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Opinion on the PhD thesis by Agnieszka Kłosowska

The PhD thesis by Agnieszka Kłosowska addresses an interesting paradox in biochemistry that has not been resolved for a long time. Many AAA+ chaperones and ATP-dependent proteases are strongly inhibited by ADP, their reaction product. For *in vitro* experiments, it has become customary to overcome this problem by the use of ATP-regeneration systems, using creatine phosphate and creatine kinase (CK) or phosphoenolpyruvate and phosphoenolpyruvate kinase (PEPK) to keep the ATPase reaction going. This practice has been justified by the explanation that the ADP concentration is "much lower" than the ATP concentration *in vivo*, due to oxidative phosphorylation by the respiratory chain. However, closer inspection shows that this widely quoted excuse for product inhibition *in vitro* and the lack of product inhibition *in vivo* cannot be right, at least for cytosolic or nuclear proteins in eukaryotes. ATP/ADP ratios are of course dependent on the metabolic state of cells (anaerobic glycolysis versus aerobic respiration). Actual values are between 10:1 and 1:1, depending on conditions. The ratio of ADP/ATP dissociation (binding) constants is much higher, showing that "low" concentrations of ADP *in vivo* do not resolve the paradox. In her PhD thesis, Agnieszka Kłosowska addresses the paradox for the special case of the Hsp104 protein from baker's yeast and provides an elegant solution for the problem that is likely to be more generally applicable.

Agnieszka Kłosowska's thesis is conventionally structured, clearly organized, and concise, yet comprehensive. The Introduction presents the proteins of interest, Hsp104, Hsp70 and Hsp40, primarily from the perspective of a biochemist or structural biologists, but nevertheless with good awareness of the cellular roles of the investigated proteins. As it should, the Introduction "homes in" from more general information to very detailed prior experimental results that are necessary to understand the experimental data. The Results section sets up an interesting paradox (that many investigators have chosen to disregard), and then presents a very logical line of experiments to resolve the paradox. Some experiments at the end of the Results section raise new questions that are not fully answered in the thesis. The Discussion is brief but clear and nicely puts the experimental results into perspective. The bibliography is extensive (about 160 citations) and shows that Agnieszka Kłosowska knows her field extremely well. In the following, I summarize the key results of the thesis and present a number of questions.

Setting up the paradox: In her PhD thesis, Agnieszka Kłosowska draws attention to the paradox that biochemical data for Hsp104 suggest that the protein should not be functional under physiological conditions. Using an HPLC assay, she confirms literature data on the ratio of ATP/ADP in yeast cells. She then goes on to demonstrate that in conditions of excess nucleotide over protein (the physiological situation), a small amount of ADP can effectively suppress the Hsp104 ATPase activity,

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even when ATP is in excess. The suppression is dependent on the ADP dose and convincing. But does the suppression also affect the chaperone activity of Hsp104? Agnieszka Kłosowska uses a very elegant, albeit somewhat artificial assay, to address this question in a quantitative manner. Hsp104, a ClpB protein, is known to interact with other chaperones (the Hsp70 machinery), but unlike ClpA, ClpX or ClpY, it does not cooperate with "cage" proteases such as ClpP or HslV (ClpQ). However, there is an engineered variant of Hsp104 developed by others, known as HAP, which interacts with ClpP and feeds its substrates to ClpP for degradation. Using this HAP variant as a "proxy" for Hsp104 itself, and a fluorescein labelled (unfolded, but non-aggregated) protein substrate, Agnieszka Kłosowska can monitor Hsp104 dependent protein degradation by the decrease in fluoresceine fluorescence anisotropy (resulting from faster tumbling as a consequence of protein degradation). The results show that ADP inhibits not only the Hsp104 ATPase activity, but also the "translocation activity" of the protein (Fig. 12), even in the presence of excess substrate (Fig. 13). ADP mediated inhibition is at least partly due to slower substrate binding, as demonstrated by another fluorescence anisotropy assay, using a tight-binding variant of Hsp104 (with a mutation in one of the two ATPase domains) and a fluoresceine labelled substrate (Fig. 14). Reassuringly, the apparent Kd values for ADP ("apparent" because of competition with ATP) are comparable (~ 0.2 mM). This finding establishes the paradox that Hsp104 should not be active *in vivo*, contrary to the experimental results (by others).

