Single-molecule studies of the conformational dynamics of ABC proteins

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Summary and outlook

9.1 Summary

ATP-binding cassette (ABC) proteins form a large family of proteins that are involved in various processes[1]. ABC transporters translocate compounds across membranes, while other ABC proteins are involved in DNA repair, mRNA translation or other processes[2]. All ABC proteins possess a pair of nucleotide-binding domains (NBDs), which bind and hydrolyse adenosine triphosphate (ATP). Besides the NBDs, ABC proteins require additional domains for function. For instance, many ABC importers require transmembrane domains (TMDs) and a substrate-binding protein (SBP)[3]. All proposed mechanisms of how ABC proteins function, are based on the precisely coordinated conformational changes in the NBDs, associated domains and interaction partners, as reviewed in **Chapter 1**. X-ray crystallography and many spectroscopic methods provided valuable insight into the conformational changes of ABC proteins[4-13]. However, the interpretation of these data can be complicated due to the fact that the measured signal comes from an enormous number of molecules. Single-molecule methods overcome this limitation and can provide insight into the conformational heterogeneity, dynamics and occurrences of rare events[14, 15]. In this thesis, I used single-molecule fluorescence methods to probe the conformational changes in SBPs and in the ABC protein ABCE1 and decipher the role of these conformational changes in function.

Type I and II ABC importers require an SBP to capture the substrate and deliver it to the translocator unit[16]. Crystal structures of SBPs suggest that substrate binding is coupled to switching between two SBP conformations, an open and a closed conformation[17-21], which has been linked to the activation of transport[22-29]. In **Chapter 2**, we assessed this hypothesis by investigating the interaction of six SBPs, namely MalE, OppA, SBD1, SBD2, PsaA and OpuAC, with a range of substrates. Based on their transport phenotype, the SBP substrates were classified as cognate or non-cognate, depending on whether they are transported or not.

Single-molecule Förster resonance energy transfer (smFRET) was used to probe the SBP conformation. These measurements led us to conclude that some SBPs, such as OppA, adopt a single closed conformation with different substrates, while others employ multiple distinct closed conformations. For instance, MalE does not acquire a single closed state, but can form at least five different closed conformations. Moreover, we observed that some SBPs, such as MalE, SBD1 and SBD2, have multiple distinct closed conformations that all activate transport. This indicates that a productive SBP-TMD interaction in Type I ABC importers can be accomplished without relying on strict structural requirements for docking.

We observed that not all SBP-substrate complexes provide the signal for transport. For instance, the binding of certain non-cognate substrates (i.e. non-transported substrates) induces a conformational change in the SBPs that is different from those that activate transport. In other cases, the binding of non-cognate substrate leaves the SBP structure largely unaltered. Thus, transport might fail in these cases, because the SBP-substrate complex adopts a conformation that has no affinity for the translocator or, even when it can still dock onto the translocator, it might not be able to activate the transporter.

For the Mn^{2+} importer of *Streptococcus pneumoniae* we identified another mechanism how substrate transport can fail. Both Mn^{2+} and Zn^{2+} trigger formation of virtually identical PsaA conformations, yet the two metal ions trigger starkly different conformational dynamics in PsaA. Contrary to Mn^{2+}, Zn^{2+} forms a highly stable closed conformation, such that PsaA cannot open and release the substrate to the translocator. These findings show that transport by ABC importers depends on the ability of the substrate to induce not only the correct SBP conformation but also the correct SBP conformational dynamics.

In **Chapter 3**, we used mathematical modelling to study the effect of non-cognate substrates on the inhibition of cognate substrate transport. Based on the results of Chapter 2, we considered three non-cognate interaction mechanisms: (i) the SBP-substrate complex is unable to dock onto the translocator, (ii) docking is still possible, but the SBP-substrate complex cannot activate the transporter and lastly (iii) the SBP cannot open and release the substrate to the translocator. We found that under low substrate concentrations, inhibition does not occur for each of the mechanisms. However, in the limit that the cognate and non-cognate substrates are available at saturating concentrations and all translocators are in a complex with an SBP, the most drastic differences in the amount of inhibition exist between the mechanisms. More specifically, transport of cognate substrates is completely inhibited when the non-cognate substrate cannot be released from the SBP. In the other two mechanisms, where substrate release is possible, no inhibition occurs when the SBP-substrate complex cannot dock onto the translocator and partial inhibition occurs when docking is still possible. The findings of Chapter 3 suggest that transport inhibition depends on the effect of the non-cognate substrate on the SBP conformation and the SBP conformational dynamics.

