Published in final edited form as:

Methods Mol Biol. 2015 ; 1329: 137–147. doi:10.1007/978-1-4939-2871-2_10.

Methods to Characterize Folding and Function of BamA Crosslink Mutants

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Summary

The utility of protein engineering, both the mutation and deletion of specific amino acids, to investigate protein structure and function has been demonstrated time and time again, and inter- and intra-molecular interactions within the BAM complex and its individual components are no exception. Extensive efforts have probed conserved and unique amino acid sequences of the Bam proteins to define their functional roles. This chapter will summarize efforts as applied to the disulfide crosslink mutants of BamA and describe experimental methods used in our studies to determine that lateral opening of the barrel domain is required for function.

Keywords

BamA; BAM complex; Outer membrane protein; Proteinase K; Disulfide crosslinking; β -barrel membrane protein

1. Introduction

BamA is the central component of the BAM complex (1,2) and a member of the TspB/Omp85 superfamily of β -barrel membrane proteins that are essential for protein transport across and/or incorporation into the outer membrane of Gram negative bacteria (3). Prior to assaying mutant BamA function, and in the event of observing a novel phenotype, one must determine whether the mutant BamA is stably folded into the membrane or not and whether any observed loss of function is due to perturbed interactions with the Bam lipoproteins or nascent OMP substrates.

As described in **Chapter 4**, heat modifiability assays can be used to access whether a β -barrel outer membrane protein is properly folded or not. Here, we will use this assay to determine if BamA mutants are being properly folded when expressed and compare the results to wild type BamA.

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Limited proteolysis of membrane proteins can be a powerful technique to assess solvent exposed regions, probe conformational changes, and monitor protein trafficking. In this application intact bacteria cells, which are heterologously expressing BamA and BamA mutants, are exposed to proteinase K (PK) (4–7). PK does not readily cross the outer membrane, and therefore only extracellular portions of the BamA are subject to the enzyme, creating conditions for limited proteolysis. When resolved on SDS-PAGE and visualized using Western blot analysis, degradation products of BamA will be observed in PK-treated samples, but full-length BamA will be observed in non-treated samples. If the exogenous BamA was not properly incorporated into the outer membrane, extracellular loops would likely not be exposed. Control experiments utilizing antibodies for soluble proteins like maltose binding protein (MBP) are performed to verify that membrane integrity was maintained during the procedure.

Structure guided disulfide crosslinking is a powerful technique to determine if a conformational change is necessary for function or not in a particular protein. Recently, we used disulfide crosslinking to show that lateral opening of the barrel domain of BamA is required for function (5, 6). Here, we will outline those experiments in more detail.

2. Materials

All solutions are prepared at 25°C using deionized water with a sensitivity of 18 MΩ-cm and are sterile filtered. Use analytical grade reagents for all solutions.

2.1. Cloning and Mutagenesis

1. Quikchange Site-Directed Mutagenesis kit (Agilent Technologies).
2. Miniprep kit (i.e. QIAprep Spin Miniprep Kit (Qiagen)).
3. Template plasmid containing BamA; Here, we will use pRSF-1b/BamA from our lab which contains an N-terminal pelB signal sequence followed by a HIS-tag (5,6).
4. Primer sets (forward and reverse) for each mutation being studied; Here, we will present protocols used for our recent work on the lateral gate of BamA as an example (5), however, these methods can be extended to study other mutations as well.
5. LB agar plates containing kanamycin (50 µg/mL) (LB/kan).
6. Inoculation loops and cell spreaders.
7. Sterile culture tubes (15 mL).
8. Plate incubator set at 37°C.
9. Laboratory incubator shaker set at 220 rpm at 37 °C.
10. Water bath set at 42°C.
11. UV-vis spectrometer for measuring DNA concentration.
12. PCR tubes.

13. PCR thermocycler.

2.2. Expression of Wild Type BamA and Mutants

1. Electrocompetent JCM-166 cells (*see Ref. (8)*): these cells have endogenous BamA under an arabinose promoter.
2. Plasmids containing BamA and BamA mutants from **Subheading 2.1**.
3. LB medium: dissolve 5 g NaCl, 10 g peptone, and 5 g yeast extract into 800 mL of water. Adjust the volume to 1 L and autoclave.
4. Arabinose (20%): dissolve 2 g in 7 mL of water, bring up to 10 mL and sterile filter.
5. UV-vis spectrophotometer capable of measuring optical density at $\lambda = 600$ nm.
6. Electroporator.
7. SOC medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose. Autoclave and let cool before use.

