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The assembly of β-barrel membrane proteins by BAM and SAM

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Summary

Gram-negative bacteria, mitochondria, and chloroplasts all possess an outer membrane populated with a host of β-barrel outer-membrane proteins (βOMPs). These βOMPs play crucial roles in maintaining viability of their hosts and therefore, it is essential to understand the biogenesis of this class of membrane proteins. In recent years, significant structural and functional advancements have been made toward elucidating this process, which is mediated by the β-barrel assembly machinery (BAM) in Gram-negative bacteria, and by the sorting and assembly machinery (SAM) in mitochondria. Structures of both BAM and SAM have now been reported, allowing a comparison and dissection of the two machineries, with other studies reporting on functional aspects of each. Together, these new insights provide compelling support for the proposed budding mechanism, where each nascent βOMP forms a hybrid-barrel intermediate with BAM/SAM in route to its biogenesis into the membrane. Here, we will review these recent studies and highlight their contributions towards understanding βOMP biogenesis in Gram-negative bacteria and in mitochondria. We will also weigh the evidence supporting each of the two leading mechanistic models for how BAM/SAM function, and offer an outlook on future studies within the field.

Keywords

Gram-negative bacteria; outer membrane; β-barrel; outer membrane protein; protein folding; envelope biogenesis; lateral gate

β-barrel outer membrane proteins

One defining feature of Gram-negative bacteria, mitochondria, and chloroplasts is that they are all enveloped by a double membrane consisting of an inner membrane (IM) and outer membrane (OM). These two membranes are home to a plethora of vitally important integral membrane proteins which play roles as gatekeepers for the cell, regulating nutrient import,

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Competing interests

Authors declare no competing interests.

virulence factor export, and maintaining the protective coat of the cell, cargo transport, membrane biogenesis and signaling transporters, enzymes, and receptors (Wimley, 2003, Koebnik *et al.*, 2000, Fairman *et al.*, 2011). More commonly, integral membrane proteins are observed with α-helical domains anchoring them within the membrane. In bacteria such as *E. coli*, however, the OM has an asymmetric composition with lipopolysaccharides in the outer leaflet and phospholipids in the inner leaflet. Here, the OM is further distinguished by the fact that it is populated by, almost exclusively, a unique class of membrane proteins called β-barrel outer membrane proteins (βOMPs) (Tamm *et al.*, 2004, Voulhoux & Tommassen, 2004, Gentle *et al.*, 2004, Schleiff & Soll, 2005, Gentle *et al.*, 2005, Walther *et al.*, 2009b). βOMPs are characterized by a transmembrane domain composed of a β-barrel fold consisting of 8 or more β-strands arranged in an anti-parallel fashion with the first and last strand coming together via a hydrogen bonding network to close the barrel (Fairman *et al.*, 2011, Wimley, 2003). Given their surface accessibility in Gram-negative bacteria, βOMPs have gained renewed interest of late not only for vaccine development, but also as promising antibiotic targets to combat multi-drug resistance (Urfer *et al.*, 2016, Choi & Lee, 2019, Imai *et al.*, 2019, Luther *et al.*, 2019, Hart *et al.*, 2019).

The biogenesis of βOMPs into the OM is mediated by the β-barrel assembly machinery (BAM) in Gram-negative bacteria, the sorting and assembly machinery (SAM) in mitochondria, and outer envelope protein 80 (OEP80) in chloroplasts (Paschen *et al.*, 2003, Gentle *et al.*, 2004, Walther *et al.*, 2009b, Webb *et al.*, 2012a) (Figure 1). These machineries are conserved from Gram-negative bacterial ancestors to the eukaryotic organelles of endosymbiotic origin (mitochondria and chloroplasts) and have retained the core component of the folding machinery. In chloroplasts, much less is known about βOMP biogenesis and therefore, will not be discussed in detail here. We do know, however, that nascent βOMPs are imported across the OM into the intermembrane space (IMS) by the translocon of the outer membrane of chloroplasts (TOC) complex (Misra, 2012, Walther *et al.*, 2009b, Hinnah *et al.*, 1997, Hinnah *et al.*, 2002, Kessler & Schnell, 2004). Then, IMS chaperones further shuttle the nascent βOMPs to OEP80 for folding/insertion into the OM. No structure of OEP80 has yet been reported, however, the structure of the POTRA domains have been reported for Toc75, the central component of the TOC complex (O'Neil *et al.*, 2017). Toc75 is in the same family, and expected to have a similar fold, as OEP80 and the core components of BAM and SAM.

βOMP biogenesis in Gram-negative bacteria

In Gram-negative bacteria, the OM protects against harsh environments and is embedded with distinct βOMPs that fulfill many essential roles in the cell (Beveridge, 1999, Rollauer *et al.*, 2015). The biogenesis of these βOMPs begins in the cytoplasm, where ribosomes synthesize polypeptide βOMP precursors (Tsirigotaki *et al.*, 2017) (Figure 2). These nascent precursor βOMPs each contain an N-terminal signal peptide that routes them to the SecYEG translocon, where the signal peptide is proteolytically removed following translocation across the IM (Crane & Randall, 2017). Once in the periplasm, the nascent βOMPs interact with several chaperones including survival protein A (SurA), seventeen kilodalton protein (Skp), and FkpA to further escort the nascent βOMPs to the OM where the β-barrel assembly machinery (BAM) then mediates folding and insertion. BAM substrates can

be quite diverse in their size and properties, having barrel domains ranging from 8–36 β-strands for a single monomeric βOMP, and includes the more complex multimeric βOMPs (Muhlenkamp *et al.*, 2015, Sklar *et al.*, 2007b, Ruiz-Perez *et al.*, 2009).

