

4. Outlook and Discussion

The main objective of my thesis is the characterization of the functional interplay of the AAA⁺ protein ClpC and its adaptor proteins. The activation of ClpC by facilitating its oligomerization via an adaptor protein is a new control mechanism among the Hsp100 proteins. This thereby differs from the homologous Clp ATPases of *E. coli*, ClpA and ClpX, whose adaptor proteins only modulate the substrate repertoire of their partner ATPase. Moreover, the adaptor protein mediated activation of ClpC only precedes the assembly of the complete proteolytic ClpCP complex. Thus, the ClpC adaptor proteins gain a vital role in the hierarchical control of the ClpCP mediated proteolysis.

Why is the activity of ClpP that tightly controlled?

It could be demonstrated that BsClpP alone displays peptidase but no proteolytic activity (Brotz-Oesterhelt et al., 2005). A shielding of the catalytic sites by an ATPase triggered oligomerization to protect the cellular environment from uncontrolled proteolysis might therefore not be an explanation for this hierarchical control. A similar ClpP activation mechanism, which is based on its assembly into its active tetradecameric state was reported for human ClpXP in mitochondria (Kang et al., 2005). hClpP requires its partner ATPase to switch from the single heptameric ring into the functional double ring structure. Likewise, heptameric hClpP exhibits only a very low peptidase and no proteolytic activity against proteins. It is assumed that the association of one heptameric ClpP ring with a ClpX hexamer induces a conformational change in the single ClpP heptamer, which then favours the formation of the double heptamer. The catalytic triad adopts thereby the active configuration, which is in the single ring in an inactive orientation, due to the high flexibility of the closely located handle region. (Yu and Houry, 2007). BsClpP differs in that it does not even form the single heptameric ring. The association with a hexamerized ClpC and probably also ClpE and ClpX leads directly to the assembly of the monomeric ClpP into the active ATPase associated ClpP tetradecamer (see 3.2.). Further structure-function analysis' would be valuable to understand the correlation of the oligomeric state and the probably unique structural features of BsClpP.

The ATPase governed control of the ClpP activity in *B. subtilis* might be crucial for two reasons for Clp proteolysis in general. (i) One can assume an equilibrium of ClpP being associated either with ClpC, ClpE or ClpX depending on the environmental conditions. This suggests a rather dynamic control of ClpP, whose activity depends on the availability of hexamerized ClpC, ClpE or ClpX. The more since the assembly of the ATPase component can become itself, as demonstrated for ClpC, a subject to regulation. (ii) Initial studies revealed that Clp ATPases also control ClpP regarding its subcellular localization. In these experiments, the *in vivo* ClpP localization was monitored via fluorescence microscopy using a *clpP-gfp* construct replacing the *clpP* gene at its chromosomal locus. It could be observed, that ClpP localizes preferentially at the cell poles already under normal growth conditions and the number and size of the foci only increase upon heat shock. Interestingly, ClpP delocalizes in a $\Delta clpX$ mutant at 30 °C but not after heat shock. This implies (i) that the 'house-keeping' Clp ATPase ClpX is required for the ClpP localization to the cell pole under normal growth conditions, whereas (ii) the loss of ClpX could obviously be compensated by the activation of ClpC and ClpE at 50 °C. Thus, the activity and localization of ClpP seem to be linked and confirm the *in vitro* data of the ATPase dependent assembly into the functional proteolytic complex (unpublished results).

Regulation by adaptor proteins

Up until now three ClpC adaptor proteins could be identified. The first, MecA was initially discovered together with ClpC in the same genetic screen for repressors of competence development (Dubnau and Roggiani, 1990). MecA forms together with ClpC a bi-partite chaperone complex, enabling the recognition and targeting of (i) misfolded and aggregated proteins and (ii) of specific substrates such as ComK.

Its paralog YpbH is the second identified ClpC adaptor protein. Although YpbH shares with MecA 52 % sequence similarity on the amino acid level, it differs from MecA in terms of the linker region between the N- and C-terminal domains (Persuh et al., 2002). This difference might be the reason, that YpbH does not target the key regulators of competence development, ComK and ComS, for degradation. However, YpbH forms like MecA together with ClpC a chaperone system exhibiting disaggregation and refolding activity and thus contribute to the protein quality control (Schlothauer et al., 2003).

