Polypeptide in the chaperonin cage partly protrudes out and then folds inside or escapes outside

Fumihiro Motojima, Masasuke Yoshida

Corresponding author: Masasuke Yoshida, Kyoto Sangyo University

Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>14 March 2010</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>13 April 2010</td>
</tr>
<tr>
<td>Revision received</td>
<td>09 June 2010</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>04 August 2010</td>
</tr>
<tr>
<td>Revision received</td>
<td>20 September 2010</td>
</tr>
<tr>
<td>Accepted</td>
<td>22 September 2010</td>
</tr>
</tbody>
</table>

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 13 April 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. As you will see, the referees all acknowledge that your findings are of interest and have the potential to advance our understanding of GroEL-GroES-dependent protein folding. At the same time, there remain however a number of important concerns regarding the experimental support for the novel conclusions, as well as several issues with the interpretation of the current data. In this respect, especially referee 2 feels that possible alternative explanations for the observed effects have not been decisively ruled out, and also raises a number of other specific technical criticisms. Referees 1 and 3 are primarily concerned about the presentation and interpretation of the data especially in light of previously established results, but also require additional control experiments, in particular with regards to the protease protection assays and their relation to earlier results reported by Weissman et al (1995). In addition, there are also various issues relating to relevant data cited only as 'not shown', and to the writing of the paper, which would benefit from careful proofreading and editing.

Taking these various points into consideration, I feel we should be able to consider a substantially revised manuscript further for publication in the case that you should be able to satisfactorily address the main issues raised by the three reviewers, with special consideration for the points reiterated above. Please be reminded that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important to diligently answer to all the various experimental and editorial points raised at this stage. Please, also make sure to carefully edit and proofread the study, ideally involving a native speaker of English in this case. Finally, when preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: 
http://www.nature.com/emboj/about/process.html). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,

Editor

The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The experiments of Motojima and Yoshida showing that unfolded proteins inside the chaperonin cage can be captured by binding partners outside the folding cage are interesting and novel. However, the physiological significance of out of cage folding is unclear - this may be an off pathway, leakage phenomenon emphasized by the use of single ring GroEL, rather than a significant part of the chaperonin mechanism. Most of the experiments had to be done on the single ring version of GroEL, in which the important, inter-ring allosteric interactions are absent. These interactions strongly affect the stability of GroES binding to GroEL. If the crosslink data are correct showing that disordered proteins escape mainly through the GroEL-GroES interface (not the biggest hole in the complex), then this is likely to involve fluctuations in GroES binding, e.g. by transient detachment of some of the 7 sites. The fraction of substrate able to escape from the encapsulated state is likely to be smaller in the native, double ring complex, as suggested by the data in Figure 6.

In emphasizing the novelty of their findings, the authors make some slightly inaccurate statements in the introduction. It is clear from the GroEL-GroES structure that the cage is not "sealed", but has holes or "windows". Cryo EM work has shown that substrate binding does not "mostly overlap with protein binding". In addition, it should be mentioned that the Horwich group has shown that bound, unfolded proteins are very disordered and can bind at many sites on the chaperonin surface. Those previous findings make it less surprising that non native polypeptide can continue interacting with GroEL during GroES binding, and also interact with external regions of the chaperonin surface.

Protease protection (p. 6): The Weissman et al 1995 study showing protection from proteinase K was not done after folding, as implied in the current paper. The protease was added to a complex made with ADP, so that the protein would not have completed folding. The authors need to analyze why they find greater protease accessibility under the conditions of their single ring complexes. For example, would chymotrypsin give a different result under the conditions used by Weissman et al, are the differences due to the use of single ring GroEL, or different nucleotide states? (The citation in this section should be to Figure 1E, not 1D).

The gold labeled EM images are very unclear. The binding appears to be on the side of the second image rather than at the GroEL-GroES interface as claimed. Most but not all of the images seem to be of "football" complexes with GroES at both ends. With only 5 particles selected it is impossible to judge whether these images really demonstrate anything.

Why are there 2 peaks for the rhodanese-trap complex in Figure 1D?

