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Tracking and Visualizing the Circadian Ticking of the Cyanobacterial Clock Protein KaiC in Solution

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(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 06 October 2010

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office. As indicated before, the delay in getting back to you with a more timely decision was based on the fact that one scientist that initially agreed to review the paper noticed quite late the strong biophysical focus of the study.
Three scientists did by now evaluate potential suitability for publication at The EMBO Journal. All three indicate that providing a more dynamic view on 'ticking' of the cyanobacterial circadian clock might indeed have some impact also for our perception of metazoan systems. Despite this general interest, all three still raise some important concerns, that would need both experimental as well as textual changes that we kindly ask you to provide during a single round of major amendments. Specifically: ref#1 demands potential further insights into ATPase activity regulation and fitting KaiA/B into the proposed model. Ref#2 requests definitive statements related to subunit exchange that seems currently conflicting between the dynamic picture presented here and your earlier structural work. Lastly, ref#3 would appreciate further clarifications towards the exact nature of the structural changes to strengthen your model.

Conditioned on satisfactorily addressing these crucial points from our referees, we would be delighted to assess a thoroughly revised paper.

Please be reminded that it is EMBO_J policy to allow a single round of revisions only and that the final decision will entirely depends on the content within the last version of your manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REVIEWS

Referee #1 (Remarks to the Author):

Work on the cyanobacterial system is at the forefront of trying to integrate biochemical and structural perspectives on how a circadian clock 'ticks'. KaiC undergoes a circadian phosphorylation cycle and also interacts with KaiA and KaiB. It is known that the assembly/disassembly of complexes containing the 3 proteins is tightly linked to the KaiC phosphorylation cycle, which is dependent on the ATPase activity of KaiC. Presumably, the phosphorylation cycles drive conformational changes that affect assembly/disassembly and the states of these complexes in turn affect the rate of KaiC ATPase activity and phosphorylation cycles. KaiC is composed of C1 and C2 domains that are thought to exhibit functional coupling. X-ray crystallographic studies appear to have yielded limited information on state-specific conformations. Here, Murayama et al., use time-resolved fluorescence (TRFL) spectroscopy and small-angle X-ray scattering (SAXS) to get a more dynamic picture of the relationship between KaiC phosphorylation state, intrinsic ATPase activity and KaiC shape. They show that global structural changes in KaiC are largely due to stepwise structural changes in the C2 ring that correlate with phosphorylation state. Essentially, the C2 ring appears to expand and contract on a circadian time scale, and importantly is linked to the ATPase activity. It is possible that their approach has uncovered more subtle changes as the intrinsic ATPase activity is very low, presumably the basis for a rather low frequency biochemical oscillator in the daily time scale. They also use mutants to support which phosphorylation states are associated with C2 expansion and contraction. Prior work has shown that ring structure rearrangements are typical for these kinds of ATPases (as mentioned above, the modest ATPase of KaiC is rather unique and likely related to its longer time-scale). The general model, supported by the data, is that the energy released from ATP hydrolysis leads to autophosphorylation of KaiC, which introduces conformational changes that lead to tension within the multi-meric structure (as a result of changes in the contraction/relaxation of the C2 ring), resulting in attenuation of the ATPase activity, until dephosphorylation releases the tension and begins the biochemical/structural cycles anew. What is not clear from the findings, but speculated upon is; 1) how C1 ATPase activity is regulated, and 2) how KaiA and KaiB fit into this model. Answers to those questions would be of high interest to obtain a more broad understanding of the biochemical oscillator. Although the cyanobacterial system is viewed as rather unique by circadian biologist, it is likely that phosphorylation/structural relationships will also be at the core of the 'ticking' in metazoan clocks. I should admit that I do not have expertise in structural biology so I will defer to other experts if they think the data is flawed. But I can say that the results are presented in a manner of interest to non-specialists.

