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Abstract

Leukotrienes are specialized lipid molecules derived from arachidonic acid that have severe pathological roles in inflammatory diseases like asthma, allergy and in the formation of cardiovascular diseases. Since these diseases can be fatal if not treated, it is significant to study leukotriene products and how they form. The formation of leukotrienes from arachidonic acid is a multi step process. This event is aided by different enzymes. Two such enzymes are 5 – lipoxygenase (5-LO) and 5 - lipoxygenase activating protein (FLAP). FLAP is an integral membrane protein and belongs to the MAPEG super family (Membrane Associated proteins in Eicosanoid and Glutathione metabolism). In the leukotriene synthesis pathway, the only function of FLAP is to increase the oxygenation reaction by assisting the transfer of arachidonic acid from nuclear membrane to 5-LO. In fact, FLAP doesn't seem to have any enzymatic/mechanistic activity on its own. Many biological questions in this pathway are unanswered. Is there any functional activity of FLAP? Is there any physical contact between FLAP and 5-LO in the leukotriene formation? To address these questions, we introduced a novel method of studying the pathway by constructing nanodiscs that are small soluble pieces of membrane of defined sizes. The aim of the project is to develop a protocol for generation of nanodiscs that contain FLAP (so called reconstitution of FLAP) and to verify the presence of FLAP in the nanodiscs by practical techniques such as blue native PAGE and Western Blot. Images from transmission electron microscopy were less conclusive but improvement can be done in future by use of antibodies to visualize FLAP nanodiscs. Ultimately, reconstitution of FLAP was successfully completed for further structural and functional studies. To conclude, this project paves way for the studies of complex formation of integral membrane proteins with soluble proteins and further applications on pharmaceutical front.

Keywords: FLAP, 5-LO, nanodiscs.

Abbreviations

S.No	Abbreviations	Acronyms
1	Adenosine Tri phosphate	ATP
2	Adenosine Mono phosphate	AMP
3	Charged coupled device	CCD
4	Cytosolic Phospholipase A_2	$CPLA_2$
5	Cysteinyl leukotrienes	$CysLT_1$
6	Dimyristoyl phosphatidylcholine	DMPC
7	Dipalmitoylphosphatidylcholine	DPPC
8	Ethylene-di-amine tetra aceticacid	EDTA
9	Five lipoxygenase activating protein	FLAP
10	High Density Lipo protein	HDL
11	5-Hydroperoxy eicosatetraenoic acid	5-HPETE
12	Isopropyl β-D-thiogalacto-pyranoside	IPTG
13	5-Lipoxygenase	5-LO
14	Leukotriene A_4	LTA_4
15	Leukotriene B_4	LTB_4
16	Leukotriene C_4 synthase	LTC_4S
17	Lipid to protein ratio	LPR
18	Lysogeny broth	LB

19	Lysogeny broth agar medium	LB agar medium
20	Membrane scaffold protein	MSP ($_1E_3D_1$) MSP ($_2N_2$)
21	His tag cleaved MSP ($_1E_3D_1$)	MSP $(_1E_3D_1)^-$
22	Membrane associated proteins in Eicosanoid and Glutathione super Family	MAPEG
23	Molecular weight cut off	MWCO
24	N-Dodecyl-β-maltoside	DDM
25	Nickel immobilized metal affinity Chromatography	NI-IMAC
26	Poly acryl amide gel electrophoresis	PAGE
27	*Pichia Pastoris*	*P.Pastoris*
28	Phosphotungstic acid	PTA
29	Sodium dodecyl sulphate	SDS
30	Superoxide dis mutase	SOD
31	Tobacco Etch Virus	TEV
32	Terrific broth	TB
33	Uranyl acetate	UrAc
34	Uranyl formate	UrFo

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Aim

This thesis project is mainly aimed at the generation of a protocol for the formation of nanodiscs containing the transmembrane 5–lipoxygenase activating protein (FLAP) and verification of the presence of FLAP in these so called reconstituted FLAP nanodiscs.

Introduction

Leukotrienes are inflammatory mediators involved in many clinical diseases such as atherosclerosis, asthma and allergy (1). Leukocytes have the capability for the complete biosynthesis of leukotrienes from arachidonic acid (fig 1a) in a biosynthetic pathway that can be activated by a variety of external stimuli such as antigens, oxidants and immune complexes (20). Briefly, immunal or allergic responses trigger an increase in the intracellular concentration of calcium that promotes the transfer of Cytosolic phospholipase A_2 ($CPLA_2$) and 5 Lipoxygenase (5-LO) to the surface of the nuclear membrane (12). $CPLA_2$ initiates the pathway by accelerating the release of arachidonic acid stored in the nuclear membrane lipids for the conversion to 5-hydroperoxy eicosa tetraenoic acid (5-HPETE) that in turn is converted to Leukotriene A_4 (fig 1b). These conversions are mainly aided by 5-LO and FLAP (fig 1b) (1, 2). LTA_4 is further involved in the formation of Leukotriene B_4 catalysed by LTA_4 hydrolase (1). On the other hand, LTC_4 synthase can conjugate LTA_4 with glutathione to form LTC_4 and successive cleavage yields Leukotriene D_4 and Leukotriene E_4 (fig 1b). The last three products such as LTC_4, LTD_4 and LTE_4 together are called cysteinyl leukotrienes. LTB_4 is the potent chemo attractant for granulocytes (4). LTD_4 increases vascular permeability and causes mucus secretion in the respiratory tract (4). There is also consensus stating that cysteinyl leukotriene antagonists have an effective role in asthma (4). These functions suggest that there are several therapeutic applications associated with leukotriene inhibitors.

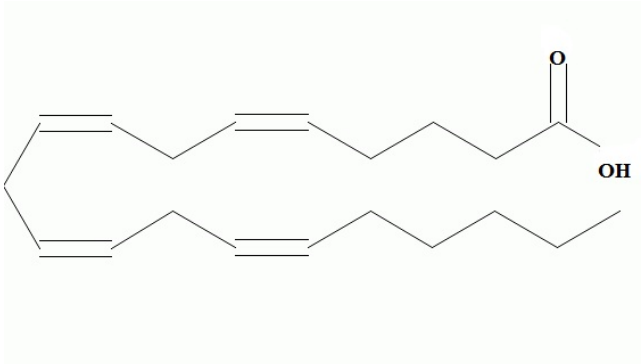


Figure 1a: chemical structure of arachidonic acid (20 carbon molecules with 4 conjugated double bonds)