The paper is clearly written but the English grammar needs a little editing.

In summary, this is a novel study which reveals interesting behavior of encapsulated substrates in the single ring model of GroEL, some of which will be relevant to the full machine. The data showing the capture and extraction of substrate molecules from the folding cage are quite remarkable and provide new insight into the dynamics of non-native proteins inside the chaperonin
Referee #2 (Remarks to the Author):

In this paper, the authors present data that they interpret as suggesting that substrate protein encapsulated in the so-called Anfinsen cage can stick out into bulk solution or even escape whilst GroES is bound. Such scenarios are not included in current models of GroE's mechanism of action but I'm not sure that their inclusion would really change the way we think about how this machine works. More importantly, I believe that the interpretation of the data is flawed since the authors ignore the possibility that substrate can escape via the equatorial plane that is flexible in SR1. Hence, (i) most of the data are not relevant to the double-ring system that exists in vivo and (ii) the model in Fig. 7 is likely to be wrong. The authors could have prevented escape via the equatorial plane by stabilizing it using cross-linking or by using larger substrates (~60 kDa). Further comments follow.

Comments:
1. The FRET experiment shown in Fig. 2C is not a measure of escape but a measure of binding to the trap in an appropriate orientation as there are modes of escape that would not result in a FRET signal. In addition, since the reaction is bi-molecular the rate constant as measured is concentration-dependent and thus not meaningful.
2. The authors do not get rid of substrate that does not enter the cavity before starting the experiments. Hence, at least some of the substrate found outside the cavity did not escape but was there from the start.
3. The stability of the GroES-GroEL (SR1 or double-ring) complex should be monitored over time under the different experimental conditions employed.
4. The fit to lag kinetics in Fig. 2D is poor and not justified given the number of data points.
5. p. 8 - the cysteine-less variant is known to be unstable and difficult to work with. Hence, I would like to see the evidence that is referred to as 'not shown' that this variant is active.
6. p. 8, bottom line - the loss in free rhodanese band intensity can also be due to aggregation the extent of which cannot be judged as the bottom of the wells in the gels are not shown.
7. Figure 4F - the differences between lanes 1-4 are not explained in the legend.

Referee #3 (Remarks to the Author):

The experiments described by Motojima and Yoshida were designed to examine the location of pre-folded (i.e. unfolded) rhodanese polypeptide within the GroEL-GroES complex as the assisted folding reaction proceeds.

In order to attempt to trap the substrate protein within the cavity the authors use a single-ring version of GroEL with an ATPase-inhibiting mutation (SR398). The observed rate of folding to the native state was slow (0.11 min⁻¹) allowing the time-resolved treatment of the reaction with proteinaceous agents incapable of penetrating the SR398-GroES cavity. Three different proteins are used as trapping agents; mutant versions of GroEL (trapGroEL), anti-rhodanese antibody and chymotrypsin. The general finding is that rhodanese becomes protected from the trapping agent at a rate which is the same as its rate of assisted folding. This means that the pre-folded cavity-held species of rhodanese is exposed to these large proteins that cannot enter, hence the rhodanese chain must be protruding from the cavity itself.

One question arises. Why do they not show the result of the experiment where proteinase-K is used as a proteinaceous trapping or degrading agent, like one done by Weissmann et al. 1995? The authors describe doing this experiment on the rhodanese-folding system, but state that a clear time course was not obtained and data not shown.

Looking at the rate of escape of substrate polypeptide from the SR398-GroES cavity it is 0.13 min⁻¹ with D87K trap, but faster with the N265A trap, 0.49 min⁻¹. The authors do not make it entirely clear why, if this is the intrinsic dissociation or escape rate for rhodanese, then why should it vary with the trap?
The authors also probed the rhodanese substrate position in the pre-folded form using thiol groups introduced by cysteine mutagenesis into the GroEL structure. Of these groups, seven were introduced into the GroEL-GroES interaction surface region and one on the inside of cavity. Residue 44 on the cavity wall reacted readily with pre-folded rhodanese, four of the GroES-interaction surface SH groups were unreactive, but three reacted with the rhodanese. Of these three groups, two are in the outer region and one on the inner region of the contact surface. Like the trapping experiments, this result also implies that the unfolded substrate can escape at least partially. It is also shown that exposed hydrophobic groups on the cavity surface have a strong effect on the rate of folding and release of the rhodanese substrate.

