Journal of Structural Biology 176 (2011) 419–424

Contents lists available at SciVerse ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Crystallization Note

AcrB contamination in 2-D crystallization of membrane proteins: Lessons from a sodium channel and a putative monovalent cation/proton antiporter

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ARTICLE INFO

Article history:
Received 30 June 2011
Received in revised form 14 September 2011
Accepted 15 September 2011
Available online 20 September 2011

Keywords:
Sparse matrix
Continuous-flow dialysis
Electron crystallography
2dx
Membrane proteins
Structural genomics
2-D Crystal

ABSTRACT

Contamination with the multidrug transporter AcrB represents a potential pitfall in the structural analysis of recombinant membrane proteins expressed in *Escherichia coli*, especially when high-throughput approaches are adopted. This can be a particular problem in two-dimensional (2-D) crystallization for electron cryomicroscopy since individual crystals are too small for compositional analysis. Using a broad 'sparse matrix' of buffer conditions typically used in 2-D crystallization, we have identified at least eight unique crystal forms of AcrB. Reference to images and projection maps of these different forms can greatly facilitate the early identification of false leads in 2-D crystallization trials of other membrane proteins of interest. We illustrate the usefulness of such data by highlighting two studies of membrane proteins in our laboratories. We show in one case (a bacterial sodium channel, NaChBac) how early crystallization 'hits' could be attributed to contaminating AcrB by comparison against our AcrB crystal image database. In a second case, involving a member of the monovalent cation/proton antiporter-1 family (MPSIL0171), a comparison with the observed AcrB crystal forms allowed easy identification of reconstituted AcrB particles, greatly facilitating the eventual purification and crystallization of the correct protein in pure form as ordered helical arrays. Our database of AcrB crystal images will be of general use in assisting future 2-D crystallization studies of other membrane proteins.

The major multidrug efflux pump of *Escherichia coli*, AcrB, is frequently found as a contaminant of membrane protein preparations (Psakis et al., 2009; Veesler et al., 2008). It has a propensity to bind, via its endogenous histidine-rich C-terminus, to metal affinity resins used in purification of His-tagged membrane proteins. Environmental stress has been identified as a factor in up-regulating transcription of the *acrB* gene (Ma et al., 1995). As a result, standard over-expression methods are likely to induce native AcrB expression.

Structures of detergent-solubilized AcrB have been determined by X-ray crystallography (e.g. Murakami et al., 2002, 2006; Seeger et al., 2006). There is also anecdotal evidence that investigations of other membrane proteins have been pursued as far as the final stage of structure determination only to reveal that the protein crystallized was AcrB (Veesler et al., 2008). Electron microscopy (EM) of membrane protein 2-D crystals is potentially vulnerable to the same problem. Indeed, small amounts of contaminating AcrB can present a greater problem since individual, microscopic crystals cannot be isolated for protein identification. If the protein sample is close to 100% pure and the crystal population is high, the experimenter can be confident they represent the protein of interest. However, if small amounts of other proteins remain as contaminants, the protein of interest within a given 2-D crystal cannot always be unambiguously identified. This is a particular concern when the crystals are relatively rare. It would be useful to have a diagnostic marker for the presence of contaminating AcrB in 2-D crystal studies of membrane proteins.

Here we report on attempts to crystallize two membrane proteins in 2-D; NaChBac, a voltage-gated sodium channel from *Bacillus halodurans* (Ren et al., 2001) and MPSIL0171, a member of the monovalent cation/proton antiporter-1 family from *Vibrio cholerae* (Brett et al., 2005). Both of these proteins appeared to give

Abbreviations: 2-D, two dimensional; 3-D, three dimensional; β-DM, n-Decyl-β-D-maltoside; β-DDM, n-Dodecyl-β-D-maltoside; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; EPLE, *E. coli* polar lipid extract; ETL, *E. coli* total lipid extract; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; LPR, lipid:protein ratio (w/w); MW, molecular weight.