Referee #2 (Remarks to the Author):

The authors provide here structural evidence and modeling of the dynamic processes within the cyanobacterial oscillator. The advantage of their experiments lies in the dynamic nature of the methods employed as opposed to previously published crystal-structure based models. They were capable to correlate their time-resolved fluorescence data (TRFL) to the circadian phosphorylation/dephosphorylation cycle of the KaiC protein. In addition, small-angle X-ray scattering revealed cycles of compaction and expansion of the KaiC C-terminal domains. These dynamic changes were used to fit the existing X-ray crystal structure of the KaiC C-terminal domain to the different phosphorylation states. These structures were usefull to shed some light on the phenomenon of temperature compensation, a characteristic of circadian clocks. In addition, they provide data on the structural changes of KaiC in response to complex formation with KaiA and KaiB. Altogether, the data are very sound and their interpretation very solid.
The question remains, whether this manuscript provides sufficient new data compared to previously published papers (e.g. Pattanayek et al., 2008, EMBO J.). In this paper, the authors described already the conformational changes of KaiC based on X-ray crystallography. I believe, the new paper not only extends the old data, but also gives much more mechanistic insights. I have a couple of minor comments:

1) The abbreviations SAXS and TRSAXS should be described in the text (I assume that both describe the same method, which was employed for multiple time points?)

2) page 11, second paragraph: There is an explanation missing, why the value for WT (which was set to 1) is intermediate compared to the others.

3) page 12, second paragraph: The authors introduce the concept of a tethering module. I would include in the text the term "potential tethering module", since the transition states are based mainly on modeling of the data with some simplifications (e.g. some missing parts of the protein structure were included as dummy residues (page 24, top paragraph) and there are no phosphorylations of the sites considered).

4) page 14, top paragraph: The authors measure Trp fluorescence with the mutant KaiA-W10F. The interpretation is that there is no difference compared to the WT KaiA. However, the scales of fluorescence intensities are different (threefold). This should be discussed somewhere.

5) General question: How would a subunit exchange affect the measurements in solution? The above-mentioned paper states that the conformational changes observed would facilitate subunit exchange. From the SAXS measurements, we would conclude that the overall composition of the KaiC C-terminal domain doesn't change (as far as I understand the I(0) value). But what if the subunit exchange occurs very rapidly? If the authors were confident that a subunit exchange would not affect their measurements, then it is probably not worth to mention this. However, if this is not the case, the impact of subunit exchange should be mentioned in the discussion.

Referee #3 (Remarks to the Author):

The manuscript by Murayama et al. makes use of time-resolved intrinsic tryptophan fluorescence intensity (TRFI) and small-angle X-ray scattering (SAXS) to characterize structural changes in the KaiC6 hexameric ring as it undergoes auto-dephosphorylation.

The amount of individual phosphorylated species was measured by SDS-page, and the overall fluorescence deconvoluted to yield fluorescence spectra for individual species, which showed that phosphorylation on T432 is a primary determinant of the relative fluorescence intensity. In parallel experiments, SAXS measurements were used to track structural changes during the auto-dephosphorylation reaction. Here again, the abundance of individual phosphorylated species was monitored by SDS-PAGE. What was observed with the wild-type protein was a relatively rapid increase in the Rg from approximately 46 Å to just over 47 Å. This is a relatively modest change, particularly for such a large protein, but the error bars in Figure 2C indicate that the change is statistically significant. The time course of this change correlates strongly with the dephosphorylation of T432 (in the context of either phospho or de-phospho S431; Figure S1). So, there are corresponding changes in the fluorescence and SAXS that accompany dephosphorylation of T432.

Mutations that mimic the phosphorylation of S431 and T432 - namely S431D and T432E - were used to investigate the structures of individual species. The Rg values for mimics of the intermediates (Figure 3C) provided a good fit to the overall Rg measured during the dephosphorylation reaction (Figure 2C, dashed curve), supporting the idea that the mimics are reasonable models for the phosphorylated intermediates, even though these mutations did not produce significant domain movements in crystal structures.

