Analysis of AcrB and AcrB/DARPin ligand complexes by LILBID MS

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ABSTRACT

The AcrA/AcrB/TolC complex is responsible for intrinsic multidrug resistance (MDR) in Escherichia coli. Together with the periplasmic adaptor protein AcrA and the outer membrane channel TolC, the inner membrane component AcrB forms an efflux complex that spans both the inner and outer membrane and bridges the periplasm of the Gram-negative cell. Within the entire tripartite complex, homotrimeric AcrB plays a central role in energy transduction and substrate selection. In vitro selected designed ankyrin repeat proteins (DARPin) that specifically bind to the periplasmic domain of AcrB were shown to ameliorate diffraction resolution of AcrB/DARPin protein co-crystals (G. Sennhauser, P. Amstutz, C. Briand, O. Storchenegger, M.G. Grutter, Drug export pathway of multidrug exporter AcrB revealed by DARPin inhibitors, PLoS Biol 5 (2007) e7). Structural analysis by X-ray crystallography revealed that 2 DARPin molecules were bound to the trimeric AcrB wildtype protein in the crystal, whereas the V612F and G616N AcrB variant crystal structures show 3 DARPin molecules bound to the trimer. These specific stoichiometric differences were analyzed in solution via densitometry after microchannel electrophoresis, analytical ultracentrifugation and via laser-induced liquid bead ion desorption mass spectrometry (LILBID-MS). Using the latter technology, we investigated the gradual disassembly of the AcrB trimer and bound DARPin ligands in dependence on laser intensity in solution. At low laser intensity, the release of the detergent molecule micelle from the AcrB/DARPin complex was observed. By increasing laser intensity, dimeric and monomeric AcrB species with bound DARPin molecules were detected showing the high affinity binding of DARPin to monomeric AcrB species. High laser intensity LILBID MS experiments indicated a spectral shift of the monomeric AcrB peak of 3.1 kDa, representing a low molecular weight ligand in all detergent-solubilized AcrB samples and in the AcrB crystal. The identity of this ligand was further investigated using phospholipid analysis of purified AcrB and AcrB variant samples, and indicated the presence of phosphatidylethanolamine and possibly cardiolipin, both constituents of the Escherichia coli membrane.

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1. Introduction

Tripartite complexes are important toxic compound resistance determinants in Gram-negative bacteria. These complexes are composed of an energy transducing pump, residing in the inner membrane, a channel in the outer membrane and a periplasmic adapter protein that establishes a stable connection between the inner-membrane pump and the outer-membrane channel. In opportunistic pathogens like Escherichia coli and Pseudomonas aeruginosa, the multidrug resistance (MDR) pumps AcrAB-ToIC and MexAB-OprM, respectively, are responsible for resistance phenotypes against a great variety of different antimicrobial agents. AcrB and MexB represent the inner-membrane located pump in these complexes and belong to the large superfamily of Resistance Nodulation and cell Division (RND) transport proteins. These homologous proteins have been studied in detail on the functional [1–6] and structural level [7–10]. AcrA and MexA act as adapter proteins and stabilize binding of AcrB and MexB to ToIC and OprM, respectively [11].

Initial crystallographic data from AcrB protein crystals provided substantial structural insights and presented the protein as a homotrimer [12], in which all of the monomers adopt the same conformation. Later, trimerization was postulated to be the physical basis for a conformational cycling mechanism for drug efflux, on basis of recent crystal structures of asymmetric AcrB, which suggested that
each monomer can adopt different conformational states loose (L), tight (T) and open (O) [6–9]. Moreover, trimerization was also observed in the crystal structure of the AcrB homolog MexB from *P. aeruginosa* [10] and the CusA silver- and copper ion RND efflux protein [13]. Biochemical experiments with covalently linked AcrB monomers indicated that trimerization of AcrB is essential for its function and that inactivation of one monomer is sufficient to block the activity of the entire AcrAB-ToLC complex [14]. Ultracentrifugation provided evidence that AcrB is most likely trimeric in its detergent solubilized state as well [9,15]. Thus, trimerization is believed a general feature of proteins of the RND superfamily.

