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Minireview

Molecular insights into the mechanism of ATP-hydrolysis by the NBD of the ABC-transporter HlyB

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Abstract The ABC-transporter HlyB is a central element of the Type I protein secretion machinery, dedicated to export the *E. coli* toxin HlyA in a single step across the two membranes of the cell envelope. Here, we discuss recent insights into the structure and the mechanism of ATP-hydrolysis by the NBD of HlyB. Combining structural and biochemical data, we have suggested that substrate-assisted catalysis (SAC), but not general base catalysis, is responsible for ATP-hydrolysis in this NBD and might also operate in other NBDs. Finally, the implications and advantages of SAC are discussed in the context of ATP-induced dimerization of the NBDs.

Keywords: ABC-transporters; Dimerization; Nucleotide-binding domain; Substrate-assisted catalysis; Crystal structures

1. Type I protein secretion – the ABC transporter pathway

Type I protein secretion in bacteria, also called the ABC-pathway [1,2], is a rather promiscuous, Sec-independent process used to translocate polypeptide toxins, hydrolytic enzymes, and surface bound proteins in one step across the double membrane of *E. coli* [3]. All information necessary and sufficient for this transport process is encoded as a targeting signal in the C-terminal part of Type I substrates. This secretion signal is not cleaved after translocation, another feature distinguishing this mechanism from the well-understood Sec-apparatus [4]. The paradigm of the Type I system is the haemolysin A (HlyA) machinery, which was discovered in the early 1980s in certain uropathogenic *E. coli* strains [5]. This system is composed of three indispensable elements, the ABC-transporter HlyB, the membrane-fusion protein HlyD, both residing in the inner membrane, and the outer membrane protein TolC [6]. It is now commonly accepted that these three membrane proteins form a continuous channel across the cell envelope [7] following engagement by the *allocrite* HlyA, our preferred term for a transported substrate [8], thereby preventing the appearance of periplasmic intermediates.

2. ABC-transporters

HlyB is a member of the continuously growing family of ABC- (ATP-binding cassette) transporters [9], that are found in all three kingdoms of life. These ATP-dependent channels or pumps ultimately use the energy of ATP-hydrolysis to achieve translocation of an astonishing variety of allocrites, ranging from small ions such as chloride (CFTR), to nutrients such as amino acids or sugars (histidine and maltose 'permeases'), up to large proteins (HlyB) [10]. Despite this diversity, including both importers and exporters, all these transporters share the same organization: two nucleotide-binding domains (NBD) and two transmembrane domains (TMD) to form a functional unit. In eukaryotes, all four domains are found in a single polypeptides, whilst in the importer systems in prokaryotes, the ABC–NBD and the membrane domain are usually found as separate proteins (e.g., HisP). On the other hand, exporters such as HlyB, or the peptide transporter TAP, in eukaryotes, are so called half-size transporters with the NBD and the membrane domain in a single polypeptide. Since the first report of an ABC-transporter in 1982 [11], we have seen tremendous achievements. However, we are still a long way from a functional understanding. For example, it is not clear how the energy of ATP is coupled to allocrite transport or how molecular signals are sent back and forth between the motor domains (the NBDs) and the TMDs [9,12].

Membrane protein research is still hampered by a significant degree by the fact that overexpression and purification of a functional protein is certainly not a straightforward task. Furthermore, transporters require reconstitution in lipsosomes in order to study vectorial translocation of an allocrite in vitro. In the case of an ABC-transporter, we took the view that it should be possible to analyze the many details of the mechanism of ATP-hydrolysis and the associated conformational changes with the isolated NBDs alone, in addition to longer-term studies with the intact protein. This approach we argued, would provide an opportunity, which should in principle more easily allow a detailed analysis of the catalytic mechanism of the NBD. However, one always has to keep in mind that in

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Abbreviations: ABC, ATP-binding cassette; Hly, haemolysin; NBD, nucleotide-binding domain; SAC, substrate-assisted catalysis; TMD, transmembrane domain

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the absence of the TMD, the tight interaction between NBD and TMD is lost, which consequently results in a free diffusion of the isolated NBD in solution, thereby increasing its mobility. Furthermore, the important communication between NBD and TMD is completely absent. Nevertheless and without question, isolated NBDs are proven valid model systems in relation to certain questions. For example, detailed mechanistic insights have been obtained from the isolated NBD of the yeast transporter Mdl1p [13] or the NBD of HlyB [14–16]. In fact, whilst it is quite feasible that the underlying kinetics will be affected by the presence or absence of the TMD, it is unlikely that a TMD will change the fundamental thermodynamics of the system. Thus, isolated NBDs are in our opinion suitable systems to derive insights into how chemical energy stored within ATP, is transformed into mechanical energy and how the NBDs, or motor domains, might fuel allocrite translocation.

