Insight into the structure and interactions of the *M. tuberculosis* Mce-associated membrane proteins Mam1A-1D

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Abstract

Tuberculosis (TB) is a major infectious disease caused by *Mycobacterium tuberculosis* (Mtb) leading to more than a million human deaths every year. Mammalian cell entry complexes (Mce1-4) play an essential role for the survival of Mtb during the latent stage by mediating the import of lipids inlcudiing fatty acids and cholesterol from the host. The proper functioning of Mce-complexes requires additional proteins such as Mce-associated membrane (Mam) proteins and lipid uptake coordinator (LucA), thus making them potential candidates for the development of anti-TB drugs. Four Mam (Mam1A-1D) proteins are coded from the *mce1* operon and two from the *mce3* (Mam3A-3B) and *mce4* (Mam4A-4B) operons. In addition, five orphaned mam (Omam) proteins have been identified which are not part of the *mce* operons but are functionally relevant for the Mce complexes. Analysis of the sequences of Mam/Omam proteins suggests that they share many common secondary and tertiary structural elements despite the low sequence identity between them. Here, we have recombinantly produced and characterized a Mam1A variant by small angle X-ray and neutron scattering. The studies indicate that Mam1A is tetrameric in solution with two disulfide bridges necessary for its stability. Similarly, a disulfide bridge has also been identified in Mam1C. Furthermore, through coexpression and copurification studies, we demonstrate that Mam1A-1D and LucA interact to form stable Mam1ABCD as well as Mam1ABCD-LucA complexes. The results obtained pave way for further studies towards understanding how the Mam1ABCD and Mam1ABCD-LucA complexes are organized and interact with the Mce complexes leading to their stabilization.

Introduction

Tuberculosis (TB) is one of the oldest diseases that has riddled mankind. Recent reports from the World Health Organization (WHO) estimated that nearly 11 million people fell ill with TB, leading to 1.4 million deaths[1]. The more recent reports also show that TB cases are rising everywhere except in the European region[2]. With the increase in multidrug-resistant strains detected and more strains resistant to conventional BCG vaccines, finding new ways to battle TB is crucial. *Mycobacterium tuberculosis* (*Mtb*), the pathogen that causes TB, has survived for centuries by adapting to the host intracellular environment. *Mtb* persists in its host in a latent form even for decades, during which *Mtb* switches from using carbohydrates to lipids as the main source of carbon and energy for its survival[3,4]. *Mtb* has an extensive repertoire of lipid-processing enzymes and protein complexes[5]. One such set of four homologous ATP-binding cassette (ABC) transporters are mammalian cell entry (Mce) complexes, Mce1-4, encoded by the *mce1-mce4* operons. The Mce1 complex encoded by the *mce1* operon is responsible mainly for *Mtb*'s palmitate and oleate intake[6]. Mutational studies have also suggested the role of the Mce1 complex in the import of mycolic acid[7]. Additionally, mutations of the *mce1* operon have been observed to cause hypervirulence[8]. The Mce4 complex is a cholesterol importer[9–11]. The Mce2 complex is likely a sulfolipid transporter[12]. The precise substrate specificity of the Mce3 complex is not yet known. The importance of the Mce proteins for virulence[13–15] and survival of mycobacteria[14–16] has been shown in animal models.

The *mce1*, *mce3,* and *mce4* operons also code for additional proteins, which are named Mce-associated membrane (Mam) proteins[17]. The *mce1* operon codes for four Mam proteins (Mam1A-1D), while the *mce3* and *mce4* operons each code for two Mam proteins (Mam3A-3B and Mam4A-4B)[17]. Additionally, five proteins homologous to Mam proteins have been identified as encoded outside the *mce* operons, and these are named orphaned Mam (Omam) proteins[17,18]. *Mtb* has five *omam* genes, labelled *omamA*, *B*, *C*, *D*, and *E*. The Mam and Omam proteins are the least understood proteins among the Mce-related proteins. Although their importance in infection has been established with mutational studies[16,19], only recently, it has been suggested that OmamA plays a crucial role in stabilising the Mce1 complex and enabling the Mce4 complex to work properly[18,20]. Moreover, it has been demonstrated that the Mam proteins are not involved in the binding of cholesterol to the Mce4 complex. Rather, they are involved in the internalisation of cholesterol at the cytoplasmic membrane. The process involves another protein named LucA which interacts with the Mam/Omam proteins and is involved in the stabilisation of the Mce1 and Mce4 complexes[6]. Deletion of LucA showed an increase in the expression of all *mce1* genes while almost completely inhibiting the palmitate lipid uptake[21,22]. Inhibiting the interaction of the Mam/Omam proteins with LucA and with the Mce complexes could be an effective strategy to impair the function of the Mce complexes, potentially reducing the survival

of *Mtb* in its latent form. Therefore, a detailed understanding of the Mam/Omam proteins and LucA is necessary. In this study, we recombinantly produced Mam1 proteins, and through biophysical characterisation, including SAXS and SANS studies, show that Mam1A most likely exists as a tetramer. In addition, we show that Mam1A-1D and LucA interact to form stable Mam1ABCD and Mam1ABCD-LucA complexes. Furthermore, we have discovered two crucial disulfide bonds in Mam1A and Mam1C that are important for their overall stability. These studies pave the way for further detailed structural and interaction studies on Mam1ABCD and Mam1ABCD-LucA complexes.

Results

Sequence analysis of the Mam/Omam proteins

Analysis of the sequences of Mam1A-1D suggests that Mam1A and Mam1C share a sequence identity of 21%, whereas Mam1A and Mam1B have only about 4% sequence identity (Supplementary Table 1). Despite the low sequence identities between them, secondary structure predictions suggest that Mam1A-1D have a common domain organisation with four key regions. The N-terminal region has a varying number of amino acids; for example, 28 amino acids in Mam1C is the shortest, and 159 in Mam1B is the longest region. The Mam1B variable region additionally contains many small α-helices and β-strands, which do not occur in the other Mam1 proteins. The variable N-terminal region is followed by a long α-helix that contains a transmembrane (TM) region. While Mam1A, Mam1C and Mam1D have only one predicted TM helix, Mam1B is predicted to have three TM helices, two of which are in the variable region. In addition, Mam1B has one more helix predicted to be buried in the membrane (Fig. 1a and d). The TM region in each Mam is about 20 amino acids long, and the α-helix containing TM is the longest in all Mam1 proteins, ranging from 43 to 55 amino acids. After the α-helix-containing TM region is a small helical region with two α-helices, each of which is between 10 to 20 amino acids long. The protein C-terminus contains a β-sheet segment with four β-strands. The β-strands are between 7 and 14 amino acids long. A schematic illustration of the various domains of the Mam1 proteins is shown below in Fig. 1a.

Sequence comparison shows that there are only four amino acids that are conserved in all of Mam1A-1D (Supplementary Figure 1). Two of these, arginine (Mam1A-R52, Mam1B-R162, Mam1C-R25, Mam1D-R77) and alanine (Mam1A-A61, Mam1B-A170, Mam1C-A34, Mam1D-A86) are located close to or inside of the α1-helix (Fig. 1b). The α1-helix is the longest helix in Mam1 proteins, which also contains TM region in the first half of the helix. The α3-helix contains conserved glutamine (Mam1A-Q131, Mam1B-Q237, Mam1C-Q103, Mam1D-Q154) and the β1-strand the conserved valine (Mam1A-V159, Mam1B-V262, Mam1C-V130, Mam1D-182) (Supplementary Figure 1). The importance of these conserved amino acids for the potential function of the proteins is not yet known. Mam1B has been

noted to contain a conserved RDD domain. This is part of the RDD superfamily, characterised by an arginine and two aspartates as well as two TM regions[23]. This superfamily's function is unknown, and no other proteins within the family have been studied. The three RDD conserved amino acids in Mam1B are R95, D133 and D144, which are absent in other Mam and Omam proteins. The RDD domain is highlighted in Fig. 1c with a black circle.