Designed ankyrin repeat peptides (DARPins) represent a novel antibody-like scaffold that is based on a consensus design of the ankyrin repeat motif [16]. In nature, the fundamental function of ankyrin repeat proteins is the establishment of specific protein–protein interactions [17]. Highly specific DARPins have been raised against a number of target protein using *in vitro* selection methods such as ribosome display [18] and phage display [19]. Selected high affinity binders have been used in co-crystallization experiments [20] and with AcrB, the first example of crystals for a membrane protein in complex with DARPins was obtained [9]. In the AcrB/DARPin co-crystal structure, two DARPins are bound to the periplasmic domain of the AcrB trimer. A single DARPin molecule binds to the surface of the loose (L) and tight (T) conformers each, but not to the open (O) conformer.

We address in this manuscript the interaction of DARPins with different variants of AcrB. During our X-ray crystallographic analysis of the AcrB/DARPin complexes we observed different stoichiometries and wondered if the protein/DARPin complex behavior is similar in solution. The method applied was laser-induced liquid bead ion desorption mass spectrometry (LILBID MS), which is a novel, native mass spectrometry, that allows detection of membrane protein complexes in a detergent-solubilized state. Depending on the laser intensity, entire protein complexes or contributing subunits can be observed [21–24]. Nano-ESI has also been applied to analyze membrane proteins [25], but the analysis of the spectra might be a challenge due to the highly charged ions and overlapping ion series, which might be solved by MS/MS and ion-chromatography. Apart from the stoichiometries, LILBID-MS gives us also insight in the stability of the DARPin/AcrB interaction, as well as the interactions (i.e. most likely the loop structure) of the monomers forming an AcrB trimer. Here, we analyze three different AcrB variants in detergent-solubilized and crystalline state, with and without bound DARPin molecules.

### 2. Materials and methods

#### 2.1. Construction of the AcrB mutants AcrB(dl), AcrB(V612F) and AcrB(G616N)

AcrB variants (AcrB(dl), AcrB(V612F) and AcrB(G616N)) were constructed by site-directed mutagenesis using the Quikchange parametric oscillator using LiNbO3 crystals and a neodymium-doped yttrium aluminium garnet and an Nd:YAG laser as pump. The wavelength of the idler component of the radiation of the optical parametric oscillator (OPO) is tuned to the absorption maximum of water at around 3 μm corresponding to an excitation of the stretching vibrations of water. At threshold intensity the droplet explodes resulting in the emission of ions from liquid into vacuum. There the ions are mass analyzed in a time-of-flight reflectron mass spectrometer. To detect very large biomolecules, a Daly type ion detector is available.

AcrB variants (AcrB(dl), AcrB(V612F) and AcrB(G616N)) were constructed by site-directed mutagenesis using the Quikchange protocol from Stratagene. In the AcrB(dl) (deleted loop) mutant amino acids 218–239 (QLGGTPPVKGQQLNASIIAQTR) were removed. Amino acid substitution and region deletion were achieved with 5′-phosphorylated primers. Cloning procedures were performed on the pET24 vector carrying the wildtype *acrB* gene as template (pET24acrB<sub>wt</sub>, [26]). The final constructs were verified by sequencing (Microsynth, Switzerland).

#### 2.2. Drug susceptibility assay

*E. coli* BW25113 ΔacrB carrying pET24acrB<sub>his</sub> (AcrB wt), pET24acrB<sub>dlhis</sub> (AcrB(dl)) and pET24 (empty vector) were used for minimal inhibitory concentration (MIC) experiments as described earlier [3].