In *E. coli*, BAM has been extensively characterized to help understand its essential role in βOMP biogenesis and consists of five components, BamA (a βOMP) and four lipoproteins termed BamB, BamC, BamD, and BamE (Kim *et al.*, 2012, Noinaj *et al.*, 2017, Konovalova *et al.*, 2017) (Figure 2). However, the exact composition of BAM can vary across other bacteria (Webb *et al.*, 2012a). BamA and BamD are essential for viability, whereas BamB, BamC, and BamE are required for efficient function of BAM, with concurrent deletion of more than one of these components also rendering cells nonviable (Wu *et al.*, 2005, Ruiz *et al.*, 2005, Vuong *et al.*, 2008, Leonard-Rivera & Misra, 2012). BAM has been proposed to recognize nascent βOMPs via their terminal strand, often referred to as the β-signal, which has been shown to be species specific and trigger conformation changes in BamA (Robert *et al.*, 2006). This β-signal contains a highly conserved phenylalanine at the C-terminus and has a consensus sequence that generally resembles HyGHyPoHyPoF (Paramasivam *et al.*, 2012). A second targeting sequence has also been proposed in β14 that binds to BamD (Hagan *et al.*, 2015). However, more work is needed to confirm this idea and to clarify if there may be other determinants contributing to recognition by BAM.

Structures of all the individual components of the BAM complex have now been reported for *E. coli* (Wu *et al.*, 2020, Kim *et al.*, 2012). BamA is a βOMP itself with an N-terminal periplasmic domain consisting of five polypeptide transport-associated (POTRA) domains and a 16-stranded C-terminal β-barrel domain (Noinaj *et al.*, 2013, Albrecht *et al.*, 2014, Ni *et al.*, 2014). Providing the first evidence in deciphering the mechanism mediating βOMP biogenesis, the structure of BamA demonstrated that lateral gating along the barrel seam was essential for BAM function (Noinaj *et al.*, 2014). Molecular dynamics simulations revealed that lateral gating occurred spontaneously, opening a path for nascent βOMPs to be routed from the periplasm directly into the OM. In addition, these studies highlighted that BamA is able to destabilize and thin the local membrane in proximity of the lateral seam, thereby acting as a folding catalyst to make βOMP biogenesis more energetically favorable (Moon *et al.*, 2013, Gessmann *et al.*, 2014, Costello *et al.*, 2016). BamB has an eight-bladed *β*-propeller fold and has recently been proposed to mediate the formation of BAM clusters/precincts (Gunasinghe *et al.*, 2018, Rassam *et al.*, 2015). These BAM clusters have been proposed to drive OMP assembly, which is active at the cell midbody, yet becomes inactive as the precincts are pushed towards the poles during division. BamC consists of a flexible N-terminal region followed by two helix-grip domains and has been shown to be partially surface-exposed in *E. coli*, although the role here is not known (Webb *et al.*, 2012b). While still a controversial notion, more recent studies have shown that in *N. gonorrhoeae*, BamE and even BamD (in the absence of BamE) are both also surface exposed, indicating broader studies are needed here in order to determine if this contributes to the function of BAM in βOMP biogenesis (Sikora *et al.*, 2018). BamD contains five tetratricopeptide repeat (TPR) domains and is an essential component for viability in many bacteria (Malinverni *et al.*, 2006, Wu *et al.*, 2005). It interacts directly with BamA and BamC and is thought to also contribute to substrate recognition (Hagan *et al.*, 2015, Lee *et al.*,

2018). BamE, the smallest component, contains an α/β globular fold and has been shown to stabilize the overall complex and increase efficiency (Sklar *et al.*, 2007a, Ryan *et al.*, 2010).

βOMP biogenesis in mitochondria

In Gram-negative bacteria, βOMPs are inserted into the membrane by a unidirectional pathway from the cytoplasm to the periplasm to the OM. In contrast, however, given the endosymbiotic nature of mitochondria and their location inside the cell, mitochondrial βOMPs are synthesized in the cytoplasm, and must first be imported across the outer mitochondrial membrane (OMM) into the IMS (Pfanner *et al.*, 2019, Paschen *et al.*, 2003, Hohr *et al.*, 2015) (Figures 1 and 3). A distinction between Gram-negative bacteria and mitochondria is the number and types of βOMPs present in the OM, with mitochondria containing only a handful of βOMPs including Por1, Sam50, VDAC (1, 2, and 3), Tom40, and Mdm10 (Hohr *et al.*, 2015). This is in contrast to Gram-negative bacteria which contain a plethora of βOMPs having a wide range of strand numbers (all even) with some containing periplasmic and/or luminal domains (Fairman *et al.*, 2011, Koebnik *et al.*, 2000, Schulz, 2000, Wimley, 2003). Additionally, the OMM contains the mitochondrial import machinery (MIM) complex that facilitates the insertion of α-helical membrane proteins, allowing for a diverse composition of both α-helical and β-barrel membrane proteins (Dimmer *et al.*, 2012, Krüger *et al.*, 2017).

Previous studies have sought to discover the distinguishing features that determine whether certain proteins over others are trafficked to the mitochondria. Mitochondrial matrix proteins all contain a positively charged presequence that facilitates their interaction with the translocase of the outer membrane (TOM) complex (Vögtle *et al.*, 2009) (Figure 3). Recent cryo-EM structures of the TOM complex have revealed that the barrel lumen of Tom40 is largely negatively charged (Araiso *et al.*, 2019, Tucker & Park, 2019). This has led to models where the import is facilitated by electrostatic interactions between the positively changed presequence and the negatively charged barrel lumen.

Nascent mitochondrial βOMPs contain two signals that mediate their import into the mitochondria (β-hairpin) and insertion into the OMM (β-signal) (Kutik *et al.*, 2008, Jores *et al.*, 2016, Hohr *et al.*, 2018, Pfanner *et al.*, 2019, Walther *et al.*, 2009a). Studies have shown that these nascent βOMPs utilize the terminal β-hairpin for trafficking to the mitochondria and across the OMM (Jores *et al.*, 2016). Studies in yeast have further demonstrated that both bacterial and chloroplast βOMPs can be targeted to the mitochondria when expressed with the C-terminal β-hairpin of mitochondrial βOMPs (Walther *et al.*, 2009a, Kozjak-Pavlovic *et al.*, 2011). The mode of recognition, however, that occurs between this β-hairpin and the TOM complex has not been well characterized.