Comments:
1. In the legend to Fig.3, it says that measurements are the "mean plus/minus SD for two or more independent measurements" - standard deviation cannot be used to characterize two measurements.
2. The conclusion made at the top of page 10, namely that changes in the C2 ring are solely responsible for the overall increase in the Rg, does not appear to be well supported. A construct with the C2 domain deleted remains static through the dephosphorylation reaction (Fig 1C), but this seems to be a trivial result since the phosphorylation sites are on the C2 domain, which is no longer present, and on this basis there can be no changes in the phosphorylation of 432, which is apparently driving the increase in the Rg. The changes in tryptophan fluorescence are attributed to W331 and/or W462 in the C2 domain, but since W331 on one subunit is quite close to the residues 431 and 432 on a neighbouring subunit, the changes in fluorescence could be due to local changes in structure or

dynamics. This is consistent with phosphorylation-dependent changes in fluorescence that are not accompanied by structural changes, as discussed on page 9.
3. Regarding deletion of the C-terminal tail (the delta500 mutant) it is said "While KaiC-Δ500 lacking the C-terminal tail displayed a gradual increase in Rgapp similar to that observed for KaiC-WT..." but in Fig 2C, it looks like the increase is much more gradual for the KaiC-Δ500 protein, and in fact it never seems to attain as large an Rg value as the wild-type KaiC.

These data are used to model the structural changes in the Kai6 hexamer. Although potentially informative, the model for the concerted change in the C2 ring is speculative because it relies on two assumptions. First, 6-fold symmetry in the hexamer is built into the model; however, given the non-symmetrical interactions with accessory proteins KaiA and KaiB, as well as the non-symmetrical phosphorylation of sites in crystal structures, the maintenance of 6-fold symmetry may be at odds with the way the system works. Second, there seems to be limited evidence that the structural changes are restricted to the C2 ring (as discussed above).

In summary, the study does a good job of documenting an increase in the Rg of KaiC that accompanies dephosphorylation of T432, but the exact nature of the structural change seems open to interpretation: a symmetrical expansion of the C2 ring represents only one possible model, and the authors need to make a stronger case for this model as opposed to others.

1st Revision - Authors' Response 26 October 2010

Our item-by-item responses to the reviewer comments are detailed below (Remarks to the Referees).

Referee #1
We would like to thank referee #1 for his/her appreciation of our work. We have improved the manuscript according to the comments as described below.

Comment 1:
What is not clear from the findings, but speculated upon is; 1) how C1 ATPase activity is regulated, and 2) how KaiA and KaiB fit into this model.

Response:
As noted by the referee, the regulatory mechanism of C1 ATPase is central to the understanding of the Kai oscillator. The present observations were interpreted as that a tension imposed on C1 ATPase is interlocked with the expansion and contraction of the C2 ring. Thus, we were interested in the structural changes of the C1 domains and also in how they were linked to the C2 ring (page 18, line 5 from the bottom). However, because of the limited spatial resolution of SAXS and fluorescence techniques, it was difficult to discuss in detail the structural mechanism by which C1 ATPase is regulated. Instead, we discussed, in line with the published literature, regarding potential structural units that may function as key elements during ATP hydrolysis (page 19, line 1).

According to the referee's suggestion, we have revised Figure 7 to include KaiA and KaiB in our model. We have also created new paragraphs describing an indication of asymmetry in the KaiC hexamer (page 20, line 3) and strengthened our discussion on the KaiC ATPase activity elevated by KaiA in relation to the asymmetry in the KaiC hexamer (page 20, line 13).

Referee #2
We would like to thank referee #2 for his/her appreciation of our work and for a critical reading of our manuscript. We have improved the manuscript in line with the reviewer's comments as described below.

Comment 1:
The abbreviations SAXS and TRSAXS should be described in the text (I assume that both describe the same method, which was employed for multiple time points?)