#### 2.3. Protein expression and purification

Overexpression and membrane preparation of AcrB<sub>wt</sub>, AcrB<sub>V612F</sub>, AcrB<sub>dl</sub> and AcrB<sub>G616N</sub> was carried out as previously described [7] with modifications. During solubilization and purification only the detergent n-dodecyl-β-maltoside (DDM, *M<sub>c</sub>* 510.9, GLYCON Biochemicals GmbH,Luckenwalde, Germany) was used throughout the procedure. Membranes were solubilized in 20 mM Tris/Cl, 150 mM NaCl, 10 mM imidazole, 10% glycerol and 25 mM DPH, pH 7.5 (15–20 ml total volume). The detergent-solubilized fraction was separated from insoluble material by centrifugation at 145,000 × g at 4°C for 1 h and was subsequently applied to a Ni<sup>2+</sup>–nitrilotriacetic acid (NTA)–agarose column (2 ml bed volume, Qiagen) pre-equilibrated with 20 column volumes (CV) of buffer 1 (20 mM Tris/Cl, 150 mM NaCl, 10% glycerol and 0.03% DDM, pH 7.5). The column was washed with 45 CV buffer 1 and subsequently washed with 30 CV buffer 2 (20 mM Tris/Cl, 150 mM NaCl, 50% imidazole, 10% glycerol and 0.03% DDM, pH 7.5). AcrB was eluted in 6 ml of buffer 3 (20 mM Tris/Cl, 150 mM NaCl, 200 mM imidazole, 10% glycerol and 0.03% DDM, pH 7.5). Purified DARPin (inhibitor 1108_19 [9]) were added to the purified AcrB in a two-fold molar excess (to AcrB monomer). After concentration in centrifugal filter devices with a cutoff size of 100 kDa (Amicon Ultra, Millipore), AcrB/DARPin protein mixture (0.7 ml) was subjected to size exclusion chromatography in buffer 4 (20 mM Tris/Cl, 150 mM NaCl, 0.03% DDM, pH 7.5) using a GE Superdex 200 10/300GL column connected to a GE Healthcare ÄKTAprime system. Peak fractions corresponding to AcrB (trimer)/DARPin complexes were collected and concentrated in centrifugal filter devices with a cutoff size of 100 kDa (Amicon Ultra, Millipore) to 10 mg ml<sup>−1</sup>. For the gel filtration run shown in Fig. 2, AcrB and AcrB<sub>dl</sub> were purified as above, but with 0.05% cyclohexyl-hexylmaltoside as detergent. Subsequent size-exclusion chromatography was performed using a GE HiLoad 16/60 Superdex 200 (120 ml pack size) with 10 mM Na-HEPES, pH 7.0, 200 mM NaCl, and 0.05% cyclohexyl-hexylmaltoside as running buffer at 0.4 ml min<sup>−1</sup>. Apparent molecular weight values were calculated on basis of a run under identical conditions with 4 marker proteins (Ferritin (440 kDa), Albumin (67 kDa), Ovalbumin (43 kDa), Ribonuclease A (13.7 kDa)). Protein concentration was determined using the absorption at 280 nm (Nanodrop ND-1000 spectrophotometer) or using the colorimetric BCA protein determination method (Pierce).

#### 2.4. LILBID MS

Before subjecting the samples to LILBID MS, a buffer exchange was carried out on purified AcrB<sub>wt</sub>, AcrB<sub>variants</sub> and the AcrB/DARPin complexes into 30 mM NH₄HCO₃, pH 7, 0.05% DDM. AcrB/DARPin co-crystals were dissolved in 20 mM Tris/Cl, pH 7.5 containing 0.05% DDM. Insoluble particles were removed by filtration through a Milliplex-GV syringe-driven filter unit (0.22 μm, Millipore). The final protein concentration was 10 μM with respect to the AcrB monomer.