3. The nucleotide-binding domain of HlyB

The first crystal structure of an NBD, HisP, was described in 1998 [17]. Since that time several structures of isolated NBDs and full-length ABC-transporters have been reported [17–30]. All ABC-NBDs are L-shaped molecules (Fig. 1) with a two domain architecture. The catalytic domain harbors the RecA-like [31] nucleotide binding site and contains various conserved motifs; the Walker A and B motifs [32], also present in other ATPases and GTPases [33], and the D- and H-loops. The second and smaller helical domain is built up entirely of α-helices and contains the C-loop or signature motif, the diagnostic feature of ABC-transporters. Both domains of the NBD are connected via the Q-loop and the Pro-loop [25]. Over the years,

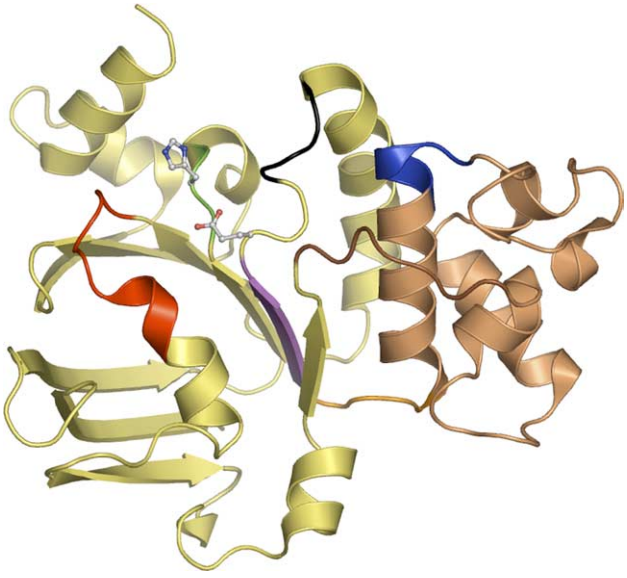

Fig. 1. Overall structure of monomeric, wildtype HlyB-NBD (pdb entry 1MT0). The catalytic and helical domains are shown in light yellow and light tan, respectively. Conserved motifs are colored in red (Walker A, residues 502–510), brown (Q-loop, residues 550–556), C-loop (blue, residues 606–610), Pro-loop (orange, residues 622–625), Walker B (magenta, residues 626–630), D-loop (black, residues 634–637), and H-loop (green, residues 661–663). The catalytic dyad (E631 and H662) is shown in ball-and-stick representation.

