limited. However, the authors do not address this interpretation. The authors also do not discuss their results in light of other kinesin studies looking at ATP binding kinetics in the absence of filaments (kinesin-1, kinesin-5, kinesin-10).

5. ADP dissociation in not the rate-limiting step (pg. 6) - The authors state that this finding "distinguishes MCAK from other kinesins", but they state in the "Discussion" section (pg. 15) that "NOD has previously been shown to have rate-limiting ATP cleavage". The authors need to clarify this contradiction and should also discuss the implications for this similarity between MCAK and NOD.

6. A highly fluorescent intermediate (pg 6) - This is not a very reliable extrapolation for the rate constant of ATP binding at 1mM ATP (probably significantly overestimated). If the authors knew the Km,ATP for MCAK, they could fit their mantATP binding data to a hyperbola and fix the Kd,ATP to the Km,ATP as determined in steady-state assays, which would be better approximating the extrapolated rate constant.

7. A highly fluorescent intermediate (pg 6) - The authors did not use single-turnover conditions. According to Johnson (1992), a single-turnover experiment must have the enzyme well in excess over substrate to allow the direct observation of the conversion of substrates to intermediates and products in a single pass of the reactants through the enzymatic pathway. The rate of substrate binding is governed by a pseudo-first order rate constant defined by the product k1[E]. Therefore, the enzyme concentration must be sufficiently high to saturate the rate of substrate binding in order to measure the rate of the chemical reaction, which the authors did not do in this study. In their experimental design, their enzyme concentration was 1uM and mATP concentration was 0.8uM. Under these conditions, there will be a significant fraction of the enzyme not bound to substrate. In addition, they have a stoichiometric concentration of ADP bound to the MCAK, so this complicates the presteady-state assumptions at the outset of these experiments. Why didn't the authors perform these experiments with nucleotide-free MCAK as they did in the mantATP binding experiments? Since these experiments were not done under single-turnover conditions, their subsequent interpretation of the rate-limiting step cannot be justified (see more below).

8. A highly fluorescent intermediate (pg 7) - If ADP release is not rate-limiting, then why is it presumed that ADP (and not ATP) would be bound to each motor domain? Their presteady-state kinetics of mADP binding suggest weak ADP binding.

9. A highly fluorescent intermediate (pg 7) - The authors conclude that "mantATP-MCAK has a higher fluorescence signal than mantADP-MCAK." This statement is not supported by data shown in Fig 2 and Fig S2. In Fig 2 panel A, a transient is shown that represents presteady-state data for 0.6uM nucleotide-free MCAK and 3.2uM mantATP. The amplitude of this transient is approx 0.25V. In Fig S2 panel A, a transient is shown that represents presteady-state data for 0.3uM nucleotide-free MCAK and 1.6uM mantADP. The amplitude of this transient is approx. 0.5V. These results are contradictory to the statement made here. The authors need to rationalize the difference.

If the mantATP and mantADP binding experiments shown in Fig 3 were performed at different PMT high voltage settings, the authors need to perform a control experiment on the stopped-flow using the same concentration of MCAK and a high concentration of mant nucleotide to show this difference in fluorescence intensity upon mantATP vs mantADP binding. This "highly fluorescent intermediate" should be observed in these assays as well. Additionally, the time required to reach peak fluorescence is dramatically different in Fig 3 (500-1000 sec) versus Figs 2 (20 sec) and S2 (10 sec). What accounts for this?

10. ATP cleavage is rate-limiting (pg 8) - The authors try to discern which step is rate-limiting in the cycle; ATP cleavage or phosphate product release. The only way to determine which of these intermediates is populated would be to experimentally determine if there is a burst of phosphate product is formed during the first turnover event. The experimental design discussed in this section does not differentiate between these two possibilities. Also, they report in Fig 4 the kinetics of substrate catalysis, which does not order the kinetic steps as to which is rate-limiting. Given the emphasis on ATP turnover being rate-limiting and the implications of this result in their overall model, the authors do not present convincing data in this manuscript demonstrating ATP cleavage is the rate-limiting step. Based on the data presented, the argument for phosphate release being rate-limiting could be equally compelling.

11. Microtubules accelerate... (pg 9) - The authors claim that the phase of decreasing fluorescence was no longer visible with polymerized MTs. How can the reader compare these transients in Fig 5B and 5C given the radically different time scales? How do we know that the slow fluorescence decay is not present in Fig 5C (which ends at 5 sec) given the decay is not apparent in Fig 5B until 20-25 seconds. Also, the amplitude of these transients is significantly different. What is the explanation of this?