Details of the experimental set-up for LILBID MS have been published previously [27]. Micro-droplets of sample solution (diameter: 50 μm) are produced at 10 Hz by a Piezo-driven droplet generator and introduced into vacuum via differential pumping stages. There the droplets are irradiated one by one by synchronized high-power mid-infrared laser pulses. These are generated in a home-built optical parametric oscillator using LiNbO₃ crystals and a neodymium-doped yttrium aluminium garnet and an Nd:YAG laser as pump. The wavelength of the idler component of the radiation of the optical parametric oscillator (OPO) is tuned to the absorption maximum of water at around 3 μm corresponding to an excitation of the stretching vibrations of water. At threshold intensity the droplet explodes resulting in the emission of ions from liquid into vacuum. There the ions are mass analyzed in a time-of-flight reflectron mass spectrometer. To detect very large biomolecules, a Daly type ion detector is available.
used, working up to an m/z range in the low MDa region. At low laser intensity, LILBID desorbs ions out of the liquid very gently (ultrasoft mode) enabling detection of the non-covalently assembled protein complexes. At higher laser intensity (soft mode) the strong interactions are still kept, the weak ones are broken. At the highest laser intensities the complex is thermolyzed into its covalent subunits (harsh mode). The signals from the detector are recorded by a transient recorder. For data acquisition and analysis a user-written labview program is used. The signal to noise ratio is improved by subtracting an unstructured background, caused by metastable loss of water and buffer molecules, from the original ion spectra. These difference spectra are smoothed by averaging the signal over a pre-set number of channels of the transient recorder.

2.5. Capillary electrophoresis

Protein samples (AcrB wt and AcrB_V612F) co-purified with DARPins via SEC were analyzed on a 2100 Bioanalyzer (Agilent Technologies Inc.) using the Agilent Protein 230 chips. Samples (approx. 10 mg ml⁻¹, 4 μl) were prepared according to the manual with omission of the sample heating step. Relative concentrations of AcrB and DARPin fractions were calculated from the integrals of the eluted protein peak areas in the recorded electropherograms by the software 2100 Expert (Agilent Technologies Inc.).

2.6. Phospholipid analysis via thin-layer chromatography

Phospholipid analysis of AcrB wt and AcrB_V612F samples was done by thin-layer chromatography. Reference phospholipids (E.coli polar lipids, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), cardiolipin, (Avanti Polar Lipids, Inc.) in 20 mM Tris/Cl, 150 mM NaCl, 0.03% DDM, pH 7.5) with an OD280 of 1,4 were applied to an An50-Ti analytical rotor (Beckman Coulter) in a chromatography tank and air dried in a fume hood for several hours. For data acquisition and analysis the highly sensitive LILBID MS as method of choice to determine the oligomeric state of AcrB (theoretical Mw of His-tagged monomer: 114.6 kDa) in detergent solution (Fig. 1). Under ultrasoft laser condition, the AcrB trimmer (charge state 2–6) is observed together with its detergent micelle, causing a broad peak ranging from approximately m/z ≈ 190 to m/z ≈ 225 with a flat maximum at m/z ≈ 215 (Fig. 1E, the second hump in Fig. 1C and 1D is assigned to an AcrB dimer in a micelle). This corresponds to a size distribution of the micelles of 70 kDa and an average size of 50 kDa. Approximately the same distribution is observed for the (AcrB₃ + micelle)³⁻ peak. With a molecular weight of DDM of 510.9 Da this translates into an average aggregation number of 98 and a maximal aggregation number of 140. The correspondence with the values from the DDM provider is excellent. With increasing laser intensity, the distribution shifted to detergent-free trimeric AcrB (apparent Mw: 355 kDa) (Fig. 1B–D). The estimated detergent micelle from these experiments would be 70 kDa, close to the size of a dodecylmaltoside detergent micelle in solution [30]. At the highest laser intensity used, mainly monomeric AcrB (apparent Mw: 116 kDa) was observed, but still a detectable amount of di- and possibly trimERIC AcrB were present in the sample (Fig. 1A). This extraordinary stability appears to be quite unique compared with other membrane protein complexes tested with LILBID MS [21,22,31]. The AcrB trimer contains an intermonomeric loop structure, protruding from one monomer to the neighboring monomer, which might be the determinant for the observed stability of the trimeric complex. Indeed, deletion of the loop resulted in the formation of mainly monomeric AcrB (apparent Mw: 116 kDa) in detergent solution (Fig. 2) and Escherichia coli harboring the deleted loop gene mutant of AcrB ([AcrB_dil]) in a ΔacrB background led to an increased sensitivity towards drugs (Table 1). Notably, activity was not reduced completely, indicating that the role of the loop region could be an exclusive structural one. Since the deleted loop variant of AcrB is still able to form trimeric assemblies (Fig. 2, apparent Mw: 267 kDa), it appears that trimerization is mandatory for the activity of AcrB, as has been postulated based on biochemical and structural data [6–8,14].