After entering the IMS, nascent βOMPs interact with the Tim9/Tim10 chaperone complex, shuttling them to SAM, which mediates their biogenesis into the OMM (Weinhäupl *et al.*, 2018) (Figure 3). All mitochondrial βOMPs share a conserved β-signal found within the terminal strand, which contains a consensus "**Po**XGXX**Hy**X**Hy**" motif, where **Po** is any polar residue, X is any residue, and **Hy** is any hydrophobic residue (Kutik *et al.*, 2008). Previous studies have found that this β-signal alone is sufficient for interaction with SAM,

interacting directly with the lateral seam of Sam50 (Kutik *et al.*, 2008, Hohr *et al.*, 2015, Hohr *et al.*, 2018).

SAM is comprised of three components termed Sam50, Sam35, and Sam37 (1:1:1 ratio), with Sam50 and Sam37 being essential for βOMP biogenesis (Waizenegger *et al.*, 2004, Milenkovic *et al.*, 2004, Kutik *et al.*, 2008, Wiedemann *et al.*, 2003, Paschen *et al.*, 2005, Stroud *et al.*, 2011) (Figure 3). Sam50 is the mitochondrial ortholog of BamA (consisting of a 16-stranded C-terminal β-barrel domain and a single POTRA domain) and is directly responsible for the insertion of mitochondrial βOMPs into the OMM (Kutik *et al.*, 2008, Wiedemann *et al.*, 2003). However, in contrast to the lipoproteins in BAM, Sam35 and Sam37 completely reside on the cytosolic side of the OMM and are not homologous to any of the BAM accessory proteins. Instead they have a GST-like folds with Sam37 containing an N-terminal α-helical domain that anchors it to the OMM, opposite to the side of substrate recognition and insertion (Waizenegger *et al.*, 2004, Milenkovic *et al.*, 2004). Given that SAM receives nascent mitochondrial βOMPs from the IMS, it is unlikely that Sam35 and Sam37 play a direct role in substrate recognition, but certainly do contribute to other aspects of βOMP biogenesis by SAM including substrate release (Chan & Lithgow, 2008, Kutik *et al.*, 2008).

Structures of BAM

The first structures of BAM from *E. coli* were reported in 2016 (Gu *et al.*, 2016, Han *et al.*, 2016, Bakelar *et al.*, 2016). These structures revealed two main differences based primarily on the conformational state of the barrel domain of BamA: outward-open (PDB IDs 5D0Q and 5EKQ) or inward-open (PDB IDs 5D0O and 5AYW) (Wu *et al.*, 2020) (Figure 4A, B, and C). The inward-open state is characterized by canonical pairing of the β-strands at the BamA barrel seam, with POTRA5 positioned away from the center of the barrel. The outward-open state is characterized by a BamA barrel seam that is open toward the extracellular side, with positioning of POTRA5 directly beneath the BamA barrel, and accompanied by a 45° counterclockwise rotation of the periplasmic ring relative to that in the inward-open state. These inward-open structures were solved with BamB bound, while the outward-open structures were solved in the absence of BamB, suggesting that conformational switching from the outward-open to the inward-open state may be mediated by BamB association. However, the cryo-EM structure of BAM (with BamB) was reported soon after in the outward-open conformation, debunking the notion that BamB may be regulating conformational states of BAM, and leaving the field with a set of conformational states with unknown function (Iadanza *et al.*, 2016). While all structures to date of BAM have been monomeric, studies have indicated that within the cell, BAM may instead function as localized precincts, which might be formed through BamB, which contains WD40-like motifs commonly associated with scaffolding proteins (Noinaj *et al.*, 2011, Gunasinghe *et al.*, 2018, Jansen *et al.*, 2012, Rassam *et al.*, 2015).

Recent cryo-EM studies of *E. coli* BAM in complex with a substrate BamA molecule (PDB ID 6V05) have revealed the first structural evidence supporting a direct interaction between the barrel domain of BamA with substrate βOMPs during biogenesis (Tomasek *et al.*, 2020) (Figure 4D and E). Here, the C-terminal strand (β16) of the substrate pairs with the first

strand (β1) of BamA of BAM, forming a continuous β-sheet consisting of an elongated asymmetric hybrid-barrel. Interestingly, the registry observed here is shifted by four residues compared to that observed for the fully closed form of a single BamA, indicating that BamA can somehow decipher substrate from self. These studies also postulate that features found in both the initial strands and last strands of substrates help stabilize the nascent βOMPs during biogenesis. Additionally, an observed overhang of the terminal strand of the substrate may trigger strand-exchange to pair with its first strand, finalizing the folding process and promoting release from BAM.

Structures of SAM

Another exciting report from 2020 was the elucidation of the first structures of SAM from *Myceliophthora thermophila* (Diederichs *et al.*, 2020). Here, Sam35 mediates the primary interaction with Sam50 (buried surface area, 1944 $Å^2$) and also forms an extensive interaction with Sam37 (buried surface area, 1892 $Å^2$). Sam37, however, has a non-essential minimal interaction with Sam50 (buried surface area, 927 $Å^2$) (Figure 5A). These structures were determined both in nanodiscs and in detergent, with the structure in nanodiscs having a monomeric form, and the detergent structures having a mix of monomers and inverted dimers (Figure 5A, B, and C). Monomeric forms were observed with the barrel in a mostly closed form with few interactions along the first and last stands. The dimers form an extended β-sheet along the barrel domain interface that extended between the dimer subunits and exhibit quasi-two-fold symmetry, with the barrel domain of Sam50 in a laterally open state. This is mostly attributed to conformational shifts along strands β1-β4, with the remainder of the barrel domain remaining relatively static. Whether or not these conformations observed in the dimeric SAM structures truly represent physiological states remains to be determined.

