A molecular understanding of the catalytic cycle of the nucleotide-binding domain of the ABC transporter HlyB

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Abstract

The ABC transporter (ATP-binding-cassette transporter) HlyB (haemolysin B) is the central element of a type I secretion machinery, dedicated to the secretion of the toxin HlyA in Escherichia coli. In addition to the ABC transporter, two other indispensable elements are necessary for the secretion of the toxin across two membranes in a single step: the transenvelope protein HlyD and the outer membrane protein TolC. Despite the fact that the hydrolysis of ATP by HlyB fuels secretion of HlyA, the essential features of the underlying transport mechanism remain an enigma. Similar to all other ABC transporters, ranging from bacteria to man, HlyB is composed of two NBDs (nucleotide-binding domains) and two transmembrane domains. Here we summarize our detailed biochemical, biophysical and structural studies aimed at an understanding of the molecular principles of how ATP-hydrolysis is coupled to energy transduction, including the conformational changes occurring during the catalytic cycle, leading to substrate transport. We have obtained individual crystal structures for each single ground state of the catalytic cycle. From these and other biochemical and mutational studies, we shall provide a detailed molecular picture of the steps governing intramolecular communication and the utilization of chemical energy, due to ATP hydrolysis, in relation to resulting structural changes within the NBD. These data will be summarized in a general model to explain how these molecular machines achieve translocation of molecules across biological membranes.

Many Gram-negative bacteria use ABC (ATP-binding cassette)-dependent type I secretion machineries for the translocation of toxins, lipases, proteases, surface layer proteins and cytolysins across both membranes in a single step [1,2]. The paradigm of these Sec-independent systems is the HlyA (haemolysin A) translocase of Escherichia coli, the genes for which were originally isolated from a human uropathogenic strain [3]. The only common feature of the great majority of the transport substrates, or allocrites [4] of the type I pathway, is the presence of Gly-rich repeats. Thus HlyA, a 1023-amino-acid protein, belongs to the family of RTX (repeat in toxins) toxins [5], containing repetitive amino acid sequences with the consensus sequence GGXGXDL/IFX, where X represents any amino acid. The repeats bind Ca2+ with high affinity and are thought to trigger refolding after successful secretion on to the cell surface prior to release to the extracellular medium [6]. In addition to the Gly-rich repeats, many studies have demonstrated that all information necessary and sufficient for the secretion of HlyA and other type I proteins is encoded in the C-terminal region of the allocrite [7,8]. Although the importance of the C-terminal secretion sequence has been clearly established, the nature of the precise recognition features remains unclear [9,10]. There are no clear indications of highly conserved residues or motifs, nor secondary structure elements that could be detected in aqueous solution [11,12]. This is in clear contrast with the Sec-machinery [13], where the signal sequence is located at the N-terminus, contains readily recognizable features and is cleaved by a signal peptidase after translocation.

The HlyA transport machinery is composed of three indispensable components [14–18]: HlyB, an ABC transporter, HlyD, the so-called MFP (membrane fusion protein), both of which reside in the inner membrane, and the outer membrane protein TolC (see Figure 1). Genetic studies have shown that removal of any one of the three components results in abrogation of allocrite secretion. TolC, whose trimeric structure was solved in 2000 by Koronakis et al. [19], forms a ‘channel-tunnel’. The β-barrel resides within the outer membrane and elongates into 12 antiparallel α-helices protruding into the periplasmic space. It is thought that TolC and HlyD form a continuous tunnel capable of accommodating an unfolded protein during passage across both membranes of E. coli. HlyD contains a small cytoplasmic domain (residues 1–60), a single transmembrane spanning helix, and a large periplasmic, helical domain (residues 81–478), which is thought to interact at some stage of allocrite transport with
The allocrite HlyA is shown in red, the ABC transporter HlyB in blue, the MFP HlyD in green and the outer-membrane protein TolC in orange. The white spheres in the extracellular media represent Ca\(^{2+}\).
within the helical domain of different NBDs that appeared to adopt an NBD-specific conformation [39]. In our proposal, this SDR is responsible for proper interaction of the subunits and signal transmission between the NBDs and TMDs, indicating that each NBD is essentially unique. This has important implications for future drug design in relation to modulating the action of human ABC transporters.

