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Small angle X-ray scattering (SAXS) characterization of the POTRA domains of BamA

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Summary

BamA is the central component of the BAM complex and contains a C-terminal β -barrel domain embedded in the outer membrane, and a soluble, periplasmic domain, made out of five polypeptide transport associated (POTRA) motifs. Structural characterization of the POTRA domains was carried out by a combination of crystallographic, NMR and solution Small Angle X-ray Scattering (SAXS) approaches. Despite its limited resolution, SAXS is an excellent complement to NMR and crystallography. It is well suited to validate high-resolution models in solution and is particularly useful to characterize flexible systems such as the POTRA domains of BamA. Here we present a protocol for sample preparation and discuss the considerations of SAXS data collection and quality control, which is applicable to most soluble proteins.

Keywords

SAXS; BamA; BAM; conformational change; structure; OMP

1. Introduction

Small angle X-ray scattering (SAXS) is a powerful technique for the structural characterization of biomolecules in solution. No labeling is necessary and, with bright sources of X-rays at synchrotron facilities and modern detectors, it requires relatively small amounts of sample at concentrations of a few mg/ml for proteins. Data collection is very quick, and simple analysis yields the macromolecular radius of gyration (R_g), which provides an initial indication of molecular size. The maximum dimension (D_{max}) as well as a histogram of interatomic distances, the $P(r)$ function, are also easily derived from the scattering data and provide additional information about molecular shape [1]. The software programs Primus [2] and ScÅtter (<https://bl1231.als.lbl.gov/scatter/>) offer user-friendly access to this basic data analysis.

While limited in terms of resolution, SAXS is an excellent complement to high-resolution techniques such as X-ray crystallography [1]. It is possible to compare experimentally determined and coordinate-derived scattering curves and $P(r)$ profiles to assess the similarity between crystal and solution structures [3]. In addition, it is often possible to use *ab initio*

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methods to calculate molecular envelopes from SAXS data [4-6]. This is very useful not only to compare crystal and solution structures, but also to allow “docking” of partial high-resolution structures into the envelopes of complete proteins or multiprotein complexes. Approaches have also been developed to evaluate the structural flexibility of macromolecules and model their conformational changes [7]. The ATSAS software package (<http://www.embl-hamburg.de/biosaxs/software.html>) provides several tools to carry out these experiments and has been instrumental in popularizing sophisticated SAXS analyses.

Structural characterization of the periplasmic domain of BamA, the central BAM subunit containing five POTRA domains, benefited from several SAXS approaches. Initial crystal structures defined alternate conformations for the first four POTRA domains due to a flexible hinge between POTRA 2 and 3 [8,9]. A subsequent crystal structure of a POTRA4-5 fragment allowed construction of spliced models containing all five POTRA domains, which was validated by SAXS [10]. Furthermore, analysis of the scattering curves and P(r) profiles experimentally determined from the POTRA1-5 fragment and derived from the splice models defined conformational flexibility of the protein, which was further characterized by the Ensemble Optimization Method [7]. Here we present the protocol for sample preparation, data collection and analysis used in these experiments, which can be used for further study of the periplasmic domain of BamA or easily adapted for other soluble macromolecules.

2. Materials

All solutions are prepared with MilliQ water and reagent of analytical grade or better. All stock solutions are filter sterilized.

1. *E. coli* Rosetta (DE3) cells (Novagen)
2. Kanamycin stock: 50 mg/mL in water
3. Luria Broth
4. Isopropyl- β -D-thiogalactopyranoside (IPTG) stock: 0.4 M in water
5. Sodium Chloride stock: 5 M
6. Imidazole stock: 1 M
7. Glycerol
8. Complete EDTA-free protease inhibitor (Roche)
9. Lysis Buffer: 25 mM Tris-HCl pH 8
10. Buffer A: 25 mM Tris-HCl pH 8, 150mM NaCl
11. SAXS Buffer: 25 mM Tris-HCl pH 8, 150 mM NaCl, 5% glycerol
12. Nickel-NTA resin (Qiagen)
13. His-tagged TEV protease [11]
14. Superdex 200 Size Exclusion Chromatography (SEC) column HiLoad 26/60 (Amersham Pharmacia Biotech)