McsB is the most recently identified and third ClpC adaptor protein (Kirstein et al., 2007). The amino acid sequence shows no similarity to MecA/YpbH, which demonstrates the difficulty to identify new adaptor proteins based on an *in silico* approach, only. The class III heat shock repressor, CtsR and the short-lived Clp ATPase ClpE seem to be the only substrates for McsB (Kirstein et al, 2007 and unpublished data). Thus, restricting the substrate spectrum of McsB to the CtsR regulon. Unlike CtsR, ClpE is not a phosphorylation target of McsB (unpublished data). Therefore, a phosphorylation of a substrate appears not to be a prerequisite for the McsB-mediated targeting to ClpCP. This raises the question whether McsB might also phosphorylate a certain protein, which is not concomitantly targeted for degradation to ClpCP. Preliminary data demonstrated that ComK, which is a ClpCP substrate targeted by MecA is a substrate for the McsB kinase, but does not get targeted to ClpCP by McsB. The role of the ComK phosphorylation and the involvement of McsB in the competence regulation is subject of an ongoing joint project with Dave Dubnau at the PHRI/ICPH in Newark, NJ (USA).

A chaperone function could not be attributed to McsB so far, however McsB does co-localize with inclusion bodies *in vivo* (unpublished data). This suggests that McsB could, as already demonstrated for the two other adaptor proteins, MecA and YpbH, be part of the protein quality control system, too.

Substrate recognition by the adaptor protein and / or ClpC?

A key question that remains unanswered is whether ClpC itself contributes to the substrate recognition, too. The diverse substrate spectrum of ClpC is achieved by its interaction with probably a number of adaptor proteins (see below). Nevertheless it is known for homologous Clp ATPases, such as ClpA and ClpX, that they exhibit an adaptor independent substrate recognition. However, none of the known ClpCP substrates is recognized and bound by ClpC alone. This failure is independent of its assembly as demonstrated by using the CTD of MecA, which still facilitates the oligomerization of ClpC, but lacks the substrate binding (Kirstein et al., 2006). Nonetheless, using solid phase supported peptide libraries, it was shown that ClpC binds to aliphatic, aromatic and / or positively charged peptides (unpublished data). The preference for a hydrophobic core enriched with aromatic and arginine or lysine residues is a common recognition motif for chaperones. Thus, such an adaptor

independent recognition and binding of presumably unfolded substrates by ClpC might then only require an (unspecific) adaptor protein for the oligomerization of ClpC. However, it could also be demonstrated that the recognition by the adaptor determines the final binding pattern of the ClpC-adaptor complex. Therefore, one can assume that ClpC might contribute to substrate binding, but basically relies on the primary recognition of a particular adaptor, emphasizing again the vital role of adaptor proteins. On the other hand it could be observed that although the ClpAC hybrid protein supported, together with MecA, the degradation of ComK, it was less efficient than the native ClpC (see 3.2.). Obviously, also other structural elements of ClpC, which are not present in the NTD and Linker domain contribute to the substrate degradation probably at the stage of the translocation. Substrate binding sites within the ATPase domains, in particular at the central pore of either the first or second AAA module, were already reported for ClpB and ClpA (Hinnerwisch et al., 2005; Schlieker et al., 2004).

In summary, the initial substrate recognition seems to depend on the adaptor protein, whereas ClpC might bind the substrate as part of the subsequent process of unfolding and translocation into the ClpP chamber.

Regulation of adaptor proteins

Interestingly, McsB as regulated adaptor protein seems not to be an exception as more and more data substantiate the assumption that adaptor proteins are generally subject to regulation. Whereas McsB is regulated by its kinase-status, exhibiting adaptor properties only when phosphorylated, the MecA mediated targeting of ComK for instance is abolished in the presence of ComS. Therefore, ComS can be regarded as a member of a new protein family, the anti-adaptor proteins (Bougdoor et al., 2006; Prepiak and Dubnau, 2007). The second member of this novel class of regulators is IraP, which antagonizes the activity of RssB, a ClpX adaptor protein targeting σ^S for degradation in *E. coli* (Bougdoor et al., 2006). Both, IraP and ComS are rather small proteins of 86 aa and 46 aa, respectively. Thus, it is possible that anti-adaptor proteins exist in a high number and are missed out so far during the genome annotation due to their small size. It is even conceivable to assume that one particular adaptor protein could be regulated by several anti-adaptor proteins. This would thereby allow a

control of the cognate adaptor-Clp ATPase-complex under a variety of environmental conditions.