The isolated NBD of HlyB was studied in detail by various biochemical, biophysical and structural approaches [24,32,39,45–48]. Most important was our observation of an ATP-induced dimerization of the NBD. For ABC transporters, it has been proposed that NBDs undergo changes in their oligomeric state during the catalytic cycle. It is now assumed that a monomeric state predominantly exists in the nucleotide-free or ADP-bound state, while NBDs dimerize upon ATP-binding. Since the isolated NBD of HlyB also displayed ATP-dependent dimerization, not only in solution, but also in the crystal structure of a mutant incapable of ATP hydrolysis (see below), we conclude that the isolated NBD can serve as an appropriate model system for full-length HlyB, at least under certain conditions. In addition to the fact that the isolated NBD displayed ATP-induced dimerization, equally important was the observation that ATP-hydrolysis displayed nonlinear kinetics, dependent on ATP and NBD concentration [45]. The sigmoidal velocity versus concentration plots (either ATP or protein) revealed a Hill coefficient of close to two. From the protein concentration dependence, an apparent dimerization constant for the NBDs of 1.2 $\pm$ 0.2 $\mu$M was also calculated. This clearly demonstrated that an oligomeric form of the NBD rather than the monomer is the active species during ATP hydrolysis.

Mutational studies have revealed the importance of certain key residues in ATP-hydrolysis by the HlyB-NBD [48]. Most important for our understanding of the catalytic cycle were mutations of Glu$^{631}$ and His$^{662}$. Based on studies initially performed with the NBDs of MJ1276 and MJ0796 from M. janashii [49], the equivalent glutamate located C-terminal to the Walker B motif, was previously proposed to act as a ‘general base’ required for catalysis [50]. In line with this model, Glu/Gln mutants locked in ATPase-activity, of various systems were reported. This was consistent with the notion that the glutamate residue orients and polarizes the nucleophilic water molecule within the active site [50–52]. However, in other studies, including our own, a Glu/Gln mutation resulted in NBDs retaining substantial amounts of ATPase activity [32,53–56], thus casting doubt on the precise role of the glutamate. On the other hand, another interesting residue is the highly conserved His$^{662}$ in the so-called H-loop of HlyB (Figure 1). Mutation of this histidine in the maltose and histidine permease systems resulted in transporters without detectable ATPase activity and an inability to translocate the allocrite in vivo [57,58]. Thus, while the E631Q (Glu$^{631}$ $\rightarrow$ Gln) mutation in HlyB retained around 10% ATPase activity [32], the H662A mutant was completely ATPase-deficient (up to a protein concentration of more than 20 mg/ml) [46]. Using the H662A mutation, we subsequently performed experiments pioneered for HisP [59], in which equal molar ratios of wild-type and H662A NBD were mixed and the ATPase activity of the corresponding mixtures was determined. ATPase activity proved to be linear, dependent on the molar amount of wild-type enzyme, and constant over time, indicating that in the HlyB-NBD dimer composed of one wild-type and one H662A subunit single-site hydrolysis was possible [45]. Such a sequential mechanism was already described for HisP, the NBD of the histidine permease [59], and the NBD of Mdl1p, an ABC transporter from yeast [51].
Based on detailed biochemical and functional knowledge of the HlyB-NBD, we succeeded in crystallizing each single state of the catalytic cycle of the NBD [32,39,46]. In the nucleotide-free state [39], an interesting conformation of the last three residues of the Walker A motif (coloured red in Figure 2) was observed. These residues form the N-terminus of helix 1 and adopt a $\beta$-helical conformation rather than the usual $\alpha$-helix, which is present in all NBD structures solved in the presence of nucleotide. The $\beta$ helix is further stabilized by a salt bridge between Lys$^{508}$ and Glu$^{631}$, which caps the helix. Such a $\beta$ helix prevents nucleotide binding because Ser$^{509}$ and the $\alpha$-phosphate moiety of any nucleotide would result in a steric clash. This alternative rearrangement might present a molecular switch used to regulate ATPase activity in vivo.