2.3. Heat Modifiability Assays

1. PBS(10x): 17 mM KH₂PO₄, 50 mM Na₂HPO₄, 1.5 M NaCl, pH 7.4.
2. N-dodecyl- β -D-maltoside (DDM) detergent stock solution (10%).
3. Lysis Buffer: 1xPBS, 1% DDM, 10 mM EDTA, 10 μ g/mL lysozyme, supplemented with 100 μ g/mL AEBSF and 10 μ g/mL DNase I.
4. Refrigerated benchtop microcentrifuge for 1.5 mL microcentrifuge tubes.
5. Rocking platform at room temperature.

2.4. Proteinase K Digestion Assays

1. Proteinase K stock solution (50 mg/mL) in 20 mM Tris-HCl, pH 7.4, 1 mM CaCl₂.
2. Phenylmethanesulfonylfluoride (PMSF) protease inhibitor: 200 mM stock solution in 100% ethanol.

2.5. Disulfide Crosslinking

1. Reaction Buffer: 20 mM Tris-HCl, pH 7.5, 100 mM NaCl.
2. Copper sulfate (CuS) (25 mM).
3. Copper-o-phenanthroline (CuP) (25 mM).
4. Dithiothreitol (DTT) (1 M).

2.6. SDS-PAGE

1. SDS-PAGE sample loading buffer (2x): 20% glycerol, 120 mM Tris-HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue (*see Note 1*).

2. SDS-PAGE sample loading buffer (2x) with DTT: 20% glycerol, 120 mM Tris-HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue, 100 mM DTT (*see* ^{Note 1}).
3. SDS-PAGE gels and native gels (*see* ^{Note 2}). Here, we will be using 5% SDS-PAGE cast in-house and precast 4–12% NuPAGE and 4–12% NativePAGE gels (Life Technologies).
4. MES-SDS Running Buffer (20x): 1 M MES, 1 M Tris base, 2% SDS, 20 mM EDTA, pH 7.3.
5. MOPS-SDS Running Buffer (20x): 1 M MOPS, 1 M Tris Base, 2% SDS, 20 mM EDTA, pH 7.7.
6. Prestained protein markers (e.g. SeeBlue Plus2 Protein Standard (Life Technologies)).
7. Polyacrylamide gel electrophoresis (PAGE) system.
8. Heat block set to 95°C.
9. Ice bucket and ice.

2.7. Western Blot Analysis

1. Anti-HIS-HRP antibodies (Sigma).
2. Anti-MBP antibodies (NEB).
3. Anti-Mouse IgG HRP secondary antibodies (Sigma).
4. PBST(1x): 1.7 mM KH₂PO₄, 5 mM Na₂HPO₄, 150 mM NaCl, 0.1% Tween-20, pH 7.4.
5. PBST(1x) + BSA: 1.7 mM KH₂PO₄, 5 mM Na₂HPO₄, 150 mM NaCl, 0.1% Tween-20, pH 7.4, 1% bovine serum albumin (BSA).
6. Transfer system for blots (e.g. Life Technologies iBlot® Gel Transfer Device or similar).
7. Western blot box.
8. ECL Western blotting substrate (e.g. Pierce ECL Western Blotting Substrate).
9. Western blot imaging system (e.g. GE ImageQuant LAS 4000 or similar).

¹For the dye, we advise against using coomassie G250 here since we have experienced odd results when substituted for bromophenol blue. We hypothesize that the coomassie G250, which is in fact used for blue native PAGE methods, interacts with the samples due to its properties while that is not the case for the bromophenol blue.

²Most native gels should work here either fixed or gradient. For the examples shown here, we used precast NativePAGE Novex 4–12% Bis-Tris protein gels (Life Technologies).