3.2. DARPin binding to AcrB and AcrB variants

AcrB-specific DARPins were selected in vitro using ribosome display, which facilitated the formation of well-diffusing (to 2.5 Å) crystals of AcrB [9]. One AcrB trimer containing two bound DARPin molecules was present in the asymmetric unit. This 3:2 (AcrB monomer to DARPin) ratio was also found in detergent solution using analytical ultracentrifugation [9].

Initial crystallographic studies of AcrB variants, which have substitutions near the drug binding pocket located in the periplasmic domain of the periplasmic part of AcrB (i.e. V612F and G616N), revealed cubic crystal forms (I23) with one AcrB monomer and one DARPin molecule in the asymmetric unit (Eicher, Cha et al., unpublished). A symmetric setup would imply that DARPin/AcrB trimer stoichiometry would have been shifted from 2 in the case of the AcrB variants. To distinguish if the different stoichiometries are only observed in crystalline state or can also be detected in solution, we analyzed the samples using LILBID-MS, capillary electrophoresis and analytical ultracentrifugation.

3.3. LILBID-MS of AcrB/DARPin complexes

For the AcrB wt/DARPin complex and the V612F/DARPin complex, similar spectra were obtained under high intensity laser conditions (Fig. 3) indicating that 2 or more DARPin molecules bind to the AcrB complex...
trimer. For the AcrB_G616N variant, the AcrB monomer and DARPin stoichiometry was determined to be 3 (Fig. 3), indicating a symmetric AcrB:DARPin structural complex commensurate to the threefold symmetry found in the AcrB_G616N crystals. Notably, Monroe et al. [32] studied binding of different DARPin variants to AcrB via surface plasmon resonance and showed that flexible stoichiometries are found indicating substantial plasticity of the AcrB/DARPin interaction.

The spectra of all three AcrB variants also revealed a broad peak which might represent an AcrB monomer containing 2 bound DARPin molecules, in contrast to what has been found in the crystal structure [9]. This observation can be best explained by the formation of DARPin dimers via a DARPin/DARPin interface and under ultrasoft laser intensities 4 DARPin molecules are attached to both the AcrB wt and V612F variant (Fig. 4A and B). When using dissolved AcrB/DARPin crystals (space group P2_12_12) for measurement with LILBID-MS (Fig. 5), the only stoichiometry which could be detected was the one observed in the crystal structure i.e. two DARPin bound per AcrB wt trimer. Under ultrasoft laser intensities, the LILBID MS spectrum of the AcrB masses shifted to higher molecular weight species of 460 kDa, representing the AcrB/DARPin complex of 380 kDa (2 DARPin per AcrB trimer) and a detergent micelle of almost 80 kDa, comparable to the difference in molecular weight found in the soluble AcrB preparation (Fig. 1).

