Title: Investigations of the solution structure of MurI for the development of bacterial cell wall biosynthesis inhibitor

Category: Biology and Biodiversity

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Research background, problem statements and research objective:

Multidrug-resistant Pseudomonas aeruginosa (P. aeruginosa) is one of the most serious threats to human health. Antibiotics used to treat P. aeruginosa infections are relatively toxic and frequently cause unwanted side effects. Additionally, antibiotics have a difficult time penetrating the layer of bacteria's peptidoglycan, a major component of their cell walls. Glutamate racemase or MurI is a cofactor independent enzyme which is an importance component of the peptidoglycan biosynthesis. Importantly, MurI is absent in humans, hence that MurI is a new attractive drug target.

In this study, we intend to investigate the solution structure of PaMurI, an enzyme involved in peptidoglycan biosynthesis, as a model to produce antibacterial drugs. Understanding PaMurI oligomerization and conformation can support researchers in the discovery and development of specific bacterial cell wall inhibitors, reducing bacteria's ability to evolve drug resistance.

Materials and methods used:

The *murI* gene from *P. aeruginosa* ATCC27853 (PaMurI) was cloned into modified pET 21d(+) vector (pSY5), to be expressed inframe with N-terminal 8xhis-tag. The cultures were grown and collected by centrifuging. Then, Cell pellets containing PaMurI disrupted by sonication. The cell was removed by centrifugation and the supernatant was incubated with pre-equilibrated nickel-NTA metal affinity resin. The non-specifically bound protein was removed by washing buffer and eluted by using elution buffer. Then purified and analyzed the homogeneity by gel filtration chromatography. The eluent fractions corresponding to the major peak observed in chromatogram were collected, pooled and concentrated in protein buffer. Each concentrated protein fraction was analyzed molecular weights by SDS-PAGE. The initial crystallization screening was performed by the microbatch under-oil method. The crystal growing was observed under the microscope for every 7 days. Finally, SAXS data from solution of PaMurI were performed at Synchrotron Light Research Institute (SLRI). The scattering data was analyzed by the

program ATSAS. Scattering pattern of the crystal structures of MurI in different stages was calculated by program CRYSOL and molecular weights were determined using PRIMUS.

Results and discussion:

Cloning and expression

PaMurI construct were overexpressed in *E. coli* as His-tagged protein. The protein was purified by affinity chromatography, PaMurI were highly soluble.

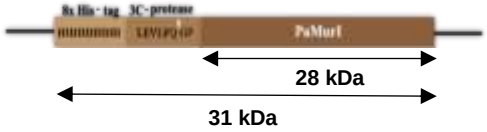

Fig.1 Fusion protein map of PaMurI

Purification

Initial SEC on an S75 10/300 GL column indicated that PaMurI showed a single peak from the chromatogram of greater than 95% purity protein (Fraction 29-33) as determined by SDS-PAGE.

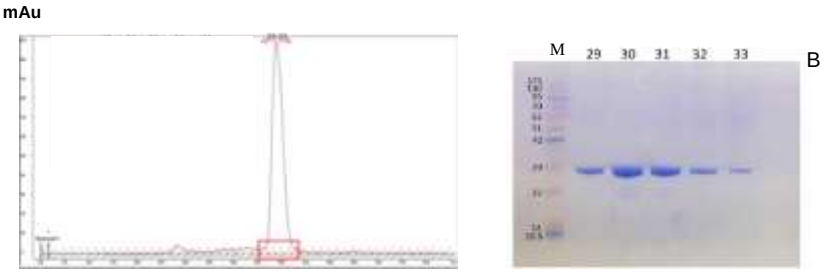

Fig.2 [A] Gel filtration chromatography of PaMurI.
[B] SDS-PAGE analysis of eluted fractions from Gel filtration chromatography. Protein molecular weight marker with 29-33 kDa are eluted fractions corresponding to the major peak

Crystallization screening of PaMurI

Precrystallization test suggested that 4 mg/ml PaMurI is suitable for crystallization screening. The small crystal protein was able to crystallize in 22% PEG 5000MME, 100 mM HEPES, pH 7.0 (Fig.3A) and in 27% PEG 5000MME, 100 mM Mes-NaOH, pH6.2 (Fig.3B). Those crystals were too small for the X-ray diffraction.