3. Methods

3.1. Designing and Cloning BamA Mutants

1. Design DNA oligo primers for the engineered mutation being studied (*see* ^{Note 3}). Dilute to 1000 ng/μL with water. Make a working solution for each primer at 100 ng/μL.
2. Use the Quikchange Site-Directed Mutagenesis kit following manufacturer's instructions. Briefly, in a PCR tube, add 50 ng DNA template (pRSF1b/BamA in our case), 125 ng of each primer, 1 μL of dNTP mix, 2.5 μL 10x Reaction Buffer, and 1 μL polymerase. Bring to 25 μL final volume with water and mix by pipetting.
3. Place into a thermocycler using a program that matches the manufacturer's instructions (*see* ^{Note 4}).
4. When finished, add 1 μL of DpnI enzyme to each tube and incubate at 37°C for 1 h (*see* ^{Note 5}).
5. Transform each into the provided competent cells using manufacturer's instructions, except only use 200 μL of SOC medium following the heat pulse at 42°C. Then spread the entire sample onto an LB/kan agar plate and incubate inverted overnight at 37°C.
6. The next day, prepare at least five 5 mL cultures from 5 different colonies from the plate in LB/kan medium. Allow to grow overnight at 37°C.
7. The next day, perform DNA plasmid minipreps using manufacturer's instructions for each culture and verify the correct mutation is present using sequencing analysis.
8. Repeat **steps 1–7** for each desired mutation to be analyzed (*see* ^{Note 6}).

3.2. Expression of BamA Mutants in JCM-166 Cells

1. To assay the mutants for expression and folding, transform plasmids for BamA and each BamA mutant prepared in **Subheading 3.1** into JCM-166 electrocompetent cells using an electroporator following manufacturer's instructions.
2. Following electroporation, resuspend the cells in 0.5 mL SOC medium and grow at 37°C for 1 h.
3. For each sample, (1) pipet 25–50 μL of cells onto a prewarmed LB/kan agar plate and spread across the plate by streaking with an inoculation loop, and (2) pipet 25–

³For designing primers for site-directed mutagenesis, one can use the online QuikChange Primer Design Tool found on the Agilent Technologies website. While convenient and time saving, it does tend to design primers that are often longer than needed. We recommend trimming the primers suggested by the tool to ~35 base pairs long.

⁴We routinely do 25–30 cycles rather than the 18–20 suggested by the kit instructions.

⁵We routinely do extended incubation times (2–5 h) for the DpnI digestion which reduces the chances of growing colonies which have no mutation.

⁶If possible, it is always easier and more efficient to do all the mutations at the same time, rather than doing them one after the other, unless multiple mutations are needed within the same construct as with our disulfide mutants.

50 μ L of the cells onto a prewarmed LB/kan agar plate containing 0.1% arabinose and spread across the plate by streaking with an inoculation loop (*see* ^{Note 7}).

4. Invert and grow overnight at 37°C.
5. The next day, for each mutant, compare the growth with and without arabinose. Those mutants showing normal colony growth in the absence of arabinose are likely functioning in a manner similar to wild type BamA. Those mutants showing a growth defect, however, are likely revealing either disrupted BamA function or folding, and can be studied further. Here, we will examine if the mutants are being folded properly and making it the outer membrane.
6. For wild type BamA and each mutant showing growth defects, prepare a 5 mL culture using LB/kan medium containing 0.1% arabinose and grow at 37°C shaking at 220 rpm to an OD₆₀₀ = ~1.5.
7. For each sample, prepare 1 mL of cells at OD₆₀₀ = 1.0 in a 1.5 mL microcentrifuge tube and spin using a benchtop microcentrifuge for 1 min at full speed. Remove the supernatant and then resuspend the cells in 1 mL cold 1x PBS by pipetting. Spin again and remove the supernatant.
8. The cells can now be used for further analysis as described in **Subheadings 3.4 – 3.6**.

3.3. SDS-PAGE and Western Blot Analyses

Before proceeding with analyzing the BamA mutants, we will present the methods required for performing SDS-PAGE and Western blot analyses, since these will be essential for determining the results of the heat modifiability, proteinase K, and disulfide crosslinking assays described next.

3.3.1. Denaturing SDS-PAGE Analysis—Denaturing SDS-PAGE analysis may be performed using standard methods. Briefly, we will describe those below.

1. Prior to preparing samples for analysis, assemble the gel apparatus according to manufacturer's instructions. Insert the gel and then fill the inner reservoir completely with 1x MES Running Buffer or 1x MOPS Running Buffer and the outer reservoir at least 1/3 full.
2. Load 20 μ L of each sample onto the gel. Be sure to also include a prestained protein standard for when analyzing results and for monitoring the transfer step during Western blot analysis.
3. Run at constant 200 volts for 35 min if using 4–12% SDS-PAGE gradient gels or 70 min if using 5% SDS-PAGE gels.

⁷Depending on the number of mutations, we routinely plate up to 6 mutations per plate by dividing the plate into 6 triangular regions, into each of which we streak ~15 μ L of cells.