Using the H662A or the E631Q mutant, crystal structures in the ATP- and ATP/Mg$^{2+}$-bound states were obtained at 2.6 and 2.5 Å ($1\,\text{Å} = 10^{-10}\,\text{m}$) respectively [32,46]. In these structures a dimeric architecture was observed, in which ATP glues together two monomers through interaction of the Walker A motif of one monomer and the C-loop of the opposing monomer. Such a sandwich dimer was also described for MJ0796 [35] and MalK from *E. coli* [60] and suggests that such a composite dimer is a universal feature of all ABC transporters. The dimer interface of the HlyB-NBD is stabilized by interaction of the extended C-terminal region of HlyB. More important, however, is the fact that the D-loops (residues 634–637) are involved in monomer–monomer communication (Figure 3) [32]. Thus the structure of the HlyB-NBD, in the presence of ATP and ATP/Mg$^{2+}$, revealed for the first time a molecular communication pathway by which the functional state of the nucleotide-binding site of monomer, e.g. ATP- or ADP-bound or nucleotide-free, could be transmitted to the other monomer. Notably, Ala$^{635}$ (SALD) of the trans monomer interacts across the dimer interface with His$^{662}$, which performs the vital function of a linchpin in catalysis (see below). In addition, Asp$^{637}$ (SALD) of the trans monomer forms a hydrogen bond with Ser$^{524}$ of the Walker A motif of the cis monomer.

What are the precise molecular functions of Glu$^{631}$ and His$^{662}$? Re-introducing the histidine *in silico* in the HlyB-NBD H662A mutant, resulted in a complex network of interactions (Figure 3) [32]. In the modelled structure, Glu$^{631}$ forms a bidentate interaction with the backbone and the side chain of His$^{662}$, thereby positioning His$^{662}$ in a proper orientation to stabilize the transition state of the HlyB-NBD/ATP-Mg$^{2+}$ complex. His$^{662}$, on the other hand, acts as a linchpin. It interacts with ATP and the D-loop of the trans monomer, and co-ordinates a water molecule, which is in the proper position to act as the catalytic water. We propose that in this structure Glu$^{631}$ acts as a platform. Without this glutamate, the conformational restriction imposed on His$^{662}$ is relaxed and the probability of obtaining the correct orientation is drastically reduced. However, the intrinsic flexibility of individual NBDs might explain the varying levels of residual ATPase activity found after mutation of the glutamate.

In contrast, substitution of the histidine removes the linchpin, ATP is not fixed in space anymore and the catalytic water is not co-ordinated. Thus, ATPase activity is abolished. This model is supported by a variety of biochemical evidence [32]. First, ATPase activity in solutions of increasing viscosity revealed that the reaction velocity did not change. This is a clear indication that a ‘chemical’ reaction is the rate-limiting step of the catalytic cycle rather than nucleotide association, dissociation or NBD-dimerization, since the latter depend on the diffusion rate, which is slowed in solutions of higher viscosity. Secondly, reaction velocities of the ATPase activity are insensitive to the presence of $^2\text{H}_2\text{O}$. This experiment
allowed us to eliminate general base catalysis as the ‘rate-limiting reaction’, since by definition such a process requires a proton abstraction or polarization step [61]. When a reaction is performed in $\text{H}_2\text{O}$, the zero-point energy changes and the activation barrier for any proton-abstraction process is raised, with a consequent reduction in the reaction velocity [61]. The absence of a deuterium isotope effect [62] clearly demonstrates that protons are not involved. This implies that ATP-hydrolysis might be the ‘rate-limiting step’ of the catalytic cycle, i.e. that the step of bond cleavage has the highest activation barrier. This model is further supported by the fact that ATPase activity is modulated by the nature of the divalent ion present, going hand in hand with the pK$_a$ value of the corresponding ion rather than with the ionic radius. This suggests that the bound cofactor influences the pK$_a$ value of the $\gamma$-phosphate moiety, resulting in modulation of the reaction velocity. As a consequence, the substrate, ATP, acts as a base and ATP-hydrolysis by the HlyB-NBD should then follow ‘substrate-assisted catalysis’ [63], a mechanism already described for another branch of P-loop enzymes, the GTPase ras$^\text{T2}$ [64] or EcoRI and V [65].

The biochemical and structural investigations performed on isolated NBDs have provided a molecular picture that allows us to start to understand the ‘mode of action’ of NBDs at the molecular level. Slowly, the pieces of a molecular puzzle are coming together and a scenario starts to emerge which explains how chemical energy is converted, via ATP-hydrolysis, into vectorial transport of the allosteric across biological membranes. Thus, it may be envisioned that hydrolysis allows us to start to understand the ‘mode of action’ of biological membranes. Thus, it may be envisioned that hydrolysis, into vectorial transport of the allosteric across

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