Thermophilic P-loop transport ATPases
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Summary and concluding remarks

Thermophilic organisms thrive at relatively high temperatures in geothermally heated regions such as hot springs and deep sea hydrothermal vents. The first extreme thermophilic organism was discovered in Yellowstone's Lower Geyser Basin in 1967 by Professor Thomas Brock, who simply placed a clean microscope slide into the waters of Mushroom Pool. After a few days, visual inspection of the slide under the microscope revealed that it was covered with cells. These cells were grown in the laboratory and the isolated organism was named *Thermus aquaticus* (229). The discovery of such thermophilic organisms was not only a new finding because it extended the previously anticipated temperature limit of life but it also initiated the successful research on thermophilic enzymes yielding for instance the "Taq" DNA polymerase of *T. aquaticus*. The search for thermophilic bacteria has lead to the discovery of whole palette of extreme thermophilic organisms and the description of the first archaeon *Sulfolobus acidocaldarius* (176).

Thermophiles are classified into obligate and facultative thermophiles. Obligate thermophiles are also called extreme thermophiles and strictly require high temperatures for growth. Facultative thermophiles (also called moderate thermophiles) can grow both at high and at lower temperatures. Hyperthermophiles are defined as organisms with optimal growth temperatures above 80 °C. Most isolated extreme- and hyper-thermophiles belongs to the domain of Archaea. Archaea were identified in 1977 by Carl Woese as a separate kingdom of life and distinct from other prokaryotes on the basis of the 16S rRNA phylogenetic tree. He renamed the groups Archaea, Bacteria and Eukarya as they comprise the three domains of life (25). The domain of the Archaea is divided into the subdomains euryarchaeota and crenarchaeota based on rRNA trees. The euryarchaeota include the methanogens, the halobacteria, which thrive in marine environments with extreme concentrations of salt, some extremely thermophilic aerobes and anaerobes, and extremely acidophilic thermophiles. Most Crenarchaea are thermophilic or hyperthermophilic organisms, some of which have the ability to grow up to 113 °C (230). Extreme and hyperthermophiles are, however, also found within the *Thermotogales* and *Aquificales*, both subdomains of the Bacterial domain (24;25).

This thesis focuses on two distinct organisms *Sulfolobus solfataricus* and *Thermotoga maritima*. *Sulfolobus* species are crenarchaeotes and have been isolated around the world from solfataric fields, and hot water and mud pools. *S. solfataricus* is an extreme thermoacidophile which grows optimally at temperatures ranging from 70 to 90 °C and pH values from 2-4 (176). *Sulfolobus* cells are aerobic, lobed cocci surrounded by a proteinaceous S-layer. *T. maritima* is one of the most
extreme thermophilic bacteria, and can grow at temperatures up to 90 °C and optimally around 80 °C (174). *T. maritima* belongs to the *Thermotogales* which form a unique subdomain of hyperthermophilic microorganisms that are phylogenetically distant from all other bacteria. They form the deepest and most slowly evolving branch in the bacterial domain currently discovered. *Thermotogales* are hyperthermophilic, rod-shaped, obligate anaerobes and have an outer sheath-like envelope, called the toga.

As a prerequisite for their survival, thermophiles contain enzymes that can function at high temperatures. Enzymes in thermophiles have been called extremozymes (231). These are capable to maintain their three dimensional structure at high temperatures by folding themselves more tightly than mesophilic enzymes. Thermophilic enzymes are not only thermostable, but also generally more stable to most commonly used protein denaturants than their mesophilic counterparts. Recent analyses of the structures of several hyperthermostable enzymes revealed different mechanisms of enzyme thermo-stabilization (33). Some of these enzymes show an increased number of hydrogen-bonds, hydrophobic residues, and a dense structural packing, which all together contribute to the stabilization. Alternatively, other thermostable proteins do not show significant changes in their structure packing density compared to their mesophilic counterparts but instead thermostability seems to be related to a rather small number of strong ionic interactions (34;35). Based on their high stability, these proteins are highly interesting for molecular and structural biology, but also for protein folding-unfolding studies and protein crystallization as they are stable under conditions that would denature mesophilic proteins. For similar reasons, there is a strong interest for these enzymes in biotechnological approaches.