3.4. DARPins stoichiometry from capillary electrophoresis

Complex formation between AcrB wt and AcrB_V612F with DARPin molecules was analyzed by co-elution using size-exclusion chromatography and further investigation on AcrB/DARPin

| Drug susceptibility of E. coli BW25113 ΔacrB expressing wt and mutant AcrB.4 |
|-----------------|--------|---------|----------|----------|
| Ery             | Oxa    | TPP      | Novo     | Cipro    |
| AcrB wt         | 32-64  | 64       | 200-400  | 16-32    | 0.004-0.008 |
| No AcrB         | 2      | 4        | 6.25     | 8        | 0.002      |
| AcrB_dl         | 4      | 4        | 50       | 8        | 0.002      |

* Numbers indicate the minimal inhibitory concentration in μg ml⁻¹. Abbreviations: Ery = Erythromycin, Oxa = Oxacillin, TPP⁺ = Tetraphenylphosphonium, Novo = Novobiocin, Cipro = Ciprofloxacin, AcrB wt = AcrB wildtype (pET24acrBHis), No AcrB = pET24 vector, AcrB_dl = AcrB with deleted loop (pET24acrB_dlHis).
stoichiometry of the peak fractions via an Agilent Lab-on-a-Chip capillary electrophoresis system 2100 Bioanalyzer. Electropherograms of AcrB/DARPin complexes were used for the determination of the concentrations of AcrB and DARPin by integration of the elution peak areas. Combined data from independent experiments (n = 5 and n = 6 for AcrB wt and AcrB_V612F, respectively) clearly show a significant stoichiometric difference in DARPin binding between the AcrB wt and the AcrB_V612F variant, indicating binding of 2 DARpins to the AcrB wt trimer and binding of 3 DARpins in the case of AcrB_V612F (Fig. 6). Whereas this data is in accordance to the observations in the crystalline assembly of the AcrB wt/DARPins and AcrB_V612F/DARPin complexes, it deviates from the LILBID measurements where higher stoichiometries of AcrB/DARPin formations were observed. However, the samples subjected to capillary electrophoresis were used directly after elution from the gel filtration column, compared to the samples used for LILBID MS which were subjected to buffer exchange and a concentration step, possibly favoring DARPin-DARPin interaction.

3.5. Analytical UC of AcrB_V612F/DARPin complex

Solubilized AcrB_V612F (trimer fraction after SEC) and AcrB_V612F/DARPin (after SEC) complexes were subjected to analytical ultracentrifugation and sedimentation velocity analysis. A distinct sedimentation velocity behavior between AcrB_V612F with and without DARPin could be observed (Fig. 7). The calculated difference in molecular weight of AcrB_V612F (373 kDa) and AcrB_V612F/DARPin complex (427 kDa) could be attributed to the mass of three DARPin molecules (theoretical molecular weight: 18.3 kDa). The difference in molecular weight between AcrB wt without (374 kDa) and DARPins bound complex (408 kDa) was explained by the binding of two DARPin molecules per AcrB wt trimer [9].
3.6. Small molecule ligand and phospholipid analysis

The consistent observation of a double peak in all of the LILBID MS spectra taken in the harsh mode (Figs. 3A and 5A) representing masses 115.6 kDa and 118.7 kDa was interpreted as a result of a stripping off (a) small molecule(s) with a (total) mass 3.1 kDa from the AcrB monomer. This additional mass was also observed in the spectra of dissolved AcrB crystals, but has not been observed in the electron density derived from any of the wildtype or variant AcrB crystal. However, AcrB wt, AcrB_V612F and their DARPin complexes crystallize in the presence of DDM (MW 511 Da). In the AcrB wt/DARPin co-crystal structure, 10 molecules of dodecyl-β-D-maltoside and 1 molecule of dodecyl-α-D-maltoside bound to the trimer are reported [9]. The spectral shift is observed on the AcrB monomers, suggesting that binding of the molecule(s) representing this 3.1 kDa mass is much stronger than the already strong binding of the individual monomers constituting the AcrB trimer. Although a sum of 6 molecules of DDM per monomer would account for the observed mass, the observed “all or nothing” shift of 3.1 kDa mass on the monomer peaks would represent similar affinities for all six DDM molecules to AcrB, which appears unlikely. Moreover, the binding of molecule(s) representing this

![Figure 5](image_url)

Fig. 5. Anion LILBID spectra of AcrB/DARPin complex from dissolved orthorhombic crystals. The laser intensity decreases from the spectrum A (harsh) to the spectrum E (ultrasoft). The bars colored in the blue range (spectrum B) represent charge distributions of the AcrB monomer with none or one DARPin bound, the bars in the green range (spectrum B) represent charge distribution of AcrB dimers with none, one or two DARPins bound and the red-colored bars (spectra B–D) represent charge distributions of the AcrB trimers with none, one or two DARPins bound. The grey bars in spectrum E represent a charge distribution of the trimer with detergent (micelle), the peak maxima correspond to a mass of 460 kDa.