12. Discussion (pg 12) - While reasonable, the model presented for the MCAK ATPase cycle with ATP hydrolysis being rate-limiting is speculative given the points discussed above.

Minor Concerns:

1. The third paragraph of the intro (pg. 3) goes into great detail about the "hand-over-hand" mechanism of kinesin-1. This has little relevance or connection with the results presented and could be eliminated (or shortened).

2. Third paragraph of intro (pg. 3) - not "all kinesins studied to date..." show tight binding to MTs in the presence of AMPPNP.

3. Third paragraph of intro (pg. 3) - reference to Sindelar and Downing is missing the year. The year is also missing in the References section.

4. Dissection of ATP turnover cycle (pg 5; paragraph 2) - The last sentence starting "Importantly, in the presence..." is redundant with the end of the preceding sentence.

5. The authors consistently use the notation for a non-covalent interaction between MCAK and nucleotide with a "dash" (e.g. mantATP-MCAK). Typically, this sort of interaction is represented with a "dot" (e.g. mantATP•MCAK).

1st Revision - authors' response 30 June 2011

Referee #1

In this manuscript the authors carry out the first extensive kinetic analysis of the ATPase cycle for a member of the Kinesin-13 family, MCAK. They find that the Kinesin-13 MCAK has an ATPase

cycle distinct from motile kinesins in that the rate-limiting step is ATP cleavage rather than ADP and Pi release. From their studies they propose a model wherein ATP bound MCAK interacts with the MT lattice, which stimulates hydrolysis putting MCAK into a diffusible ADP-Pi state, which facilitates MT end targeting. Upon end binding nucleotide exchange of ADP for ATP is stimulated, and this ATP binding can result in the removal of a tubulin dimer. ATP hydrolysis of MCAK is stimulated on tubulin dimer resulting in the dissociation of tubulin and MCAK-ADP + Pi. This unique catalytic cycle is ideally suited for the depolymerization activity of MCAK. Overall the studies are very well done and should be of interest to the large number of scientists interested in motor proteins. There are several points that the authors should need to address before publication.

1. For ATP binding in Fig 2, other kinesins show a linear increase in rate followed by a plateau that follows an exponential/hyperbolic model. The authors tested up to 20μM, which may be insufficient to see this effect. More points should be assayed to make the extrapolation described on page 6 more accurate.

We were unable to measure the possible saturation in the rate constant in Fig2B because we could not go to higher mantATP concentrations. The mantATP concentration is limited by the highest concentration at which it is possible to purify MCAK (~10μM: a final [MCAK] of 5μM in the assay, given 1:1 mixing). The assay is performed with [mantATP]:[MCAK] ratio of 5:1 resulting in the maximum [mantATP] measurable of 25μM. It is not possible to use a greater excess of mantATP as the signal change observed decreases as the excess of mant-nucleotide over MCAK is increased.

The reviewer has a particular problem with the linear extrapolation to physiological concentrations of ATP. **We have changed the wording of the sentence on page 6 to reflect that what the exact rate constant may be at physiological [ATP] is not the point we wished to make; the important point is that these data show that ATP association is faster than the basal ATPase rate, even at lower concentrations of ATP, and so is not rate limiting.** Thus we don't need to go to higher mantATP concentrations to make this point.

2. The authors attribute the fast phase of the biphasic stop-flow traces in Fig 3 and 5 to association or dissociation of ADP or ATP. Isn't it possible that this fast phase reflects direct nucleotide exchange and not (for example) ADP-MCAK to apo-MCAK to ATP-MCAK? What is the rate associated with the fast phase in Fig 3?

We cannot determine whether there is an apo-MCAK intermediate or whether the mantATP directly displaces the ADP. The rate constant of the fast phase measured using stopped-flow mixing in Figure 5B was 0.148 ± 0.015 s-1 (Table 1), which likely reflects nucleotide exchange limited by the rate constant for ADP release.

Using the data in Figure 3A, the rate constant of the fast phase cannot be accurately determined as the manual mixing technique is not rapid enough to allow measurement of reactions on the appropriate time-scale and also because the sampling frequency (10 s) results in a low number of data points in the fast phase. We have now plotted these data as individual points (new Figure 3A), rather than a line, so that the number of data points available can be seen.