This thesis describes the characterization of transport ATPases of thermophilic organisms. These transport ATPases drive the transport of their substrates across the membrane by the hydrolysis of ATP. Conformational changes in these ATPases upon binding, hydrolysis and release of the nucleotides results in structural changes in the transport channel and allows the subsequent transport of the substrates across the membrane. Based on highly homologous sequences and the conserved phosphate-loop sequence motifs in the nucleotide binding domains, transport ATPases are often termed P-loop ATPases (1). Beside sequence homology, tertiary structure analysis of these ATPases revealed the prototype of a typical NBD (2) termed the RecA fold. **Chapter II** describes the study of the SecA, SecYE and SecYEG components of the protein secretion complex of *T. maritima*. SecA is a transport ATPase with the typical RecA fold and it functions as a motor protein that mediates the translocation of proteins through the SecYEG protein conducting channel. The goal of these studies was to obtain a functional Sec translocase from a thermophilic source for protein crystallization. The Sec-components were overexpressed in *E. coli*, purified and the SecYE and SecYEG complexes were reconstituted in proteoliposomes to validate
their functionality. The isolated SecA showed a basal ATPase activity with a maximum at 74 °C which was close to the growth temperature of the organism. This basal activity was four-fold stimulated in the presence of isolated *Thermotoga* lipids. A purified precursor protein and SecYE containing vesicles or proteoliposomes stimulated the ATPase activity of SecA 2-fold whereas the activity was further enhanced when SecG was present. SecG has in particular been implicated in *E. coli* to be needed for protein translocation at low temperatures. However, our observation that the SecG protein stimulates the ATPase activity of the *T. maritima* SecA also at high temperatures indicates that the stimulatory effect of SecG will not only be limited to low temperatures. Imaging of small two-dimensional crystals of the SecYE complex using electron microscopy showed square-shaped particles with a side-length of about 6 nm, which likely represent dimers of the SecYE complex. The two-dimensional crystallization made use of a lipid monolayer that contained a low concentration of a Ni²⁺-chelating lipid that allowed oriented binding of the His-tagged SecYE complex to the monolayer and the formation of small crystalline patches. Because of the presence of the His-tag at the C-terminus of SecY, the SecA-binding site of the SecYE complex likely faces the monolayer thus being masked from the solution. To visualize the SecA-SecYE complex, it would be interesting to obtained a reversed orientation of SecYE, for instance by the presence of a His-tag at the periplasmic C-terminus of SecE. Unfortunately, because of low expression, attempts to obtain such a complex were unsuccessful.

The second class of transport ATPases studied in this thesis is the ATP-binding cassette (ABC) Transporters. ABC transporters constitute a large group of proteins found in all domains of life. These proteins are involved in many cellular processes and they transport a wide variety of substrates, i.e., sugars, amino acids, hydrophilic drugs, vitamins, lipids and so forth. Mutations in genes encoding for ABC transporters can cause dysfunction and different diseases, for example, hypercholesteremia, adrenoleukodystrophy, stargardt disease or cystic fibrosis. Moreover several ABC transporters were found to be responsible for multidrug resistance, presenting a major problem in cancer chemotherapy.

ABC transporters consist of either homo- or heterodimeric nucleotide binding domains (NBDs) which bind the membrane spanning domains (MSD). The MSDs form the transport channel, can also be either homo- or heterodimeric. Transport is driven by binding and hydrolysis of ATP by the NBDs, which is thought to drive conformational changes in the MSDs. In eukaryotes these domains are often fused on a single polypeptide while in bacteria and archaea they often are found as separate domains. The NBDs share a high degree of sequence homology which suggests a conserved mechanism. The NBDs contain characteristic motifs, e.g. the Walker A and Walker B and C-loop motifs, which are found in all ATP-binding proteins and are structurally conserved in
the typical RecA-like fold. Due to their wide spread through all phyla of life and their importance in human diseases they are one of the best studied active transporter protein family. Together the numerous biochemical studies and several available X-ray crystal structures of ABC NBDs have strongly deepened our understanding of these transporters.

In Chapter III the mechanism of ATP hydrolysis by GlcV, the NBD of the glucose ABC transporter of *S. solfataricus* was studied using Isothermal Titration Calorimetry (ITC). This method directly measures the heat of the reaction (enthalpy, ∆H), the stoichiometry of substrate binding (n) and the binding affinity of the substrate (Kₐ). From these values the Gibbs free energy of the association (∆G = -RTlnKₐ) and the entropy (T∆S = ∆H-∆G) can be calculated. Furthermore, based on the dependence of the enthalpy on the temperature, changes in the heat capacity (∆Cₓp = ∆∆H/∆T) can be determined. The use of a thermostable protein such as GlcV in such studies is favorable as it allows data acquisition at a wide range of temperatures, and thus a more accurate determination of the thermodynamic parameters. To analyze the thermodynamics of the ATPase cycle of GlcV, mutants that are blocked at different stages of the catalytic cycle were used. The G144A and E166A mutants, which carry mutations in the C-loop and in the residue after the Walker B binding motif, have a strongly reduced ATPase activity. The G144A mutant is unable to dimerize, while the E166A mutant is still able to dimerize, but is defective in dimer dissociation. It was shown that these mutations had no effects on the nucleotide binding stoichiometry and only small effects on the binding affinity. The use of these three proteins allowed the determination of thermodynamic parameters of different steps of the ATP hydrolytic cycle. The data shows that the different consecutive steps in the ATP hydrolysis cycle are all energetically favorable, except possibly for the last step of nucleotide dissociation. Therefore, only the final step in the cycle, ADP dissociation from the NBD appears as an energetically unfavorable process. This step is, however, driven by the more favorable binding of ATP in a next round of catalysis.

