Structural and biochemical studies of GlcV, a transport ABC-ATPase from sulfolobus solfataricus

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ABC (ATP-binding cassette) transporters perform the active translocation of a broad spectrum of molecules across cellular membranes, and participate in essential processes, like the uptake of nutrients, and the removal of toxic compounds. These transporters consist minimally of two membrane domains forming a translocation channel, and two cytosolic ATP-binding cassettes (or ABC-ATPases) driving transport through their ATPase activity. In exporters binding of a substrate molecule to the membrane domains stimulates the activity of the ABC-ATPases. In bacterial uptake systems the cognate, loaded substrate-binding protein triggers this stimulation by binding to the channel on the extracellular side of the cytoplasmic membrane. The energy-transduction mechanism exploited in these transporters is believed to involve dimerization of the ABC-ATPases, coupled to cooperative binding and hydrolysis of ATP. These mechanisms of allosteric control of the ABC-ATPases, and of energy-transduction involve conformational changes that are transmitted between the membrane domains and the ABC-ATPases.

The work presented in this thesis focuses on the structural and functional properties of GlcV, the ABC-ATPase of the glucose ABC transporter from *Sulfolobus solfataricus*. Using X-ray crystallography combined with biochemical techniques, we aimed at determining the contacts that form at the ATP-Mg^{2+} binding site, and the conformational changes that occur during the catalytic cycle of this mechano-enzyme. Such data are essential to understand the functioning of ABC-ATPases as molecular engines in ABC transporters and other mechanical systems.

Chapter 1 reviews the current knowledge on the functional and structural properties of ABC transporters and ABC-ATPases. X-ray crystallographic studies have revealed the conserved molecular architecture of ABC-ATPases. Moreover, the structures of the ATP-bound forms of the ABC-ATPase domain of Rad50, involved in DNA-repair, and the transport ABC-ATPase LolD, showed that the active state of the ABC-ATPases is a head-to-tail dimer, in which two active sites form at the dimer interface.

The experimental work involved in the structure determinations of GlcV in nucleotide-Mg^{2+}-free and nucleotide-Mg^{2+}-bound states, is presented in **Chapters 2** and **3**. GlcV was expressed in *Escherichia coli* and purified using ion exchange, hydrophobic interactions and dye-affinity chromatography. Two crystal forms (A and B) were obtained under identical conditions in the absence of nucleotide and Mg^{2+}. Experimental phases were derived from single-wavelength anomalous diffraction data collected on an iodine derivative (form A), and improved to 2.1 Å resolution by density modification. These phases were combined with data collected to 1.65 Å resolution on a similar derivative, extended, and further improved by density modification. A model was built, refined, and used to solve, by molecular replacement, the structures of GlcV (form B) in nucleotide-Mg^{2+}-free, AMPPNP-Mg^{2+}-bound, and ADP-Mg^{2+}-bound states at 2.1, 1.95, and 2.1 Å resolution, respectively.

As presented in **Chapter 3**, the GlcV structure consists of a typical ABC-ATPase domain and a C-terminal domain of unknown function. The structure of this latter domain is similar to that of the equivalent domain in MalK, the ABC-ATPase subunit of the maltose ABC transporter, suggesting a similar function of the C-terminal domain of GlcV in regulating the transcription of the transporter operon, as found for MalK. The ABC-ATPase domain of GlcV contains two subdomains (ABCα/β; ABCα) featuring the conserved motifs that typify ABC-ATPases. The Walker A and B residues are located in the ABCα/β-subdomain, and form most of the ATP-Mg^{2+} binding site. The ABC signature motif, of which several residues have been shown to participate in binding and/or hydrolysis of ATP, is found in the ABCα-subdomain at about 25 Å from the ATP-Mg^{2+} binding site.