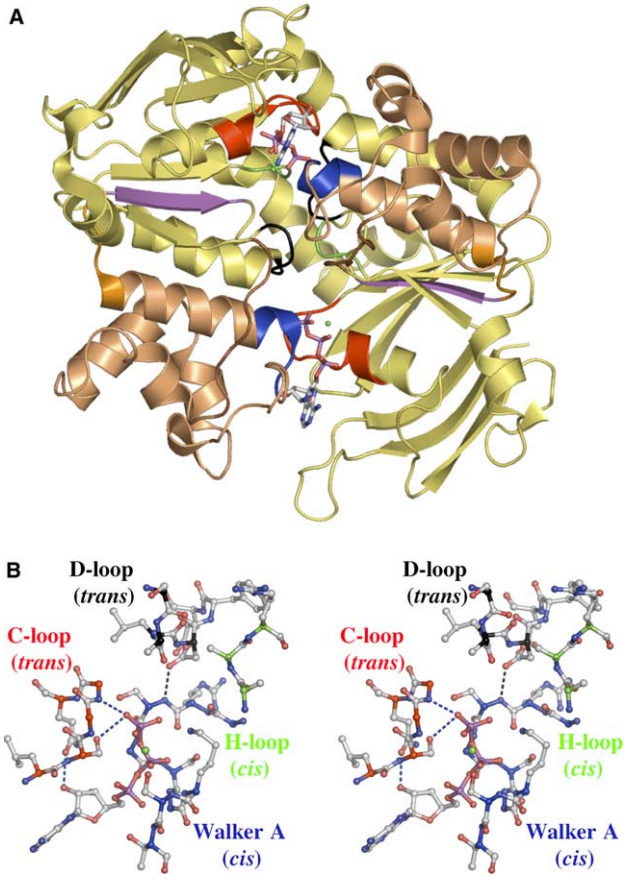

Fig. 2. (A) Cartoon representation of the crystal structure of the dimeric ATP/Mg^{2+}-bound form of HlyB-NBD H662A (pdb entry 1XEF). Color-coding is identical to Fig. 1. ATP is shown in ball-and-stick representation and the cofactor Mg^{2+} as a green sphere. (B) Stereoview of the architecture of the composite ATP/Mg^{2+}-binding site in the HlyB-NBD H662A. For simplicity only the interactions of the C-loop amino acids of the *trans* monomer and the D-loop of the *cis*-monomer are shown. To visualize conserved motifs, only Ca-atoms of the motifs have been color-coded according to Fig. 1.

structural work with the isolated NBDs demonstrated that ATP binding to the catalytic domain, induces a rigid body motion of the helical domain of roughly 20° [16,20,23,30] and consequently dimerization.

The architecture of the NBD-dimer, although initially controversial, is now solidly established due to recent biochemical and structural data [16,20,23,30,34]. Thus, in the dimer, ATP acting as a molecular glue, is sandwiched between the Walker A motif of the *cis* monomer and the C-loop motif of the opposing *trans* monomer (Fig. 2A). However, with the exception of a single interaction with the hydroxyl moiety of the ribose, all the other interactions of the C-loop of the *trans* monomer with the bound ATP are directed towards the γ-phosphate (Fig. 2B). This explains why this composite dimer is only stable in the presence of ATP but not ADP.

4. ATP-induced dimerization and the mechanism of ATP-hydrolysis

Recent studies have indicated that even in the absence of nucleotides, the two NBDs are in close proximity [35], and

indeed in the case of the BtuCD crystal structure, the NBDs (BtuD) are in contact but cover only a small buried surface area [24]. However, structural [20,27,30] and biochemical data [13,16,34,36] obtained for isolated NBDs have now clearly established that ATP binding is required to trigger functional dimerization and that dimer stability depends solely on the presence of the γ-phosphate moiety of ATP. It is important to note that these conclusions were derived from studies of NBDs bearing mutations of essential amino acids or NBDs such as MalK [37] or OpuAA [38] containing a third additional domain. This latter provides an additional degree of stability to the NBD dimer. The first reported mutation stabilizing the ATP-bound dimer came from the laboratories of John Hunt and Philip Thomas [39]. Here, a glutamate next to the Walker B motif, E171, was mutated to Q and this increased the stability of the dimer by orders of magnitude. This approach finally resulted in the first crystal structure demonstrating the composite dimer arrangement [30], which is identical to the one shown for the HlyB-NBD in Fig. 2A. Parallel studies suggested that this glutamate, as in the case of F_1-ATPase [40] acts as the 'catalytic base' [39,41], i.e., that this residue abstracts a proton from the catalytic water molecule in the *rate-limiting step* of the reaction [42]. However, data from other and our laboratories challenged this proposal [16,43–45]. On the other hand, mutations of the conserved histidine (H662 in HlyB) of the H-loop [15,16], which were pioneered for the histidine and maltose importers [46–48], resulted in proteins without any ATPase, or transport activity above background levels. These studies indicated therefore that two amino acids (E631 and H662 in HlyB) might play key roles in ATP-hydrolysis. Combining structural and biochemical data, we have recently proposed the linchpin model of ATP-hydrolysis operating in HlyB and we suggest perhaps in all other ABC-NBDs [15,16,27]. Here, E631 and H662 form a catalytic dyad (Fig. 3) required for proper orientation of the two side chains. In this model, the carboxyl moiety of E631 forms two vital interactions with H662, one with the backbone amide and one with the nitrogen of the imidazole ring. H662 on the other hand polarizes the putative catalytic water molecule and interacts with the γ-phosphate group of ATP. This latter interaction likely serves to stabilize the transition state. In other words, H662 represents the most important residue essential for hydrolysis, while E631 adopts a platform-like function orienting the side chain of H662 in a productive configuration.