The final sections of the paper deal with a comparison of the escape mechanisms of dihydrofolate reductase and rubisco and lastly with the forced escape of rhodanese from the double-ring GroEL-GroES system where the ATPase had been inhibited by the D398A mutation. The authors show that in this system folding can also be trapped on a short time scale.

The quality of the work is good and new findings are produced. I recommend acceptance given some consideration of the points made above, but some careful editing is needed to perfect the English usage.

RESPONSE TO REFEREE #1

Comment by Referee #1
The experiments of Motojima and Yoshida showing that unfolded proteins inside the chaperonin cage can be captured by binding partners outside the folding cage are interesting and novel. However, the physiological significance of out of cage folding is unclear - this may be an off pathway, leakage phenomenon emphasized by the use of single ring GroEL, rather than a significant part of the chaperonin mechanism.

Our response
Although physiological significance is not a major content of this paper, potential physiological relevance is indicated by Fig. 4G in which more fraction of rhodanese failed folding in E. coli cell when GroEL was leakier (more natural escape occurred). The hydrophobic interaction between denatured protein and GroEL is important for in-cage folding and hence successful folding at high yield.

Comment by Referee #1
Most of the experiments had to be done on the single ring version of GroEL, in which the important, inter-ring allosteric interactions are absent. These interactions strongly affect the stability of GroES binding to GroEL. The fraction of substrate able to escape from the encapsulated state is likely to be smaller in the native, double ring complex, as suggested by the data in Figure 6.

Our response
Yes, fraction of the natural escape of rhodanese is smaller in the double-ring GroEL than in the single-ring version of GroEL. However, it may depend on substrate protein as in the case of single-ring GroEL. Important point is that all non-native substrate proteins in the double-ring GroEL are subjected to the forced escape as shown in Fig. 6, and, therefore, polypeptide is protruding partly outside of the cage of the double ring during assisted folding. So far, the scenario inferred from single ring is essentially valid for double-ring GroEL.

Comment by Referee #1
If the crosslink data are correct in showing that disordered proteins escape mainly through the GroEL-GroES interface (not the biggest hole in the complex), then this is likely to involve fluctuations in GroES binding, e.g. by transient detachment of some of the 7 sites.
Our response

Horwich group reported that two or three GroES-binding-active GroEL subunits in the GroEL heptamer ring are enough to bind GroES stably (Farr, 2000, Cell) and we also reported that three or four GroEL-binding-active GroES subunits in the GroES heptamer are enough to bind GroEL stably (Nojima, 2008, JBC). Therefore, among seven GroEL-GroES binding sites, transient unbinding of three or four sites can occur while other four or three sites retain intact binding. This is a good candidate of the conformational state for the natural escape although poor crosslink with T261C (located underneath GroES mobile loop that has tight contact with GroEL) makes it ambiguous.

Comment by Referee #1

1. In emphasizing the novelty of their findings, the authors make some slightly inaccurate statements in the introduction. It is clear from the GroEL-GroES structure that the cage is not "sealed", but has holes or "windows".

Our response

We agree. In the revised manuscript, we changed the word "sealed" to "covered".

Comment by Referee #1

Cryo EM work has shown that substrate binding does not "mostly overlap with protein binding".

Our response

We read the above sentence as "Cryo EM work has shown that GroES binding does not "mostly overlap with protein binding"". In Horwich-Saibil’s paper (2007, Mol Cell), cryo EM images of MDH contacting three or four consecutive GroEL apical domains were shown. In this case, GroES occupies other four or three GroEL apical domains and "GroES binding does not mostly overlap with protein binding". When looking at a single apical domain, most of the hydrophobic residues in the apical region of GroEL contribute to binding of substrate protein and GroES as well (Fenton, 1994, Nature; Motojima, 2000, JBC). In this case, the apical residues for GroES binding mostly overlap with those for substrate protein binding.