More adaptor proteins?

Besides the protein quality control, ClpC is involved in a variety of signaling and cell differentiation processes. The proteolysis of regulatory proteins such as ComK, CtsR, MurAA and SpoIIAB enables ClpC to control key steps of developmental pathways. The broad substrate spectrum is covered by the employment of several adaptor proteins. Whereas the cognate adaptor protein for ComK (MecA) and CtsR (McsB) is known, no adaptor protein could be assigned for the ClpC-dependent degradation of MurAA and SpoIIAB. The identification of these so far missing adaptor proteins would be valuable especially with respect to their specific features regarding ClpC-interaction (binding sites, affinity, competition or simultaneous/concerted action with one of the known adaptors), their substrate spectrum (unique or overlapping with one of the known adaptors) and their regulation (modification, anti-adaptor). The current knowledge of the ClpC adaptor protein network is illustrated in figure 24.

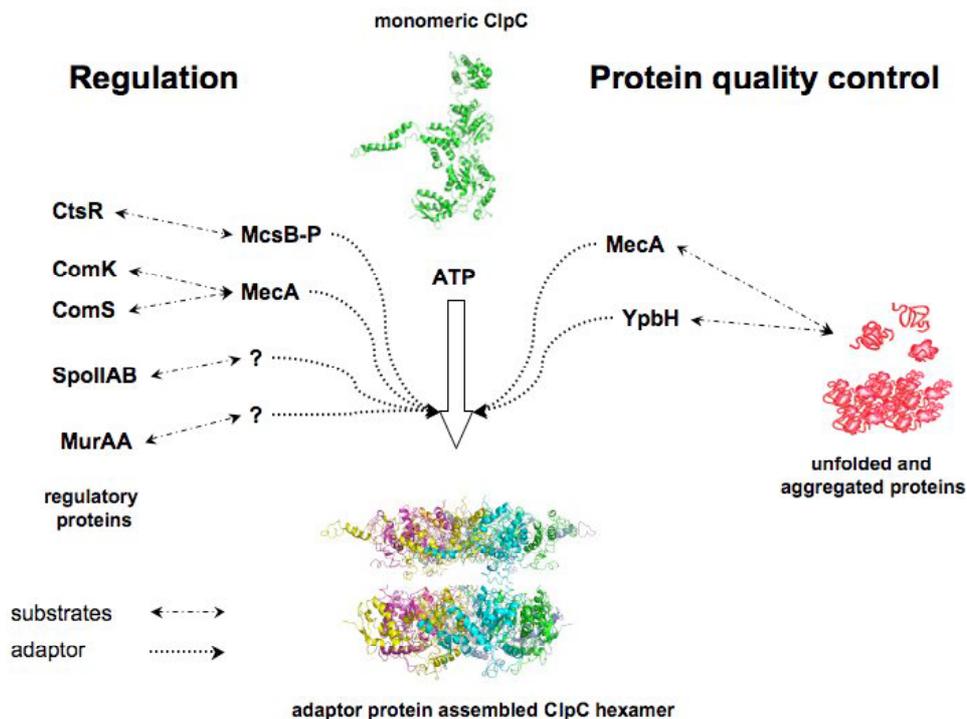


Fig. 24 ClpC adaptor protein network. The adaptor protein mediated assembly precedes the association with the proteolytic core (not shown; see 3.2.). The two branches reflect the dual function of ClpC: regulatory proteolysis (left) and general proteolysis as part of the protein quality control (right).