15. Centrifugal concentrators
16. Dialysis tubing
17. 0.1 mm filters
18. Sonicator
19. Centrifuge capable of spinning 30,000 x g
20. SAXS data collection facility. We assume a synchrotron beamline facility such as Beamline 12.3.1 of the Advanced Light Source (Lawrence Berkeley National Laboratory)

3. Methods

3.1 Protein Expression and Purification

1. Coding sequences for fragments of the periplasmic domain of E. coli BamA (amino acids 21-424 for POTRA1-5; 175-424 for POTRA3-5 and 264-424 for POTRA4-5) were cloned into a variant of pET28 vector as described in [10], creating N-terminal fusions to a His-tag followed by a TEV protease cleavage site. These plasmids are used to transform Rosetta DE3 cells (Novagen) for expression.
2. Pick one colony to inoculate a 100 mL of LB with 50 µg/mL kanamycin and grow at 37°C overnight, shaking at 160 RPM.
3. Inoculate 4 x 1 L LB cultures containing 50 µg/mL kanamycin with 10 ml (per 1L) of the overnight culture and grow at 37°C until they reach OD₆₀₀ of ~0.7.
4. Induce expression adding IPTG to the cultures for a final concentration of 0.4 mM and incubate for an additional 1.5 hours at 37°C.
5. Harvest cells by centrifugation. Resuspend cell pellet in Lysis Buffer supplemented with Complete EDTA-free protease inhibitor tablets (Note 1).
6. Lyse cell by sonication using a macro tip at full power and 2 second pulses followed by 5 second pauses until the cells are broken (Note 2).
7. Add sodium chloride to a final concentration of 0.3 M.
8. Remove cell debris by centrifugation at 30,000 x g for 30 minutes at 4°C, keeping the supernatant.
9. Pack a 10 ml Ni-NTA column and equilibrate with Buffer A.
10. Load the supernatant into the Ni-NTA column (Note 3). Wash with 2 column volumes of Buffer A, followed by 20 column volumes of Buffer A supplemented

¹The resuspended cell pellet can be used immediately or frozen at -70°C until needed.

²We resuspend the cells in enough lysis buffer so that a 1/60 dilution in water yields an OD₆₀₀ of approximately 0.8. Lysis is deemed to be complete when the OD₆₀₀ of the 1/60 dilution drops below 0.1 or does not change after additional 1 minute sonication periods.

³Keep the flow through and analyze by SDS PAGE for expressed protein content. Constructs expressing POTRA domains 3-5 and 4-5 express at high enough levels that can saturate the 10 ml column.

with 25 mM imidazole. Elute the protein with Buffer A supplemented with 200 mM imidazole.

11. Pool protein-containing elution fractions and measure protein concentration. Add His-tagged TEV protease at a ratio of 1 mg to 20 mg of POTRA domain and DTT at a final concentration of 10 mM. Incubate for 24 hrs at 4°C to allow cleavage of the Histag and dialyze overnight against Buffer A to remove the imidazole (Note 4).
12. Pass the preparation through a Ni-NTA column again to remove the tag, the His-tagged TEV protease and any POTRA domain fragment that remains uncleaved by the protease. The purified, untagged POTRA domain protein will be in the flow-through.
13. Concentrate the protein (Note 5) and load onto a Superdex 200 SEC column equilibrated with SAXS buffer and collect the elution in 0.4 ml fractions.
14. Set aside the fraction corresponding to the center of the protein peak eluting from the SEC column, which has the highest protein concentration. Pool the other protein containing fractions and concentrate using centrifugal concentrators followed by filtration through 0.1 μ m centrifugal filters (Note 6).