Stopped-flow mixing, as used in Figure 5, is required to accurately measure the rate constant of this phase.

3. The authors say in the discussion "Because the proposed tubulin binding site on MCAK is distant from the nucleotide-binding pocket (Ogawa et al, 2004), this stabilization likely occurs via an allosteric mechanism rather than a direct interaction of residues from tubulin with the nucleotide-binding site." Isn't this true for all kinesins - ATP hydrolysis in the nucleotide pocket is stimulated by MT binding a distance from the pocket? This should be reworded so as not to suggest that this is a unique feature of the Kinesin-13 family members.

The reviewer correctly states that the ATP binding pocket has been shown to be distant

from the MT binding site for a number of kinesins and this is likely to be true for most, if not all, kinesins. **We did not intend to suggest that this was unique to kinesin-13s and have reworded this section to clarify this**.

4. In the introduction, please clarify the conformational change that you're referring upon ATP binding to kinesin - changes in switches or neck linker docking

The conformational change referred to, according to the work of Sindelar & Downing, is now described in more detail.

5. Moore & Wordeman (2004) also show that MCAK ATPase activity is coupled to MT ends. They should be cited along with Desai et al, Hunter et al, and Wagenbach et al. in intro and in discussion.

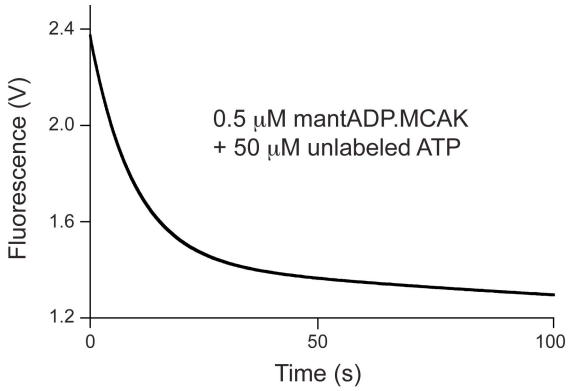
We have added this citation.

6. Waganbach et al also identify unique aspects of the MCAK catalytic cycle. This should be emphasized more in the discussion.

We have added more detail about this work in the introduction and the discussion.

7. Stop flow signals are not a smooth line as seen in the figures. Please show the raw data with the best fit line.

Stopped-flow signals can have very low noise depending on the size of the signal change being observed and the amount and concentration of sample available, e.g. Friel et al (2004) J. Mol. Biol.; Hackney (2002) Biochemistry; Kuhlman & Bagshaw (1998) J. Muscle Res. Cell Motility. The stopped-flow traces shown in the manuscript are an average of 3-5 raw traces, an example of an unaveraged trace is shown below.



8. Please be consistent within the manuscript and figures with how you report your values in terms of significant digits and standard deviation/error. It got very confusing to try and match numbers in the text to the table because the text often had SD where the table is SEM.

We would prefer to retain the use of SD and SEM currently in the manuscript. Throughout the text the error is quoted as SD when it results from a number of replicate experiments, but as SEM when it results from a fit to a number of data points. In Table 1 the quoted error is then standardized for clarity by quoting all errors as SEM, we felt this was the most appropriate way to quote the relevant error throughout the manuscript.

9. Incorporation of Scheme 1 into the model would be helpful so that appropriate comparisons can be made.

The steps in Scheme 1, which correspond to transitions shown in the model in Figure 7A, have now been stated in the legend.

10. Overall the paper is fairly wordy. The text could be cut by 10-20% without a loss of clarity.

We have tried to be as concise as possible and have cut the text in a number of places.

11. Organizational considerations
a. Combine Figure 1A, C, D and Figure 2. Figure 1B better suited in supplemental

The figures have been reorganized to accommodate new data. Figure 1B is now in supplemental information. Figure 2 remains separate, as we think the combination of Figures 1 and 2 would be too busy.

b. Supplemental Fig 2 is more suited as a figure in the body of the paper because it is new work and represents steps in the cycle being determined

Figure S2D, which contains data on monomeric MCAK, has been moved from supplementary information to the new Figure 3.

c. Combine figure 3 and figure 4
Figures 3 and 4 have been combined and are now Figure 3A and C, respectively. Figure S2D has been added as Figure 3B and also a panel showing new data requested by Reviewer #3 (Figure 3D).