SBPs are not only associated with ABC importers but are also part of other protein complexes. In **Chapter 4**, we assessed the effect of substrate on the conformation of SiaP and the regulatory domain (RD) of CynR. The SBP SiaP is part of a tripartite ATP-independent periplasmic (TRAP) transporter[30] and the SBP CynR belongs to the LysR-type transcriptional regulator (LTTR) family[31]. By using smFRET, we observed that the

substrate switches SiaP from an open to a closed conformation. In contrast, no such conformational changes could be detected for CynR. Thus, transcription activation by CynR is most likely based on minor and/or localized structural changes in the RD, rather than being based on large rigid-body rearrangements that are common to SBPs of ABC importers, TRAP transporters and other membrane protein complexes.

In the single-molecule measurements of Chapter 2 and 4 only the conformational changes in the SBP could be studied and not directly the binding and unbinding of substrate. To circumvent this limitation, and to directly relate the SBP conformational changes with the binding of substrate, we developed a single-molecule assay in **Chapter 5** to simultaneously probe the FeuA conformation via FRET and the substrate Fe^{3+}-bacillibactin via fluorophore quenching. The SBP FeuA is part of the Type II importer FeuABC. We observed that FeuA is predominantly in the open conformation without substrate and is closed when the substrate is bound. However, we found that FeuA can also close without the involvement of the substrate, demonstrating that substrate interactions are not required to close FeuA. Rare intrinsic closing events were also observed for SBD1, SBD2 and OppA (Chapter 2). These observations question the precise mechanism of substrate binding by the SBP. Two proposed mechanisms are the induced-fit[32] and the conformational selection mechanism[33]. In the induced-fit mechanism, the open state binds the substrate and subsequently closes the SBP. In the conformational selection mechanism, the substrate is directly bound by the intrinsic closed state. By examining the protein conformation and the moment the substrate binds, we directly observed that FeuA uses the induced-fit mechanism. However, the mechanism deviates from Koshland's original induced-fit formulation[32], in the sense that closing also occurs intrinsically.

In **Chapter 6**, we further investigated the SBPs SBD1 and SBD2, which belong to the Type I ABC importer GlnPQ. This importer has the intricate feature that both SBPs are directly linked to the TMD subunit. We investigated the effect of length and structure of the linkers, which connect the SBPs to each other and to the translocator, on transport. By combining transport assays, single-molecule data and mathematical modelling, we concluded that at high substrate concentrations, transport is slower when the linkers become longer, because of the extra time needed to find and dock onto the translocator. However, at low substrate concentrations, the probability to release the substrate has an additional impact on transport. Under these conditions, a released substrate from the SBP is not rapidly replaced by a new one. So depending on the timescales of substrate release relative to the time needed for docking, variations in linker length have an additional impact on transport. These findings, combined with the results of Chapter 2, show how

nature might fine-tune the uptake of essential nutrients by varying the conformational dynamics of the SBPs and the linkers that connect them to the translocator.

Most ABC proteins function in membrane transport, while others are part of cellular complexes that are involved in functions such as mRNA translation and DNA repair[2]. In **Chapter 7**, we studied the soluble ABC protein ABCE1 at the single-molecule level. ABCE1 plays a vital role in ribosome recycling, by splitting the archaeal ribosomes (70S) into large (50S) and small (30S) subunits[11, 34]. Confocal microscopy was used to determine the conformations of the two ATP binding sites (termed site I and site II) via FRET and simultaneously probe the association with the ribosome by measuring the diffusion constant of ABCE1. In contrast to any of the deterministic models of ABC proteins, we found that both sites of ABCE1 are always in a dynamic equilibrium between three conformations: open, intermediate and closed. The conformational behaviour of the two sites is asymmetric, allowing, for example, one site to close, while the other remains open. Thus, the NBDs of ABCE1 do not switch between an open and closed state as proposed for many ABC proteins but can acquire many more conformations due to the asymmetry between the two ATP binding sites. Moreover, we observed that the interaction with the ribosome influences the conformational equilibria of both sites differently. For instance, the binding of ABCE1 to 70S ribosomes biases the conformational equilibrium of site II towards the intermediate state, whereas the equilibrium of site I remains largely unaffected. The X-ray crystal structure and cryo-EM data suggest, however, that free ABCE1 and ABCE1 in complex with 70S or 30S, each adopt a unique conformation[11, 12, 35]. Our findings are in strong disagreement with such tight correlation and reveal instead a remarkable conformational plasticity of the highly conserved NBDs of ABCE1.