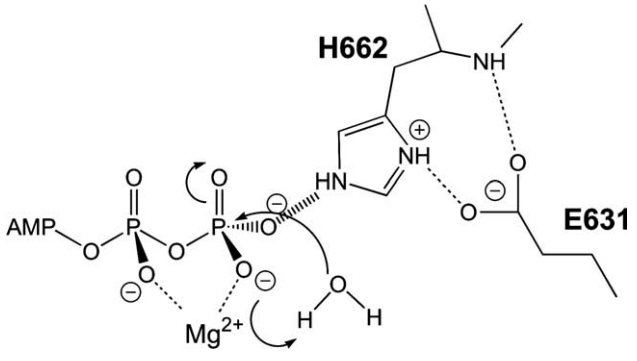

Fig. 3. Schematic representation of the linchpin model. Reprinted from [16] with permission.

ATPase experiments in the presence of D_2O were equally important for the understanding of the molecular mechanism of ATP-hydrolysis in the HlyB-NBD. 'General base catalysis', by definition, requires that the reaction velocity is decreased in the presence of increasing amounts of D_2O [42]. No such effect was observed for the HlyB-NBD, and therefore at least for this NBD, 'general base catalysis' has to be ruled out. Based on the influence of divalent ions on ATPase reaction velocity and other experimental evidence, we proposed that 'substrate-assisted catalysis' (SAC) [49] operates in HlyB-NBD. Such a mechanism of nucleotide triphosphate cleavage is reminiscent of that proposed for other P-loop containing enzymes such as the GTPase ras^{p21} [50] *Eco*RI or *Eco*RV [51]. Although important from a mechanistic point of view, one has to ask the question whether or not these alternative mechanisms are merely semantically different or whether consequences of SAC, rather than general base catalysis, are fundamental to the coordinated action of ABC-proteins. We shall return to this point later.

5. The D-loop: a 'forgotten trademark' of ABC-transporters

The D-loop, typical sequence SALD (residues 634–637 in HlyB), was recognized as a diagnostic feature for the identification of ABC-transporters from the first sequencing, as equally important as the C-loop motif. However, during the subsequent 20 years, no distinct function could be assigned to this conserved feature, thus it became a somewhat forgotten diagnostic for ABC-transporters. Mutational studies performed on many different ABC-transporters never touched the D-loop, leaving its functional conservation an open question. In contrast, however, the first dimeric ATP-bound structures of ABC-proteins, MJ0796 [30] and MalK [20] from *E. coli*, revealed a notable interaction of the D-loop with the Walker A motif of the *opposing* ATP binding site. We have confirmed this observation with the structure of the HlyB–NBD in complex with ATP/Mg^{2+} and have proposed a function for the D-loop. As shown in Fig. 2B, amino acids of the conserved D-loop not only interact with the Walker A of the *cis* monomer but are also in a position to sense, via the H-loop of the *trans* monomer, the functional state of the opposing ATP-binding site. Thus, any change within one of the ATP-binding sites should be immediately transmitted to the other ATP-binding site via the D-loop. This observation might be crucially linked to the observed cooperativity of many ABC-transporters, full-length or isolated NBDs (such as the HlyB-NBD) on a molecular level, with the D-loop *trans*-monomer interaction representing one of the communications lines between the two spatially distant ATP-binding sites.

6. The rationale of dimerization and SAC

ATP-induced dimerization of isolated NBDs in most cases has only been observed after certain key amino acids were mutated. Importantly, in the case of the HlyB-NBD, we also showed that the dimerization step was not rate limiting, if we assume that the thermodynamic boundaries were fulfilled [27], i.e., that the conditions employed were near or above the apparent dissociation constant for dimer formation. As

discussed above, the presence of the TMDs will drastically influence the mobility of the NBD, thereby modulating ATP-induced dimerization. On the other hand, the fact that ATP-induced dimerization could be observed with the isolated NBDs points to the mechanistic importance of this step. This leads intuitively to the expectation that in the cell, ATP binds to a 'monomeric' NBD, induces dimerization and subsequently ATP hydrolysis will take place. In a related scenario, one could imagine that the TMDs impose a lock on the NBD, preventing dimerization – but not ATP binding – in the absence of the allocrite. The interaction of the Q-loop (NBD) with the L-loop of the TMDs, which was observed in the crystal structures of the bacterial importer BtuCD [24] and the Lipid A exporter MsbA [19,52], might represent this lock.