Referee #2

This is excellent work that establishes that MCAK, a depolymerase kinesin, has a kinetic cycle that differs radically from that of translocase kinesins, in that (a) ATP cleavage is suppressed until the enzyme binds to microtubules and (b) microtubule-activation of ADP release is partially suppressed until the enzyme encounters a microtubule tip. These findings very substantially advance our understanding of the catastrophase mechanism of MCAK, and for this reason I certainly think this belongs in EMBO J.

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[1] The manuscript is written in didactic style that carefully assembles each component of the argument. I appreciated this, but on the other hand I think to lecture the reader in very basic kinetics is a bad idea. Fine to put this in but please move to supplementary, and use the freed space to move back some vital material that is currently in supplementary - for example the kinetic comparison of 1-headed and 2-headed MCAK.

As the reviewer states, we tried to be very clear in our presentation of the data and in the definitions of our nomenclature. In manuscripts describing kinetic data, the terms used for certain concepts vary making the work difficult to understand. However, we would not like to fall into the trap of being patronizing and so **we have shortened some of the sections we think the reviewer may be referring to.**
The data from monomeric MCAK has been moved to the main text as Figure 3B.

[2] After lattice binding stimulated hydrolysis, what happens to Pi? I would like to know about the ADP.Pi state - my reading is that Pi release is assumed fast in the presence and absence of microtubules, which differs considerably from previous models from this lab, yet is not discussed - we need to see discussion of this, if only to make it clear that the possible suppression of Pi release

from lattice-bound MCAK remains a moot point. Figure 7 is noncommital.

The data (including new data requested by Reviewer #3, Figure 3D) indicate that P_i release is not rate limiting. The precise rate constant for P_i release is still an open question, although the diffusive properties on the MT lattice are similar in ADP or ADP.P_i (Helenius et al, 2006). **We have made this point in the discussion.**

Data previously published from the lab have shown that the gamma phosphate remained associated with MCAK much longer than for conventional kinesin. Whether this was due to slow Pi release or slow ATP cleavage was unknown. We have now shown that this is due to slow ATP cleavage.

[3] Acknowledgement needs to be made of the prior work of Taylor, who identified a Mant signal associated with ATP cleavage by kinesin-1 (Ma & Taylor Biochem 1995, JBC 1997). The present work establishes that for MCAK, this signal-generating change is indeed coupled to hydrolysis.

Citations added on Page 7.

[4] Nucleotide free MCAK is described, please indicate how stable the nucleotide free state of MCAK is in the absence of MTs. Most kinesins do not like having an empty active site - if MCAK is stable when empty, that is an interesting difference.

Nucleotide-free MCAK is stable for about 1 hr at 4ºC in the buffer used for this work (BRB80, 75mM KCl, 0.05% Tween20, 1mM DTT). When nucleotide-free MCAK was required the nucleotide was removed, as described in the Methods, and the protein used directly. **Further information, describing in which assays nucleotide-free MCAK could or could not be used, is now supplied in the Supplementary Information.**

[5] Consider also acknowledging the work of Naber and colleagues, who detected a change in the kinesin-1 active site that they interpreted as a closure of the switch regions on microtubule binding, consistent with the acceleration of ATP cleavage by microtubule binding. In MCAK, it might be that this effect is exaggerated to the point that ATP cleavage in the absence of MTs is substantially inhibited.

This citation has been added in the discussion.

[6] Regarding the coupling of depolymerisation to ATP turnover, consider referring to work from the Wordeman lab in which a hydrolysis-incompetent MCAK mutant can depolymerise (GMPCPP) microtubules, albeit very slowly (Wagenbach JCB 2008).

Further reference to the work has been added to the introduction and the discussion.

[7] MCAK is referred to throughout as a depolymerase, but it must be remembered that so far as we know it only depolymerises GMPCPP microtubules and GTP caps. With dynamic microtubules, MCAK is a catastrophase, destabilising caps and causing catastrophes. I am sure this is familar territory for MCAK specialists, but this reader at least was left wondering why catastrophase activity is not reported. Is it because with dynamic microtubules, free tubulin competes with microtubules for MCAK? Can the authors say something about this, based on their measurements of the activation of MCAK's ATPase by tubulin and by MTs?

All the work described in this manuscript was carried out with stabilized microtubules (using GMPCPP or GMPCPP+taxol). **The catastrophase activity is now explained in the Introduction.**

Other work in the lab with dynamic microtubules has shown that MCAK is indeed a catastrophase; however, this is well beyond the scope of this study.