Resolving the paradox: The contradiction between the biochemical properties of Hsp104 on the one hand and the cellular activity on the other hand is solved in an elegant way. The key experiment of the thesis is the demonstration that Hsp70 (in collaboration with the Hsp40 co-chaperone) allows Hsp104 to overcome ADP inhibition in protein disaggregation (Fig. 15). Multiple explanations for this finding are possible: The Hsp70 system might be involved in recruiting Hsp104 to substrates, could be involved in feeding substrates to Hsp104, or could affect the biochemical properties of Hsp104, for example by allosteric activation of Hsp104 in the presence of ADP. In order to distinguish between these mechanistic possibilities, Agnieszka Kłosowska uses variants of Hsp104 differing from the wild-type protein in various respects. First, Agnieszka Kłosowska concentrates on a variant of Hsp104 (D484K), which is hyperactive as an ATPase and can dissolve aggregates in addition to being able to translocate proteins (in the HAP experiment). As perhaps expected, ADP inhibits this protein to a similar extent as the wild-type protein (Fig. 21). However, very surprisingly, disaggregation of heat-aggregated GFP by the hyperactive Hsp104 variant in the presence of the Hsp70 system remains efficient (Fig. 22). This suggests that Hsp70 might be involved in recruitment of Hsp104 to aggregates, as indeed confirmed (Fig. 23). Efficient recruitment may then make up for impaired substrate binding of Hsp104 in the presence of ADP. If this model was correct, an Hsp104 variant that cannot interact with the Hsp70 system should be blocked by ADP. Here, Agnieszka Kłosowska has the problem that a mutant has been described, but turns out not to have the desired properties. By elegant sequence analysis and comparison of sequence features with known properties of Hsp104 orthologues, Agnieszka Kłosowska finds a variant that she predicts will abolish Hsp104 Hsp70

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interaction. Indeed, the F508A mutation abolishes Hsp104 D484K (hyperactive Hsp104) Hsp70 cooperation in disaggregation (Fig. 26).

A new paradox: Up to this point in the thesis, the results can be straightforwardly interpreted. However, a few additional experiments are then reported that do not easily fit with the previously described data. First, Agnieszka Kłosowska shows that Hsp70 does not support Hsp104 in processing of non-aggregated proteins (Fig. 28). Second, and more confusingly, Agnieszka Kłosowska finds that at least the ATPase activity of Hsp104 can be rescued from ADP inhibition by excess substrate (Fig. 30). This result does not directly contradict the earlier finding that ADP inhibition of the translocation activity cannot be overcome by an excess of substrate (Fig. 13), but the two findings are hard to reconcile. If both findings are correct, the chaperone system would be "wasting" ATP under conditions of stress, and by producing more ADP, undermine its own activity. Agnieszka Kłosowska is aware of the paradox and discusses possible solutions (discussion on p. 75). I (partially) understand the mechanistic model to reconcile the findings. Nevertheless, the impression remains that the combined results suggest Hsp104 behaviour that would be undesirable in cells. Therefore I suspect that something is still missing from the model.

I have a very high opinion of the presented science and of the editorial standard of the thesis. Apart from the above question regarding a possible contradiction in the findings, I have only few technical questions or suggestions.

- Commercial ADP preparations are known to be impure. Has the quality of the ADP preparations been independently checked prior to use?
- When the inhibitory effect of ADP is monitored, the assays are started with a given ADP concentration. However, during the assay additional ADP is generated. First order kinetics should not be affected, but in practice, slopes of progress curves are measured at time points other than the reaction start. Is Agnieszka Kłosowska sure that the "additional" ADP is always negligible? If not, what computational corrections have been made?
- Auto-inhibition by the M-domain: I would have expected that auto-inhibition affects the binding constant (in the same way that a competitor changes the Kd to an apparent Kd), but I would not have expected the kcat to change. The de-repressed Hsp104 variant does the exact opposite. Does Agnieszka Kłosowska have an explanation?
- I have missed a direct demonstration that the Hsp104 F508A mutation is indeed disrupting the interaction between Hsp104 and the Hsp70 machinery. All presented assays are indirect. Has the interaction been tested directly (by comparing pull-downs, ITC, competition experiments)?
- I have some reservation with regard to the substrates. All substrates used in the study are non-physiological model proteins. I know that this has been widespread practice, but are there really still no physiological substrates that could be used instead of these model proteins?

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The results described in Agnieszka Kłosowska's PhD thesis have been very deservedly published in high impact journals. Agnieszka Kłosowska's work is the basis of a publication in eLife (she is first author), in EMBO J. (she is second author). I suspect that her answer to the ATP/ADP paradox will become the prototype for the resolution of similar paradoxes in the case of other AAA+ ATPases. Due to the rigor of the work, the novelty of the insights, and the potential applicability of the general ideas to a larger class of proteins, I advocate to not only award her a PhD, but to also consider her PhD thesis for a distinction, and possibly the award of the Polish Prime minister for the best PhD thesis.

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