Mam proteins from the *mce3* and *mce4* operons and OmamA-E have a similar secondary structure organisation. The variable N-terminal regions for Mam3B and Mam4B are much shorter, with less than 6 amino acids, immediately followed by the TM region (Fig. 1a). Additionally, OmamB has three TM regions similar to Mam1B. However, OmamB does not have as long variable region and the helix is predicted to be buried in the membrane (Fig. 1a and d). Comparison of all the Mam/Omam sequences shows that overall, Mam/OmamAs/Cs and OmamE fall under one branch of the phylogenetic tree while Mam/OmamBs/Ds fall under another branch (Fig. 1e). Between Mam/Omam proteins, the sequence similarity is much higher towards the C-terminus of the proteins, where the alignment of the secondary structure elements is better. Overall, the highest identity is 31% between Mam3B and OmamD, and the lowest is between Mam1A and Mam1B (Supplementary Table 1). The TMHMM2.0[24] prediction provides information on the TM region as well as the overall topology. The prediction confidence for Mam/Omam orientation varies between proteins (Supplementary Table 2).

A comparison of the AlphaFold2[25] predicted structures of each of the Mam/Omam proteins showed that they have many common features and are generally in line with the secondary structure predictions (Fig. 1 a-d). In the predicted structures, the β-sheet partially surrounds the backbone α1-helix and the following α2 and α3 helices to form a V-shaped hinge, indicating flexibility in the structure. The β-sheet with the α2 and α3 helices creates a pocket for possible interactions. It has been shown that OmamA has a similar predicted structure as VirB8 using a Phyre 2 web service[18]. Like Mam and Omam proteins, VirB8 is a membrane protein with a single TM region. The structure of VirB8 has been determined by crystallography[26]. The similarities between VirB8 and Mam/Omam proteins are threefold: i) they have one long α-helix that is partially surrounded by the β-sheets at the C-terminus, which also contains the TM region, ii) the longer α-helix is followed by two shorter α-helices that form a V-shape turn, and iii) the protein ends in a β-strand rich region with short loops connecting each.

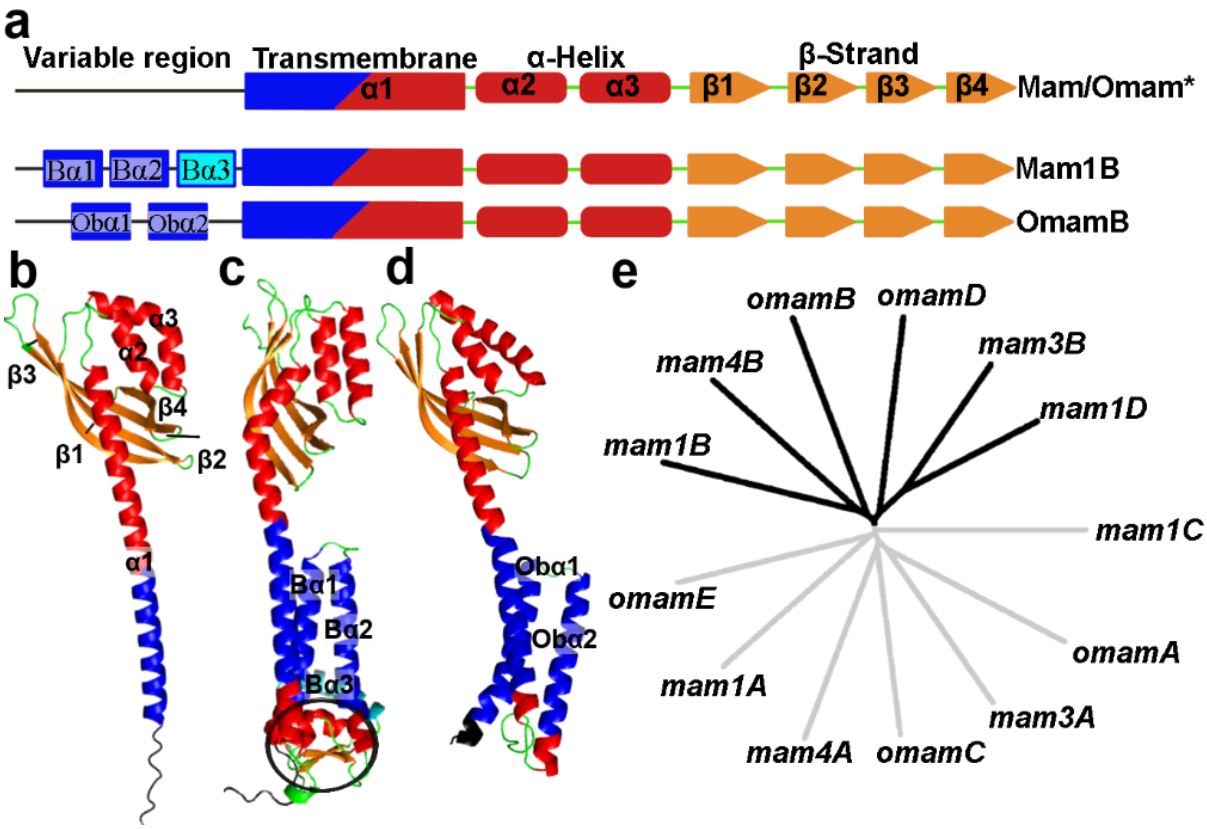

Fig 1. Sequence analysis and secondary and tertiary structure predictions of Mam and Omam proteins. a A schematic illustration of the general conserved secondary structure organization of all *Mtb* Mam/Omam proteins excluding Mam1B and OmamB (denoted as Mam/Omam*). The four regions are named i) variable region, ii) transmembrane iii) α-helix and iv) β-strand. Additional secondary structure elements present in Mam1B and OmamB are shown in the second and third rows. **b** AlphaFold2-predicted tertiary structure of Mam1C, a representative of general Mam/Omam protein. The color coding is the same as that used in Fig. 1a. **c** AlphaFold2-predicted tertiary structure of Mam1B. The RDD domain is highlighted by a black circle. **d** AlphaFold2-predicted tertiary structure of OmamB. **e** Phylogenetic tree generated from the sequence alignment of all Mam/Omam proteins of *Mtb* highlighting that Mam/Omam As, Cs and E cluster on one side of the tree and Mam/Omam Bs and Ds cluster together on the other side of the tree dividing the tree into two halves.

Mam1AΔ106 forms a homodimer with an intermolecular disulfide bridge

All the Mam/Omam proteins have a predicted transmembrane helix in the N-terminus. To increase the solubility, a variant of Mam1A was created by removing 106 residues from the N-terminus (Mam1AΔ106), expressing only the C-terminal region outside the membrane. Preliminary expression and purification indicated that Mam1AΔ106 still required the presence of detergents, similar to the full-length Mam1A. Dodecyl nonaethylene glycol ether (C12E9) was chosen as the most optimal detergent for further purification as the use of this detergent for purification resulted in the lowest

polydispersity measured by the dynamic light source (DLS) (Supplementary Figure 2) and the best unfolding curve, as observed by Nano differential scanning fluorimetry (NanoDSF) suggesting a well-folded protein (Supplementary Figure 3). Mam1AΔ106 required the detergent only in the lysis step of the purification for obtaining a well-folded homogenous protein sample (Fig. 2a). The identity of the purified protein was confirmed by peptide mass fingerprinting using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS).