Comment by Referee #1

In addition, it should be mentioned that the Horwich group has shown that bound, unfolded proteins are very disordered and can bind at many sites on the chaperonin surface. Those previous findings make it less surprising that non native polypeptide can continue interacting with GroEL during GroES binding, and also interact with external regions of the chaperonin surface.

Our response

In Horwich-Saibil’s paper (Mol Cell, 2007), a residue E315C located at external region was crosslinked with unfolded protein. However, after GroES binding, the same E315C was not crosslinked with unfolded protein (Fig. 3B). It appears that what we observed is not simple continuation of interaction of polypeptide with GroEL during GroES binding.

Comment by Referee #1

2. Protease protection (p. 6): The Weissman et al 1995 study showing protection from proteinase K was not done after folding, as implied in the current paper. The protease was added to a complex made with ADP, so that the protein would not have completed folding.

Our response

Previously we reported that GroES was unable to bind to GroEL-rhodanese complex in ADP when hexokinase and adenylate kinase inhibitor were present (Motojima, 2003, JBC). Therefore, we suspect that, in Weissman’s experiment, trace amount of ATP contaminating in or generated from ADP could support rhodanese folding in the cage. However, the issue is not settled and we deleted the sentence “However, in most of the previous reports, proteinase K treatment was carried out after the substrate protein already completed folding in the cage” in the revised version.

Comment by Referee #1

The authors need to analyze why they find greater protease accessibility under the conditions of their single ring complexes. For example, would chymotrypsin give a different result under the conditions used by Weissman et al, are the differences due to the use of single ring GroEL, or different nucleotide states? (The citation in this section should be to Figure 1E, not 1D).
Our response
Weissman et al used proteinase K (0.2 g/ml). We found that proteinase K (50 g/ml) digested rhodanese in the early phase of the assisted folding and resistance increased with a time course similar to chymotrypsin (new Fig. S1C), although the data was not fully satisfactory because free native rhodanese was also susceptible to proteinase K at this concentration. We modified the description on proteinase K in the text accordingly.

As pointed, the citation in this section should be to Figure 1E, not 1D. We corrected.

Comment by Referee #1
3. The gold labeled EM images are very unclear. The binding appears to be on the side of the second image rather than at the GroEL-GroES interface as claimed. Most but not all of the images seem to be of "football" complexes with GroES at both ends. With only 5 particles selected it is impossible to judge whether these images really demonstrate anything.

Our response
We observed 22 images of the gold-labeled GroEL-GroES complex. Among them, 20 had a gold particle clearly at the GroEL/GroES interface region and two at ambiguous region (new Fig. 6D). In supplemental data (new Fig. S5C), we showed images of streptavidine-labeled GroEL-GroES that contained biotin-labeled rhodanese. The images were not very clear but streptavidine appeared to bind to GroEL/GroES interface region.

Recent works (Koike-Takeshita, 2008, JBC; Sameshima, 2008, JBC; Nojima, 2009, JBC; Sameshima, 2010, JBC) revealed that the majority of the complexes formed in ATP was "football".

Comment by Referee #1
4. Why are there 2 peaks for the rhodanese-trap complex in Figure 1D?

Our response
From electron micrographic observation, we found that rhodanese-trapGroEL(N265A) complex can form a dimer.

Comment by Referee #1
5. The paper is clearly written but the English grammar needs a little editing.

Our response
The English was polished by a native speaker.

Comment by Referee #1
In summary, this is a novel study which reveals interesting behavior of encapsulated substrates in the single ring model of GroEL, some of which will be relevant to the full machine. The data showing the capture and extraction of substrate molecules from the folding cage are quite remarkable and provide new insight into the dynamics of non-native proteins inside the chaperonin cage.

Our response
We appreciate this positive comment.