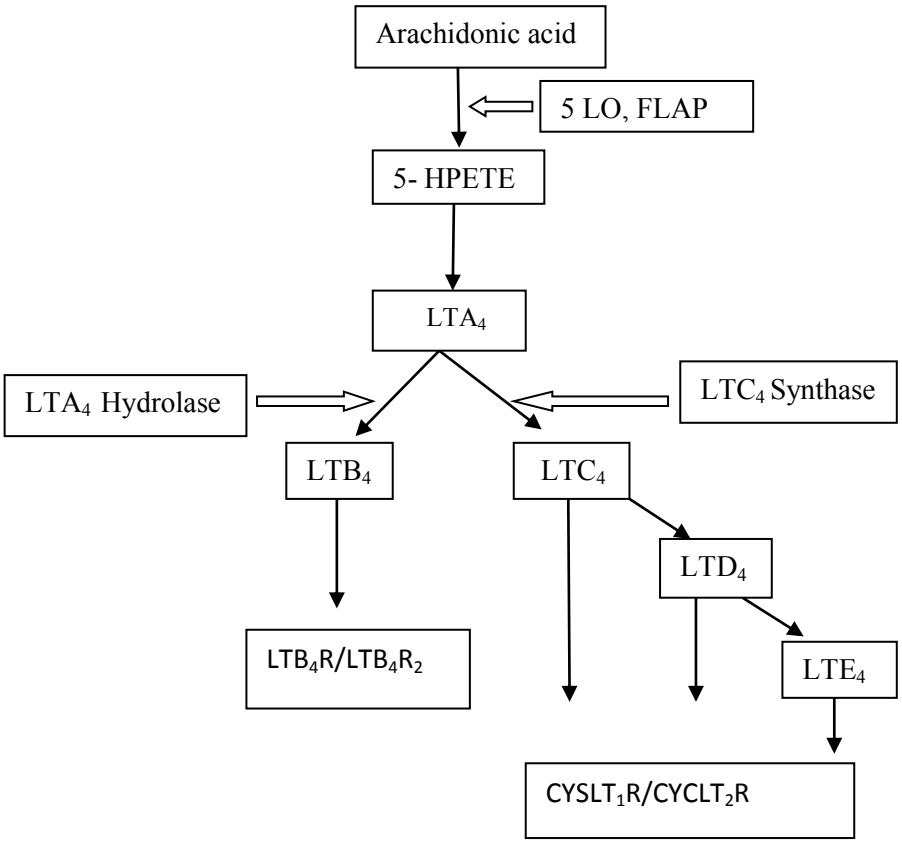


Figure 1b: Role of FLAP in the synthesis of Leukotrienes (2)

As said before, leukotrienes in general have been identified as mediators for a variety of diseases (1). Furthermore, 5-LO was investigated in several diseases such as asthma, atherosclerosis, and bone related diseases such as osteoporosis, rheumatoid arthritis, and even prostate cancer (17). Knockout mice experiments suggests that 5-LO plays a significant role in rise of airway hyper responsiveness (19) as well as in atherosclerosis (17). Mice with a 5-LO knockout allele was found to show decrease in aortic lesions growth. These researchers also observed that in aortic lesions of Apo-lipoprotein E (-/-) lacking mice, 5-LO prolifically expressed especially in macrophages (17). Similarly, knock out studies were carried out in mice on osteoporosis, a bone related disease (19). It was observed that mice which lack the 5-LO gene showed dramatic increase in cortical bone thickness and also many differences in mechanical properties of bones.

Knock out studies in FLAP were also carried out with mice as an experimental model. Lack of this activating protein was identified to give vulnerability to cardiovascular diseases such as atherosclerosis and stroke (17). FLAP deficient mice clearly proved an involvement of leukotrienes in the pathogenesis of respiratory diseases such as asthma (17). To combat against these diseases, 5-LO inhibitors and FLAP inhibitors proved to be very effective for producing drugs. To name a few, zafirlukast, zileuton, pranlukast were the potential inhibitors for the respiratory diseases. FLAP inhibitors such as MK 886, MK 0591 and BAY X 1005 inhibit leukotriene synthesis in isolated human polymorphonuclear cells (4).

Five lipoxygenase, 5-LO

Human 5-LO is a monomeric enzyme with 673 amino acids (fig 2) and a molecular weight of 78 kDa. The crystal structure of human 5-LO was published recently and can be seen as a cylinder about 100 Å long and 60 Å in diameter (PDB: 308Y) (16). The structure consists of an N-terminal domain with 114 amino acids and a C terminal domain which has amino acid residues 121 to 673. The N-terminal β-sandwich domain confers calcium induced membrane binding, whereas the C-terminal domain is mainly alpha helical and contains a catalytic iron ion (16). The structure shows that the iron is anchored by two conserved Histidine residues, the C–terminal Ile-673, Asn-554 and a water molecule (16). Electron paramagnetic studies suggests that recombinant 5-LO iron is in ferrous state (Fe^{2+}) and when it interacts with 5-HPETE, it turns into the ferric state (Fe^{3+}) (15). The stability of human 5-LO is significant to study since this enzyme turns hypersensitive and loses activity when exposed to oxygen (15). Mutant 5-LO was proven to be highly successful in terms of stability and has a longer half-life compared to the wild type 5-LO (16) and this was, in fact, the key for the crystallisation of human 5-LO (16). For the wild type enzyme to remain active, stabilizing agents such as ATP and superoxide dismutase were added. If either of these is not added, enzyme loses its activity within 10 hrs after purification (15, 16).

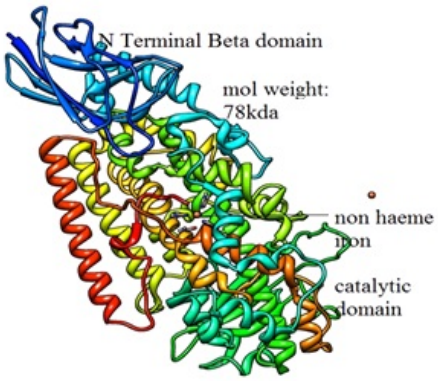


Figure 2: Structure of 5-LO (Ferrous form of human 5 LO) PDB: 308Y (16)

The N-terminal β-sandwich is similar to the C2 domain of $CPLA_2$ and amino acids in certain loops adheres to calcium and embeds in the membrane and it was shown that a minimal concentration of calcium is sufficient for this activation (15). The function of Calcium is to recruit 5-LO, $CPLA_2$ and many other enzymes to the nuclear membrane (12). In some cases, when doing in-vitro experiments, Mg^{2+} (Magnesium) may activate 5-LO (15). Furthermore, the interaction with membrane phospholipids increases the activity of 5-LO in vitro (15).

Five-lipoxygenase activating protein, FLAP

Membrane proteins present in our body promotes significant contribution to cell functions such as cell communication or acts as transport machinery (3). Statistics say 50 % of the drug targets are membrane proteins (3). One among these membrane proteins is FLAP (Five Lipoxygenase Activating Protein). In the late 1980s, scientists identified an indole class

compound named MK 886 that inhibited the leukotriene synthesis without affecting 5-LO (4). The use of photo affinity analogue of MK 886 identified an 18 kDa integral membrane protein called FLAP (21). The function of FLAP is, as the name suggests, to activate 5-LO and does so by helping and presenting arachidonic acid to 5-LO (1, 2, 4) with an unknown mechanism. A proper enzymatic activity of this protein is not known till now. The total sequence length of FLAP is 161 amino acid residues and is similar to human LTC_4 synthase as it shares 33 % sequence identity to it (1).