![Figure 6](image_url)

Fig. 6. The molar ratio of DARPins per AcrB trimer (ordinate) is plotted for wildtype AcrB and AcrB_V612F (indicated on the abscissa). The mean ratios are 2.26 (white bar) and 2.83 (dotted bar) DARPin per AcrB wt and AcrB_V612F variant, respectively. Errors are calculated as standard error of the mean (SEM). Unpaired t-test resulted in a p-value of 0.022, thus imply a significant difference of relative amount of DARPins bound to AcrB wt and AcrB_V612F.

![Figure 7](image_url)

Fig. 7. Sedimentation velocity experiments with AcrB_V612F with and without bound DARPin after SEC. The calculated mass difference between AcrB_V612F trimers with (dotted line, 426.6 ± 1.9 kDa) and without DARPin (solid line, 373.1 ± 1.7 kDa) corresponds to the mass of three DARPin molecules.
spectral shift are clearly distinct from the initial stripping of the detergent micelle, which occurs at very low laser intensities and before the release of AcrB monomers from the trimeric assembly. The unsatisfactory explanation concerning the identity of the 3.1 kDa spectral shift triggered the analysis of the presence of phospholipids in our AcrB samples. During the purification process, AcrB is gradually removed from the bacterial membrane and its hydrophobic domains are integrated into detergent/lipid micelles. Phospholipids originating from bacterial membranes have often been reported to be co-purified with integral membrane proteins [33,34].

We initiated thin-layer chromatography (TLC) analysis with purified samples of AcrB wt and AcrB_V612F to qualitatively identify lipids which might have been co-purified. A number of phospholipid standards, phospholipid mixtures, DDM solution and sample buffer were run in parallel to the protein samples. The chromatography plates then were stained with iodine (Fig. 8) and molybdatophosphoric acid solution (not shown). The chromatography pattern of the two protein samples (AcrB wt and AcrB_V612F) were basically identical (Fig. 8, lanes 8 and 9). Two distinct spots are visible that may represent phospholipids. The signal with the lower retention factor corresponds with the migration pattern of DDM or cardiolipin, the latter being an E. coli lipid component. The high retention factor signal observed in the lanes containing protein samples (Fig. 8, lanes 8 and 9) may represent phosphatidylethanolamine (PE), commensurate to the samples in lane 1 (polar lipid extract of E. coli) and lane 4 (dioleoyl-phosphatidylethanolamine, DOPE), Considering the mass of each of the potential ligands which could give rise to the signal, several combinations of each of the detected molecules could explain the spectral shift of 3.1 kDa e.g. 6 molecules of DDM (Mₜ 511 Da), or a combination of cardiolipin (1435.9 Da), PE (743.5 Da) and DDM. The exact combination and ligand identities remain, however, elusive.

4. Conclusions

We analyzed three different AcrB variants in detergent-solubilized and crystalline state, with and without bound DARPin molecules. The AcrB/DARPin complex stoichiometry differs depending on the AcrB variant used, consistent with preliminary crystallographic data on AcrB variant/DARPin co-crystals. Moreover, AcrB trimerization is due to strong binding of the monomers, most likely via the periplasmic loop (amino acids 218–239) protruding from one AcrB monomer into the neighboring monomer. DARPin binding appears to be even stronger than the interaction between the AcrB monomers, since interaction of DARPins is observed to trimeric, dimeric and mono-meric AcrB. We also observed the consistent release of a 3.1 kDa mass after high intensity laser exposure, which might represent very strong binding of detergent and/or lipids.

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