Response:
To avoid any confusion, we have replaced the abbreviations "FL," "TRFL," and "TRSAXS" with "fluorescence," "time-resolved fluorescence," and "time-resolved SAXS," respectively, throughout the revised manuscript. We have clearly defined the abbreviation "SAXS" at the end of the INTRODUCTION (page 6, line 8).

Comment 2:
page 11, second paragraph: There is an explanation missing, why the value for WT (which was set to 1) is intermediate compared to the others.

Response:
We recorded the time-dependent increase in the concentration of ADP from an incubation time (IT) of 4 h to 30 h at 30°C (Terauchi *et al*. *PNAS* 104, 16377-16381, 2007). After incubation at 30°C for 24 h, KaiC-WT was found to exist as a mixture of 4 different phosphorylation states (Figure 1A), and thus, it is reasonable that the ATPase activity of KaiC-WT is intermediate between the lowest KaiC-DE activity and the highest KaiC-AA activity. To explain this situation, we have added the following statement in the legend of Figure 4C (page 31, line 12): Because KaiC-WT includes 4 different phosphorylation states during the assay (e.g., Figure 1A), its ATPase activity set to unity is intermediate between the lowest $KaiC^{D/E}$ activity (0.75 ± 0.11) and the highest $KaiC^{A/A}$ activity (1.85 ± 0.32). We should have presented our results more carefully, and we again thank the reviewer for his/her fruitful suggestion.

Comment 3:
page 12, second paragraph: The authors introduce the concept of a tethering module. I would include in the text the term "potential tethering module", since the transition states are based mainly on modeling of the data with some simplifications (e.g. some missing parts of the protein structure were included as dummy residues (page 24, top paragraph) and there are no phosphorylations of the sites considered).

Response:
According to the referee's suggestion, we have edited a number of sentences (e.g. page 13, line 5) by replacing the term "tethering module" with "potential tethering module" throughout the revised manuscript.

Comment 4:
page 14, top paragraph: The authors measure Trp fluorescence with the mutant KaiA-W10F. The interpretation is that there is no difference compared to the WT KaiA. However, the scales of fluorescence intensities are different (threefold). This should be discussed somewhere.

Response:
The reduced Trp fluorescence upon KaiA-W10F mutation is simply due to the removal of the fluorescent contribution of Trp10 in KaiA-WT. Under our experimental condition, the fluorescent contribution from KaiA-WT was approximately 4.50×10^{-3} μM^{-1}. Thus, given that the Trp fluorescence from all molecules (3.26 μM) of KaiA-WT remained unchanged during the cycle, their contribution to $FI(340)^{AbC}$, as shown in Figure 6C, was calculated to be approximately 14.7×10^{-3}. This roughly matched the difference in the scales of the fluorescence intensity shown in Figure 6C.

A brief description, which was included in the legend of Figure 6C in the original manuscript, probably did not sufficiently explain the 3-fold difference in Trp fluorescence. Therefore, we have rewritten the second sentence of the legend of Figure 6C. Furthermore, we have added the following sentence in the main text (page 14, line 1 from the bottom): A removal of the fluorescent contribution from KaiA greatly diminished the *FI*(340) (see legend and right axis in Figure 6C). Moreover, we have explained the diminished Trp fluorescence to be a result of the removal of the fluorescent contribution of Trp10 in KaiA-WT.

Comment 5:
General question: How would a subunit exchange affect the measurements in solution? The above-mentioned paper states that the conformational changes observed would facilitate subunit exchange.

From the SAXS measurements, we would conclude that the overall composition of the KaiC C-terminal domain doesn't change (as far as I understand the I(0) value). But what if the subunit exchange occurs very rapidly? If the authors were confident that a subunit exchange would not affect their measurements, then it is probably not worth to mention this. However, if this is not the case, the impact of subunit exchange should be mentioned in the discussion.

Response:

First, we would like to define the phenomenon of the subunit exchange between 2 KaiC hexamers. The N-terminal domain (C1) in each KaiC monomer is covalently linked to its C-terminal domain (C2), and thus, every exchange of a KaiC monomer should accompany a simultaneous exchange of its C1 and C2 domains in a biologically relevant time scale.