Structure of FLAP

The X-ray crystal structure of FLAP in complex with the inhibitor MK 591 and the iodinated analogue of MK 591 was solved to 4.25 Å (PDB: 2Q7M) and 4.00 Å (PDB: 2Q7R) (5). Each monomer consists of four transmembrane helices (α1-α4) that are connected by two cytosolic loops and one luminal loop (5). The functional unit of FLAP is a trimer of molecular weight 54 kDa.

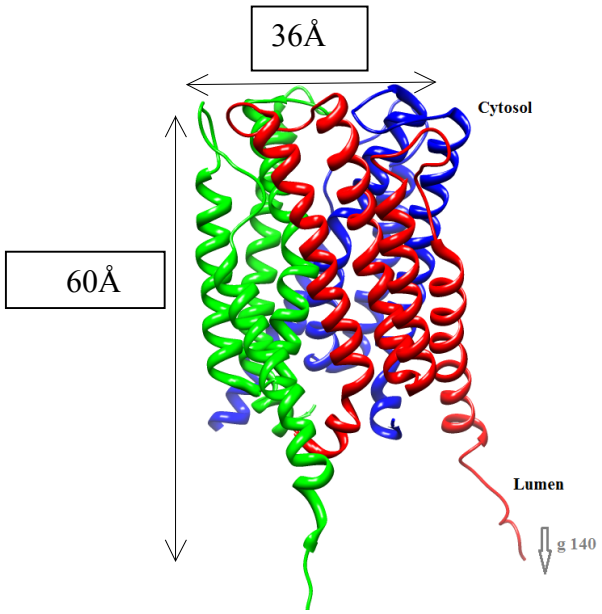


Figure 3a: FLAP structure as trimer (PDB: 2Q7M)

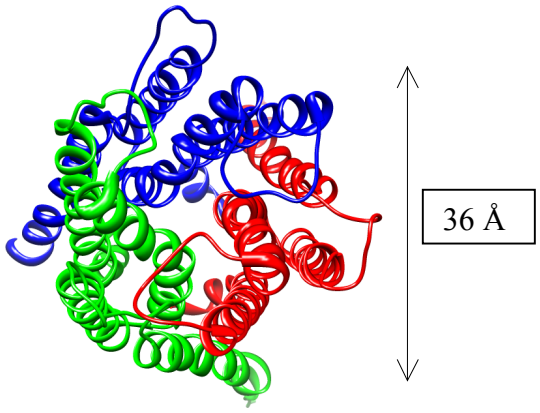


Figure 3b: Top view of FLAP structure (PDB: 2Q7M)

Fig 3a, 3b shows the graphical representation of the FLAP structure generated with the chimera software (inhibitors not shown but are located in pockets between each subunit). Fig 3a reveals the side view and fig 3b is the top view of human FLAP (PDB: 2Q7M). The whole

trimer is cylindrical in shape which measures about 60 Å high and 36 Å in diameter. It is found that the C-terminus of the FLAP structure is highly disordered beyond G140 residue and continues in the lumenal region (5). As mentioned before, although the X-ray crystal structure of FLAP is already known, the mechanism for the activity of FLAP is not yet identified. In this project, the FLAP protein is studied using a novel approach called nanodiscs.

Nanodiscs and Membrane proteins

Just as for soluble proteins, it is important to have membrane proteins isolated in a stable form and free from impurities for structural as well as functional studies. However, membrane proteins are located in lipid environment. Hence, they need to be solubilised from the membranes by the use of detergent and can be maintained stably and kept active in a solubilised state in mixed protein-detergent micelles (7). Many membrane proteins require specific types of phospholipids for active function, sometimes a requirement which is not satisfied even by mixed detergent-lipid micelles. Altogether, detergent solubilisation may sometimes lead to activity loss and problems in analysing the protein due to heterogenecity and aggregation. For stabilisation, reconstitution of membrane proteins into liposomes is considered to be successful despite some limitations such as the size factor (large in size) and that they are difficult to prepare with exact stochiometry of protein per vesicle (7). Nanodisc is another approach, where the enzymatic function as well as the structural aspects of membrane proteins can be studies in a membranous environment (fig 4) (6).

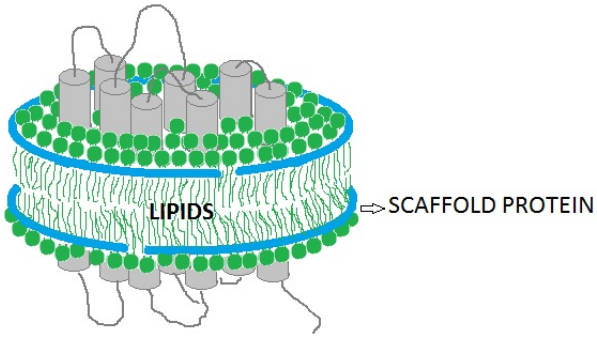


Figure 4: Schematic view of nanodiscs. Lipids (green) with a schematic protein (grey) stabilized by two amphipathic belts of membrane scaffold proteins (blue)

Structure of nanodiscs

Nanodiscs are circular fragments of phospholipid bilayer which are encircled by two copies of membrane scaffold proteins (MSP) and the longer MSP the larger diameter of the disc (6, 7). An illustration of how membrane protein oligomers can be assembled depending on the size of nanodiscs is how monomeric rhodopsin reconstituted into smaller MSP_1 D_1 whereas trimers reconstituted in the longer MSP_1 E_3 D_1 (6). The choice of MSP, lipids and even detergents is significant for preparing nanodiscs and has to be optimised for correct stochiometric ratios (6, 7). For this project, it is important that one FLAP and one 5-LO can form a complex and hence the size of the nanodisc is correctly chosen.

Origin of MSPs

Membrane scaffold proteins are derived from genetically engineered versions of apolipoprotein A_1 which have the property of forming circular amphipathic belts upon accurate addition of lipids and complete removal of detergents (6, 23). This forms nanodiscs called High Density Lipoprotein (HDL) that are involved in the cholesterol transport (24). The apo-lipo protein A_1 is predicted to contain ten amphipathic alpha helices (H_1-H_{10}) and an N-terminal stretch which does not have the property of disc formation but seemed to make nanodisc preparations less homogenous. Hence, researchers cut away that region and created a version of MSP named MSP_1 D_1 yielding a disc size 9.7 nm, same as with the original apolipoprotein A_1 (6). Later, for efficient reconstitution of dimers, trimers and complex studies with larger proteins that require more disc space not compatible with the shorter version MSP_1 D_1, an extended version of MSP_1 D_1 was made (6). Larger disc size was achieved by insertion of a repeat of helices 4, 5 and 6 (denoted E3) between helix 3 and helix 4 which yielded a disc of 12.8 nm. The variant is named MSP_1 E_3 D_1. Furthermore, these MSPs contain an N-terminal hepta-histidine tag followed by a TEV protease cleavage site, useful for separating reconstituted discs from empty discs. Membrane proteins may not have this specific engineered TEV protease cleavage site, even if they contain a His-tag. Hence, purification of reconstituted discs can be achieved by using commercially available engineered TEV proteases. In this project, two different types of TEV proteases were used. Furthermore, an extended version of MSP_1 E_3 D_1 was made named MSP_2 N_2 with disc size of 16.5 nm (6). These larger discs were achieved by fusion of MSP_1D_1-11 and MSP_1 D_1-22 tagged with a GT-linker. In this project, both MSP_1 E_3 D_1 and MSP_2 N_2 were used. The molecular weight and extinction coefficients of MSP_1 E_3 D_1 and MSP_2 N_2 have been estimated at 32 kDa, 45 kDa and 29,910 M^{-1} cm^{-1} and 39,430 M^{-1} cm^{-1} respectively (6).