Superpositions of the monomeric structures of GlcV emphasize its conformational flexibility. It is apparent from re-orientations of the ABC-subdomains accompanied by structural changes in the Q-loop, a region that connects the ABCα-subdomain to the ATP-Mg^{2+} binding site. The orientations of the ABC-subdomains are influenced by the crystal contacts and are nucleotide-

independent. The conformation of the Q-loop is clearly dependent on the orientations of the ABC-subdomains, and on the absence or presence of nucleotide-Mg^{2+} at their binding site. In the nucleotide-free structures the ubiquitous Q-loop glutamine is positioned away from the ATP-Mg^{2+} binding site, while in the nucleotide-bound structures it interacts with the Mg^{2+} ion.

Alignments of the GlcV nucleotide-Mg^{2+}-bound structures with those of other isolated ABC-ATPases show large conformational differences. Most notably, comparison of the monomeric GlcV-AMPPNP-Mg^{2+} structure with that of the LolD ATP-bound dimer reveals a rigid-body re-orientation of the ABCα-subdomain relative to the ABCα/β-subdomain (~ 20°). Although it is unknown whether this conformational change occurs in transporters, it could represent a conformational switch between a non-productive conformation, unsuitable for dimerization, and the conformation suitable for dimerization. Interestingly, the structure of the transporter BtuCD reveals that each membrane domain binds one ABC-ATPase near the interface of the ABC-subdomains. Therefore, it is conceivable that these relative re-orientations of the ABC-subdomains are controlled by the membrane domains, and participate in the mechanism of allosteric control of the ABC-ATPases and/or that of energy-transduction. Additionally, our analysis indicates that the direct contact of the Q-loop glutamine with the Mg^{2+} ion is strictly ATP-dependent. This interaction keeps the ABC-subdomains in close positions, and therefore could restrain their re-orientations along a defined conformational path.

While the ABC-ATPase domain of Rad50 forms dimers in the presence of AMPPNP-Mg^{2+} or ATP alone, GlcV behaves as a monomer in these conditions. To resolve the oligomeric state of the active form of GlcV, we mutated the putative catalytic base, a conserved glutamate that follows the Walker B motif (E166) and the most conserved ABC signature residues (S142 and G144). The effects of each mutation on the activity and oligomeric state of GlcV were studied by means of ATPase activity assays and gel filtration experiments, as presented in **Chapter 4**.

In the presence of Mg^{2+} mutants E166Q, E166A, G144A, and S142A still bind ATP, but are defective in ATP hydrolysis. Our experiments also revealed that mutants E166Q, E166A, and S142A dimerize in an ATP-dependent manner. Since in the presence of ATP alone wild-type GlcV is inactive and does not form dimers, we concluded that GlcV forms transient dimers in an ATP-Mg^{2+}-dependent manner, and that the dimer is its active state. Moreover, although the G144A and E166A mutants alone are inactive, and the G144A mutant is unable to dimerize, together they can form an active hetero-dimer. Our data agree with a head-to-tail arrangement of this dimer, as observed for Rad50 and LolD, in which two active sites form at the dimer interface, by juxtaposition of the ABC signature residues of one monomer with the ATP-Mg^{2+} binding site of the other monomer. In each active site the ATP γ-phosphate group is stabilized by the Mg^{2+} ion, and by two hydrogen bonds formed with the conserved glycine and serine residues of the ABC signature motif across the dimer interface. Such contacts further stabilize the negative charge that develops on the γ-phosphate group and lower the energy of the transition state, thus facilitating ATP hydrolysis. The residual activities of the E166Q and S144A mutants show that some conserved active site residues are not essential. It suggests that ABC-ATPases with partially degenerated machineries, as they occur *in vivo*, still form active dimers to drive transport.

Overall, our analysis provides evidence for the occurrence of conformational changes along the catalytic cycle of ABC-ATPases, involving essentially the re-orientations of the ABC-subdomains. Such changes could be part of the mechanism of allosteric control of the ABC-ATPases, and that of energy-transduction in ABC transporters. Additionally, we showed that the active state of GlcV is a dimer, most likely with a head-to-tail arrangement, which forms in an ATP-Mg^{2+}-dependent manner. In **Chapter 5** we propose to use mutants to trap the ATP-bound monomeric and dimeric states of GlcV, either in its isolated form or incorporated into

the transporter, as an approach to conduct a more detailed structural study on the functioning of this ABC-ATPase.