As shown in a previous study (Hayashi *et al*. *Biochem Biophys Res Commun* 348, 864-872, 2006), the C1 domains can form the hexametric ring by themselves, unlike the C2 domains. This means that, at least in solution, the C2 domains of KaiC are marginally arranged in the hexameric configuration on the scaffolding C1 ring (page 12, line 5 from the bottom). Therefore, subunit exchange would not be hindered by the expansion of the C2 ring, but it is uncertain whether the expansion of the C2 ring itself is sufficient to accelerate the physical exchange of the KaiC monomer.

In the paper referenced by the referee (Pattanayek *et al*. *EMBO J.* 27, 1767-1778, 2008), the authors explained the enlargement of the central channel to be a result of the extension of the C-terminal loop. In our opinion, what they really observed is the enlarged central channel in the C2 domain of the KaiBC complex, which is not necessarily identical to either the expansion of the C2 ring or the protomer-protomer separation we observed. Currently, it is not certain which more effectively promotes the subunit exchange, the channel enlargement (extension of the C-terminal loop), or the ring expansion (protomer-protomer separation). Because the detailed molecular mechanism of the subunit exchange remains unknown, we sought to carefully discuss our findings by comparing with those of the studies mentioned by the reviewer.

Three extreme scenarios might be hypothesized for the physical exchange of a KaiC monomer. In the first model, a KaiC monomer transiently released from a KaiC hexamer collides with another KaiC hexamer, and thereby the exchange takes place. In the second model, a KaiC hexamer collides with another KaiC hexamer to form a transient dimer of the KaiC hexamer, in which subunit exchange is promoted. In the third model, the transient species such as the KaiC monomer and the dimer of the KaiC hexamer are insufficiently stable to accumulate, and thus, the exchange occurs apparently without detectable intermediates.

Although the KaiC monomer was identified in several studies, there is little evidence to show its stable accumulation under physiological solution conditions containing an excess amount of ATP. In fact, our gel chromatographic analysis of KaiC-WT in the presence of ATP (3 mM) showed that more than 99% of KaiC formed the hexamer, and that the transient species, if any, comprised less than 1%. Among the 3 extreme scenarios, therefore, the third model is most likely under our experimental condition with abundant ATP.

It is evident that the third model, in which subunit exchange will occur very rapidly without detectable intermediates as referee #2 assumed, does not affect the $I(0)$ value of SAXS measurements. This is consistent with our experimental data shown in Figure 2C. At the same time, subunit exchange could induce a slight change in the shape (R_g) of the hexamer in some cases. However, any rapid processes within the acquisition time (typically 1 s) should be averaged, and a rapid pre-equilibrium of the exchange reaction would be achieved at any measurement time point (Figure 2C). If this is the case, subunit exchange could make only a *minor* contribution to the time-dependent increase in R_g of KaiC-WT (Figure 2C), whereas the interconversion among the 4 phosphorylation states could make a *major* contribution (see below).

The major contribution of the change in the phosphorylation state is supported by the larger R_g values observed for $KaiC^{D/T*}$ and $KaiC^{S/A*}$ mutants, each of which should be free from the effects of subunit exchange. The R_g values of these phospho-mimicking mutants gave rise to a good fit to the time-course of R_g recorded during the auto-dephosphorylation reaction (Figure 2C, dashed curve), further supporting the major contribution of the interconversion of the phosphorylation to the time-dependent increase in R_g of KaiC-WT.

As described above, our answer to the referee's comment is that subunit exchange would not affect SAXS measurements to a great extent under the current experimental condition, and

that the detailed discussion on the subunit exchange was not within the scope of this study. To explain this situation, a brief sentence has been included in the revised manuscript (page 9, line 9): This agreement also implies that subunit exchange between 2 KaiC hexamers (Kageyama et al, 2006) will make only a limited contribution, if any, to the observed increase in the R_g values. We again thank the reviewer for highlighting these difficult but important issues.