3.2 Consideration for SAXS sample preparation and data collection

1. SAXS is a contrast technique where scattering from the macromolecule has to be isolated from that of the solvent. Accurate buffer subtraction is therefore essential so we use the SEC eluted protein and the SEC buffer to carry out the experiments.
2. As the scattering is proportional to the size (and shape) of the particles it is important to avoid even small amounts of aggregated material in the sample. Therefore, whenever possible we use the most concentrated fraction from the SEC elution or dilutions of this fraction to collect data without further manipulation.
3. Glycerol has been shown to protect the sample against radiation damage. Therefore, 5% glycerol is incorporated in the SAXS Buffer used to develop the SEC column.
4. Concentration dependent effects are often observed in SAXS data. We thus create concentration series of the proteins with samples at approximately 2, 5 and 10 mg/ml. We use the most concentrated fraction from the SEC elution as one of the concentrations in the series and, if the protein is concentrated enough, make dilutions from this fraction. If the protein does not display a tendency to aggregate

⁴Tag cleavage and imidazole removal can be done simultaneously by dialyzing against Buffer A supplemented with 10 mM DTT to keep the protease active during dialysis. This approach is sometimes advantageous as Ni²⁺ released into the eluted fractions from the column can induce aggregation of some proteins.

⁵We concentrate as much as possible before the SEC column so that the elution fractions can be concentrated enough to use directly for data collection without additional concentration. For constructs coding POTRA domains we concentrate to 8 mg/ml POTRA1-5, 10 mg/ml for POTRA3-5 and 40 mg/ml for POTRA4-5.

⁶Centrifugal concentrators and filters are often impregnated with preservatives. We pre-run these devices with SAXS Buffer twice before using them with sample to minimize their impact in buffer matching.

during centrifugal concentration, as is the case for POTRA4-5 fragments, we also prepare higher concentration samples at 20 and 40 mg/ml.

5. Even with glycerol as a radical scavenger is the SAXS buffer, some samples display radiation damage. We thus collect images at increasingly long exposures to X-rays and analyze the data for signs of radiation damage. The exposure times will depend on the X-ray source. At the Advanced Light Source beamline 12.3 (Sibyls beamline) we routinely collect 0.5, 1, 2 and 4 second exposures.
6. To maximize the chances of accurate buffer subtraction, we organize data collection as recommended by the Sibyls beamline protocol [12]: (i) wash sample cuvette with water, (ii) wash with SAXS Buffer; (iii) collect data on SAXS buffer at all exposures; (iv) collect on protein samples at all exposures in order of increasing concentration; (v) wash sample cuvette with buffer; and (vi) collect data on SAXS buffer at all exposures. This allows buffer subtraction to be carried out with two independent buffer measurements.

3.3 SAXS data quality control

The raw SAXS images contain radially symmetric scattering whose intensity decays with scattering angle. Initial image processing depends on the specific detector setup and is typically carried out at the beamline. It involves masking out direct beamstop shadow(s), radial averaging of the integrated intensities and use of detector calibration data to convert the data into a plot of intensity (I) vs q ($q = 4\pi\sin\theta/\lambda$, where 2θ is the scattering angle and λ is the X-ray wavelength). We then perform an evaluation of data quality as described below using the program PRIMUS [2].

1. With the two independent measurements of buffer scattering collected before and after the protein sample, subtract the buffer signal from the total signal to isolate the scattering from the macromolecule. Both subtractions should result in superimposable curves. If this is not the case, additional measurements of buffer scattering can be used for the subtraction to identify the outlier. Buffer subtracted curves are used for all the subsequent analyses.
2. Compare the curves corresponding to each exposure. The curves should have the same shape, with the intensity scaling linearly with the exposure. Radiation damage can be identified by an upward deviation of the curves at low q values.
3. Compare curves corresponding to each concentration in the series. A protein has a characteristic decay of I vs q and the curves should thus have the same shape and scale linearly with concentration. However, oligomerization and interparticle repulsion are concentration dependent effects observed for some protein samples. Data collected at concentrations displaying these artifacts are not used for further analysis.
4. Inspect the curves collected at the highest protein concentrations for signs of detector saturation. This is easily identified by a flattening of the signal at low q values. Data from the non-saturated region of the curve is nevertheless useful.

5. Curves passing the quality controls described above are scaled together and averaged to generate a consolidated plot of I vs q with error estimates that is used for all further analysis.

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