Structural comparison of BAM and SAM

The structures of BAM and SAM share little outside of the conserved core components BamA and Sam50 (Paschen *et al.*, 2003, Jiang *et al.*, 2012, Ulrich & Rapaport, 2015, Gentle *et al.*, 2005). Yet, even with these differences, a commonality in BAM and SAM is observed, as some bacterial OMPs are able to utilize the mitochondrial SAM/TOM architecture for insertion into the OM and vice versa (Jiang *et al.*, 2012). A structural alignment of monomeric Sam50 with BamA reveals a mostly closed state for the barrel domain which best compares to the inward-open state of BamA found within the BAM structures (RMSD of ~2.2 Å), however, with fewer hydrogen bonds, if any, between the first and last strands of the Sam50 barrel (Figure 5D). It may be that without the presence of IMS accessory proteins, that Sam50 has evolved an intermediate state where the POTRA domain does not need to move at all and bypasses the requirement of a significantly sheared β-barrel domain observed in the BamA outward-open state. This may be possible, in part, because the catalog of substrates that SAM needs to assemble is much less than that of BAM. To date, all substrates of SAM, except for Sam50 itself, have only 19 β-strands (Tom40, VDAC (1, 2, and 3), and Mdm10) (Araiso *et al.*, 2019, Tucker & Park, 2019, Ujwal *et al.*, 2008, Imai *et al.*, 2011), whereas BAM needs to insert a larger pool of higher diversity substrates with monomeric βOMPs ranging from 8 to 36 strands. It is worth also noting that without

the nineteenth strand, the SAM substrates could possibly be inserted inverted, somewhat mimicking what was observed in the dimer structures (Figure 5C and F). Further, despite the interface between the dimers being inverted, they still closely resemble the interface observed in the recent BAM/BamA cryo-EM structure, possibly indirectly demonstrating a shared mechanism for βOMP biogenesis with BAM, as is expected. Additionally, the open state of Sam50 may represent a state analogous to the BamA outward-open state, but with some notable differences. For example, the closed state of Sam50 is nearly identical to its open state, except for the first four strands of the β-barrel, which are curled out ~35–40° to open the Sam50 barrel creating an ~20 Å opening (Figure 5E). Additionally, there is only ~3–4 Å shift in the position of the POTRA domain between the two states. Despite the observed differences discussed here, BAM and SAM, as well as OEP80, are all thought to share a common conserved mechanism for the biogenesis of βOMPs into the membrane.

Models for βOMP biogenesis by BAM and SAM

Despite the wealth of structural information for BAM reported in 2016, and more recently for SAM in 2020, direct structural evidence reporting the molecular details of βOMP assembly has remained elusive for the most part. Reported biochemical and structural data narrowed the field down to two overarching models describing how βOMPs may be assembled into the OM (Noinaj *et al.*, 2017, Wu *et al.*, 2020, Konovalova *et al.*, 2017). Variations have been proposed, but the most distinguishing feature among models is whether or not β-strand templating at the BamA/Sam50 β-barrel seam plays a significant role in the assembly of βOMP substrates. In the model sometimes referred to as the budding model, the βOMP substrate utilizes the exposed β-strand backbone at the barrel seam of BamA/Sam50 as a template onto which it attaches its own β-strands, beginning with its C-terminal strand (β-signal) and systematically adding β-strands until the entire protein is assembled and buds away (Kim *et al.*, 2012, Gruss *et al.*, 2013). In the non-templating model, also referred to as the assisted model, the nascent βOMPs utilize BAM/SAM for localization and possibly stabilization to the membrane, but then spontaneously inserts into a primed portion of the membrane produced by a thinned hydrophobic region flanking the BamA/Sam50 lateral gate. While evidence exists in support of both models, recent structural and functional reports have provided new and exciting data directly supporting the budding model, tipping the scales in favor of this previously controversial hypothesis.

Critics of the budding model point out that just because disulfide crosslinking at the BamA seam is lethal is not conclusive evidence that lateral gating exists or that templating must occur. Rather, alternative explanations are that it slows down the folding of βOMPs and/or causes assembly defects to a sufficient degree to result in an overaccumulation of unfolded βOMPs in the periplasm. Another explanation is that crosslinking BamA prevents conformational changes in BAM which are essential for its role as a catalyst on the membrane. Evidence supporting the assisted model includes the fact that some βOMPs readily assemble into liposomes spontaneously, and that locking the lateral gate of BamA closed does not prevent its ability to accelerate the folding of small OMPs (Burgess *et al.*, 2008, Iadanza *et al.*, 2016, Kleinschmidt, 2015, Doerner & Sousa, 2017). However, several of these studies suffer from the challenging task of finding proper experimental conditions such as: (*i*) having suitable controls to account for the presence of a membrane

destabilization factor itself, which is another separate role of the barrel domain of BamA, and (*ii*) reporting the percentage of crosslinking efficiency in BamA mutants. Other *in vitro* studies have shown that OmpA can fold spontaneously into diverse membrane environments and that the thickness of the membrane can have a significant effect on the rate of folding (Burgess *et al.*, 2008). *In vivo* crosslinking studies with LptD and EspP have also been presented as supporting evidence of the assisted model, where folding of both were proposed to have been mostly folded within the periplasm in route to BAM, rather than directly by BAM itself (Lee *et al.*, 2016, Pavlova *et al.*, 2013, Ieva *et al.*, 2011). These observations together seemingly support the notion that BAM was simply needed as a membrane disruptase rather than serving as a conductor orchestrating the entire biogenesis process.

In order for BamA/Sam50-mediated β-strand templating to occur, as has been proposed in the budding model, the interaction between the β1 and β16 strands of the barrel domain would need to first be disrupted, thus creating a separation generically referred to as the lateral gate. While normally this would be energetically unfavorable, the structures of BamA/Sam50 have demonstrated that each is tailored to be stable despite few to no hydrogen bonds at the barrel seam. Further, crosslinking studies initially performed on SAM and more recently for BAM, have demonstrated direct crosslinking of the barrel domains of Sam50 and BamA and their respective substrates, VDAC1 and EspP (Ieva *et al.*, 2011, Pavlova *et al.*, 2013, Doyle & Bernstein, 2019, Hohr *et al.*, 2018). In both cases, the crosslinks were consistent with trapping the substrates in intermediate states which supported a hybrid-barrel intermediate. Further, dimers of Sam50 directly demonstrated β-templating between subunits, albeit unconventionally given the inverted topologies (Diederichs *et al.*, 2020). The most striking evidence published to date in favor of the budding model, however, is the recent structure of a late-stage assembly intermediate of BAM in complex with a substrate BamA molecule consisting of 14 strands bound at the lateral gate; the first direct structural evidence of a hybrid-barrel being formed during βOMP biogenesis (Tomasek *et al.*, 2020). This structure alone, though, does not allow differentiation between whether the substrate is inserted as a partial or full barrel, or if the substrate is inserted systematically strand by strand; more studies are needed here to make this distinction.