Mam1AΔ106 eluted at a retention volume of 13.5 ml in a Superdex 200 10/30 GL column (GE Healthcare), much earlier than expected for a protein with a theoretical monomeric molecular mass of 14.8 kDa. SDS-PAGE analysis of Mam1AΔ106 showed that it runs at a higher molecular mass (~30 kDa) close to that of a dimer in the absence of beta-mercaptoethanol (BME). In contrast, in the presence of BME, the bands run close to the theoretical monomeric molecular weight (Fig. 2a). Both bands were confirmed to be Mam1AΔ106 from the peptide mass fingerprinting analysis. The primary sequence of Mam1AΔ106 has only one cysteine (Cys123), which should, therefore, be involved in the disulfide bridge formation stabilising the homodimer. To test this, a variant Mam1AΔ106-C123S was generated by mutating the Cys123 to Ser. This variant indeed ran as a monomer in SDS-PAGE both in the presence and absence of BME, further confirming the involvement of Cys123 in disulfide bridge formation. Moreover, the Mam1AΔ106-C123S variant was less stable and was prone to severe aggregation during concentration, suggesting the importance of this disulfide bridge for the overall stability of Mam1AΔ106 (Supplementary Figure 4). The yield and stability of the Mam1AΔ106-C123S variant were too poor for further characterisation.

Analysis of the circular dichroism (CD) spectrum suggested that Mam1AΔ106 contains about 29% helices, 13% antiparallel, and 9% parallel sheets with 18% beta turns (Supplementary Figure 5). The protein exhibited a melting temperature (T_m) of 56.5 °C, calculated from NanoDSF data with MoltenProt. Analysis of the molecular mass of Mam1AΔ106 by size-exclusion chromatography (SEC)-multi-angle light scattering (MALS) followed by protein-conjugate analysis showed that the total molecular mass of the eluted Mam1AΔ106 was around 68 kDa, of which 54 kDa was protein and 14 kDa was C12E9 detergent. The measurement revealed that although the detergent was used only in the first lysis step, it was strongly bound to Mam1AΔ106 (Supplementary Figure 6). Furthermore, these results suggest that the oligomeric state of Mam1AΔ106 is tetrameric. Additional hydrodynamic radius calculation conducted by DLS measurement of the SEC-MALS run gave a radius of 3.45 nm, which corresponds to the combined mass of Mam1AΔ106 tetramer surrounded by a detergent micelle of around 72 kDa. Therefore, the tetramer is proposed to contain two intersubunit disulfide bridges formed between the two pairs of Cys123.

Mam1AΔ106 SEC-Small Angle X-ray Scattering (SAXS) and Small Angle Neutron Scattering (SANS) analysis reveal protein-detergent complex

Mam1AΔ106 was further analysed by SEC-SAXS (Table 1), from which signature detergent signal was observed as a bump or oscillation at a q of 0.18 $Å^{-1}$ in the measured intensities $I(q)$ (Fig. 2b) frequently characteristic of core-shell or mecellar structures. Additionally, the Kratky plot suggested a globular shape for the protein (Fig. 2c), possibly due to surrounding detergent formation. The estimated maximal particle distance (D_{max}) was 90 Å, and the radius of gyration (R_g) was 32.6 Å. A dummy atom model was generated from the SAXS data using the DAMMIN program[27] (Fig. 2f and h). Due to the lack of information about the complex formation from homologues, AlphaFold3 was used to model possible modes of tetrameric assembly. Of the multiple possible assemblies predicted by AlphaFold3, there were two cases, in which the cysteines in the α2 helix were placed close enough to one another to allow disulfide bridge formation. The experimental evidence on disulfide bridge formation was critical in selecting these potential oligomer models with high confidence. Interestingly, with one prediction, referred to here as Model 1, the bridge formation was possible with a domain swap arrangement of α2 and α3 helices between the chains A and C (Fig. 2f). The four-chain arrangement gave the χ^2 fit of 10.5. The other predicted tetramer, referred to here as Model 2 (Fig. 2h), had domain swaps with respective neighbouring chains, namely, chain A α2 helix with chain B, chain B α2 helix with chain C and so forth. In both models, alterations were made to the N-terminal tail and the best-fitting χ^2 score against the data was 6.9 for Model 2. The SAXS data collection parameters and analysis are listed in Table 1.

The disulfide bridge between chains in Model 1 forms between chains A and B and chains C and D. In Model 2, the cysteines are located so that they can form the disulfide bridge between chain A to either chain B or chain D. The neighbouring chain cysteines are positioned so that it is not possible to determine the disulfide bridge orientation. Nevertheless, for modelling purposes, the disulfide bridge in Model 2 was considered to be between chains A and D and between chains B and C