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doi:10.1016/j.jsb.2011.09.005

promising early 'leads' in the crystal screening process. We demonstrate how these subsequently turned out to be false leads after comparison against 2-D trials of pure AcrB. We report on the crystallization of AcrB in 2-D under a broad range of conditions that reflect those typically used in 2-D trials. As part of this effort we have developed an array of conditions analogous to those used in sparse matrix screens of proteins for 3-D crystallization.

The NaChBac purification protocol (Suppl. Text) yielded a preparation of approximately 95% purity as judged by the optical density of the bands revealed in SDS–PAGE (Fig. 1A). Under a number of crystallization conditions, some based on conditions given in Table S2, we observed lipid vesicles containing randomly dispersed protein particles and occasionally small ordered patches (e.g. Fig. 1B). These exhibited sufficient order to process crystallographically (Fig. 1C), the result of which shows threefold symmetric objects with a triangular envelope. The presence of crystalline patches appeared to be correlated with the presence of a weakly stained SDS–PAGE band, of variable intensity between batches, at an apparent molecular weight of ~100 kDa (Fig. 1A). In this case, because the oligomeric state of the protein is known (Nurani et al., 2008), it was at first thought that this band arose from a small amount of tetrameric NaChBac (predicted MW of ~119 kDa). We should emphasize here that other protocols for NaChBac (Nurani et al., 2008; Powl et al., 2010), which included additional purification steps, never showed this band and did not contain AcrB.

In our earliest 2-D crystallization trials of MPSIL0171, similar protein particles were observed reconstituted into close-packed assemblies (Fig. 1D). A faint additional band was again observed on SDS–PAGE at ~110 kDa (Fig. 1E). The individual particles appeared to have a triangular appearance and single particle averaging indicated threefold symmetry (Fig. 1F) and dimensions similar to those found in the putative 'NaChBac crystals' (Fig. 1C). We were unable to reproduce these assemblies upon refinement of the

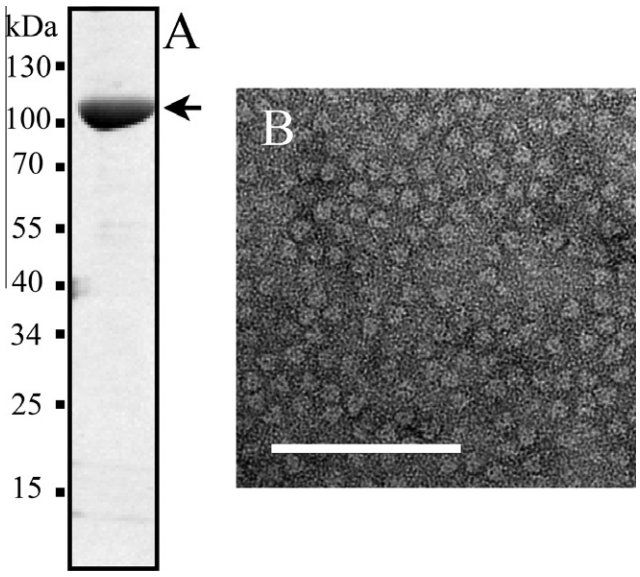

Fig. 2. (A) Coomassie stained SDS–PAGE of purified AcrB(His)$_8$, 5 μg total protein loaded. The strong band at ~110 kDa indicates AcrB(His)$_8$. (B) EM of purified solubilized AcrB(His)$_8$ in negative stain. Scale bar represents 100 nm.

purification protocol by replacing the initial Ni–NTA step with His-Pur (Pierce) cobalt resin (Suppl. Text). Pure MPSIL0171 (Suppl. S1C) was found to exclusively assemble into helical arrays (Suppl. S1A, B).