3.3.2. Semi-native SDS-PAGE Analysis

1. Prior to preparing the samples for analysis, assemble the gel running apparatus so that the gel may be loaded immediately after the samples are ready. Place the gel apparatus tank into a bed of ice within an ice bucket. Insert the gel and then fill the tank completely with cold 1x MES running buffer. It is important to keep the buffer within the tank cold during the entire experiment (*see*^{Note 8}). Once assembled, you can move to the next step.
2. Load 25 µL of each sample onto the native gel assembled in **step 1** (*see*^{Note 9}).
3. Run the gel for 60 min at constant 150 volts.

3.3.3. Western Blot Analysis

1. Once the gel has finished running from **Subheadings 3.3.1 and 3.3.2**, transfer to either nitrocellulose or polyvinylidene difluoride (PVDF) membrane using manufacturer's instructions (*see*^{Note 10}).
2. Trim the excess membrane and if probing with more than one antibody, use the protein standards to guide cutting the membrane at the appropriate locations. For the proteinase K susceptibility assay, we cut the membrane in half around 55 kDa and probe the top (higher molecular weight) portion against anti-HIS-HRP antibodies and the bottom (lower molecular weight) portion against anti-MBP antibodies.
3. Place the membrane into a blot box and add 20 mL of 1x PBST + BSA blocking solution and incubate for 1 h on a rotating or rocking platform.
4. Then add either anti-HIS-HRP (1:6000) or anti-MBP (1:5000) antibody and allow to incubate an additional 1 h. Pour off supernatant.
5. Wash each membrane 3 times for 5 min each using 1x PBST.
6. If using the anti-MBP antibody, proceed to incubate with anti-Mouse HRP secondary antibodies (1:15,000) in 20 mL 1x PBST with 1% BSA for 30 min. Pour off supernatant and wash membrane 3 times for 5 min each using 1x PBST.
7. Wash each membrane 3 times for 5 min each using 1x PBS.
8. Add ECL Western blotting substrate to the membrane and image using a Western blot imaging system, following manufacturer's instructions for both.

⁸Since we keep our electrophoresis equipment at room temperature, it was easier for us to do everything at room temperature and put the gel apparatus in ice to keep it cool. However, a gel apparatus set up in a cold room would be just as good and would not require the ice bucket or ice.

⁹If need, additional sample can be loaded here for better visualization. Alternatively, adjust the volume of Lysis Buffer used to yield a more concentrated sample.

¹⁰We recommend using a blotting system such as the iBlot system (Life Technologies) which can do transfers in 7 min, significantly speeding the time to attaining results.

3.4. Heat Modifiability Assay to Characterize BamA Folding

Here we present a protocol for heat modifiability assays for BamA using whole cells. For more details about heat modifiability assays for purified protein and in general, please see **Chapter 4**.

1. For each sample from **Subheading 3.2, item 8**, resuspend cells in 500 μ L of Lysis Buffer.
2. Rock for 15 min at room temperature.
3. Centrifuge at full speed at 4°C for 10 min using a refrigerated benchtop microcentrifuge.
4. Pipette 100 μ L of the detergent solubilized lysate supernatant into two new microcentrifuge tubes, labeling one 'boiled' and the other 'unboiled'.
5. Add 100 μ L of 2x SDS-PAGE sample loading buffer to each tube.
6. Incubate the 'boiled' sample tube at 95°C for 5 min while keeping the 'unboiled' sample at room temperature.
7. Proceed with semi-native SDS-PAGE analysis as outlined in **Subheading 3.3.2** using a 4–12% NativePAGE gel in 1x MES Running Buffer and Western blot analysis as outlined in **Subheading 3.3.3** using anti-HIS-HRP antibodies.
8. Compare the 'boiled' sample to the 'unboiled' sample, looking for a shift in the gel to indicate folded and unfolded states (Fig. 1A).

3.5. Proteinase K Susceptibility Assays to Characterize Surface Exposure

1. For each sample from **Subheading 3.2, item 8**, resuspend cells in 500 μ L of 1x PBS and aliquot 250 μ L into two new tubes, labeling one as 'PK+' and the other as 'PK-'.
2. Add proteinase K to the 'PK+' tube at a final concentration of 500 μ g/mL and incubate at 37°C for 30 min. To stop the reaction, add PMSF to a final concentration of 5 mM and gently invert to mix.
3. Spin both samples at full speed for 1 min using a benchtop microcentrifuge and carefully remove the supernatant.
4. Resuspend the cells in 200 μ L 1x SDS-PAGE sample loading buffer and boil for 5 min.
5. Spin at full speed for 10 min using a benchtop microcentrifuge.
6. Proceed with denaturing SDS-PAGE analysis as outlined in **Subheading 3.3.1** using a 4–12% NuPAGE gel in 1x MES Running Buffer and Western blot analysis as outlined in **Subheading 3.3.3** using anti-HIS-HRP and anti-MBP antibodies probing for both BamA and MBP, respectively.