[8] Regarding the scheme and comparison with other motors (figure 8) consider emphasizing that in all kinesins, K.ADP is weak and K.ATP is strong. I find this scheme a simplficiation too far, since for MCAK two steps, ATP cleavage and ADP release, are envisaged to be accelerated by the 'filament', but by different parts of the filament.

Figure 8 (now Figure 7) aims to show how the change in position of rate limiting step in the absence of unpolymerised tubulin or microtubules, relative to tranlocating kinesins (Kinesin-1 is highlighted specifically) alters the change in MT binding that occurs upon the initial encounter with the MT lattice. The further comparison with myosins (Myosin-V highlighted specifically) aims to show how this distinguishes MCAK, a non-translocating, MT depolymerase, from translocating motors. **We have clarified this in the legend to Figure 7 and in the discussion.**

[9] Regarding the proposal that curved PFs are required for full activation of ADP release from MCAK, is it not also possible that separation of PFs is the key thing? The idea that the tips of GMPCPP microtubules have curved PFs is hard to swallow for me, considering that GMPCPP is supposed, like taxol, to straighten tubulin heterodimers. It is much easier for me to believe that MCAK cannot grip the lattice properly until neighbouring PFs are unzipped.

The reviewer make a good point and we had, in fact, not intended to suggest that curved protofilaments are the only MT end-specific feature that may accelerate ADP dissociation from MCAK. However, the work of Ogawa et al (2004) suggests that protofilament curvature is the means by which kinesin-13s distinguish the microtubule lattice from the end. We feel we can say with certainty only that a degree of protofilament flexibility, which exists at or near the MT end but not within the MT lattice, is required to accelerate ADP dissociation. **We have clarified this in the discussion.**

[10] On this same point, it has been shown that the loop2 of MCAK is crucial to get it to target to MT tips and destabilise tips. Could loop2 be a device for suppressing lattice-activation of ADP release?

This is an interesting idea and may be correct. However, the work of Ogawa et al (2004) suggest that it is the neck linker that hinders kinesin-13s interaction with the MT lattice.

Referee #3

The kinesin-13 MCAK has an unconventional ATPase cycle adapted for microtubule depolymerization.
Friel C.T. and Howard J. EMBO J

In this research article, Friel and Howard present a series of experiments designed to monitor the kinetics of several steps in the ATPase cycle of a kinesin-13, MCAK. Their aim for this study was to elucidate the rate-limiting step in the ATPase cycle and how the binding of unpolymerized tubulin dimer or microtubule polymer affects various kinetic steps in the overall cycle. The major point that was emphasized throughout the entire document was that the MCAK cycle was limited by ATP cleavage rather than product release, and that tubulin or microtubules accelerates this step in the cycle. A second significant point illustrates how ADP product release is the rate-limiting step in the tubulin- or MT-stimulated cycle, with the ends of MTs being necessary and sufficient to stimulate ADP release in the absence of MT depolymerization.

While the latter experiments are clear and conclusive, there are significant issues with the design of the kinetic experiments addressing the rate limiting step of the MCAK cycle in the absence of microtubules. Primary among these are that the kinetic experiments described as being done under single turnover conditions were not actually performed under single turnover conditions, which require enzyme concentrations to be significantly (normally 10x) over substrate concentrations, with

substrate concentrations at least 2-3x over the Km. A second major concern is the lack of determination of the Km, ATP for MCAK in the absence of microtubules. Determination of this important parameter would allow the subsequent design of the kinetic experiments to produce unambiguous conclusions. Unfortunately, these issues make one of the main conclusions of the paper, that is, that ATP hydrolysis is rate limiting in the absence of MTs, unproven by the studies described.

In order to be suitable for publication, the authors need to repeat the experiments under true single-turnover conditions. However, this requires a great deal of protein and may not be achievable. In that case, they should utilize pre-steady state kinetic techniques to look for a burst of phosphate product formed during the first turnover event using acid quench methods that have been described for mysoins as well as other kinesins.