Advantages of nanodiscs

There are several distinct advantages with reconstitution of membrane proteins into nanodiscs. Nanodiscs are very stable and planar. They are render membrane proteins soluble in aqueous solutions and provide native like bilayered surroundings. Membrane proteins reconstituted in the nanodiscs are maintained monodisperse and highly active (3, 6, 7). Membrane proteins inside the disc are easy to access from either side of the membrane. The overall focus of the project in the group is to reconstitute FLAP into nanodiscs for subsequent analysis of structure and activity of 5-LO with the FLAP-containing discs in the presence and absence of calcium. This might open up for understanding the role of FLAP in the biosynthetic pathway (fig 1b).

Limitations of nanodiscs

Apart from advantages, there are also some limitations of nanodiscs. From the experimental results of this project, it is identified that the prepared nanodisc sample is stable only for a month at a temperature of 4 °C. However, recent results from reconstitution experiments on a membrane protein proved that discs could be stored at -80 °C with no harmful effects due to thawing (Natalya Fedosova, Department of Biomedicine, Aarhus University). When compared to vesicles, nanodiscs are more costly and preparation consumes more time. In transmission electron microscopic images, the diameter of the disc may vary slightly when compared to the actual disc size. Nanodiscs are not suitable for activity measurements of transporter proteins that need different environments for the two sides of the protein.

Methods

Bacterial expression of $MSP_1E_3D_1$ and MSP_2N_2 (strain used: BL21 (DE3))

LB Agar medium (appendix 2) was prepared and autoclaved. Plasmids (pET 28a, Addgene Co) were thawed and incubated in ice for 30 minutes. Ten micro litre of plasmid was taken and mixed with competent cells and incubated on ice for 30 minutes. The cells were heat shocked at 42 °C for a minute and immediately put on ice for a minute. LB medium (900 µl) was taken to grow these cells at a temperature of 37 °C for one hour. Transformed cells were poured uniformly in each LB agar plate containing 20 µg/ml ampicillin antibiotic and kept at 37 °C overnight.

Preparation of starting culture

LB medium (5 ml) was prepared with 100 µl of kanamycin antibiotic (Duchefa biochemie Inc) was added to the culture. Streaks of $MSP_1E_3D_1$ and MSP_2N_2 plasmid (Addgene Co) was added to the medium containing antibiotic in separate plates and kept in a rotary shaker at a temperature of 37 °C for nearly 20 hours. Meanwhile, one litre Terrific broth medium was prepared by mixing 47 g/l commercially available terrific broth powder (Invitrogen) with distilled water and autoclaved at 121 °C.

Expression of $MSP_1E_3D_1$ and MSP_2N_2

Overnight cultures were inoculated in one litre terrific broth medium containing kanamycin antibiotic and incubated in a rotary shaker at 37 °C, 200 rpm. OD values were checked at an interval of one hour. When the OD value was 0.275-0.3, 1 mM isopropyl β-D-thiogalactopyranoside was introduced to induce protein expression. In the case of MSP_2N_2, after IPTG induction the temperature was reduced to 28 °C and kept in shaker for another three hours. However, for $MSP_1E_3D_1$ temperature remained unchanged at 37 °C after induction with IPTG. After three hours, the cells were centrifuged at 7500 rpm for 15 minutes and the pellet containing protein was resuspended with phosphate buffer (appendix 2, pH: 7.4) and stored at -80 °C.

NI-IMAC

Since, His-tagged proteins have high binding affinity for nickel ions, Nickel sepharose column (GE healthcare) was chosen for purification. In immobilised metal ion affinity chromatography, the interaction takes place between metal ions and histidine tagged proteins. Bound proteins can be eluted by adding high concentration of imidazole.

Purification of MSP (Both $MSP_1E_3D_1$ and MSP_2N_2)

Cell pellets stored at -80 °C were taken from the deep freezer and were kept on ice. EDTA-free protease inhibitor cocktail tablet (Roche) was added to prevent proteolysis. Triton-X-100 detergent of concentration 1 % was also added to the mixture. For the cell to lyse, sonication was performed with ice surrounding the mixer for 12-13 times at an interval of 10 minutes. After sonication, the lysed culture was centrifuged at 17000 rpm for 45 minutes. The supernatant was collected and kept on ice in order to avoid degradation. The nickel sepharose column was regenerated with 50 mM EDTA/50 mM Tris buffer. Then, it was washed with 4

column volume of water. 0.1 M nickel chloride was prepared and loaded into the column. After constant washing with water, the column was equilibrated with 4 column volumes of phosphate buffer. After equilibration, the supernatant was loaded onto the column followed by wash buffer 1, 2 and 3 (appendix 2) collected in separate tubes. Finally, Proteins were carefully eluted with elution buffer containing high concentration of imidazole.

Buffer exchange and concentration

Using PD -10 column (GE Healthcare), buffer exchange was conducted of protein solutions to MSP elution buffer (GE Healthcare procedure, appendix 2). The eluted protein was concentrated using AMICON ultra tube (10,000 Molecular weight cut off). The concentration of protein was measured with the help of quartz cuvette using UV spectrophotometer (Cary-4E UV Spectrophotometer) at 280 nm. The extinction co-efficient of $MSP_1 E_3 D_1$ is 29910 M^{-1} cm^{-1} and of $MSP_2 N_2$ it is 39430 M^{-1} cm^{-1}. $MSP_1 E_3 D_1{}^{(-)}$ where the His-tag has been cleaved off has a molecular weight of 29.9 kDa and an extinction coefficient of 26600 M^{-1} cm^{-1}.

SDS-PAGE

The protein purification ($MSP_1 E_3 D_1$, FLAP and 5-LO) was monitored by SDS-PAGE using 12 % homogeneous gels. After the run, proteins were stained with Coomasie reagent and destained with methanol/acetic acid alternatively with water. $MSP_1 E_3 D_1$, FLAP and 5-LO were detected based on the molecular weight of 32.6 kDa, 18 kDa and 78 kDa respectively (see fig 7).

