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Name of the PD/PI: Schmidt-Krey, Ingeborg

Final Progress Report

Summary of progress made toward the achievement of the originally stated aim

The funded aim targeted functional studies of human leukotriene C_4 synthase (LTC_4S) via structural studies requiring over-expression and purification of wild-type and mutant human LTC_4S, two-dimensional (2D) crystallization, inhibition and augmentation of enzymatic function, and electron crystallography.

Over-expression and purifiction of wild-type and mutant protein: The fission yeast *Schizosaccharomyces pombe* was successfully employed for the over-expression and purification of the wild-type human LTC_4S and its mutants R30L, R31A, and R104A. Previously we had shown that the wild-type human enzyme could be purified via one-step S-hexylglutathione affinity column chromatography. The resulting protein was active and could be crystallized in 2D arrays, which were frequently several microns in diameter and highly ordered (Schmidt-Krey *et al.*, 2004, Zhao *et al.*, 2010). As the R31A and R104A mutants show significant reduction in function, a Ni-NTA affinity column, rather than the S-hexylglutathione affinity column, was employed for purification. This change of column was also applied in the purification of the wild-type enzyme, when newer batches of S-hexylglutathione affinity column beads appeared to contain less S-hexylglutathione and thus resulted in significantly lower purification yields. Both wild-type and mutant human LTC_4S could be purified reproducibly in the milligram-range with the Ni-NTA affinity column.

2D crystallization: To induce 2D crystallization, the detergent-solubilized (1% Triton X100), purified enzyme was mixed with dimyristoyl phosphatidyl choline (DMPC) at low lipid-to-protein (LPR) ratios and dialyzed against detergent-free buffer. By optimization of the washing step of the column, additional removal of the lipid from the purified protein provided further control of the LPR (Johnson & Schmidt-Krey, in preparation). Initial batches of purified protein resulted in 2D crystals at unusually low LPRs, indicating that lipid must have been co-purified (Zhao *et al.*, 2010). Additional washing steps during the purification allowed for a higher concentration of lipid to subsequently be added for crystallization. The thoroughly washed protein at higher LPRs for 2D crystallization, resulted in the same 2D crystal form, morphology, and high crystal quality as previously observed. While occasional adjustment of the LPR was necessary in a newly purified protein batch, this now consistently ranges closely around LPR 15 for the wild-type and mutant enzyme. Initially small ordered arrays of the mutants were observed within larger membranes, which could then be increased significantly in size by lowering the LPR, the same approach that was successful for the wild-type enzyme. Activity measurements of the newly purified and solubilized LTC_4S as well as the protein after 2D crystallization, regardless of LPR for samples where membranes were observed, corresponded to previously established activity levels. Incubation of wild-type LTC_4S 2D crystals with Mg^{2+}, which increases activity, and *N*-ethylmaleimide, which inhibits activity, demonstrated that the 2D crystals retain order, which is a critical step towards cryo-EM data collection (Johnson *et al.*, unpublished results).

While we had previously observed some stacking of 2D crystals of LTC_4S, this appeared to occur mostly over time during storage, and could be circumvented for data collection by either selecting single-layered 2D crystals of newly prepared crystals, or by storage of freshly-made

2D crystals in a -80°C freezer. A major obstacle appeared when all recent purification batches resulted in a large number of stacked crystals during and after dialysis. Neither biochemical manipulation during the purification, during dialysis, nor after dialysis could reduce or eliminate the stacks. We were, however, successful with a new cryo-EM grid preparation protocol we developed to 'unstack' and thus reduce stacked crystals of LTC_4S to mostly single layers, corresponding to one lipid bilayer containing the protein 2D crystal.

Data collection: While single-layered LTC_4S 2D crystals can now be prepared easily and reproducibly, in the fall of 2013 we experienced an unexpected delay due to installation of direct electron detectors on our collaborators' cryo-EMs. This is proving overall very beneficial though as the time of data collection is greatly reduced (days vs. months) since the enormous effort that previously had to be invested in the collection of images from tilted samples appears to have been overcome with the combination of direct electron detectors and automated drift correction (Scherer *et al.*, 2014). In addition, we are taking a two-pronged approach for the structure-function studies with both electron crystallography and microED (Shi et al., *2013*).

List of significant results

1. The over-expression and purification of mutants R30L, R31A and R104A of LTC4S was successful and reproducible.
2. Mutants R31A and R104A, which show significantly decreased activity, can be induced to form 2D crystals comparable to wild-type LTC_4S 2D crystals (Zhao *et al.*, 2010; Johnson *et al.*, 2010; 2013; Johnson & Schmidt-Krey, in preparation).
3. Wild-type LTC_4S 2D crystals retain order upon incubation with Mg^{2+}, which increases acitivity, and *N*-ethylmaleimide, which inhibits activity (Johnson *et al.*, unpublished results).
4. A significant set-back occurred when LTC_4S formed a large majority of stacked 2D crystals, which at that time could not be analyzed.
5. A new protocol was developed for LTC_4S, to reduce the stacked LTC_4S 2D crystals to single bilayers suitable for data collection (Johnson & Schmidt-Krey, in preparation).

Data sharing

Any resulting data will be deposited in the PDB and the EMDB.

List of publications

Zhao, G., Johnson, M.C, Schnell, J.R., Kanaoka, Y., Irikura, D., Lam, B.K., Austen, K.F. and Schmidt-Krey, I. (2010) *J. Struct. Biol.* 169, 450-454. PMID: 19903529

Johnson, M.C., Rudolph, F., Dreaden, T., Zhao, G., Barry, B.A. and Schmidt-Krey, I. (2010) *J. Vis. Exp.* 44. PMID: 21085097

Schmidt-Krey, I. and Rubinstein, J.L. (2011) *Micron,* 42, 107-116. PMID: 20678942

Johnson, M.C., Dreaden, T.M., Kim, L.Y., Rudolph, F., Barry, B.A., and Schmidt-Krey, I. (2013) *Methods in Molecular Biology*. 955, 31-58. PMID: 23132054

Dreaden, T.M., Metcalfe, M., Kim, L.Y., Barry, B.A. and Schmidt-Krey, I. (2013) *Methods in Molecular Biology.* 955, 73-102. PMID: 23132056

Johnson, M.C. and Schmidt-Krey, I. (2013) *Meth. Cell Biol.* 113, 325-337. PMID: 23317909

Johnson, M.C. and Schmidt-Krey, I. The main bottleneck in 2D crystallization of membrane proteins: Taking control to lower the lipid-to-protein ratio of human leukotriene C_4 synthase. *In preparation.*

Johnson, M.C. and Schmidt-Krey, I. Towards a general protocol for the preparation of single-layered two-dimensional crystals with sheet morphology for electron crystallography. *In preparation*.