We accept these criticisms. The "single-turnover" experiments are really "low turnover" experiments because MCAK is not in great excess over ATP, and the presence of ADP (due to the technical problem of the stability of nucleotide-free MCAK) further reduces the fraction of ATP that binds to MCAK in the first turnover. We calculate that this fraction is ~44%, so the fluorescence changes corresponds to ~2 cycles, and not a single turnover. Due to the difficulty of using nucleotide-free MCAK in this assay, **we have performed the presteady-state experiments requested and found that there is no product burst (Figure 3D), supporting our main conclusion that ATP cleavage is the rate-limiting step in the absence of microtubules.**

We have also determined the K_M for ATP in the absence of microtubules (Figure 1B).

With these additional experiments, we believe that the main conclusions of the paper are now firmly established. We thank the reviewer for pointing out the earlier weaknesses; the paper is now much stronger.

Following is a list of major and minor concerns:

Major Concerns:

1. The authors do not set the context in the introduction for what is already known about the ATPase cycle of kinesin-13 motors (e.g. Hertzer et al. 2006, which is not even cited). This is a critical component that is essential to placing the current work into the literature.

Hertzer et al (2006) describes the difference between dimeric and monomeric MCAK rather than aspects of the cycle of ATP turnover. Therefore, this work is not cited in the introduction. However, **we have added a section commenting on other work (including Hertzer & Walczak 2008), which deal more directly with the ATP turnover cycle, in order to set the current work in context.**

2. Basal ATPase of MCAK (pg. 4; paragraph 2) - the authors do not report the steady-state kinetics of ATP turnover by MCAK (no tubulin) as a function of ATP concentration. Hertzer et al. 2006 report the MT-stimulated steady state parameters for MCAK (kcat and Km,ATP), but no study that presents these kinetics in the absence of polymer. It is essential to know these values for the interpretation of the presteady-state kinetics of mantATP binding that are presented later in this manuscript.

We have now done this experiment (Figure 1B); the K_M is 0.4 μM.

3. MT-stimulated ATPase of MCAK (pg. 4; paragraph 2) - What was the average length of MTs used for these steady-state assays? It seems that there is a discrepancy between ATPase rates presented in this manuscript and it is not clear how the experiments were performed. It says in the "Methods" section that "MTs of average length of 2.1um....were used for all assays, unless otherwise stated". And in the second to last section of the "Results" section ("The MT end stimulates ADP

dissociation from MCAK"; pg 11) says the ATPase rate for 2.1um MTs was 3.13 s-1. Based on Fig. S1C, the rate at 5.5 uM microtubules should be ~2.2 s-1. The authors need to clarify these internal inconsistencies.

For the curve the reviewer discusses (Figure S1C) V_{max} = 5.0 ± 0.5 s^{-1} and K_M = 5.9 ± 1.8 μM, from these parameters we can calculate an expected ATPase rate at 5.5 μM polymerized tubulin and the associated error (SE) of 2.4 ± 0.5 s^{-1}. By a t-test this is not significantly different from the value of 3.13 ± 0.17 s^{-1} quoted in the text.

4. Dissection of ATP turnover cycle (pg 5/6; paragraph 3) - These data suggest a two-step nucleotide binding mechanism (at a minimum) as the rates observed in Fig. 2B and S2B are far too slow to be diffusion limited. However, the authors do not address this interpretation. The authors also do not discuss their results in light of other kinesin studies looking at ATP binding kinetics in the absence of filaments (kinesin-1, kinesin-5, kinesin-10).

Whatever the precise binding mechanism of mantATP or mantADP, it is clear that the rate constants determined are much too fast for either of these steps in the ATP turnover cycle of MCAK to be rate limiting. This is the aspect of these data in which we were interested for the purpose of this study and, therefore, this is the only interpretation we have made regarding these data.

5. ADP dissociation in not the rate-limiting step (pg. 6) - The authors state that this finding "distinguishes MCAK from other kinesins", but they state in the "Discussion" section (pg. 15) that "NOD has previously been shown to have rate-limiting ATP cleavage". The authors need to clarify this contradiction and should also discuss the implications for this similarity between MCAK and NOD.

The reviewer is quite correct – **we have clarified this issue**.

6. A highly fluorescent intermediate (pg 6) - This is not a very reliable extrapolation for the rate constant of ATP binding at 1mM ATP (probably significantly overestimated). If the authors knew the Km,ATP for MCAK, they could fit their mantATP binding data to a hyperbola and fix the Kd,ATP to the Km,ATP as determined in steady-state assays, which would be better approximating the extrapolated rate constant.

See response to Reviewer #1, point 1.