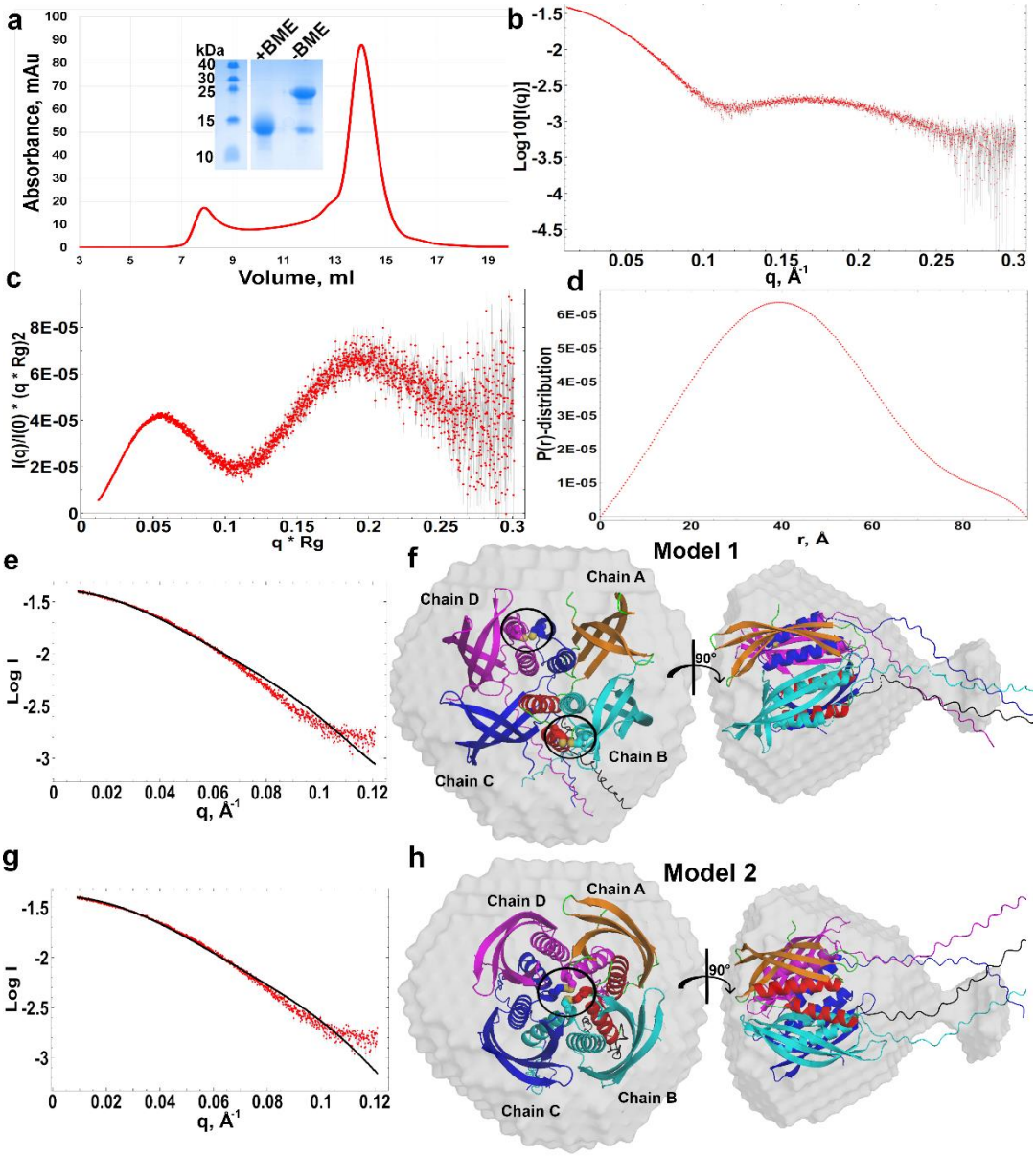

Fig 2. Purification profile and SAXS analysis of Mam1AΔ106. a SEC profile of Mam1AΔ106 showing a minor aggregation peak around 8 ml and the main peak at 14 ml. SDS-PAGE analysis of peak fractions at 14 ml in the presence and absence of BME is shown in the inset. **b** SAXS curve of Mam1AΔ106. The intensity curve has a characteristic bump/oscillation at *q* of 0.18 $Å^{-1}$ indicating the presence of the detergent micelle with the protein. **c** Kratky plot of Mam1AΔ106 suggesting a folded globular molecule. **d** The *P*(*r*) curve of Mam1AΔ106 SAXS data truncated at *q* of 0.12 $Å^{-1}$. **e** Fit of Model 1 generated by AlphaFold3 against the truncated Mam1AΔ106 SAXS data as calculated by FoXS. **f** Model 1 superimposed to the calculated DAMMIN model in a top view and a rotated side view. The disulfide bridge-forming cysteines are shown as sticks and highlighted in a black circle. **g** Fit of Model 2 generated by AlphaFold3 against the truncated Mam1AΔ106 SAXS data as calculated by FoXS. **h** Model 2 superimposed to the calculated DAMMIN model in a top view and a rotated side view. The disulfide bridge-forming cysteines are shown as sticks and highlighted in a black circle.

To better understand the tetrameric assembly, SANS measurement was conducted on Mam1AΔ106 (Table 1). The detergent signal was contrast matched (or "masked") by exchanging the C12E9 into D-C12E9. The estimated R_g and D_{max} values for the SANS data were 25.8 Å and 85.0 Å, respectively, which were lower than the corresponding values obtained from SAXS data. This gave the first indication that the detergent signal had been matched out. The DAMMIN[27] model calculated from the SANS data possessed different shape than that calculated from the SAXS data (Fig. 3b). The model suggests a region of protein forming a tail-like segment pointing away from the main body suggesting there is a disordered potion in the system. Model 1 and Model 2 obtained from AlphaFold3 had a better fit against the SANS data when compared to the fits against SAXS data. Among them, the two models had similar CRYSON calculated fit. Model 1 had a χ^2 of 4.4 versus Model 2 having χ^2 of 4.6 (Fig. 3c and e). However, by moving the helix of the model pointing downwards to follow the calculated dummy atom model more precisely and optimising the tail conformation with DADIMODO[28], the CRYSON calculated fit did not improve. The data collection parameters and analysis are listed in Table 1.

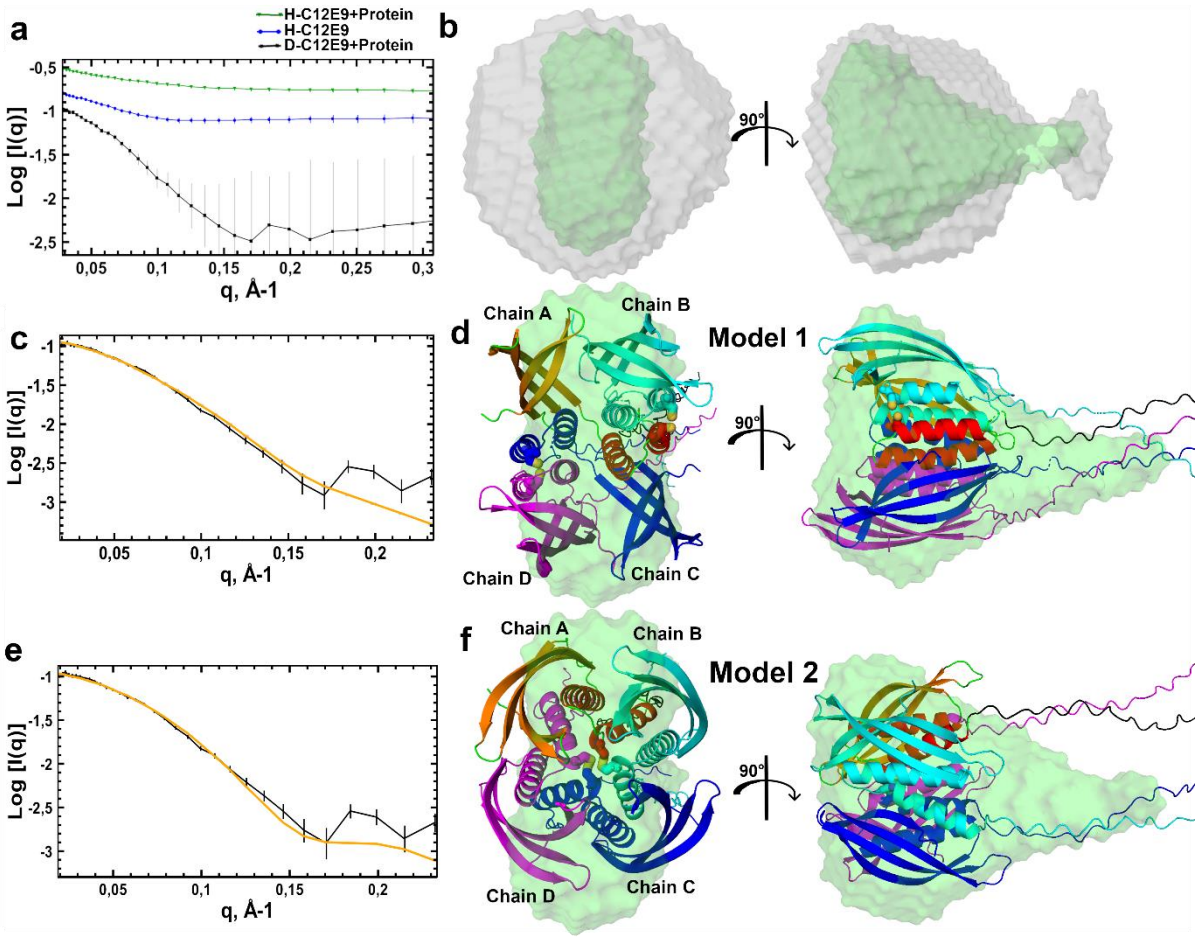

Fig 3. SANS data analysis of Mam1AΔ106. a SANS curve of C12E9 (blue), Mam1AΔ106 in C12E9 (green) and Mam1AΔ106 in D-C12E9 (black). A comparison of these curves shows that the detergent signal is

masked by the use of D-C12E9 resulting in the scattering of only protein molecules. **b** Superposition of DAMMIN calculated dummy atoms models from SAXS data (grey) and SANS data (green). **c** Fit of Model 1 against the SANS data as calculated by CRYSON with a χ^2 of 4.4. **d** Model 1 superimposed on the calculated SANS dummy atom model in both top and side views. **e** Fit of Model 2 against the SANS data as calculated by CRYSON with a χ^2 of 4.6. **f** Model 2 superimposed on calculated SANS dummy atom model in both top and side views.