Purification of FLAP

The expressed human FLAP with a hexa-histidine tag from *Pichia pastoris* species (MBB, KI Solna) pellet stored at -20 °C was taken for purification. Since, membrane proteins degrade easily pellets taken from the freezer were stored in ice in the cold room. Cell lysis was performed with glass beads of 0.1 mm using blender for 7 times with 10 minutes interval. After cell lysis, the solution was filtered through filter paper. The filtered solution was centrifuged at 8000 rpm (JA-10 rotor, Beckman J2-MC centrifuge) for 20 minutes at 4 °C. The supernatant was collected and the pH was adjusted to 7.6. To the solution, 0.1 % DDM and 5 mM β-mercaptoethanol was added. β-mercaptoethanol acts as a reducing agent. The mixture was kept one hour on ice to solubilise and was then centrifuged at 12000 rpm for 20 minutes at 4 °C and the supernatant was kept on ice for purification. The nickel-IMAC column was equilibrated with FLAP buffer (appendix 2) which contains 0.1 % DDM. 10 mM imidazole was added to the collected supernatant and loaded into the equilibrated column. The solution was collected as crude. Then, series of washes were performed with wash buffer 1 and 2 (appendix 2) and the FLAP protein was eluted using FLAP elution buffer (appendix 2). Buffer exchange was completed using PD-10 column (GE Healthcare) and concentrated using AMICON (Millipore) tubes. The concentration was checked using UV spectrophotometer. The extinction co-efficient of FLAP is 19031 M^{-1} cm^{-1}.

5-LO expression (Strain used: BL21 (DE3))

Starter culture (*E. coli*) containing 0.1 mg/ml ampicillin as antibiotic was prepared and kept at 37 °C for 20 hours. After sufficient bacterial growth, 5 ml of the overnight culture was

inoculated to the prepared medium (M9, minimal medium) containing 0.1 mg/ml ampicillin and kept at rotary shaker at 25 °C. After six hours, 0.25 mM IPTG was added for induction and the culture was incubated in rotary shaker for 14-16 hours. The grown culture was centrifuged at 7000 rpm for 20 minutes (Beckman J2-MC, rotor JA: 10). Pellet containing protein was collected and resuspended with the prepared lysis buffer (Appendix 2) and stored at -80 °C.

5-LO purification

The cell pellet stored at -80 °C was thawed and kept on ice for purification. 0.001 g of DNase, 0.5 mg/ml lysozyme, half a tablet of EDTA free protease inhibitor and 5 mM β-mercaptoethanol was added to the lysis buffer. Sonication was performed for the cells to lyse for 14 times at an interval of 10 minutes each. The final clear lysed culture was centrifuged at 18500 rpm for 100 minutes. Supernatant was collected separately.

ATP-agarose column

Since 5-LO has strong binding affinity with ATP, ATP-agarose column (Sigma Aldrich) was used for purification, and equilibrated with 3 column volumes of lysis buffer. After equilibration, 10 ml of sample was loaded into the column and collected as crude. 5 ml of wash buffer 2 (appendix 2) was poured into the column followed by 5 ml of wash buffer 1. After these steps, one column volume of wash buffer 1 (appendix 2) was loaded for final wash followed by AMP elution (appendix 2) with 20 ml of AMP elution buffer. 5-LO was eluted with 30 ml of ATP elution buffer (appendix 2) and collected separately. The collected samples were concentrated using AMICON (Millipore) tubes (10,000 MWCO) followed by a buffer exchange (PD-10 column, GE Healthcare, MSP standard buffer) and concentrated (if needed) and stored in ice. 20 µl of the purified protein was taken for loading in SDS PAGE and the protein was detected at expected molecular size (78 kda) as shown in fig 7. Since 5-lipoxygenase is very unstable and loses its activity quickly when exposed to oxygen, the stabilizing agents glutathione peroxidise 0.15 µg/ml (Sigma-Aldrich Inc), superoxide dismutase 1.5 µg/ml (Sigma-Aldrich Inc) and 12 mM ATP were added and the material was stored at -20 °C for future use (9).

Choice of lipids and MSPs

Researchers in the past worked on reconstitution of membrane proteins with different lipids such as DPPC, DMPC, and POPC (6). In this project, the lipid used was POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (Avanti Co). The main reason for choosing POPC among other lipids is 5-LO binds to phospholipids specifically containing an unsaturated acyl chain at the sn-2 position with the strongest binding to PAPC but the binding to POPC is also very strong since the oleoyl group contains one unsaturated binding. Hence, POPC was considered as an ideal choice in this project for preparing nanodiscs since this lipid has been used in the MSP research (6,7). The molar ratio of lipid to proteins (LPR) is also very significant in the preparation of empty discs. In this project, the molar ratio of POPC: MSP_1 E_3 D_1 for empty discs was xxx:1. There are different versions of engineered MSPs available for the research purpose (6). In this project, we first tested making empty discs with MSP_2N_2. However, for reasons described later in the discussions, MSP_1 E_3 D_1 was used for reconstitution. For the purpose of reconstitution, the surface area of the nanodisc

phospholipid bilayer needs to be matched to the size of the target protein chosen to be assembled.

Preparation of detergent solubilized lipids

Lipid stocks in chloroform (25 mg/ml) were stored at -20 °C. Since POPC is dissolved with chloroform, it was added in a round bottomed flask with appropriate volume and was dried under nitrogen gas. After a period of time, a thin white film was seen around the round bottomed flask ensuring the removal of chloroform. To remove it completely, the flask containing dried lipid was kept under high vacuum in a vacuum desiccator overnight. This ensures that the flask contains only a known amount of lipid to the desired concentration. Sodium cholate (Sigma-Aldrich Inc) was added to the lipid in the molar ratio of 2:1. The tubes were heated in a hot water and sonicated in an ultrasonic bath until it appeared as a clear solution. After a few minutes, tubes containing detergent solubilised phospholipids were taken out and stored at -80 °C for future use. Sodium cholate is recommended (6) but other detergents can be tested if a target membrane protein is not stable in cholate.

Preparation of empty discs

Membrane scaffold proteins were added to the cholate solubilised phospholipids taken from the deep freezer according to the calculated lipid to protein ratio. The calculated LPR (lipid to protein ratio) for preparing empty disc is 120 for $MSP_1 E_3 D_1$ and 240 for $MSP_2 N_2$. The final cholate concentration in the mixture should be between 12-40 mM (6). In this project, the final concentration of sodium cholate was optimised and found to be xx mM for empty $MSP_1 E_3 D_1$ discs. The assembly mixture was kept on ice for one hour. After one hour, two third volumes of bio beads (SM-2 adsorbent, Bio-Rad) were added to remove the detergent. The tubes were kept in a rotary shaker at 4 °C overnight. The tubes were taken out the next day for removing bio beads from the solution. Empty discs get assembled automatically upon complete removal of detergent. The solution was stored at 4 °C and 10 µl was taken for blue native PAGE analysis (4-12 % Bis-Tris gel, Invitrogen Inc). The calculated molecular weight of an empty $MSP_1 E_3 D_1$ disc and empty $MSP_2 N_2$ disc is 242 kDa and 454 kDa respectively. The prepared empty discs can be stored at 4 °C for 30-40 days. However, later it was found that deep freezing the empty discs at -80 °C is also a possible option for long term use.

Reconstitution of FLAP

Reconstitution of FLAP into $MSP_1 E_3 D_1$ nanodisc was performed according to the protocol designed as stated in appendix 3. Better results were obtained when incubated at 4 °C than at 37 °C due to the use of POPC (6).