RESPONSE TO REFEREE #2

Comment by Referee #2
In this paper, the authors present data that they interpret as suggesting that substrate protein encapsulated in the so-called Anfinsen cage can stick out into bulk solution or even escape whilst GroES is bound. Such scenarios are not included in current models of GroE's mechanism of action but I'm not sure that their inclusion would really change the way we think about how this machine works. More importantly, I believe that the interpretation of the data is flawed since the authors ignore the possibility that substrate can escape via the equatorial plane that is flexible in SR1. Hence, (i) most of the data are not relevant to the double-ring system that exists in vivo and (ii) the model in Fig. 7 is likely to be wrong. The authors could have prevented escape via the equatorial plane by stabilizing it using cross-linking or by using larger substrates (~60 kDa).
Our response

The stringent substrate protein that requires the assistance of chaperonin to fold into native state is always smaller than 60 kDa. Even though fraction of the natural escape of rhodanese from double-ring GroEL is smaller when compared with single-ring GroEL, polypeptide of non-native rhodanese in the double-ring GroEL is protruding partly outside of the cage because non-native rhodanese is subjected to the forced escape (Fig. 6). In this case, the hole at the bottom of the equatorial plane is not open to outside and cannot be a pathway of protruding polypeptide.

Comment by Referee #2

1. The FRET experiment shown in Fig. 2C is not a measure of escape but a measure of binding to the trap in an appropriate orientation as there are modes of escape that would not result in a FRET signal.

Our response

When denatured rhodaneseAlexa and trap(D87K)TexasRed were mixed, FRET efficiency jumped immediately and a slight, slow decrease followed (Supplemental Fig. S1D, red curve). Therefore, slow (0.10 min⁻¹) FRET change in Fig. 2C reflects the binding itself, but is not a measure of the binding in "appropriate orientation".

Comment by Referee #2

In addition, since the reaction is bi-molecular the rate constant as measured is concentration-dependent and thus not meaningful.

Our response

Yes, it is bi-molecular reaction. However, the binding of denatured rhodanese to GroEL (and trap(D87K) as well) is very rapid as Rubisco (Rye, HS et al., Cell, 1999, ~10⁷ Mol⁻¹s⁻¹) (see new Fig. S1D, red curve) and it should finish within 1 sec under the conditions of our experiments. Indeed, even when 2-fold trap(D87K) was mixed, FRET increase was not accelerated (we added this data in new Fig. 2C). Therefore, a slow rate (0.10 min⁻¹, (Fig. 2C, new Fig. S1D, green curve)) reflects the escape event.

Comment by Referee #2

2. The authors do not get rid of substrate that does not enter the cavity before starting the experiments. Hence, at least some of the substrate found outside the cavity did not escape but was there from the start.

Our response

When the trap(D87K) was added before addition of GroES and ATP, small rapid FRET increase (6 % of the maximal FRET change) was observed (new Fig. S1D, blue curve). This change would reflect the binding of free, denatured rhodaneseAlexa to trap(D87K) TexasRed. However, this change was much smaller than that induced by the escaped rhodaneseAlexa from the cage after the start of the reaction (26 % of the maximal FRET change, see new Fig. S1D, green curve). Therefore, FRET induced by the free rhodanese outside the cavity before the start is negligible in estimation of the escape rate of rhodanese after the start. We added explanation on this in the revised text.

Comment by Referee #2

3. The stability of the GroES-GroEL (SR1 or double-ring) complex should be monitored over time under the different experimental conditions employed.

Our response

We showed the stable association of GroESAEDANS with GroEL (single-ring or double-ring) in the presence of excess amount of non-labeled GroES after a 30-min incubation under the different experimental conditions employed (Figure 1C, Supplemental Figure S3, S4A and S5A). More than 90 % of rhodanese activity was already recovered at 30 min. We think these data are sufficient to show that the escape of denatured rhodanese is not caused by GroES release.

Comment by Referee #2

4. The fit to lag kinetics in Fig. 2D is poor and not justified given the number of data points.

Our response
We think that the fit in Fig. 2D, line O is rather good, if not perfect, in this kind of experiments, especially taking into account of a small extent of the escape (20 %) and time required for isolation of the escaped fraction (~5 min). The data for SR398(Y203C) with large extent of the escape (40 %) showed a lag phase more clearly (Fig. 4B, line O).