Whatever locking device is used, ATP-hydrolysis should inevitable follow dimerization. Equally, it seems obvious that ATP-hydrolysis within a monomeric NBD should be prevented since it would represent a waste of energy. If we now consider that 'general base catalysis' operates within ABC-transporters as in the F_1-ATPase [40], it is extremely difficult to imagine how ATP-hydrolysis within a monomeric NBD could be avoided, since all catalytically relevant amino acids for general base catalysis are already in place and properly oriented to hydrolyze ATP. In contrast, SAC depends on the substrate having the proper pK_a value. Interestingly, this could provide an explanation for the need for the LSGGQ motif to complete the composite catalytic site. Within a binding site, the microenvironment imposed by the protein will affect the pK_a value of ATP. Thus, we can easily envision that dimerization and the resulting interaction of amino acids of the C-loop motif with the γ-phosphate group will modify the intrinsic pK_a values of the bound ATP molecule. Changes in the pK_a values of amino acids, in the monomeric and dimeric state of HlyB-NBD are shown in Fig. 4. Here, it is evident that dimerization induces drastic changes in the individual pK_a values. Interestingly, these changes are restricted to certain areas of the dimer. For example, the region of the Walker A motif and the region covering the Walker B – D-loop–H-loop, show the most drastic changes in pK_a values when comparing monomeric and dimeric forms. The latter region contains many of the catalytically important amino acids such as Asp630, which coordinates the cofactor Mg^{2+}, Glu630, part of the catalytic dyad, and finally H662, which acts as the linchpin of ATP-hydrolysis. Thus, dimerization generates changes in the physical properties of key amino acids, necessary for productive function of the NBD as an ATPase, i.e., mobility, hydrogen bonding capabilities and salt bridges, due to the different environment within the dimer. The recently reported 3_{10} helix adopted by the last three residues of the Walker A motif in the nucleotide-free state of HlyB–NBD [25] or the unusual conformation of the Walker A motif in the crystal structure of the nucleotide-free state of GlcV [26] might serve as additional backup systems for preventing futile hydrolysis in the monomer. These conformations prevent ATP-hydrolysis by raising the energy barrier for productive ATP-binding to the active site.

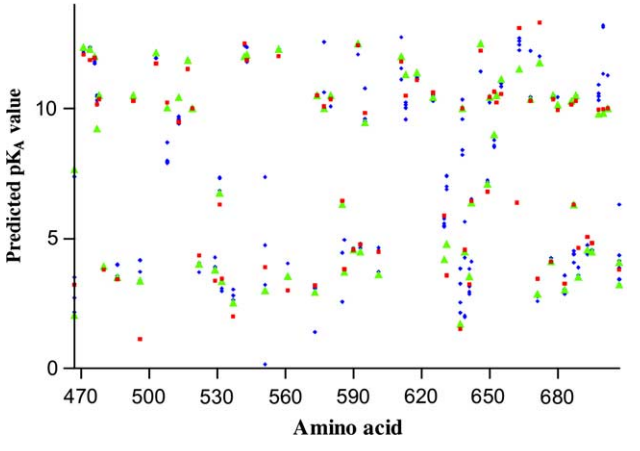

Fig. 4. Plot of the predicted pK_a values of amino acid side chains of the monomeric nucleotide-free wild type HlyB-NBD (pdb entry 1MT0, red symbols), dimeric ATP/Mg^{2+}-bound (pdb entry 1XEF, blue symbols), and a hypothetical monomeric ATP/Mg^{2+}-bound HlyB-NBD with H662 mutated to A (green symbols). pK_a values were calculated at the PROPKA web interface (http://propka.chem.uiowa.edu/). The upper layer around a pK_a value of 10 corresponds to Lys, while the lower layer around 4–5 corresponds to the side chains of Asp and Glu.

The appealing simplicity of SAC operating in HlyB derives from the fact that this provides an explanation for how dimerization of the NBDs and ATP-hydrolysis can be coupled without futile ATP-hydrolysis. Furthermore, we note that the apparent K_D of dimerization for the HlyB-NBD was determined to be 1.2 ± 0.1 μM [16], which corresponds to 33 kJ/mol. This is comparable with the energy derived from ATP-hydrolysis (36 kJ/mol). Thus, two sources of potential energy are available – dimerization dependent on the rigid body motion of the helical domain, which induces mechanical motion, and ATP-hydrolysis, which releases chemical energy. This strongly suggests that mechanochemistry might be the driving force to fuel different steps of allocrite translocation by ABC-transporters.

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