7. Compare the 'PK+' sample to the 'PK-' sample, looking for degradation of the BamA band in the presence of PK. Monitoring MBP verifies that the membrane was not permeabilized during the analysis (Fig. 1B).

3.6. Disulfide Crosslinking of BamA Barrel Domain

3.6.1. Visualizing the Natively Formed Disulfide Crosslinks

1. For each sample from **Subheading 3.2, item 8**, resuspend cells in 200 μ L of 1x PBS and aliquot 100 μ L into two new tubes, labeling one as 'DTT+' and the other as 'DTT-'.
2. Add 100 μ L of 2x SDS-PAGE sample loading buffer with DTT to the 'DTT+' tube and 100 μ L of 2x SDS-PAGE sample loading buffer without DTT to the 'DTT-' tube.
3. Boil samples at 95°C for 5 min and then spin for 10 min at full speed using a benchtop microcentrifuge.
4. Proceed with denaturing SDS-PAGE analysis as outlined in **Subheading 3.3.1** using a 5% SDS-PAGE gel in 1x MOPS Running Buffer and Western blot analysis as outlined in **Subheading 3.3.3** using anti-HIS-HRP antibodies probing for BamA.
5. Compare the 'DTT+' sample to the 'DTT-' sample looking for a shift in the BamA bands to indicate the formation of a disulfide bond which can be reduced in presence of DTT (Fig. 1C).

3.6.2. Visualizing the Oxidizer Enhanced Disulfide Crosslinks—To further enhance the disulfide crosslinks, incubation with CuS or CuP can be performed prior to processing the cells for SDS-PAGE and Western blot analyses.

1. For each sample from **Subheading 3.2, item 8**, resuspend cells in 200 μ L of Reaction Buffer and aliquot 100 μ L into two new tubes, labeling one as 'CuS' and the other as 'CuP'.
2. Add CuS and CuP to a final concentration of 100 μ M to each tube, respectively, and incubate at 37°C for 30 min.
3. Spin samples at full speed for 1 min using a benchtop microcentrifuge and carefully remove the supernatant.
4. Resuspend the cells in 200 μ L of Reaction Buffer and repeat **step 3**.
5. Resuspend each sample in 200 μ L 1x SDS-PAGE sample loading buffer without DTT.
6. Boil samples at 95°C for 5 min and then spin for 10 min at full speed using a benchtop microcentrifuge.
7. Proceed with denaturing SDS-PAGE analysis as outlined in **Subheading 3.3.1** using a 5% SDS-PAGE gel in 1x MOPS Running Buffer and Western blot analysis as outlined in **Subheading 3.3.3** using anti-HIS-HRP antibodies probing for BamA.

8. Compare the 'CuS' and 'CuP' samples to the 'DTT+' and 'DTT-' samples from **Subheading 3.6.1, item 4** to determine if disulfide bond formation was enhanced or not (Fig. 1D).

4. Notes

¹For the dye, we advise against using coomassie G250 here since we have experienced odd results when substituted for bromophenol blue. We hypothesize that the coomassie G250, which is in fact used for blue native PAGE methods, interacts with the samples due to its properties while that is not the case for the bromophenol blue.

²Most native gels should work here either fixed or gradient. For the examples shown here, we used precast NativePAGE Novex 4–12% Bis-Tris protein gels (Life Technologies).

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Acknowledgments

This research was supported by the Intramural Research Program of the NIH, The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

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Fig. 1. BamA crosslink mutants are folded and incorporated into the outer membrane Heat modifiability (panel **A**) and proteinase K (PK) digestion (panel **B**) assays were performed on whole cells expressing the BamA crosslink mutants. All crosslink mutants showed heat modifiability and were susceptible to PK, indicating presentation on the surface and proper folding. **C**. To analyze disulfide crosslink formation under native conditions, cell lysates were resolved by SDS-PAGE +/- DTT. All of the engineered disulfide mutants formed disulfide crosslinks within the cell. Additionally, treatment with 100 μ M copper sulfate (CuS) or 100 μ M copper o-phenanthroline (CuP) enhanced disulfide formation (panel **D**). BamA was probed using anti-HIS-HRP antibodies while MBP was probed using anti-MBP antibodies. Reproduced from **Ref** (5) with permission from Elsevier.