Comment by Referee #2
5. p. 8 - the cysteine-less variant is known to be unstable and difficult to work with. Hence, I would like to see the evidence that is referred to as 'not shown' that this variant is active.

Our response
We added the recovery of wild-type rhodanese activity assisted by cysteine mutants used in this study (new Fig. S2A). The data for F44C, Y203C and V263C were shown in Fig. 4.

Comment by Referee #2
6. p. 8, bottom line - the loss in free rhodanese band intensity can also be due to aggregation the extent of which cannot be judged as the bottom of the wells in the gels are not shown

Our response
The samples were mixed with a buffer containing 1 % SDS before electrophoresis as usual SDS-PAGE procedures. Thus, if aggregation was formed, it should be solubilized. We did not find any large protein band at the bottom of the sample wells or in stacking gels. Presence of rhodanese in cross-linked product was confirmed as shown in Supplemental Figure S2B and S2C.

Comment by Referee #2
7. Figure 4F - the differences between lanes 1-4 are not explained in the legend.

Our response
These lane numbers correspond to those of Figure 4G. We added the explanation in the legend.

RESPONSE TO REFEREE #3

Comment by Referee #3
The experiments described by Motojima and Yoshida were designed to examine the location of pre-folded (i.e. unfolded) rhodanese polypeptide within the GroEL-GroES complex as the assisted folding reaction proceeds.

In order to attempt to trap the substrate protein within the cavity the authors use a single-ring version of GroEL with an ATPase-inhibiting mutation (SR398). The observed rate of folding to the native state was slow (0.11 min-1) allowing the time-resolved treatment of the reaction with proteinaceous agents incapable of penetrating the SR398-GroES cavity. Three different proteins are used as trapping agents; mutant versions of GroEL (trapGroEL), anti-rhodanese antibody and chymotrypsin. The general finding is that rhodanese becomes protected from the trapping agent at a rate which is the same as its rate of assisted folding. This means that the pre-folded cavity-held species of rhodanese is exposed to these large proteins that cannot enter, hence the rhodanese chain must be protruding from the cavity itself.

One question arises. Why do they not show the result of the experiment where proteinase-K is used as a proteinaceous trapping or degrading agent, like one done by Weissmann et al. 1995? The authors describe doing this experiment on the rhodanese-folding system, but state that a clear time course was not obtained and data not shown.

Our response
Weissman et al used proteinase K at 0.2 g/ml in their experiments. As explained in the response to Referee #1, we found that, similar to chymotrypsin, when we used proteinase K at high (50 g/ml) concentration, rhodanese was susceptible to digestion at early phase of the assisted folding and the resistance increased with a time course similar to chymotrypsin (new Fig. S1C).

Comment by Referee #3
Looking at the rate of escape of substrate polypeptide from the SR398-GroES cavity it is 0.13 min-1 with D87K trap, but faster with the N265A trap, 0.49 min-1. The authors do not make it entirely
clear why, if this is the intrinsic dissociation or escape rate for rhodanese, then why should it vary with the trap?

Our response
FRET change of trap(D87K) reflected the natural escape and that of trap(N265A) reflected the forced escape. We showed that low concentration of trap(D87K) did not reduce yield of the folded rhodanese in the cage but trap(N265A) strongly reduced it (Figure 2A). The titration experiment of trap-GroELs vs recovered rhodanese activity (Figure 2B) also indicated that even low concentration of trap(N265A) halted the folding of rhodanese in the cage. Therefore, trap(D87K) binds denatured rhodanese escaped by itself from the cage ("natural escape"), but trap(N265A) binds to polypeptide portion protruding out of the cage and finally drags out whole polypeptide from the cage ("forced escape"). The faster rate of FRET change of trap(N265A) than that of trap(D87K) should reflect the binding of trap(N265A) to polypeptide before natural escape. The text of the revised manuscript is modified to make this point clear.