Table 1 SAS data collection parameters and analysis for Mam1AΔ106

Protein	Mam1AΔ106	
Source organism	Mtb, H37rv	
Expression organism	*E. coli*	
Experiment	**SAXS**	**SANS**
Beamline parameters		
Beamline	B21, Diamond Light Source, UK	ZOOM, ISIS Neutron and Muon Source, UK
Detector	EigerX 4M(Dectris)	3He gas tube detector
Wavelength, Å	0.9464	1.75-16.5
Detector distance, mm	3732.9	4000
q-measurement range, $Å^{-1}$	0.0045 – 0.34	0.006 – 0.78
Sample volume, µl	50	300
Sample concentration, mg ml^{-1}	8.8	-
Structural parameters		
I(0) (Guinear), cm^{-1}	$0.03953 \pm 0.4458 \times 10^{-4}$	$0.126 \pm 0.9651 \times 10^{-3}$
R_g (Guinear), Å	$32.67 \pm 0.4251 \times 10^{-1}$	25.8 ± 0.3431
I(0) (P_r), cm^{-1}	0.03953	0.126
R_g (P_r), Å	32.67	25.78
q*R*g range, $Å^{-1}$	0.012 – 0.039	0.0224 – 0.0458
Limits, qR_{gmin} – qR_{gmax}	0.34 – 1.3	0.58 – 1.16
q range, $Å^{-1}$	0.009 – 0.119	0.0231 – 0.1708
D_{max}, Å	90	85
Porod volume, $Å^3$	177045	69729
Mass, kDa (expected)	74	74
Mass, kDa (Porod volume/1.7)	104	41
Mass, kDa (SaxMow2)	39	-
Modelling		
Shape modelling software	Primus DAMMIN/DAMAVER	DAMMIF/DAMFILT
q-range for fit ($Å^{-1}$)	0.009 – 0.119	0.0231 – 0.1708
Symmetry assumption	P1	P1
No. of individual model reconstructions	20	5
Fitting parameters	FoXS	CRYSON
χ^2 of Tetramer Model1/Model2	10.5 / 6.9	4.4 / 4.6

Mam1A forms a complex with Mam1C

Following the studies on Mam1AΔ106, individual expression tests were done for each of the Mam proteins from the *mce1* operon cloned from the *Mtb* genome. Among them, the expression of Mam1A and Mam1C were observed, both of which belong to the same branch of the phylogenetic tree (Fig. 1e). Mam1A and Mam1C were individually purified using His-tag affinity chromatography. Like Mam1AΔ106, the full-length Mam1A showed the presence of a disulfide bridge, likely involving the same cysteine residue (Cys123). In addition, full-length Mam1C also revealed the presence of a disulfide bridge (Fig. 4a). Mam1C has only one cysteine residue in its sequence (Cys46 present in the α1 helix), which is not homologous to the Cys123 of Mam1A, present in the α2 helix. This suggests that Mam1C forms at least a homodimer via a disulfide bridge. A variant of Mam1C was created by removing the first 78 amino acids (Mam1CΔ78), which are predicted to form the TM helix. Expression and purification of Mam1CΔ78 showed that this variant is soluble without the need for any detergent and SEC-MALS studies showed that the variant is mixture of different oligomeric states in solution (Supplementary Figure 7). However, the protein started to precipitate at a concentration higher than 1 mg ml^{-1}. The variant does not contain the disulfide bridge-forming cysteine, which is probably a contributing factor to the observed instability and heterogeniousity. Mam1A and Mam1C were then co-expressed, with His-tag on Mam1A. Purification studies followed by analysis of the bands from SDS-PAGE by mass spectrometry showed that Mam1C co-eluted with Mam1A. Further analysis of the purified fraction in the presence and absence of BME showed that disulfide bridges were still present in both Mam1A and Mam1C (Fig. 4b). There was no indication of a disulfide bridge between Mam1A and Mam1C. These studies suggest that Mam1A and 1C interact to form at least a $Mam1A_2C_2$ heterotetramer (or a higher oligomer).

Mam1ABCD forms a stable complex with and without LucA

To test if the other two Mam proteins also interacted with Mam1A and Mam1C, all four Mam1 proteins were co-expressed with Mam1A, with the His-tag on Mam1A. The genes were codon optimized to improve the recombinant expression of these proteins in *E. coli*. Immobilised metal affinity chromatography (IMAC) purification results showed that Mam1B, Mam1C, and Mam1D co-eluted with Mam1A, demonstrating that the four Mam1 proteins interact to form a stable complex. (Supplementary Figure 8a). Further, SEC analysis showed two peaks at 13.2 and 16.7 ml in a 24 ml Superose6 column. The SDS-PAGE analysis of the fractions from each of these peaks suggested that both peaks contained all four Mam1 proteins. The intensity of Mam1A was the highest and of Mam1D was the lowest among the Mam1A-1D in the SDS-PAGE (Supplementary Figure 8a). As the expression of Mam1D was lower compared to the other Mam1 proteins, a construct was created such that only

Mam1D contained a Strep-tag at the C-terminus. Co-expression and co-purification studies confirmed that Mam1A-1D interacted to form a stable protein complex. With the Strep-tag on Mam1D, the purity and homogeneity of the Mam1ABCD complex improved significantly (Supplementary Figure 8c).

In a previous study, Nazarova et al.[6] showed that LucA interacts with Mam/Omam proteins using two-hybrid assays. Mam1C is one of the proteins that interact with LucA. After observing the formation of the Mam1ABCD complex, the interaction of LucA with the Mam1ABCD complex was tested as well. For that, an expression system to co-express all five proteins with His-tag coding sequences on LucA was created. IMAC purification studies showed that LucA co-elutes with other Mam1 proteins, forming a stable Mam1ABCD-LucA complex. Like the Mam1ABCD complex, the Mam1ABCD-LucA complex showed two peaks in SEC at 12.9 ml and 16.5 ml in a 24 ml Superose6 column (Supplementary Figure 8b). The expression of LucA containing His-tag produced a more intense band in SDS-PAGE compared to that of the Mam1 proteins (Supplementary Figure 8b). Therefore, a construct with all five proteins, in which only Mam1D had a Strep-tag, was created. Further Strep-tag affinity purification and analysis of the peak fractions by SDS-PAGE demonstrated the formation of the stable Mam1ABCD-LucA complex (Supplementary Figure 8d). The purity was higher and the ratio of each of the five proteins was nearly equal using this purification strategy. Mam1A ran slightly lower in the SDS-PAGE in the Mam1ABCD-LucA complex compared to Mam1ABCD (Supplementary Figure 8c and d). Next, the presence of the disulfide bridges identified in Mam1A and Mam1C during their expression and co-expression was tested in the Mam1ABCD and Mam1ABCD-LucA complexes. Both complexes were analysed by SDS-PAGE in reducing and non-reducing conditions. Boiling the complexes in an SDS sample buffer caused protein precipitation and most of the protein was retained in the stacking gel. The unboiled samples incubated in the SDS sample buffer in reducing (with BME) and non-reducing (without BME) conditions were then analysed by SDS-PAGE. The proteins still contained a disulfide bridge and formed homodimers. Both Mam1A and Mam1C were detected as higher oligomers when no reducing agent was used for the analysis of the Mam1ABCD and Mam1ABCD-LucA complexes. These experiments demonstrate that Mam1A-1D can form a stable complex with or without LucA and that LucA interacts with the Mam1ABCD complex. The disulfide bridges were observed in Mam1A and Mam1C proteins as well as in the Mam1ABCD and Mam1ABCD-LucA complexes.

