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8 Discussion

Spore germination in *Bacillus subtilis* is a highly complex molecular pathway that enables dormant spores to rapidly resume rapid metabolism then vegetative growth in response to favourable environmental cues²⁷. Central to this process are germinant receptors, which translate the presence of specific germinants into a cascade of molecular events leading to spore revival⁴⁴. Despite their fundamental importance, the molecular mechanisms by which these receptors function remain poorly understood, largely due to the lack of high-resolution structural and dynamic information.

In this thesis, we investigated the germinant receptor subunit GerAB as a model system to dissect how structural features, protein dynamics, and small-molecule interactions contribute to spore germination. To be specific, this thesis analyses the putative water channel formation within GerAB (Chapter 3, 4), the dynamics of its transmembrane (TM) regions (Chapter 5), and GerAB's interactions with monovalent cations and L-alanine (Chapter 5, 6). Structural prediction tools including RaptorX^{45,67} and AlphaFold^{71,72,141} provided us GerAB structural modelling, Molecular Dynamics simulations enabled us to study GerAB dynamics at high resolution¹⁹¹. Molecular modelling provided insights that were verified by constructing mutant spores and tracking spore germination at the single-cell level. However, the exploration of this research topic remains an open book rather than a closed one. Key questions remain: Is GerAB truly a water channel? Are there additional layers to GerAB dynamics? And how do these dynamics contribute to the overall germination cascade?

8.1 Water “channel”

In this thesis, substantial effort was made to characterizing potential water channel formation in GerAB. These efforts included identifying putative water pathways and blocking residues by pulling water through GerAB with steered molecular dynamics (SMD) simulations (Chapter 3), pinpointing transmembrane regions that constitute the water pathway and residues contacting permeating water molecules (Chapter 4), and uncovering a possible gating segment within GerAB (Chapter 5). In Chapters 3 and 4, molecular simulations were employed as hypothesis-generating tools to explore the possibility that GerAB functions as a water channel. These simulations served to guide subsequent experimental validation through mutagenesis.

An important empirical observation emerged: nearly all GerAB mutations constructed in this work, regardless of whether they involved large or small side-chain alterations, all the mutagenesis attempt resulted in the loss of detectable GerAB (using GerAA presence as a proxy of detectable GerA presence). This phenomenon is not unique to

this thesis. Previous attempts to investigate GerAB through have encountered similar difficulties^{44,55}. In practice, retaining an intact GerAB (and GerA complex) after mutation appears almost a matter of “luck” where even gene copy-number may play a determining role.

Several explanations may underlie this extreme sensitivity. One possibility is that intra-protein residue interactions contribute to GerAB stability. In Chapter 3, we attempted to probe residue–protein interactions and observed that L199 and F342 (GerAA-negative mutants) exhibited higher total protein interaction number than Y97 (GerAA-positive, albeit reduced relative to wild type). Although this analysis was based on a limited number of simulation snapshots and therefore lacks broad statistical significance, it suggests that mapping residue contact networks¹²⁶ may be a useful strategy for identifying structural hotspots in GerAB. A second possibility involves interactions between GerAB and its partner subunits GerAA and GerAC. Mutations in GerAB that resulted in the loss of GerA also produced varying degrees of impairment in AGFK-induced germination, indicating varied effects on GerB and GerK function within the germinosome. This raises the question of whether different GerAB mutants retain distinct capacities to assemble with GerBA/BC and/or GerKA/KC into complexes. If so, mutations in GerAB may alter subunit assembly sensitivity or even change its affinity for GerB and GerK. Nevertheless, the near-complete disassembly of mutant GerAB from the GerA complex ultimately prevented definitive functional conclusions for the water channel.

Given our observations we acknowledge the challenges in using mutagenesis to experimentally verify whether GerAB functions as a water channel. When experimental validation is constrained, additional insight can be gained by considering GerAB in the context of its protein family. GerAB is an APC transporter protein and likely functions as a tranceptor, coupling ligand binding to downstream signal transduction. To date, no member of this protein family has been demonstrated to function as a water channel^{45,60,110}. More commonly, such transporters facilitate water movement to stabilize ligand binding, as observed in GkApcT⁶⁰. From this perspective, it would be unexpected for GerAB to operate as a water channel. This also holds true given that the water passage rate identified in Chapter 4 ranges from 10^5 to 10^7 s⁻¹, which is two to four orders of magnitude lower than that of AQPI, whose water permeation rate can reach 10^9 s⁻¹¹³². Rather than facilitating bulk or water uptake during germination, the water permeation observed in simulations may play a subtler role. If water contributes to ligand binding or release, then mutations in water-contacting residues (as identified in Chapter 4) would be expected to share similar germination phenotype as mutations in the ligand-binding pocket by indirectly altering GerAB-ligand interactions and, consequently, germination efficiency.