Referee #3

We would like to thank referee #3 for his/her critical reading of our manuscript and an appreciation of our work. We have improved the manuscript as described below.

Comment 1:
In the legend to Fig.3, it says that measurements are the "mean plus/minus SD for two or more independent measurements" - standard deviation cannot be used to characterize two measurements.

Response:
We apologize for our inappropriate presentation of the experimental data in Figure 3. The R_g values for both $KaiC^{D/E}$ and $KaiC^{D/T*}$ mutants were obtained from 2 independent measurements, and those for the other mutants were obtained from 3 or more experiments. Figure 3 was thus improved by plotting the raw data for both $KaiC^{D/E}$ and $KaiC^{D/T*}$ as open circles together with the mean as bars. We have also modified the legends of Figure 3 (page 30, line 9) and Supplementary Table S1 in line with the improvement of Figure 3.

Comment 2:
The conclusion made at the top of page 10, namely that changes in the C2 ring are solely responsible for the overall increase in the Rg, does not appear to be well supported. A construct with the C2 domain deleted remains static through the dephosphorylation reaction (Fig 1C), but this seems to be a trivial result since the phosphorylation sites are on the C2 domain, which is no longer present, and on this basis there can be no changes in the phosphorylation of 432, which is apparently driving the increase in the Rg. The changes in tryptophan fluorescence are attributed to W331 and/or W462 in the C2 domain, but since W331 on one subunit is quite close to the residues 431 and 432 on a neighbouring subunit, the changes in fluorescence could be due to local changes in structure or dynamics. This is consistent with phosphorylation-dependent changes in fluorescence that are not accompanieds by structural changes, as discussed on page 9.

Response:
We believe that the C2 ring undergoes a much more significant structural change than the C1 ring. First, the ring architecture of the C2 domains is far less stable than that of the C1 domains (Hayashi *et al*. *Biochem Biophys Res Commun* 348, 864-872, 2006). Therefore, the structural unit more susceptible to changes in the phosphorylation state should be the C2 ring. Second, the result for the KaiC-W92F mutant (Supplementary Figure S3) clearly shows 2 Trp residues in the C2 ring as the origins of the total fluorescence change of KaiC-WT during auto-phosphorylation (Figure 1A). Although referee #3 agrees with the second point, we were encouraged to reconsider the possibility that the fluorescence change of KaiC-WT reflects only a local structural change near W331, and that the increase in R_g is influenced by a conformational change in the C1 ring.

As described below, drastic rearrangements of the C1 ring are unlikely. The C1 half is similar to the C2 half in amino acid sequence, size, and shape, and W92 is the C1 counterpart of W331 located in the C2 domain. If the local change around W331 causes the total fluorescence change of KaiC-WT, as referee #3 suggested, the fluorescence from W92 of KaiC-WT has to be almost unchanged even upon a global rearrangement of the C1 ring architecture. In other words, the local change causes the noticeable fluorescence change of W331, but the global change oddly causes little fluorescence change of W92. This is practically unlikely, considering the similarity of the structural environment between W92 and W331.

On the other hand, we have no intention to exclude any small-scale structural changes in the C1 ring, and thus, we agree with referee #3 on this point. In fact, we proposed that key amino acid residues in the C1 domain would be repositioned to regulate the ATPase activity (page 18, line 3 from the bottom). Thus, we have revised the original sentence “These SAXS and

fluorescence observations are largely attributed to stepwise structural changes of the C2 ring in KaiC," as "These SAXS and fluorescence observations suggest that the C2 ring undergoes a drastic structural change in a stepwise manner compared to the C1 ring" (page 10, line 5 from the bottom).

Comment 3:
Regarding deletion of the C-terminal tail (the delta500 mutant) it is said "While KaiC-Δ500 lacking the C-terminal tail displayed a gradual increase in R_g^{app} similar to that observed for KaiC-WT..." but in Fig 2C, it looks like the increase is much more gradual for the KaiC-Δ500 protein, and in fact it never seems to attain as large an R_g value as the wild-type KaiC.