Separation of empty discs from reconstituted FLAP nanodiscs

The prepared reconstituted FLAP disc sample contains both FLAP nanodiscs and empty discs. It is very difficult to analyse the structural properties of any proteins when it is not in pure state. Hence, it is important to separate the empty discs from the reconstituted FLAP nanodiscs. Since the engineered MSPs contain a TEV cleavage site after the hepta-histidine part in its sequence, a TEV protease was used to cleave the His-tag and the disc samples were purified using a histidine spin trap column (GE Healthcare). The FLAP protein does not have a TEV protease cleavage site and hence the reconstituted FLAP nanodiscs are retained in the column while the empty discs elute. In this project, TEV proteases from both Invitrogen and

Sigma-Aldrich Inc were used where both contained hexa-Histidine tags but had different molecular weights.

AcTEV protease from Invitrogen

Purified MSP_1 E_3 D_1 was used for cleavage with this engineered TEV protease. The idea is to cleave the His-tag before performing the reconstitution. MSP_1 E_3 D_1 was diluted with TEV buffer (50 mM Tris pH: 8.0, 0.5 mM EDTA and 1 mM DTT) mixed with AcTEV protease in the ratio of 1 unit enzyme to 3 µg of substrate. The mixture was prepared to the final volume of 150 µl and incubated at 30 °C for 3 hours. After 3 hours of incubation, the sample was taken for purification using histidine spin trap column (GE Healthcare). After regenerating the spin trap column, 0.1 M $NiCl_2$ was added and column was equilibrated with MSP buffer (appendix 2). Crude containing empty disc and reconstituted FLAP was added to the column and centrifuged at 1000 rpm for 30 seconds. Cleaved MSP_1 E_3 D_1 elutes out retaining the histidine tag from the protein and some TEV protease in the column. Cleaved protein was analysed using 12 % SDS PAGE. The molecular weight of TEV protease is 25-28 kDa and cleaved MSP_1 E_3 D_1 is 29 kDa.

TEV protease from Sigma-Aldrich

Previously, purified MSP_1 E_3 D_1 was taken for the cleavage reaction. But, the protocol was slightly changed for the cleavage reaction performed with this TEV protease (Sigma Aldrich Inc). In this method, FLAP protein was reconstituted in the disc and then the TEV protease was added to perform the cleavage reaction in the ratio of (1:100 w/w), i.e. one mg of TEV protease to 100 mg of target protein and the crude was incubated at 4 °C for 16 hours. During the incubation time, TEV protease initiates its activity by cleaving the His-tag from the MSP protein where it leaves the FLAP protein His-tag which does not contain the specific cleavage site. After the specified time, separation was completed using a His-column, where the empty discs elute first using the MSP wash buffer 1 (appendix 2) and FLAP discs using Zeba spin desalting column (40k MWCO). The equilibration buffer used was 25 mM Tris, pH: 8, 150 mM NaCl, 14 mM β-Mercaptoethanol.

Western blot

After reconstitution of FLAP, it is necessary to do the confirmation tests to indicate the presence of FLAP in the nanodiscs. For this, an analytical technique western blot was preferred. The principle behind this technique is to detect the proteins with the help of the specific antibodies. This involves several steps such as preparation and running of PAGE gel, visualization of proteins in the gel, transfer of proteins from the gel to PVDF or nitrocellulose membrane, blocking the membrane, incubation with primary and secondary antibody and development. In this experiment, we used one hour western blot detection kit (Genscript Inc). It reduced the time and increased the sensitivity of the technique when compared to classical western blot method. The primary antibody anti ALOX 5AP / FLAP from rabbit IgG was purchased from Acris Gmbh, Germany.

Confirmation of FLAP nanodisc by SDS-PAGE

The first experiment was to compare the preparation of FLAP in nanodiscs to FLAP in detergent micelles using denatured gel electrophoresis. The process is initiated by running SDS-PAGE. 12 % gel was prepared from 40 % bis – acryl amide (Bio-Rad, CA). 0.01 g Ammonium persulfate and TEMED was added to initiate polymerization. After the preparation of separation and stacking gel, the protein sample was mixed with 2x SDS buffer and heated at 60-70 °C for the proteins to denature and loaded on the gel. The entire set up was kept at 100 kV for five to ten minutes to allow the sample to migrate from stacking gel to separation gel and then changed to 150 kV for 70-90 minutes. During the running time, 1X Tris-glycine buffer (with 0.1 % SDS) was prepared. Blotting pads were soaked with the buffer for 30-45 minutes. It is necessary to remove all the bubbles sticking on the pads as it may disturb the transfer of proteins. In this experiment, nitrocellulose membrane (7.5-8.0 cm) was used. It was soaked in methanol for 30 seconds prior washing with water and membrane was incubated in 1X transfer buffer for nearly 60 minutes. 125 ml of 1X wash solution was prepared from 5X wash (Genscript Inc) taken from the kit and kept ready for further washing steps. 10 µg of anti-FLAP (Acris Gmbh, Germany) which is a primary antibody was prepared by mixing with WB-1 Solution (Genscript Inc) and kept at room temperature for 40 minutes. This is called mixture 1. This step is completed either during protein transfer or before the transfer and incubated as said above. As soon as the SDS-PAGE is complete, the gel was carefully taken from the glass plate and kept for transfer. The soaked blotting pads were kept at the cathode end. A Filter paper was placed above the blotting pads with gel facing the top and then the nitrocellulose membrane. Care must be taken to avoid bubble formation on the membrane. It is necessary to use the glass pipette to roll over the membrane, blotting pads to ensure the set up is completely free from bubbles. Again, the same arrangement was placed on the top of the membrane with blotting pads on the anode end. The entire set up was packed tightly and kept at 25 V for 90-120 minutes. With the one hour western blot kit, blocking and binding of secondary antibody steps was quick with the help of pre treat solutions and WB-2 solution (Genscript Inc). Pre treat solution mixture was prepared by mixing pre treat solution A and pre treat solution B (Genscript Inc). As soon as protein transfer to membrane is complete, it was placed in the prepared pre treat solution mixture and rinsed with 1X wash solution (15). The primary antibody with WB-1 (Mixture 1) was mixed with 10 ml of WB-2 (Secondary antibody) and membrane was kept in the solution on a shaker for overnight. After the specified time, membrane was rinsed with 1X wash solution (15).

For development, Lumisensor™ chemiluminescent HRP substrate (Genscript Inc) was used. 1.5 ml of reagent A was mixed with reagent B and vortexed for few seconds. The working solution was prepared. 10 µl -20 µl of the working solution was sufficient to cover the entire membrane. Membrane was immersed with the working solution and kept for three minutes. Tissue papers were used to drain the excess solution. After few minutes, the membrane was pictured using Gel doc scanning machine (Bio-Rad Inc) with different exposures at subsequent intervals.

Confirmation of FLAP nanodisc by native PAGE

The main difference was that the gel runs in a native non-denaturing environment. Native 1X Tris-glycine buffer (without SDS) was used for the initial gel run. The remaining steps till development are the same (15).