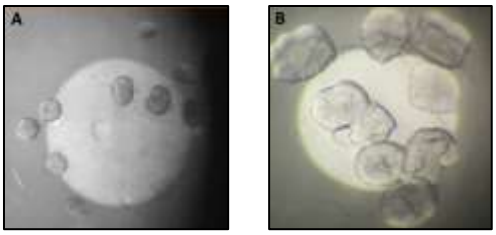

Fig.3 Initial screening of crystallization conditions of PaMurI, with equal volumes of protein and precipitation

Small angle X-ray scattering (SAXS)

The protein was severely aggregate in standard protein purification buffer (Fig.4A), suggested that the protein was unstable and very sensitive to the X-ray. Glycerol, Triton X-100, and supplement of metal ions (Na+) did not reduce the protein aggregation (Fig.4B). However, we found that L-glutamate concentration (4 mM) reduced the protein aggregation (Fig.4C) as observed in Guinier region (red square).

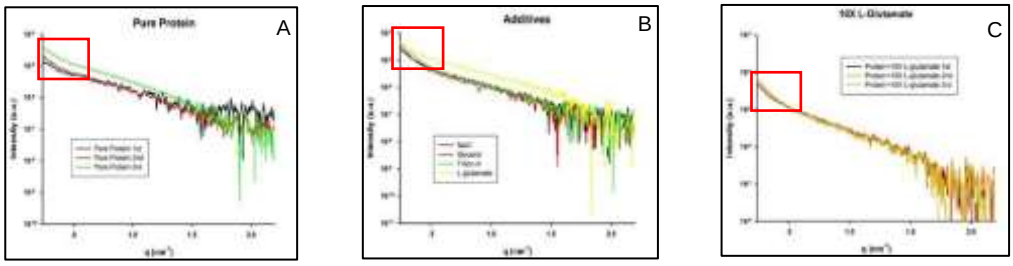

Fig.4 An X-ray scattering profile of PaMurI pooled of fraction 29-33 from gel filtration chromatography. [A] PaMurI in protein buffer (150 mM NaCl, 20mM tris-HCl, pH 7.5, 10% glycerol). [B] PaMurI in protein buffer and difference additives. [C] PaMurI in protein buffer and 10X L-glutamate.

Oligomeric state determination of PaMurI by SAXS

The protein samples (4, 6.5, and 10 mg/ml) used to determine whether there is any concentration-dependent intermolecular interaction. PaMurI gave normal scattering profiles with no sign of aggregation. Additionally, a Kratky analysis was conducted PaMurI exhibits a bell shape curve, indicating compact globular fold and flexibility with all samples. As a result, we used a sample concentration of 10 mg/ml for this research because it has the lowest noise. Guinier approximation (Fig.5C) showed that PaMurI had R_g = 2.62 nm and D_{max} = 8.78 nm (Fig.5D). The molecular weight of PaMurI was calculated as 34 kDa rather than 28 kDa.

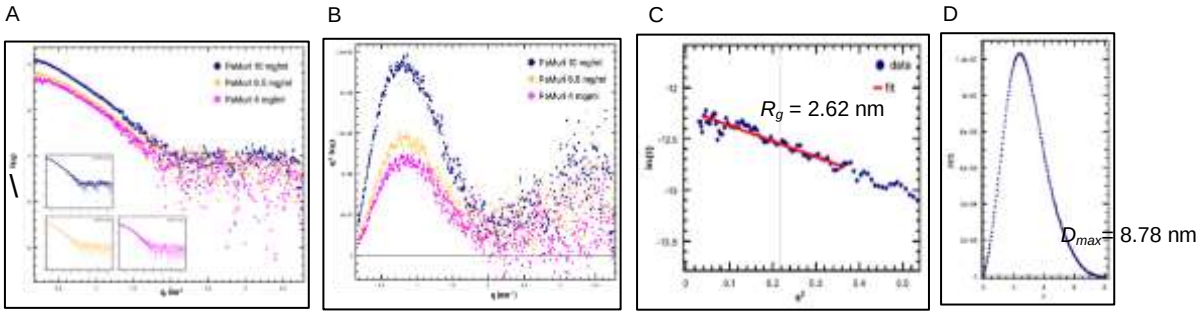

Fig.5 SAXS data of PaMurI. [A] Scattering patterns of PaMurI of 3 concentrations in 20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM D,L glutamate, 10% glycerol; [B] Kratky plot. [C] Guinier plot of 10 mg/ml. [D] Pair distribution functions, P(r) of 10 mg/ml.