Surprisingly, McsB seems to act as an adaptor for ClpE as well (unpublished data). *In vitro* experiments could demonstrate that ClpEP and McsB-P form a proteolytic complex enabling the degradation of CtsR. This finding is unexpected since ClpE, which partially undergoes autoproteolysis after heat shock is also degraded by ClpCP in a McsB-dependent manner. ClpE is therefore a substrate and a partner ATPase for McsB. This ATPase-adaptor interplay differs from the one with ClpC in that it depends completely on the phosphorylation of McsB. The autodegradation of McsB in the absence of a substrate requires the kinase activation by McsA. Moreover, McsB targets the same substrate, CtsR, for degradation by ClpEP, although at a slower rate than for ClpCP. It is not known yet, whether ClpE employs more adaptor proteins and if it requires for all its activities McsB. ClpE is the most tightly regulated member of the CtsR regulon and it has with a half life time of 1' after heat shock only a short time window of action (unpublished data). Interestingly and unlike ClpC, it already exhibits a high ATPase rate, comparable to those of the adaptor induced ClpC-ATPase activity, arguing for an independent assembly into a hexamer. Whether the interaction with McsB and potentially more adaptor proteins is required for the substrate interaction remains elusive.

Concerted activity or competition of adaptor proteins?

Since all three known adaptor proteins use the same binding sites on ClpC (Kirstein et al., 2007; Kirstein et al., 2006) a competition for binding to ClpC seems very likely. Indeed, it could be observed that the presence of McsB-P abolished the MecA- and YpbH-mediated substrate degradation (Kirstein et al., 2007). A mixed complex of different adaptor proteins interacting with different subunits within one ClpC hexamer is therefore rather unlikely but cannot be excluded, yet.

Structural changes upon adaptor binding

Although there is a difference in the binding preference, all three known ClpC adaptor proteins bind to both accessory domains, the NTD and the Linker domain. The interaction with both domains might be necessary to position the NTD and the Linker in a way to favour oligomerization and / or substrate recognition / translocation by ClpC. It was shown in a recent study that the middle domain of ClpB, a homologous Hsp100 protein from *E. coli*, undergoes movements upon nucleotide binding to the first AAA module (Watanabe et al., 2005). In the absence of nucleotides the orientation of the coiled coil domain is parallel to the main body of ClpB, but it takes a leaning position upon ATP binding. This movement of the middle domain results in a stabilization of the hexamer and triggers the ATP hydrolysis in the second AAA domain. The Linker of ClpC is approximately half the size of the homologous middle domain of ClpB. Based on a structural model, both accessory domains could be arranged in close proximity. It is therefore tempting to speculate that the adaptor bridges the contact of the NTD and the Linker domain and thereby fixing the position of the Linker, which might facilitate the hexamerization of ClpC.

Interestingly, the cyanobacterial ClpC homologue does not require an adaptor protein for its basic functions (Andersson et al., 2006), thus questioning the structural determinants for the adaptor facilitated oligomerization within BsClpC. Therefore, future studies should address the variations of the ClpC domains for a precise assignment of its functions regarding adaptor binding and oligomerization.

Concluding remarks

Taken together, the hierarchical control of ClpC allows an interference with the regulated proteolysis of a certain substrate at different stages along the degradation process e.g. at: (i) the recognition by a specific adaptor protein, (ii) the adaptor mediated oligomerization and thereby activation of ClpC, (iii) the further assembly of ClpCP and finally at (iv) the unfolding, translocation and subsequent degradation process. Especially the first two steps seem to be attractive targets for regulation given that the successful targeting to ClpCP requires (i) a sufficient concentration of the particular adaptor protein at the time / condition of the proteolysis, (ii) a high affinity to ClpC to allow an effective competition with other adaptor proteins and (iii) the subcellular co-localization with the substrate. Finally, the adaptor protein itself could be a subject to regulation as well and thus be either activated e.g. by post-translational

modification as demonstrated for RssB and McsB (see 3.4.; Bouche et al., 1998; Kirstein et al., 2007; Mika and Hengge, 2005) or antagonized by an anti-adaptor protein as shown for MecA and RssB (Bougdour et al., 2006; Prepiak and Dubnau, 2007). However, the preliminary data of the interaction network of ClpE, McsB and ClpC point out that different AAA+ proteins might share a subset of adaptor proteins and thus competition could take place at that level as well. Therefore, the identification of new adaptor proteins would be valuable to gain more insight into the various regulation modes of AAA+ proteins.