Summary and future outlook

βOMPs serve essential roles in Gram-negative bacteria, mitochondria, and chloroplasts, however, the process for the biogenesis of these βOMPs has remained elusive. Now, more than two decades since the first component of BAM was initially discovered, we are finally at the cusp of being able to describe the molecular mechanism for how these folding machineries function. Recent *in vivo* and *in vitro* reports have provided a wealth of evidence demonstrating a hybrid-barrel intermediate, directly supporting the budding model. But due to accumulating evidence for both the budding and the assisted models, the notion that perhaps these folding machineries are able to apply each mechanism in a βOMP-dependent manner cannot be disregarded. Other questions about the mechanism that remain unanswered include: how are substrates initially recognized, are substrates inserted partially folded or strand by strand, and what is the role of the accessory proteins? These questions will likely be answered through the elucidation of a larger number of insertion

intermediates, as well as, new biochemical studies that pinpoint the molecular features of BAM that are essential for folding βOMP substrates. While certainly an exciting time for the field, more studies are needed to solidify exactly how BAM and SAM function and to address the lingering questions outlined above. With the innovations in experimental design within these systems and the advancements in structural biology methodologies, there is reason to be optimistic that we may soon be able to describe step-by-step exactly how these fascinating machineries operate.

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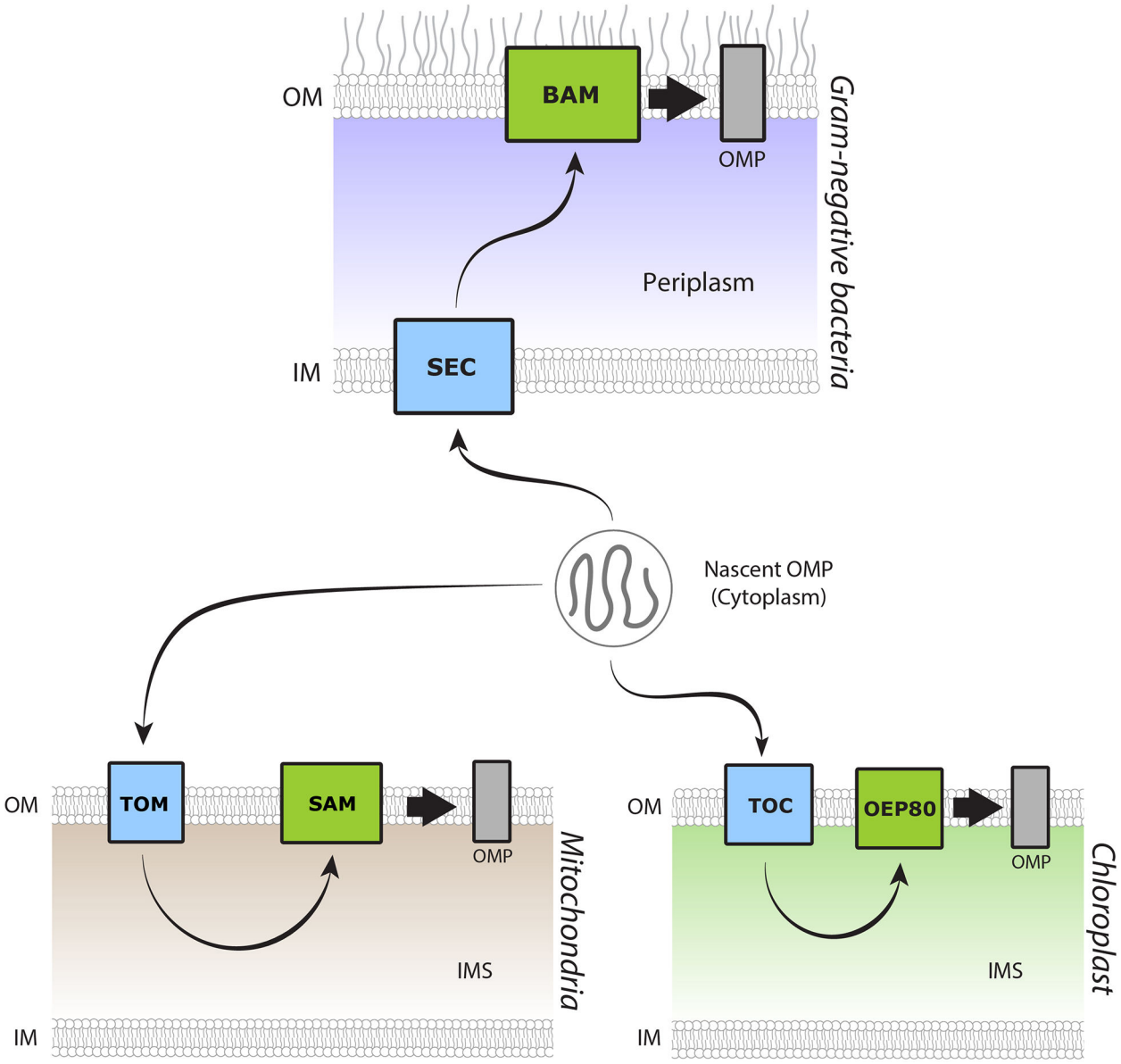

Figure 1. Paths of βOMP insertion into membranes.

In Gram-negative bacteria, mitochondria, and chloroplasts, βOMP follow a similar general path from nascent polypeptide to insertion into the OM. βOMP are translated in the cytoplasm. They are then translocated across the IM in Gram-negative bacteria or the OM in mitochondria and chloroplasts, mediated by the SEC, TOM, and TOC complexes, respectively. After crossing the membrane, βOMPs are directed to the OM for folding and insertion, mediated by BAM, SAM, and OEP80, respectively.