AcrB was cloned with a C-terminal His-tag and purified using affinity chromatography (Suppl. Text). SDS–PAGE (Fig. 2A), revealed a sharp band at ~110 kDa (predicted MW 114.6 kDa). AcrB's apparent mass on SDS–PAGE was found to vary depending on the gel system used (between 85 and 110 kDa, data not shown). EM of

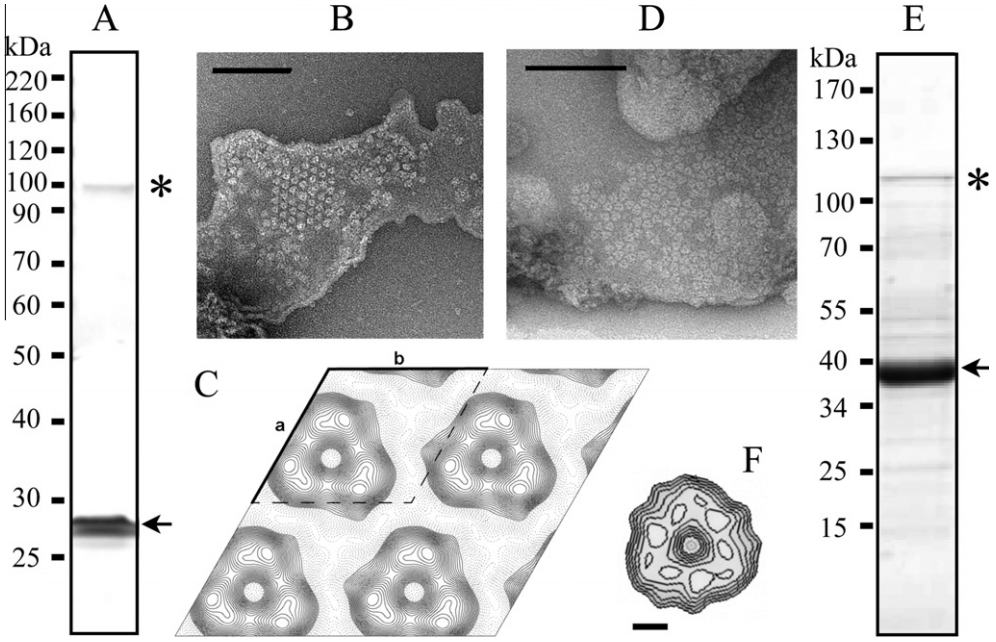

Fig. 1. (A) Coomassie stained SDS–PAGE of the NaChBac preparation used for 2-D crystallization trials. 13.2 μg total protein was loaded. NaChBac migrates as a single band at ~28 kDa (indicated by arrow). The band at ~100 kDa (*) is AcrB. (B) EM of a negatively stained 'NaChBac' crystal later confirmed to be AcrB. Crystallization buffer contained 20 mM Hepes pH 7.0, 100 mM NaCl, 5% glycerol, 100 μM mibefradil, 0.05% NaN_3, DOPC:DOPG lipids at 49:1 ratio and LPR 0.4 at 30 °C. Scale bar represents 100 nm. (C) Projection map of a typical crystalline patch obtained from putative 'NaChBac crystals', with *p*3 symmetry applied. Stain-excluding areas are represented by solid contours. One unit cell is outlined, $a = b = 136$ Å. (D) EM of a negatively stained lipid vesicle containing protein particles, from an early MPSIL0171 preparation. The particles were later confirmed to be AcrB. Crystallization buffer contained 20 mM Tris pH 7.0, 100 mM NaCl, 0.05% NaN_3, ETL at LPR 1.7. Scale bar represents 100 nm. (E) Coomassie stained SDS–PAGE of purified MPSIL0171, 1 μg total protein loaded. The arrow indicates MPSIL0171. The asterisk indicates AcrB migrating at approximately 110 kDa. (F) Averaged map of trimeric objects observed in initial MPSIL0171 2-D trials, calculated from 500 single particles. Scale bar represents 25 Å.