7. A highly fluorescent intermediate (pg 6) - The authors did not use single-turnover conditions. According to Johnson (1992), a single-turnover experiment must have the enzyme well in excess over substrate to allow the direct observation of the conversion of substrates to intermediates and products in a single pass of the reactants through the enzymatic pathway. The rate of substrate binding is governed by a pseudo-first order rate constant defined by the product k1[E]. Therefore, the enzyme concentration must be sufficiently high to saturate the rate of substrate binding in order to measure the rate of the chemical reaction, which the authors did not do in this study. In their experimental design, their enzyme concentration was 1uM and mATP concentration was 0.8uM. Under these conditions, there will be a significant fraction of the enzyme not bound to substrate. In addition, they have a stoichiometric concentration of ADP bound to the MCAK, so this complicates the presteady-state assumptions at the outset of these experiments. Why didn't the authors perform these experiments with nucleotide-free MCAK as they did in the mantATP binding experiments? Since these experiments were not done under single-turnover conditions, their subsequent interpretation of the rate-limiting step cannot be justified (see more below).

We agree with these points. When one takes account of the ADP present (coming from MCAK) we estimate that 44% of the mantATP binds in the first turnover after mixing, and so the rate constant for the fluorescence decrease (which represents ATP cleavage) will be reduced by a factor of two compared to the basal ATPase rate. As described above, **we have performed pre-steady-state experiments suggested by the reviewer (Figure 3D), which confirm that ATP**

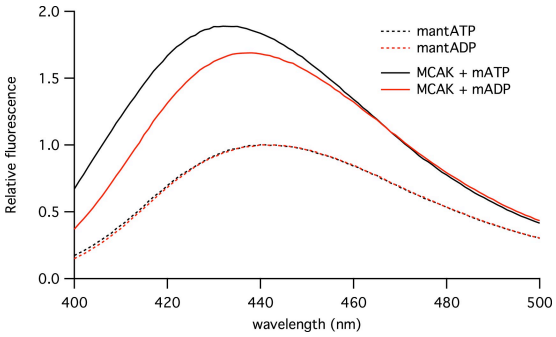
cleavage is indeed rate limiting.

8. A highly fluorescent intermediate (pg 7) - If ADP release is not rate-limiting, then why is it presumed that ADP (and not ATP) would be bound to each motor domain? Their presteady-state kinetics of mADP binding suggest weak ADP binding.

Post purification MCAK has an ADP bound as the excess ATP added at the start of the process has been hydrolyzed and fresh ATP is not available to replace it. We have confirmed that the nucleotide bound to MCAK post purification is ADP by denaturing the protein and identifying the released nucleotide using HPLC.

9. A highly fluorescent intermediate (pg 7) - The authors conclude that "mantATP-MCAK has a higher fluorescence signal than mantADP-MCAK." This statement is not supported by data shown in Fig 2 and Fig S2. In Fig 2 panel A, a transient is shown that represents presteady-state data for 0.6uM nucleotide-free MCAK and 3.2uM mantATP. The amplitude of this transient is approx 0.25V. In Fig S2 panel A, a transient is shown that represents presteady-state data for 0.3uM nucleotide-free MCAK and 1.6uM mantADP. The amplitude of this transient is approx. 0.5V. These results are contradictory to the statement made here. The authors need to rationalize the difference. If the mantATP and mantADP binding experiments shown in Fig 3 were performed at different PMT high voltage settings, the authors need to perform a control experiment on the stopped-flow using the same concentration of MCAK and a high concentration of mant nucleotide to show this difference in fluorescence intensity upon ma
***ntATP vs mantADP binding. This "highly fluorescent intermediate" should be observed in these assays as well. Additionally, the time required to reach peak fluorescence is dramatically different in Fig 3 (500-1000 sec) versus Figs 2 (20 sec) and S2 (10 sec). What accounts for this?*

The data in Figure 2 and Figure S2 are performed with different concentrations of both MCAK and nucleotide and using different settings of the stopped-flow instrument. We have confirmed the difference in fluorescence of mantATP·MCAK and mantADP·MCAK by collecting spectra on a fluorimeter using identical settings and normalizing the data to the peak signal of the nucleotides in buffer to account for differences in concentration (below). The spectra for the MCAK bound nucleotides were collected 1 min after mixing.