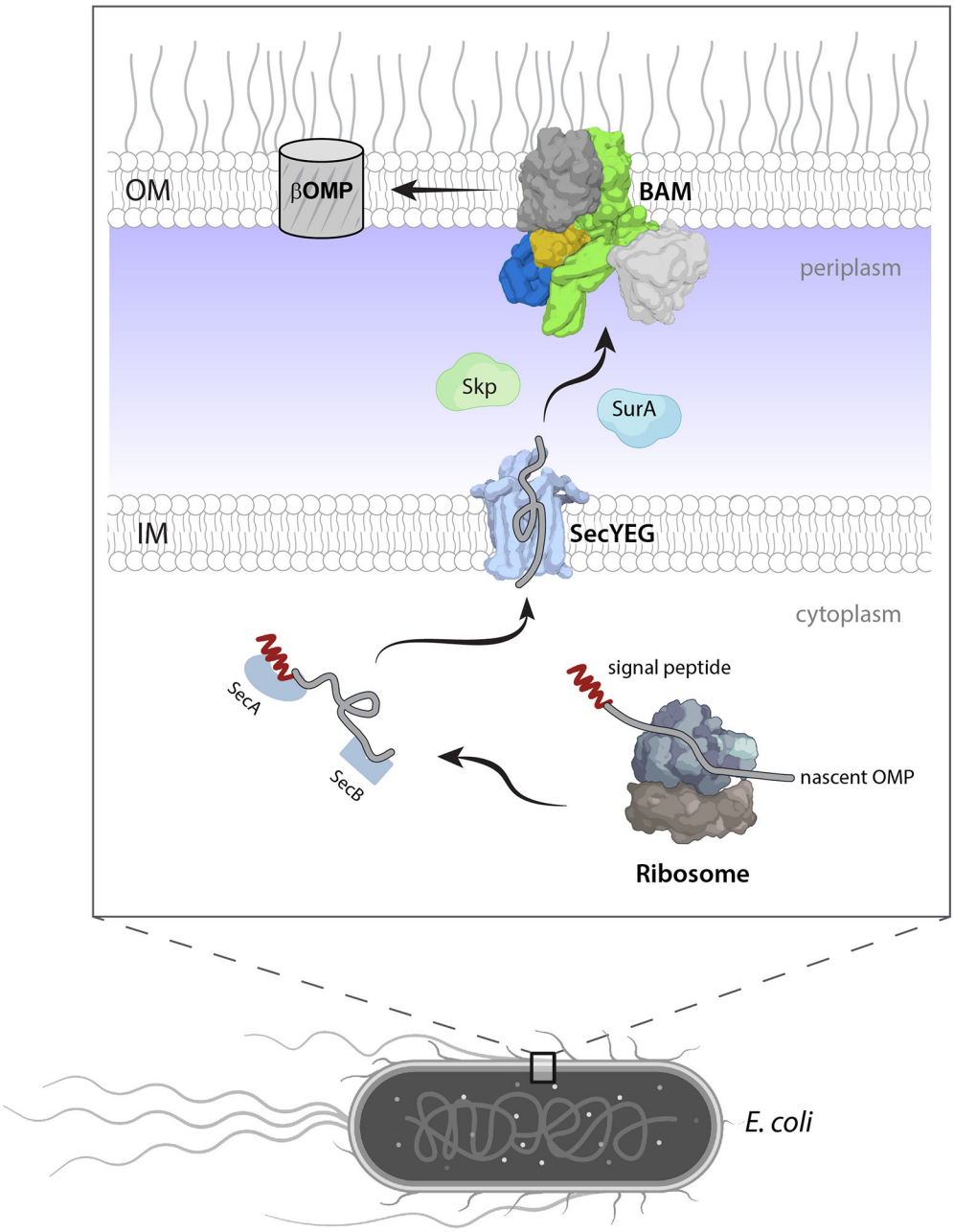

Figure 2. Biogenesis of βOMPs in Gram-negative bacteria.

Nascent βOMPs (gray) with N-terminal signal peptide (red) are synthesized in the cytoplasm by the ribosome (gray/slate) and then routed by SecA/SecB chaperones (slate) to the SecYEG translocon (light blue) for transit into the periplasm. The periplasmic chaperones SurA (light blue) and Skp (light green) further usher nascent βOMPs to BAM, where they are folded and inserted into the OM.