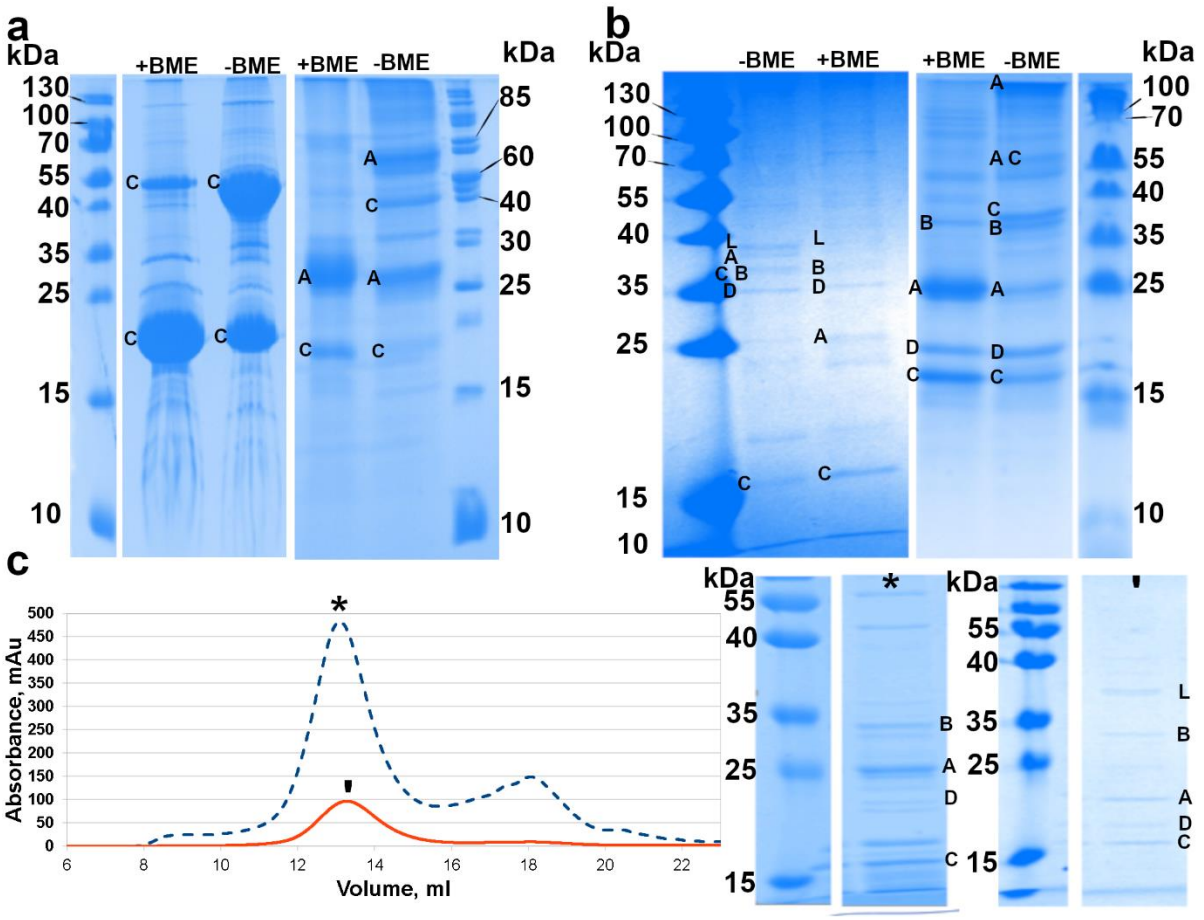

Fig 4. Complex formation of Mam1AC, Mam1ABCD and Mam1ABCD-LucA. a SDS-PAGE analysis of Mam1C (left gel) in the presence (+BME) and absence (-BME) of BME. The -BME lane has a higher amount of Mam1C at a molecular weight close to 55 kDa showing the presence of an intermolecular disulfide bridge between two Mam1C molecules. SDS-PAGE analysis of Mam1A and Mam1C co-expressed and purified showing the formation of Mam1AC complex (right gel). In the -BME, both Mam1A and Mam1C run as higher oligomers suggesting the presence of a disulfide bridge in each of them separately. **b** SDS-PAGE analysis of Mam1A-1D with (left gel) and without (right gel) LucA co-expressed and purified showing the formation of Mam1ABCD and Mam1ABCD-LucA complexes. In the -BME, both Mam1A and Mam1C run as higher oligomers suggesting the presence of a disulfide bridge. **c** SEC profile of Mam1ABCD (blue dotted line) and Mam1ABCD-LucA (red solid line) and the corresponding SDS-PAGE analysis of the indicated peak fractions. The bands in the SDS-PAGE gel corresponding to Mam1A-1D and LucA confirmed by peptide mass fingerprinting analysis are designated as ‘A’, ‘B’, ‘C’, ‘D’ and ‘L’.

Discussion

Mtb has adapted to switch from using carbohydrates to lipids as the primary energy and carbon source during the intracellular stage of infection[29]. For this, *Mtb* has a large number of lipid-processing enzymes and proteins, including four lipid-importing complexes now known as Mce complexes (Mce1-Mce4). Mce complexes are shown to import fatty acids, mycolic acids, and cholesterol from the host and its lipid-rich cell wall. The import is not only essential for the survival of *Mtb* in nutrient-depleted environments but also helps *Mtb* to remodel its cell wall in response to its surroundings, playing a critical role in survival, virulence and pathogenicity. Recent studies[6,18,20] have suggested that the Mam/Omam proteins and LucA have a crucial role in the stability and function of the Mce complexes. OmamA is necessary for the function of the Mce4 complex in cholesterol import[18], while LucA similarly stabilises the Mce1 and Mce4 complexes[6]. The precise mechanism of this stabilisation is not yet known. However, the interaction of these proteins with the Mce complexes is likely required. To understand this, we characterised the Mam proteins of the *mce1* operon at a molecular level. The sequence analysis revealed that they have conserved secondary structure features despite having low sequence identity (Supplementary Table 1). Further characterisation of Mam1AΔ106 suggested that Mam1A is a tetramer with two disulfide bridges between the pairs of Cys123 when expressed individually. Mutation of this cysteine decreases the protein stability.

Furthermore, we discovered an intermolecular disulfide bridge between Mam1C molecules formed by the sole cysteine Cys46. The disulfide bridge of Mam1C resides in the α1-helix within the TM region of the protein. Deletion of this region (Mam1CΔ78) produced a soluble variant of the protein. However, the protein was observed to be mixture of oligomeric states and aggregated during concentration, suggesting the potential importance of disulfide bridge for its stability. Mam1A and Mam1C interact with each other to form the Mam1AC complex. Furthermore, the four Mam1 proteins and LucA, when co-expressed, form stable Mam1ABCD and Mam1ABCD-LucA complexes. The disulfide bridges discovered in Mam1A (Cys123) and Mam1C (Cys46) are retained even in Mam1AC, Mam1ABCD and Mam1ABCD-LucA complexes, highlighting their importance in the overall stability of the complex. The oligomerisation state of Mam1ABCD is thus higher than heterotetrameric. Considering the presence of a disulfide bridge, the minimal unit is likely $Mam1A_2BC_2D$.

The oligomeric state obtained from SEC-MALS and the identification of the disulfide bridge played a crucial role in selecting the appropriate AlphaFold3 models to fit the SAXS and SANS data for Mam1AΔ106. As the protein was purified in the presence of a detergent, SANS was used to mask the detergent signal via contrast matching and obtain two equally well-fitting low-resolution models, which provided insights into the possible mode of oligomerization. In both models, the disulfide

bridges were formed between domain-swapped helices. However, further high-resolution studies are needed to conclude which of these two models is biologically relevant.