Although this hypothesis cannot be verified through mutagenesis due to GerAB instability, it provides a framework for interpreting the simulation results. Future

work will therefore need to explore alternative experimental and computational approaches to elucidate whether, and how, water passage contributes to GerAB function during spore germination.

8.2 TM dynamics

To understand protein functionality at the molecular level, high-resolution structural information is essential. With the rapid development of computational protein structure prediction tools, such structural models can now be readily obtained. However, experimentally determined protein structures are still considered “the golden standard”, and predicted structures cannot be used directly as optimal approximations of *in vivo* protein structure^{71,141,178,192}. Moreover, current structure prediction tools are generally unable to capture multiple functional conformational states of proteins, such as activated versus inactivated states or inward-facing (IF) and outward-facing (OF) conformations in APC transporters^{72,110,142,143}.

This study was carried out in the absence of experimentally resolved structures and well-defined functional states of GerAB. Under these circumstances, molecular simulations provided a powerful framework for generating and refining mechanistic hypotheses about GerAB dynamics. Using MD simulations, we were able to pinpoint two key dynamic features of GerAB: bending of TM1 and gating of water passage mediated by the C-terminal segment of TM6 (TM6C) (Chapter 5). While TM1 bending has been reported in several APC transporters and other LeuT-fold proteins¹¹⁰, this work represents the first observation of such bending captured dynamically along a continuous trajectory. Furthermore, we identified that the L-alanine dissociation pathway from GerAB coincides with different bending state of TM1. Notably, this dissociation direction appears reversed relative to the IF and OF states described for other APC transporters: when TM1 is bent, GerAB adopts an outward-facing-like state (as observed in other experimentally characterized OF structures of APC transporters), yet L-alanine escapes toward the inner membrane side of the protein, and vice versa. This unexpected observation raises again questions on the exact membrane organization of GerAB in *Bacillus* spores. Interaction with the A and C subunits of the GerA germinant sensor might play a role in this too.

Ultimately, the dynamic behaviours of GerAB TM regions revealed by MD simulations suggest that GerAB function is based on conformational changes rather than fixed structural states. An open question remains as to how these dynamics contribute to, or are coupled with, ligand sensing and further, downstream signal transduction within the germinosome. These findings provide clear directions for future research.

8.3 Bigger picture

Considering the size difference between GerAB (nanometre scale) and the spore (micrometre scale), this work necessarily involved extensive “zooming in” to identify the L-alanine sensor within the spore. It is now appropriate to step back and place these findings into a “bigger picture”, a broader biological context. To do so, GerAB must first be considered within its native germination molecular machinery, namely the GerA germinant receptor (GR).

Previous work from the Rudner group, combining AlphaFold-based structure prediction with mutagenesis of GerAA, suggests that the GerA complex is organized as a pentamer composed of a trimer of GerAA, GerAB, and GerAC subunits³⁵. In the present study, GerAB was studied in MD simulations as a monomer embedded in a lipid membrane; however, *in vivo* GerAB exists as part of a larger receptor complex. It is therefore highly relevant to ask how the mechanisms identified for GerAB in this thesis contribute to the dynamics of the GerA GR as an assembled complex.

As discussed earlier, GerAB exhibits an unusually high sensitivity to mutagenesis, the molecular basis of which remains unclear. One possible direction for future work would be to explore inter-subunit interactions between GerAA, GerAB, and GerAC to determine whether mutations in GerAB alter its interactions with the A and C subunits, and whether such alteration contributes to unsuccessful GerA assembly of GerA. Such questions could be addressed using MD simulations of the full GerA complex. Importantly, investigating the ABC subunit interactions is not only relevant for understanding GerAB instability but also for elucidating signal transduction within the receptor. More specific, whether the GerAB dynamics identified in this thesis can trigger conformational changes in GerAA. In the study of Rudner lab, mutations that alters amino acid side chains size on GerAA’s centric TM ring (V362A and V362L) were shown to manipulate the open and close states of a GerAA-formed ion channel, leading to the proposal that GerA functions as a nutrient-gated ion channel that opens upon L-alanine sensing via GerAB³⁵. Future studies could aim to probe also this proposed mechanism dynamically using MD simulations, to help fill this important knowledge gap.

Finally, GerAB can be placed within an even broader framework: the germinosome. As GerB and GerK share a same subunit composition with GerA, including the presence of GerBB and GerKB subunits, it would be informative to examine whether the dynamic features observed for GerAB—such as water passage and TM dynamics—are also observed in GerBB and GerKB. Exploring these behaviours across multiple germinant receptor subunits may reveal shared mechanisms. Should future work investigate the putative water “channel” function of GerBB and GerKB, it is tempting to hope that mutagenesis in them may prove more experimentally fruitful.