The molecular model calculated by DAMMIF is shown as *ab initio* bead reconstructions (gray surface) in Figure 6. Superimposition of the crystal structure of MurI (PDB:1b73, pink) demonstrated that the molecular envelope (gray surface) was much larger than the single molecule of MurI.

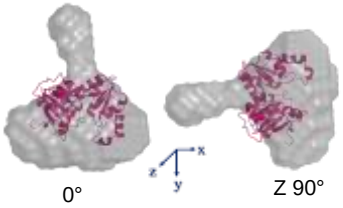

Fig.6 Averaged and filtered DAMMIF model (Gray surface) overlaid with the crystal structure (PDB:1b73, pink). Rotated model at 0 degree and at 90 degree on Z-axis.

Structural analysis by comparing the experimental scattering curve of PaMurI with the calculated scattering curves of MurI from the different crystal structures (Fig.7A). In order to monitor the structural changes of the molecule, showed that the scattering pattern of PaMurI from the experiment was not fit to the simulated scattering patterns calculated from the crystal structures of MurI in complex with the substrate. However, it is fit to the simulated scattering patterns substrate free MurI of *Aquifex pyrophilus* (ApMurI), PDB: 1b73. The superimposition of our ligand bound PaMurI shows a best fit with monomeric ligand free ApMurI with Chi^2 value of 0.99 as shown in Figure 7B, as indicated by the residuals that are relatively evenly distributed around 0 in the range of +/-4 (lower panel of Fig.7B).

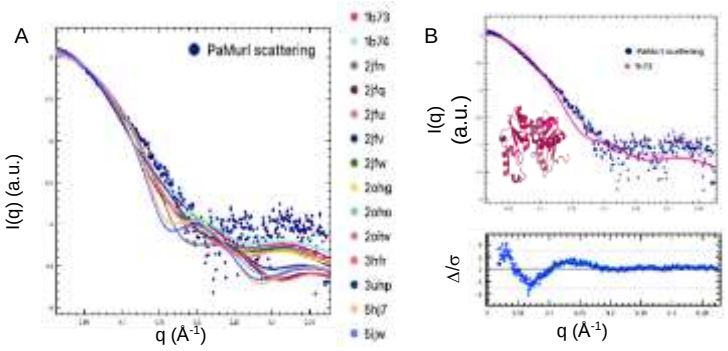

Fig.7 Structural analysis by CRYSOL. [A] Superimposition of the scattering model of the known crystal structures. [B] CRYSOL-derived models (pink solid lines) for MurI (PDB:1b73), fitted to *I(q)* versus *q*. The upper plot shows *log I(q)* versus *q*, while the lower inset plot is the error-weighted residual difference plot $\Delta/\sigma = [I_{exp}(q) - cI_{mod}(q)]/\sigma(q)$ versus *q*.

Conclusion and suggestion

The glutamate racemase encoding gene or murI of P. aeruginosa amplified by PCR was successfully cloned into an expression vector, pSY5. The SEC revealed a single peak chromatogram with a purity of 95% and a molecular weight of approximately 31 kDa, according to SDS-PAGE.

Crystallization screening of PaMurI showed that the small crystal protein was able to crystallize in 2 conditions. However, the X-ray diffraction quality is insufficient for structure determination. SAXS data showed that PaMurI displayed monomer in solution with the Rg = 2.62 nm and Dmax = 8.78 nm. The structural analysis demonstrated the different conformation between substrate bound (PaMurI) and substrate free (ApMurI). The results suggest that conformation changes of the MurI upon substrate binding is a part of enzyme activity, this may restrict other substrates (such as drug) accessing to the active site. The finding of this work which revealed structural characteristic of MurI as oligomerization and conformation can support researchers in the discovery and development of specific bacterial cell wall biosynthesis inhibitors.

Acknowledgements

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