Analysis of sample by Electron Microscope

Negative staining

Negative staining is not a novel technique for protein structural studies, it has a long history and it is still proven to be useful for studying several macromolecular assemblies (10). There are both limitations and advantages of using this method. Image resolution is restricted since the heavy metal stains replicates the outer rim of the protein and thus makes the structural information very limited. Another problem is artifacts and distortions in the images due to the stains. Despite some limitations such as positive staining, uneven staining, this method has many advantages. This method yields high contrast images due to strong scattering of the heavy metals in the staining agents (10). The stained sample is less prone to radiation damage compared to ice grafted samples. In this experiment, negative stains such as PTA (Phosphotungstic acid), uranyl acetate (UrAc) and uranyl formate (UrFo) were used. Each and every stain has different properties and it is reflected in the EM pictures of the empty discs as well as reconstituted FLAP.

Specimens were made by using circular net (400 mesh) coated with carbon called "grids" made as follows. A thin layer of carbon was evaporated on the top of automatically flat mica sheets. The carbon film was then transferred from mica to the grids. Since the carbon is hydrophobic material, it was made hydrophilic to make the surface of the grid more approachable to water as well as stained sample. This can be done by a method called "Glow discharging". Carbon coated grids were placed in a vacuum filled chamber connected to power supply. After placing the grid, high voltage at the range of 30 mA for 20 seconds was applied. After the application of high current, the electron potential ionizes the gas inside the chamber, which makes the negative charged ions to fix on the carbon to make a hydrophilic material.

Staining procedure for empty discs and reconstituted FLAP discs

Empty disc samples of both MSP_2 N_2 and MSP_1 E_3 D_1 were kept on ice. Concentrations of both disc samples were determined using UV spectrophotometer and were found to be 0.65 mg/ml and 0.56 mg/ml respectively. Carbon coated grids were taken from the glow discharging and fastened with the help of tweezers (Dumont Inc). 4 µl of empty disc sample was incubated on the grid for 60 seconds. After this step, grids were gently washed with three droplets of water and drained immediately using Whatman filter paper no 4. This step is to remove any salt content or impurities other than proteins fixed on the grid. Before drying out, the grids were then stained with 1.5 % PTA, 1 % UrAc or 1 % UrFo and then air dried at room temperature for 5-10 minutes. The staining procedure for reconstituted FLAP discs was similar to that of empty disc preparations. However, later it was found that the concentration of reconstituted FLAP discs was too high to see the discs clearly in the microscope. The optimal concentration of reconstituted FLAP nanodiscs used in this experiment was 0.15 mg/ml or even lower for clear microscopic images.

Electron microscopy

Nanodiscs images were captured with the help of transmission electron microscope CM120 at 120 kV and JEOL2100F at 200 kV using a CCD camera with either (1024 x 1024) pixels or (4096 x 4096) and 25 µm and 15 µm pixel size respectively.

Results and Discussion

MSP_2N_2

The aim of this project is the reconstitution of FLAP into nanodiscs, however, in the overall project to study complex formation with 5-LO the area of the nanodisc needed to be chosen to have space for both FLAP and 5-LO. The diameter of FLAP is about 36 Å (fig 3b) and the area it occupies in the membrane calculated to be about 1020 $Å^2$. The 5-LO was reported to be about 100 Å long and 60 Å wide (16) and that an area of at most 9600 $Å^2$ makes contact with the membrane although the effective area may be only 7200 $Å^2$ (14). The $MSP_2 N_2$ was initially chosen for the large disc size of 145 Å inner diameter (bilayer area of 16500 $Å^2$) (11).

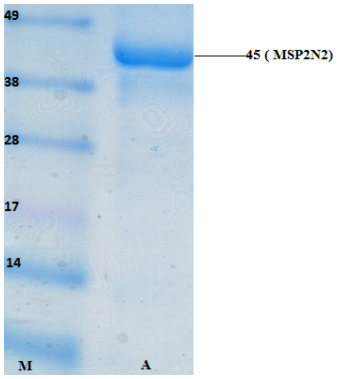


Figure 5a: purified $MSP_2 N_2$ (12 % SDS PAGE)

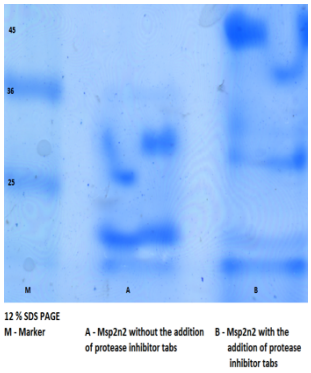


Figure 5b: $MSP_2 N_2$ Proteolysis effect (12 % SDS PAGE

Fig 5a indicates the purified $MSP_2 N_2$ at 45 kDa. This sample is the freshly purified protein prepared with the addition of EDTA free protease inhibitor tablets. The sample was stored at 4 °C for preparing empty discs and reconstitution studies. The same sample was tested in SDS PAGE after few weeks of storage at 4 °C. Unfortunately, it was found that there was a problem with instability of the $MSP_2 N_2$ preparation and that SDS-PAGE analysis indicates that proteolysis occurred as seen in lane B, fig 5b. Simultaneously, another $MSP_2 N_2$ sample was purified without the addition of protease inhibitor tablets to check the nature of $MSP_2 N_2$. Proteolytic degradation was seen clearly as shown in lane A, fig 5b. Therefore, it was found that this $MSP_2 N_2$ is not stable enough for the reconstitution studies and hence $MSP_1 E_3 D_1$ was considered for further studies in this project. Though the size of the $MSP_1 E_3 D_1$ disc is small compared to $MSP_2 N_2$, there is enough space for reconstitution of FLAP and binding of 5-LO. Considering the size of $MSP_1 E_3 D_1$ disc of 10.8 nm inner diameter (9600 $Å^2$) and sizes of FLAP and 5- LO (as shown in Fig 3a, 3b), it is assumed that one can incorporate one FLAP (and probably one 5- LO) inside the $MSP_1 E_3 D_1$ nanodisc. Considering the time factor and also the stability of the $MSP_1 E_3 D_1$, reconstitution studies of FLAP into nanodisc were performed on this 12.8 nm (outer diameter) sized scaffold protein.