Response:
We should have explained the difference between KaiC-WT and KaiC-Δ500 in a more detailed manner. In the revised manuscript, the relative abundances of KaiC-Δ500$^{S/pT}$, KaiC-Δ500$^{pS/pT}$, KaiC-Δ500$^{pS/T}$, and KaiC-Δ500$^{S/T}$ have been included in Supplementary Figure S1. Although the fraction of KaiC-WT$^{pS/T}$ increased immediately and peaked around 4 h, the fraction of KaiC-Δ500$^{pS/T}$ increased gradually and gave rise to a smaller peak at around 8 h (Supplementary Figure S1). Because the pS/T state possesses the largest R_g among the 4 phosphorylation states (Figure 3C), the gradual increase in R_g^{app} for KaiC-Δ500 was attributed to the slow and limited accumulation of KaiC-Δ500$^{pS/T}$ during the auto-dephosphorylation reaction.

At the same time, it is reasonable to observe the slightly small R_g value for KaiC-Δ500 compared to that for KaiC-WT throughout the present observation because KaiC-Δ500 is expected to be more compact than KaiC-WT upon truncation of the C-terminal tails. In fact, the R_g value calculated using a crystal structure (1TF7) of KaiC without the C-terminal tail is smaller by approximately 0.6 Å than that calculated using a crystal structure (2GBL) of KaiC with some C-terminal tails.

To explain the difference between KaiC-WT and KaiC-Δ500 clearly, we have added the following 2 sentences: The gradual increase in R_g^{app} for KaiC-Δ500 relative to that for KaiC-WT is attributable to a slower and limited accumulation of KaiC-Δ500$^{pS/T}$ during the auto-dephosphorylation reaction (Supplementary Figure S1). The slightly smaller R_g value for KaiC-Δ500 than that for KaiC-WT is qualitatively consistent with compaction upon truncation of the C-terminal tails (page 10, line 8).

Comment 4:
These data are used to model the structural changes in the Kai6 hexamer. Although potentially informative, the model for the concerted change in the C2 ring is speculative because it relies on two assumptions. First, 6-fold symmetry in the hexamer is built into the model; however, given the non-symmetrical interactions with accessory proteins KaiA and KaiB, as well as the non-symmetrical phosphorylation of sites in crystal structures, the maintenance of 6-fold symmetry may be at odds with the way the system works. Second, there seems to be limited evidence that the structural changes are restricted to the C2 ring (as discussed above).

Response:
We appreciate the scientific importance of the referee's comment on potential asymmetries of the protomer configuration and phosphorylation states in the KaiC hexamer. The concerted transition of the KaiC hexamer is described in our model in Figure 7A, because both the fluorescence and SAXS observations during the auto-dephosphorylation (Figures 1A and 2C) could be explained by considering only the 4 different symmetric states. We understand, however, that these are not sufficient to exclude the involvement of the asymmetric species. In fact, although we may be able to conduct complex simulations involving a variety of asymmetric KaiC hexamers, a unique solution of the model (e.g., from the fitting in Figure 2C) would not be expected, considering the quality of the present experimental data and inherently limited resolution of SAXS to slight asymmetries. Our model shown in Figure 7A is purely intended to present the simplest model reconciled with the present observation and *not* intended to exclude non-symmetrical interactions within the KaiC hexamer.

To clearly explain the situation, we have added the following statement in a new paragraph (page 20, line 3): To present the simplest model reconciled with the current observation, potential asymmetries of phosphorylation and protomer configuration within the KaiC hexamer are not included in Figure 7A. Moreover, we have discussed a sign of the

asymmetry induced by KaiA and/or KaiB in terms of the difference in $FI(340)^{abC}$ between the experiment and simulation (Figure 6C, page 20, line 7 from the bottom). To clearly discuss the possible asymmetries within the KaiC hexamer, we have revised original Figure 7 as well as its legend.