Previous studies have shown that LucA interacts with Mam1C[6]. It is possible that even in the Mam1ABCD-LucA complex, the interaction of LucA is through Mam1C, which further confirms that the stabilising effect of LucA to the Mce complexes comes through the interaction with Mam/Omam proteins. Based on our results, we propose that the other Mam/Omam proteins encoded from the same operon can interact to form complexes such as Mam3AB, Mam4AB, OmamAB and OmamCD. It is also possible that the Mam1ABCD complex has a critical role in the stabilisation of the Mce1 complex as they are encoded by the same operon. However, LucA, which forms a stable complex with Mam1ABCD, has also been shown to be associated with other Mam/Omam proteins, such as OmamA and Mam4B[6]. This points towards the complex interplay of interaction between different Mam/Omam proteins and their complexes with different Mce complexes. For example, OmamA has been suggested to be essential for the stability of the Mce1 and Mce4 complexes[18]. Our results demonstrate that Mam1A and Mam1C can exist individually and as a part of the Mam1ABCD and Mam1ABCD-LucA complexes. More studies are needed to show if all these forms exist or only as Mam1ABCD and Mam1ABCD-LucA complexes in the cell. The Mam/Omam proteins are structurally related to VirB8[18], which is also shown to have multiple interacting partners[30,31]. It is possible that Mam/Omam proteins also behave in a similar manner.

Despite the importance of Mam/Omam proteins and LucA for the functioning of the Mce complexes, these proteins were not present in the recently reported *M. smegmatis* Mce1 complex structure[32]. The interaction between Mam/Omam proteins and LucA with the Mce complexes may be dynamic and transient in nature. These interactions are probably necessary at different points in the assembly and functioning of the Mce complexes. Interruption of these interactions can be an effective strategy to destabilise the Mce complexes and pave the way for exploring therapeutic applications for treating latent TB infection. The demonstration and production of the Mam1ABCD and Mam1ABCD-LucA complexes are an important stepping stone for studying their structures and interactions further and provide insights into how these complexes are organised, how they interact with the Mce complexes and ultimately reveal the mechanism of their action.

Methods

Biochemicals

The genomic DNA of *Mtb* H37Rv was purchased from ATCC. Phusion DNA polymerase and the restriction enzymes used for cloning were purchased from Thermo Scientific (Massachusetts, USA) and New England Biolabs. The Ni–NTA chromatography resin for 6xHis-tag purification was obtained

from Qiagen (Hilden, Germany). Streptavidin beads for Strep-tag purification were obtained from IBA Lifesciences (Göttingen, Germany).

Sequence analysis of the genes of interest

Protein sequences were obtained from https://mycobrowser.epfl.ch/. For the sequence analysis and secondary structure predictions, TMHMM2.0 online server[24], DeepTMHMM1.0[33], and praline online service tools (Praline) were used. For tertiary structure prediction, AlphaFold2.0[25] and AlphaFold3.0[34] were used.

Cloning *mam1A-1D*, *lucA* and their variants

mam1AΔ106 was cloned using the restriction-free cloning method. The gene of interest was amplified from the *Mtb* H37Rv genome using gene-specific primers (forward primer: 5'-AATCTTTATTTTCAGGGCGCCATGGCACCCGATGCTGGGGCG-3', reverse primer: 5'-GGTGGTGGTGGTGCTCGAGTCACTTCGTCACCTGGTCGAG-3'). *mam1CΔ78* was cloned similarly using the forward primer: 5'- CTTTATTTTCAGGGCGCCATGGCGTCACCGGATCCGTTTC-3' and the reverse primer: 5'- GGTGGTGGTGGTGCTCGAGTCAGATCACCGGAAGCAG-3'. The primers from the first PCR reaction created a megaprimer containing the gene and overhangs to the pETM11 recombinant expression vector. The underlined region of the primer is the pETM11-specific sequence. *mam1AΔ106_C123S* was cloned using forward primer 5'-CAGAAGATCATCGAGTCTGGCACCGGTGATTTC-3' and a reverse primer that was complementary to the forward primer. The underlined region is the mutation site. The mutation was introduced in a single PCR reaction with m*am1AΔ106* pETM11 as the template by replacing cysteine (TGT) with serine (TCT).

For the co-expression of Mam1A-1D, codon-optimised clones were purchased from GenScript. *mam1A* and *mam1C* were cloned in pET-Duet, while *mam1B* and *mam1D* were cloned in pCOLA-Duet vectors. Altering the protein complex assembly in plasmids was done using the Golden Gate assembly, and *luca* was inserted into the vector with *mam1A* and *mam1C*. Additionally, a pCDF-Duet plasmid containing all the genes of interest, *mam1ABCD* and *luca,* was created using the Golden Gate assembly[35,36].

Expression and purification of Mam1AΔ106 and Mam1CΔ78

The recombinant protein expression was performed in the *E. coli* BL21 (DE3) strain, and the overexpression at 20 °C for 16 h was induced with 0.3 mM to 0.5 mM β-D-1-thiogalactopyranoside (IPTG), once OD_{600} of 0.6-0.9 was reached. Harvested cells were resuspended in lysis buffer A (500 mM NaCl with 50 mM Tris at pH 8.5) supplemented with 20 µg ml^{-1} of DNase and RNase with an added 0.1 mg ml^{-1} lysozyme and 5 mM $MgCl_2$ and lysed by passing the suspension through a cell disruptor at 34.8

kPSi twice. In the case of Mam1AΔ106, the lysate was incubated with C12E9 (0.1%) to solubilise the protein. Mam1CΔ78 was found to be soluble without the use of detergents. The sample was clarified by centrifugation at 32000 *g* for 45 minutes at 4 °C. The supernatant was bound to the Ni-NTA IMAC column and washed with buffer A containing 5 and 50 mM imidazole. The sample was eluted with lysis buffer A with 600 mM imidazole and directly injected into Superdex200 16/600 pg SEC column, which was pre-equilibrated with the appropriate SEC buffer A (500 mM NaCl with 50 mM Tris at pH 8.5). The fractions were analysed by SDS-PAGE for purity. The pure fractions were pooled, concentrated, and flash-frozen in liquid nitrogen for storage at -70 °C.

Expression and purification of Mam1AC complex

The codon-optimised constructs were expressed in BL21 (DE3) or BL21 (DE3) RIPL, and the overexpression of proteins was initiated by adding 0.5 mM IPTG to the media once OD_{600} of 0.6-0.9 was reached. Protein overexpression was done at 20 °C for 16 hours. The Mam1AC complex purification followed the same protocol as Mam1A purification with 5 mM FosCholin-12 (FC12) supplementation to the buffer. Mam1AC purification required detergent in the following steps of IMAC and SEC, for which the buffers were supplemented with 0.05% C12E9.