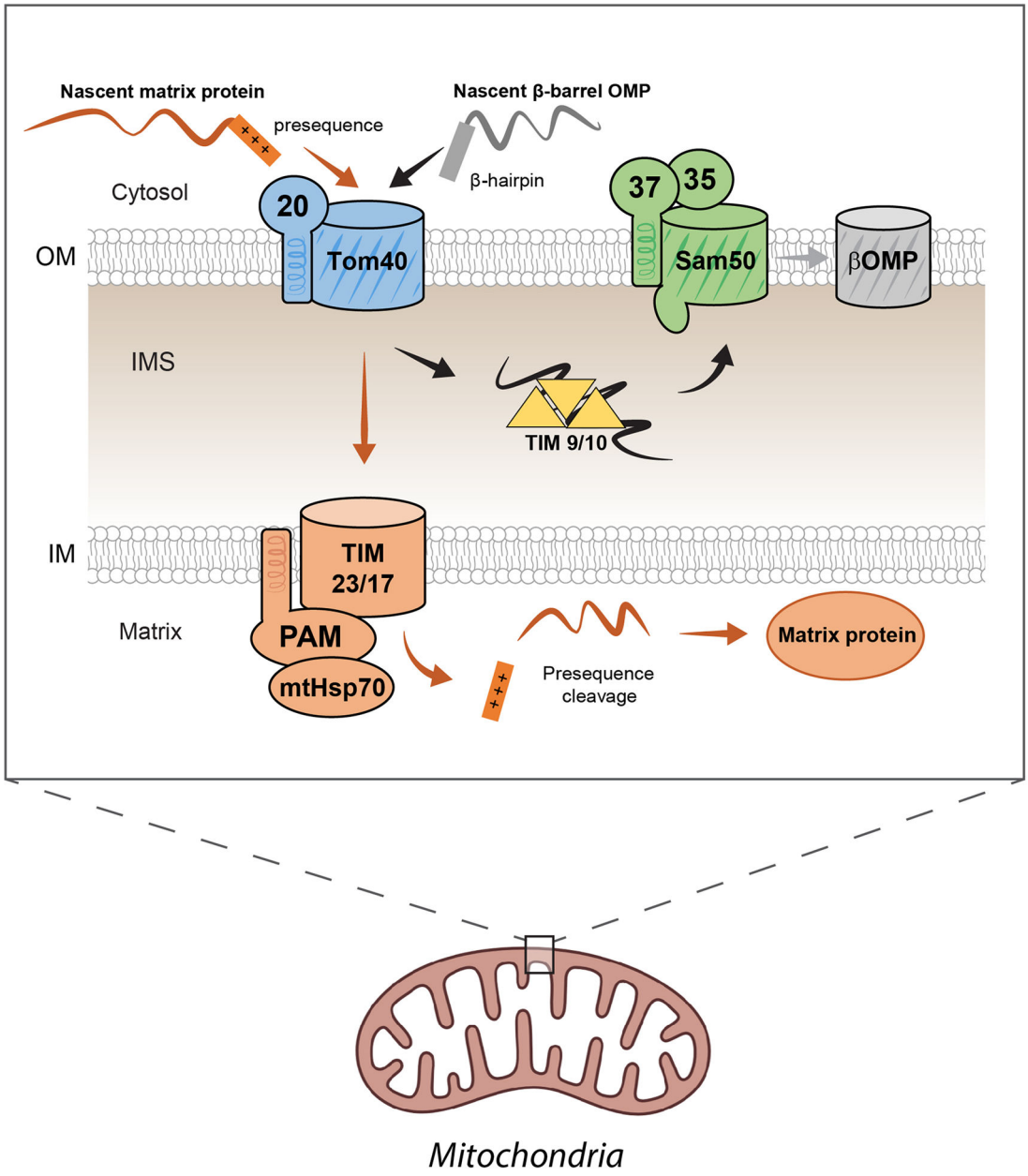

Figure 3. Protein import into the mitochondria.

Proteins trafficked to the mitochondria are first translated in the cytoplasm. Proteins designated for import in the mitochondrial matrix contain a positively-charged presequence peptide, which is hypothesized to facilitate protein import by TOM across the outer mitochondrial membrane (OMM). After entering the intermembrane space (IMS), the polypeptide then interacts with the translocon of the inner membrane (TIM) machinery. The membrane potential across the inner mitochondrial membrane (IMM) activates the complexes' translocation activity which is assisted by the presequence translocase associated motor (PAM) and matrix heat shock protein 70 (mtHSP70). This drives the polypeptide across the IMM towards the negatively-charged environment of the matrix. The presequence is then cleaved by the mitochondrial presequence protease and the protein is folded within

the matrix. Nascent βOMPs form an N-terminal beta hairpin that facilitates their trafficking to the mitochondria. After translocation through TOM, the nascent proteins are stabilized by the TIM9/10 chaperones within the IMS and shuttled to SAM, which facilitates folding and insertion into the OMM.

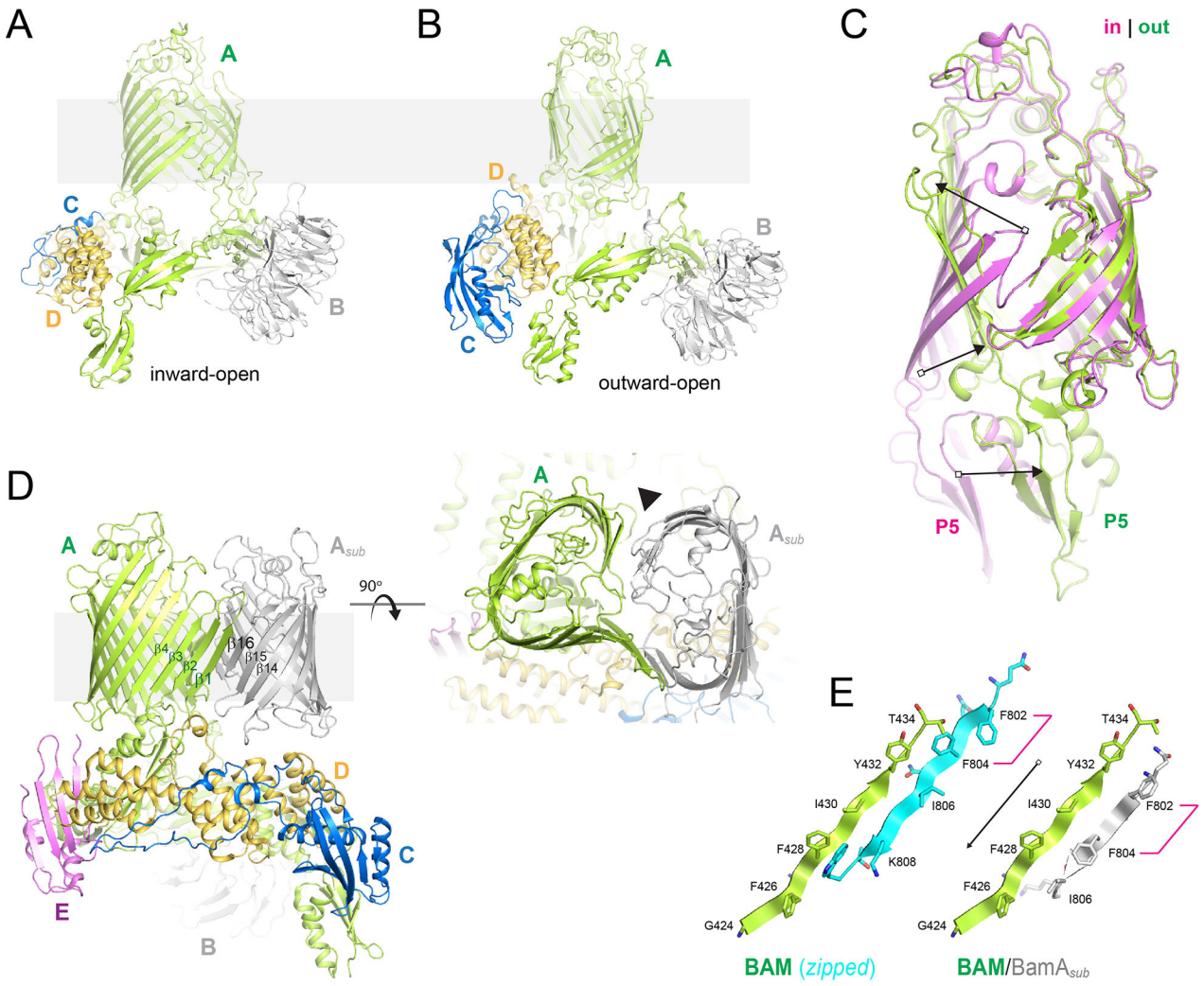

Figure 4. The structure of the β-barrel assembly machinery (BAM).