After determination of the thermodynamics of the ATP hydrolysis cycle, the protein structural packing density, domain organization and the effect of nucleotides on the structural stability of GlcV were studied. Differential scanning calorimetry (DSC) measurements in Chapter IV describe the thermal unfolding of wt and mutant GlcV in the absence and presence of nucleotides. Unfolding of the wild-type enzyme resulted in a single transition that was followed by rapid precipitation. Unfortunately, the latter precluded the quantitative analysis of the DSC thermograms. Both sequence alignment and the crystal structure of GlcV showed a two domain organization of the protein wherein the typical RecA like NBD domain is linked to the β-barrel shaped C-terminal domain via a linker region. A similar C-terminal extension was also described for other ABC
ATPases (13;147;154;155), where it was described to function as a regulatory domain. Since nucleotide dependent stabilization of the single unfolding event was observed, it was proposed that this unfolding corresponds to the NBD only, and that the C-terminal domain has a higher $T_m$ value which in the DSC experiments remained undetected due to the precipitation of the protein. The DSC thermogram of the mutant with a mutation in the ABC signature motif (G144A) showed that this mutation does not lead to a different unfolding behavior. In contrast, the E166A and E166Q mutants showed two thermal transitions indicating the uncoupling of the unfolding of the two domains when the glutamate was changed to alanine or glutamine. The DSC results thus suggest that the wild-type GlcV is at least a two domain protein wherein only the NBD domain unfolds prior to protein precipitation. Mutagenesis of E116 results in a disruption of the NBD structure which results in unfolding of the NBD domain in two steps.

Thermophilic ATPases employ different approaches to increase their structural stability and maintain their function at high temperature. GlcV showed a high structural stability against trypsin digestion, and was found to be more susceptible to proteolysis by trypsin in the presence of ATP or AMP-PNP. It remained trypsin resistant in the presence of ADP. This result is consistent with conformational changes observed in ABC ATPases (13;151;152) whereupon ATP binding a large conformational change and a rigid body movement in the NBD occurs. Upon these movements structural changes in the monomeric NBD leads to the formation a dimeric interface. Moreover, using photoaffinity crosslinking with a nucleotide analog along with trypsin digestion we were able to detect the formation of the dimeric state of GlcV. All together our data show that GlcV has a rigorous protein structure leading to a highly stable ATPase.

Our thermodynamic analysis involved the full length GlcV and did not address the question how the regulatory domain affects protein stability and functionality. For that purpose, genetic vectors were constructed that allow the overexpression of the GlcV protein lacking the C-terminal regulatory domain. Although a high level of overexpression could be obtained, only inclusion bodies were obtained. Apparently, without the regulatory domain, the heterologously expressed GlcV is unable to fold into its native state for reasons which remain elusive. In this respect, it would be of interest to test a series of GlcV truncates that differ in length, as a lack of soluble expression could be a trivial observation and simply be due to an unfortunate splitting point that separated the N- and C-terminal regions of GlcV.
Outlook

In this thesis the function, energetics, thermodynamics and folding of thermophilic transport ATPases were extensively studied. The ATPase activity of the heterologously expressed and purified *T. maritima* SecA together with the reconstituted SecYE has shown that these proteins are functionally active and thermostable. In *in vitro* translocation of the precursor protein into the membrane vesicles or reconstituted proteoliposomes, however, were unsuccessful, most likely due to a rapid preprotein folding and aggregation at higher temperatures. In *E. coli*, the molecular chaperone SecB, keeps the precursor protein in a translocation-competent state and targets this protein to the translocone via an interaction with the C-terminal domain of SecA. *T. maritima* and other thermophilic bacteria lack a SecB homolog and also the C-terminal region of SecA lacks the conserved SecB binding domain. This suggests that *T. maritima* either employs another molecular chaperone or translocation of preproteins in this organism occurs mostly co-translational which would be an effective means to prevent premature folding. The *in vitro* assay now allows for a direct biochemical identification of such putative chaperones and will deepen our insight in the protein translocation reaction at high temperatures.

Throughout the three phyla of life, ABC transporters are involved in many different physiological processes. The high sequence and structural homology of ABC ATPases suggests a conserved mechanism for the coupling of ATP hydrolysis and the transport reactions. We have studied the thermodynamics of the ATP hydrolysis cycle in the isolated NBDs. It should be stressed that these studies only partially mimic the physiological conditions as they do not involve the interactions of the NBDs with the MSDs. Therefore, it will be interesting to study the thermodynamics and kinetics of the ATP hydrolysis cycle with the full-length transporter in order to determine how the interplay between both domains affects the catalytic cycle. Other remaining questions are: “How does ATP binding and hydrolysis drive the conversion between an inward and an outward facing conformation?”, “Do half size transporters follow a similar mechanism as full length ABC transporters?”, “What determines the specificity of the interactions between the four (five for exporters) domains?”, “How can a uniform mechanism account for the transport of so many different substrates with different polymeric lengths?” and, “Are there similar or different mechanism used for uptake and excretion systems?”. Future experimental assessment of these questions will gain a deeper understanding of the function and mechanism of the important class of ABC transporters.