Expression and purification of Mam1ABCD and Mam1ABCD-LucA complexes

The expression of the Mam1ABCD and Mam1ABCD-LucA complexes was done in the Rosetta (DE3) pLysS strain. The expression was initiated with 1% primary culture in the terrific broth-auto induction medium (TB-AIM) and grown at 37 °C until OD_{600} of 0.25-0.3 was reached, after which the temperature of the media was brought down to 22 °C and expression was allowed to proceed for 20-22 hours. The cells were harvested by centrifugation and resuspended in lysis buffer B (500 mM NaCl and 10 % glycerol with 50 mM Tris at pH 8.0) supplemented with 20 µg ml^{-1} of DNase and RNase, 0.1 mg ml^{-1} lysozyme, 10 mM $MgSO_4$, 25 mM DDM, one tablet Protease COCKTAIL (Catalog #S8830 Sigma Aldrich), and 25 mM ATP. The His-tag purification continued by supplementing lysis buffer B with 5 mM imidazole. The cells were lysed by sonication, and cell debris was removed by centrifugation at 28800 *g* for 40 minutes at 4 °C. IMAC purification was initiated by incubating the supernatant with Ni-NTA beads equilibrated in lysis buffer B supplemented with 1 mM DDM and 5 mM imidazole for one hour. The sample was eluted by adding elution buffer (500 mM NaCl, 10% glycerol, 1 mM DDM and 500 mM imidazole with 50 mM Tris at pH 8.0) to the beads. In the case of Strep-tag purification, the cells were resuspended in lysis buffer B with the same supplementation except for the imidazole, and they were similarly lysed and clarified as in Ni-NTA purification. The complex was bound to Strep-beads and eluted in lysis buffer B supplemented with 70 mM biotin. In the case of both Ni-NTA and Strep-tag purification, the eluted flowthrough was concentrated by centrifugation to the volume of around 1 ml

using 100 kDa cutoff centricons. The concentrated sample was injected into the Superose6 10/300 GL SEC column, equilibrated in SEC buffer B (500 mM NaCl, 5% glycerol and 1 mM DDM with 50 mM Tris at pH 8.0). The peak fractions were analysed by SDS-PAGE, and the identity of the bands was confirmed by peptide mass fingerprinting analysis using MALDI-TOF MS.

CD spectrum analysis of Mam1AΔ106

For secondary structure analysis, the protein was diluted to 0.1 mg ml^{-1} in 10 mM potassium phosphate buffer at pH 7.6 and measured between 180 and 260 nm. Deconvolution into secondary structure information was done using the DNSS program with smoothed data of three measurements. CD measurements to calculate T_m were performed with a temperature ramp of 1 °C per minute from 22 to 94 °C at a wavelength range of 190 to 250 nm. All measurements were done using 1 mm quartz cuvettes from Hellman Analytics.

Unfolding of Mam1AΔ106 by NanoDSF

The instance of aggregation as well as T_m was calculated at a concentration of 0.1 mg ml^{-1}, 0.3 mg ml^{-1}, and 0.6 mg ml^{-1} from 20 °C to 90 °C with 1 °C per minute temperature increase, using standard NanoTemper Prometheus capillaries (PR-C002)**.** NanoDSF data were analysed using the MoltenProt online service program[37,38].

Polydispersity analysis of the purified Mam1AΔ106 by DLS

The polydispersity of the sample at concentrations 0.2 mg ml^{-1}, 0.4 mg ml^{-1} and 0.6 mg ml^{-1} in triplicates were measured by DynaPro Platereader-II and analysed using Dynamics version 10.1.0.

Mam1AΔ106 SEC-MALS analysis

50 µl of purified protein at 5 mg ml^{-1} was injected into a Superdex 200 10/300 GL column, connected to a Shimadzu purification system, a Shodex refractive index detector, and a Wyatt mini-DAWN Treos MALS detector. The results were analysed by Astra 7.0 software. UV and RI measurements were used to calculate the molecular weight of the protein complex.

Mam1AΔ106 SEC-SAXS sample preparation, data collection and processing

SAXS measurements were carried out at the Diamond Light Source. 50 to 100 µl of the sample at concentrations ranging from 1 to 8 mg ml^{-1} were injected into a UHPLC coupled system (SEC-SAXS). Inline SEC-SAXS sample was injected into Superdex200 3.2/300 GL column pre-equilibrated in SEC buffer A. Measurement of the sample was done with PILATUS 2M two-dimensional with a sample to detector distance of 3732.9 m at a wavelength of 0.9464 Å at the beamline B21 in Diamond Light Source UK.

SAXS data analysis and buffer substraction were done using the Scåtter III and IV programs[39,40]. With the Scåtter R_g, D_{max} and forward scattering $I(0)$ were calculated. The produced output file was used for online ATSAS services to model the DAMMIN dummy atom model[27,41]. The predicted AlphaFold2.0 and AlphaFold3.0 models[25,34] were then fitted against SAXS data using the FoXS online server[42].

Preparation of deuterated C-12E9 (D-C12E9)

Tetraethylene glycol (25 g) was dried under vacuum at 60° C for 24 hours. *t*-BuOK (1.11 g, 10% excess) was then added, and the mixture was stirred under vacuum at 60°C for 2 hours. Subsequently, 1-bromododecane (3 g) was introduced, and the reaction mixture was heated to 110° C under vacuum for 2–3 hours. After completion, a few drops of HCl were added to neutralize the reaction, followed by extraction with ether. The crude product was concentrated via rotary evaporation, yielding 4.94 g before purification by flash column chromatography.

The chain-deuterated species were synthesized using the same procedure as the protonated counterpart, replacing 1-bromododecane with 1-bromododecane-d_{25}. Deuteration was achieved from dodecanoic acid, where 25 g of protonated dodecanoic acid was mixed with 10% Pt/C (10 wt%) in 300 mL of D_2O and subjected to a Parr reactor at 180° C for three days. After two H/D exchange cycles, the deuteration level was confirmed by nuclear magnetic resonance (NMR) (Supplementary Figure 9). The resulting deuterated dodecanoic acid was then reduced to the corresponding alcohol using $LiAlD_4$. Bromination was performed by reacting the deuterated alcohol with 48% HBr in the presence of TBAB as a phase transfer reagent. The product was extracted with diethyl ether, concentrated under vacuum, and purified via column chromatography.

Mam1AΔ106 SANS sample preparation, data collection and analysis

200 µl of protein sample at the concentration of 10 mg ml^{-1} were mixed with 0.2 % deuterated C12E9 (D-C12E9) suspended in 50 mM Tris with 500 mM NaCl at pH 8.5 in a 1:1 ratio. D-C12E9-protein mixture was kept on ice for an hour before injecting the sample into the Superdex 200 10/300 GL column. The column was pre-equilibrated in SEC buffer with twice the critical micelle concentration (CMC) of C12E9 (CMC = 0.003%). For the experiment, it was assumed that the CMC of D-C12E9 was the same as that for H-C12E9.

SANS Data were collected on the Zoom time-of-flight SANS instrument (ISIS Neutron and Muon Source, Harwell, UK) using wavelength range 1.75-16.5Å. The source to sample and sample to detector distance was 4 m and the beam size was 12 mm. From the highest point of the peak, 3 fractions were selected. Each of these fractions was loaded to Starna Scientific quartz round cuvette (match code 6, path length 1, type 32/Q/1) and SANS data were collected from each fraction with an exposure time of 1 h in 5 cycles (5 h total exposure time for each fraction) at 6 °C in a temperature controlled sample rack. The detector images were reduced to 1-D using Mantid software, including normalisation, detector correction, absolute scaling and buffer subtraction. SANS data were processed using the same method outlined above for the SAXS data. The predicted Alphafold3 structures were fitted against the SANS data using the CRYSON from online ATSAS[43].

Data availability

The relevant data associated with the published study are present in the paper or attached as Supplementary data.

Author contributions

R.V. with M.H. planned the study. M.H. executed the cloning, purification, and characterization regarding the Mam1A, Mam1C, Mam1AC and their variants. P.P. and N.T.H. created the Mam1ABCD and Mam1ABCD-LucA expression constructs. P.P. and M.H. executed the purification and identification of Mam1ABCD and Mam1ABCD-LucA-related complex profiling. K.M. prepared the C12E9 and D-C12E9 for the SANS experiment. J.D. assessed the SANS data and modelled the respective dummy atom model from the dataset. R.V., M.H. and N.T.H. wrote the manuscript with contributions from other authors. R.V. acquired the funding for the work.

Competing interest

The authors declare no competing interests.

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