A. Shown is BAM in the inward-open (PDB ID 5D0O) and outward-open states (PDB ID 5LJO; **panel B**), with BamA in green, BamB in gray, BamC in blue, and BamD in gold. **C**. A zoomed view of the barrel domain of BamA comparing the conformational changes observed in the inward-open (magenta) and outward-open states (green); POTRA5 is indicated by 'P5', while the arrows indicate the conformational changes. **D**. The structure of the hybrid-barrel BAM/BamA$_{sub}$ complex (PDB ID 6V05) showing orthogonal views; BamA from BAM is in green, while the BamA$_{sub}$ in in gray. Strands at the interface are labeled, while the outer edges of the barrels mediating the curling of the BamA$_{sub}$ is indicated by the black solid triangle. **E**. A structural alignment at the BamA barrel seam for the BamA$_{sub}$ (*right*; green and gray), compared to BamA-zipped (*left*; green and cyan), which shows a four residue shift producing a C-terminal extension on the substrate that may play a role in the final step of βOMP biogenesis by BAM.

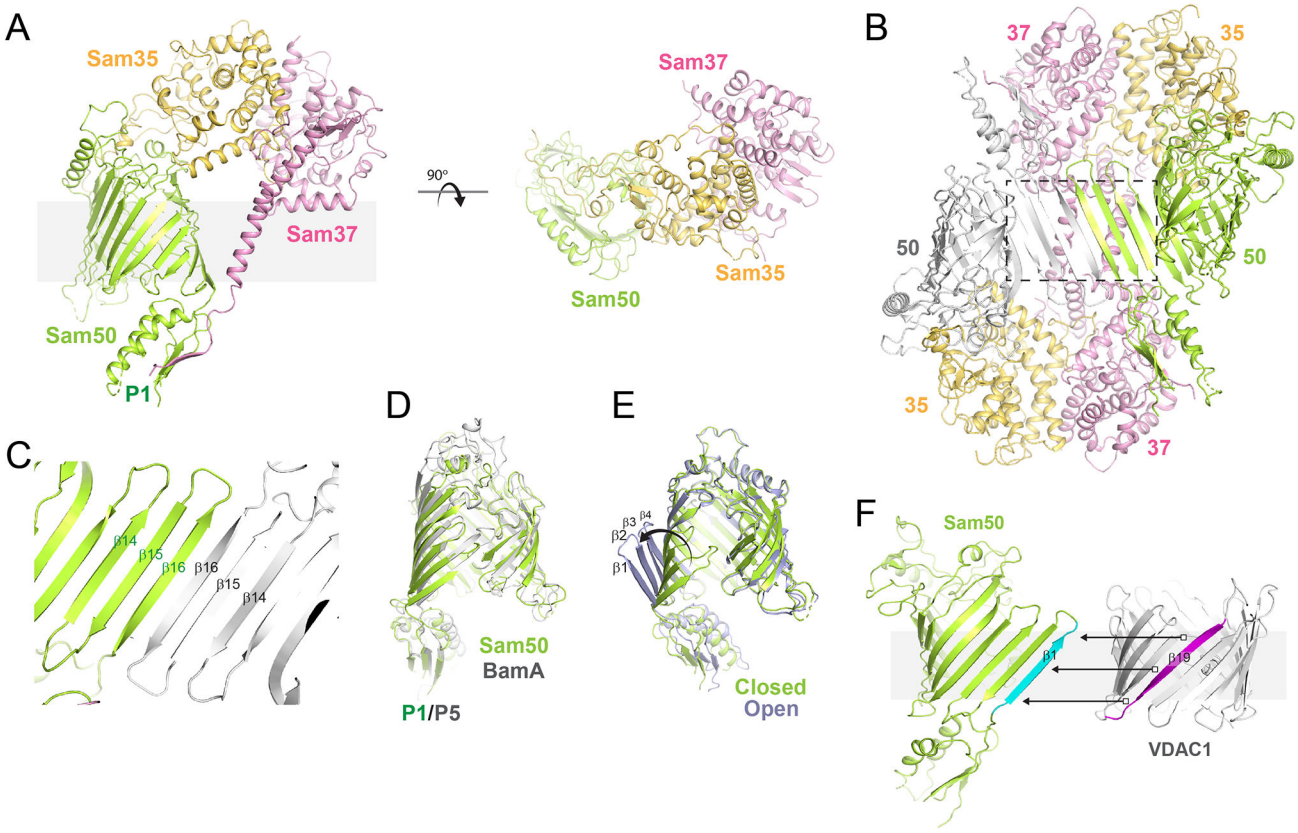

Figure 5. The structure of the sorting and assembly machinery (SAM).

A. Shown are orthogonal views of SAM (PDB ID 6WUL) with Sam50 in green, Sam35 in gold, and Sam37 in magenta. **B**. Several dimer forms were reported of SAM and shown here is the form which may mimic β-strand templating (PDB ID 6WUM), similar to what was observed for BAM in complex with $BamA_{sub}$ (PDB ID 6V05), albeit with the dimer form having pseudo two-fold symmetry (inverted). **C**. Zoomed view of the templating interface, a view turned 180° along y-axis from the region within the dashed box in panel B. **D**. Orthogonal view of the structural alignment of BamA (gray) and Sam50 (green). **E**. A structural comparison of the open (gray) and closed (green) states of Sam50, depicting the movement of β1-β4. **F**. A model showing how templating may occur between Sam50 (β1 shown in cyan) and VDAC1, which contains 19 β-strands, with the nineteenth strand (magenta) being required to ensure proper topology during biogenesis.