A picture emerges from our data on SBPs (Chapters 2 and 5) and ABCE1 (Chapter 7) and of data on other proteins[33], that suggest that many proteins always exist in an equilibrium of different conformations. Ligand binding only seems to redistribute the conformational equilibrium of the protein, instead of inducing completely new conformations. In **Chapter 8**, we used classical statistical mechanics to derive the equilibrium probabilities to form a protein conformation with and without ligand. By assuming that the Hamiltonian function is additive, these probabilities could be related to each other. We concluded that a shift in the apo conformational equilibrium biases the conformational equilibrium of the holo protein in the same direction. Furthermore, changes in the apo conformational equilibrium can increase or decrease the affinity for the ligand. These theoretical findings provide insight into how the apo and holo conformational equilibrium are related and how changes in these equilibria affect the affinity for the ligand.

9.2 Outlook

The findings of this thesis provide new insight into the working mechanism of ABC proteins. However, with any new information, also new questions arise. For instance, it is not entirely clear how the conformational and dynamic plasticity of an SBP (Chapter 2) influences the interaction with the translocator. For example, it remains unclear if the different closed conformations of MalE, SBD1 and SBD2, that all productively interact with the translocator, have the same affinity for the translocator and induce the same conformational change in the translocator. Moreover, it remains unknown if SBPs with a non-cognate substrate can still dock onto the translocator. Single-molecule approaches are particularly attractive to study the SBP-translocator interactions, since not only thermodynamic constants, such as the dissociation constant K_D, but also the dynamics of this interaction could be studied.

Another aspect of major interest are the intrinsic conformational changes of the SBPs (Chapters 2 and 5), ABCE1 (Chapter 7) and other proteins[33]. First of all, it remains elusive if other intrinsic SBP conformations exist that have a lifetime below the microsecond timescale. Such conformations could be, for example, a partially closed state, as has been observed for MalE by NMR[13]. For such scenarios, the ligand-binding mechanism could be more complex as presented in Chapter 5 and might involve elements from both the conformational selection and induced-fit mechanism. To further elucidate this, methods with high temporal resolution such as NMR[13] would be required. Secondly, the theoretical predictions of Chapter 8 about the relationship between the conformational equilibria of the apo and holo protein and the K_D should be tested experimentally. However, it must be noted that a few observations already support them (Section 8.3). By using protein mutagenesis to perturb the stability of the protein conformations and using single-molecule tools to probe the conformational equilibria, it would be possible to test the predictions of Chapter 8 in more detail. This could give important insight into the functional relevance of intrinsic conformational changes in ABC proteins.

From our findings in Chapter 7 many questions arise about the conformational crosstalk and asymmetry between the two ATP binding sites of ABCE1. For instance, does the conformational equilibrium of site I depend on the conformation of site II and vice versa? Three- or four-colour smFRET experiments[36, 37] could be used to determine the conformations of both ATP sites simultaneously, and thus directly reveal the crosstalk and asymmetry between them. Alternatively, by exploiting the fact that the conformational equilibrium can be frozen at room temperature, multiple distances could be measured with switchable FRET[38]. Besides the conformational changes of the ATP sites, the FeS cluster domain movement also requires further investigation. Insight into FeS cluster domain

movement is needed to better understand how ABCE1 drives the ribosomal subunits apart and, in particular, reveal how the FeS cluster domain movement is coupled to the conformational changes in the ATP sites.

9.3 References

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