The time to reach peak fluorescence in Figure 3, Figure 2A and Figure S2B is not expected to be the same. These are different assays performed with different concentrations of nucleotide, which changes the kinetics of binding (Figure 2B and S2C), and also with ADP·MCAK (Figure 3) or nucleotide-free MCAK (Figure 2A and S2B).

10. ATP cleavage is rate-limiting (pg 8) - The authors try to discern which step is rate-limiting in the cycle; ATP cleavage or phosphate product release. The only way to determine which of these intermediates is populated would be to experimentally determine if there is a burst of phosphate product is formed during the first turnover event. The experimental design discussed in this section does not differentiate between these two possibilities. Also, they report in Fig 4 the kinetics of substrate catalysis, which does not order the kinetic steps as to which is rate-limiting. Given the

emphasis on ATP turnover being rate-limiting and the implications of this result in their overall model, the authors do not present convincing data in this manuscript demonstrating ATP cleavage is the rate-limiting step. Based on the data presented, the argument for phosphate release being rate-limiting could be equally compelling.

We have performed the suggested experiments (Figure 3D) and confirmed that there is no burst of product indicating that ATP cleavage is indeed rate limiting.

11. Microtubules accelerate... (pg 9) - The authors claim that the phase of decreasing fluorescence was no longer visible with polymerized MTs. How can the reader compare these transients in Fig 5B and 5C given the radically different time scales? How do we know that the slow fluorescence decay is not present in Fig 5C (which ends at 5 sec) given the decay is not apparent in Fig 5B until 20-25 seconds. Also, the amplitude of these transients is significantly different. What is the explanation of this?

The data in Figure 5C have been measured for up to 50 s and no decay in the fluorescence is observed. To allow good visualization of the fast phase of the transient, it is necessary to plot the data on a shorter time scale. The time scale used is a compromise to allow visualization of both the fast and slow phases of this double exponential. **We have now shown the first 10 s of the transient.**

The amplitude of the transient in Figure 5C is different from that of the upper and middle transients in Figure 5B as the highly fluorescent intermediate state, observed when ATP cleavage is slower than ADP dissociation, is not populated in Figure 5C. This amplitude should be compared with that of the lower transient in Figure 5B, from which it is not significantly different.

12. Discussion (pg 12) - While reasonable, the model presented for the MCAK ATPase cycle with ATP hydrolysis being rate-limiting is speculative given the points discussed above.

Given the new data supplied as requested by the reviewer, we feel that the model is now well supported.

Minor Concerns:

1. The third paragraph of the intro (pg. 3) goes into great detail about the "hand-over-hand" mechanism of kinesin-1. This has little relevance or connection with the results presented and could be eliminated (or shortened).

We have shortened this section.

2. Third paragraph of intro (pg. 3) - not "all kinesins studied to date..." show tight binding to MTs in the presence of AMPPNP.

"All kinesins studied to date use nucleotide turnover to alternate between states of high and low microtubule affinity".

3. Third paragraph of intro (pg. 3) - reference to Sindelar and Downing is missing the year. The year is also missing in the References section.

This has been corrected.

4. Dissection of ATP turnover cycle (pg 5; paragraph 2) - The last sentence starting "Importantly, in the presence..." is redundant with the end of the preceding sentence.

This paragraph has been reworded due to the addition of new data.

5. The authors consistently use the notation for a non-covalent interaction between MCAK and nucleotide with a "dash" (e.g. mantATP-MCAK). Typically, this sort of interaction is represented with a "dot" (e.g. mantATP•MCAK).

We now use the notation suggested by the reviewer.

2nd Editorial Decision 13 July 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2011-77119R. It has now been seen again by referee 3, whose comments are enclosed below. As you will see, he/she finds the manuscript to be substantially improved and is now fully supportive of publication here. I am therefore pleased to be able to tell you that we will be able to accept your manuscript, pending a few minor editorial issues:

- We require "Author Contributions" and "Conflict of Interest" statements - please can you add these below the Acknowledgments?
- Your figure files currently comprise both Figure and Legend. We need individual figure files of just the figures only, and the legends should be put into the main text (below the references). Please can you change this and upload new versions?

Once we have these final changes, we should then be able to accept the paper for publication without further delay.

Many thanks for choosing EMBOJ for publication of this study, and congratulations on a fine piece of work!

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #3 (Remarks to the Author):

The authors have done a heroic job in addressing essentially all of the previous concerns expressed; the premise of the manuscript and the underlying kinetic experiments are now much more convincing.