Comment by Referee #3
The authors also probed the rhodanese substrate position in the pre-folded form using thiol groups introduced by cysteine mutagenesis into the GroEL structure. Of these groups, seven were introduced into the GroEL-GroES interaction surface region and one on the inside of cavity. Residue 44 on the cavity wall reacted readily with pre-folded rhodanese, four of the GroES-interaction surface SH groups were unreactive, but three reacted with the rhodanese. Of these three groups, two are in the outer region and one on the inner region of the contact surface. Like the trapping experiments, this result also implies that the unfolded substrate can escape at least partially. It is also shown that exposed hydrophobic groups on the cavity surface have a strong effect on the rate of folding and release of the rhodanese substrate.

The final sections of the paper deal with a comparison of the escape mechanisms of dihydrofolate reductase and rubisco and lastly with the forced escape of rhodanese from the double-ring GroEL-GroES system where the ATPase had been inhibited by the D398A mutation. The authors show that in this system folding can also be trapped on a short time scale.

The quality of the work is good and new findings are produced. I recommend acceptance given some consideration of the points made above, but some careful editing is needed to perfect the English usage.

Our response
We appreciate the above positive comment. English was checked by a native speaker.
REFEREE REPORTS:

Referee #1 (Remarks to the Author):

There is one scientific point that I didn't raise explicitly the first time round: in the bottom paragraph of page 8, the experiment describing how they tested the site of escape by cross linking, it seems that they only tested residues around the GroEL-GroES interface. There are larger holes in the sides of the complex, between adjacent GroEL subunits. Those holes could provide a more likely site for exposure and trapping of the unfolded polypeptide. They don't seem to have tested residues in that region.

Another experimental point that I don't find entirely satisfactory is the gold labelling. It is still a very small amount of data, and there are no controls mentioned. Gold particles often bind non specifically. If they want to show the gold labelling they should include in the supplementary figures a negative control with streptavidin gold particles added to complexes without biotin. It is hard to deduce anything at all from the 4 panels of streptavidin labelling without gold.

2nd Revision - authors' response

Response to comments by referee #1

Comment by Referee #1
There is one scientific point that I didn't raise explicitly the first time round: in the bottom paragraph of page 8, the experiment describing how they tested the site of escape by cross linking, it seems that they only tested residues around the GroEL-GroES interface. There are larger holes in the sides of the complex, between adjacent GroEL subunits. Those holes could provide a more likely site for exposure and trapping of the unfolded polypeptide. They don't seem to have tested residues in that region.

Our response
Yes, a possibility of the escape through space between adjacent GroEL subunits remains and we did not deny this possibility in the original text. Rather, we stated (page 9, line 12) "It is noteworthy that Y203 and V264 are exposed outside of the cage and located near the interface between GroEL subunits, while R231 is exposed inside of the cage near the GroEL/GroES interface. These results indicate that a portion of the polypeptide chain of rhodanese protrudes through a narrow space near the interfaces between the GroEL subunits and between GroEL and GroES". The context of the sentence includes, though not directly, the possibility of the holes of the side wall and may be enough to remain the possibility.

Comment by Referee #1
Another experimental point that I don't find entirely satisfactory is the gold labeling. It is still a very small amount of data, and there are no controls mentioned. Gold particles often bind non specifically. If they want to show the gold labeling they should include in the supplementary figures a negative control with streptavidin gold particles added to complexes without biotin. It is hard to deduce anything at all from the 4 panels of streptavidin labelling without gold.

Our response
Specificity of the binding of streptavidin-coated gold particle to biotinylated protein has been well established. Indeed, when we observed electron microscopic images of a control specimen in which non-biotinylated rhodanese was used as a substrate protein, gold particles were all free and did not associate with GroEL-GroES-rhodanese complex (see attached figures "rhodanese", free gold
particles are indicated by white arrows). In contrast, when biotinylated rhodanese was used as a substrate, about half of gold particles were associated with GroEL-GroES-rhodanese complexes (see attached figures "biotin rhodanese", indicated by red arrows). Therefore, non-specific association of streptavidine-conjugated gold particle with protein did not occur. I don’t think these figures are necessary in the supplementary figures because specificity of biotin and streptavidin are well known.