Empty nanodiscs from MSP_2N_2

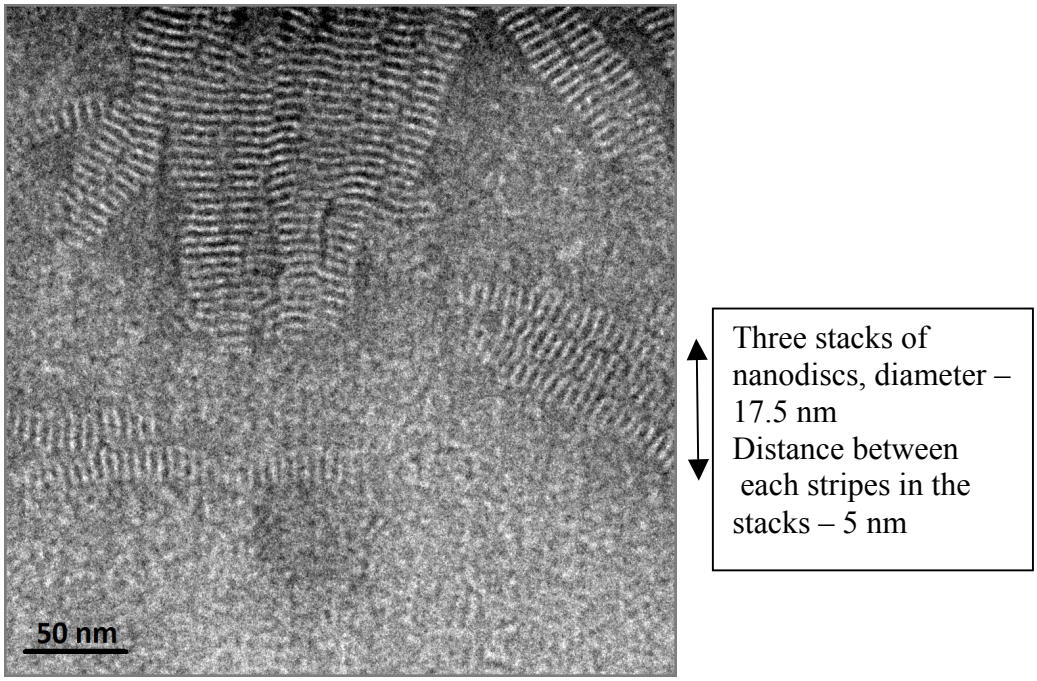


Figure 6: EM picture of empty discs with MSP_2N_2 (35 kX) stained with 1 % PTA

Earlier when researchers worked with nanodiscs such as HDL (24) they used the negative stain PTA (Phosphotungstic acid), and found more or less aggregation of the discs bilayer to bilayer in long "stacks" and not free lying discs on the grids (24). Later when Uranyl acetate was used they found that the discs could separate (24). For this thesis project the stacking could be a useful tool. Empty discs should stack but for nanodiscs containing a membrane protein that sticks out from the membrane there should be no stacking.

Fig 6 shows the stacking formation of MSP_2N_2 empty discs stained with 1 % PTA. The arrow in the picture depicts the homogeneous pattern of side view of the stacked discs, one over the other. The width of the long stacks are about 17.5 nm as expected for the MSP_2N_2 discs and the distance between the stripes is about 5 nm as expected for stacks of membrane bilayers (6). The concentration of the empty disc was 0.65 mg/ml and the LPR used for the preparation of empty disc was "240". The molecular weight of the MSP_2N_2 empty nanodisc was calculated at 450 kDa.

Results from expressed and purified proteins

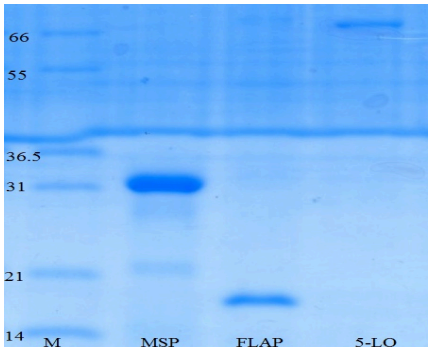


Figure 7: 12 % SDS PAGE analysis of Purified $MSP_1 E_3 D_1$, FLAP and 5-LO

Due to the stability problem with $MSP_2 N_2$, $MSP_1 E_3 D_1$ was used for purification and further reconstitution studies. The necessary proteins used in this project $MSP_1 E_3 D_1$, FLAP and 5-Lipoxygenase were purified and analysed with SDS-PAGE as shown in fig 7. To explain precisely, Lane 1 shows the $MSP_1 E_3 D_1$ scaffold protein at molecular weight 32 kDa. Lane 2 shows the FLAP protein at 18 kda and lane 3 shows 5-LO at 78 kDa.

Empty nanodiscs from $MSP_1 E_3 D_1$

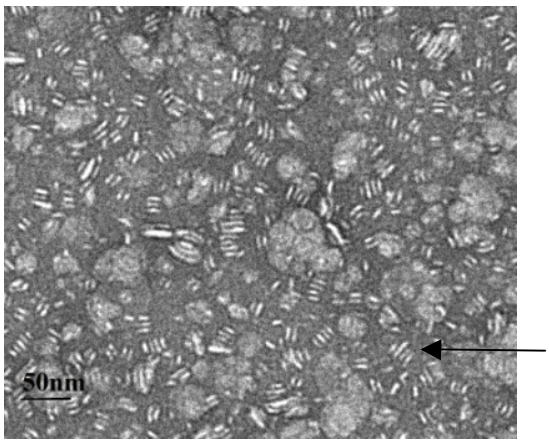


Figure 8: EM image of empty MSP1 E3 D1 nanodisc (1 % PTA, 40 kX) (arrow indicates a stack of four discs of varying length seen from the side

Figure 8 shows the EM pictures of empty $MSP_1 E_3 D_1$ nanodics (white bands against the dark background) prepared with 1 % PTA. The concentration of empty nanodiscs was found to be 0.56 mg/ml and the LPR used for this preparation was "120". It is observed that there are differences in diameter when compared with empty $MSP_2 N_2$ nanodiscs showed in fig 6 and empty $MSP_1 E_3 D_1$ nanodiscs. The sizes of empty $MSP_2 N_2$ nanodisc were 16-18 nm and the stacks were very long (fig 6). The empty $MSP_1 E_3 D_1$ nanodiscs sizes varied in size (<10-14 nm) within the stacks that were short (e.g. Fig 8, arrow). This variation in size and the presence of large aggregates (that could be short stacks seen from top) needs to be solved.

Practical limitations due to TEV protease size

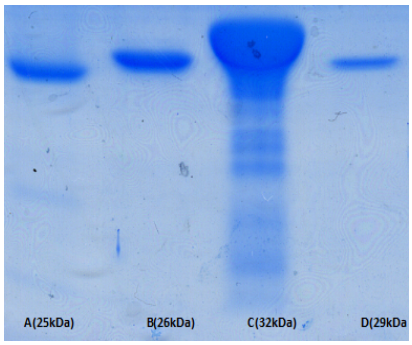


Figure 9a: 12 % SDS-PAGE analysis of AcTEV protease and $MSP_1 E_3 D_1$ from Invitrogen

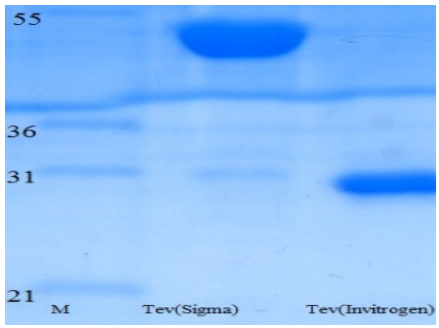


Figure 9b: 12 % SDS-PAGE analysis of molecular weight difference of TEV protease (Sigma: 54 kDa, Invitrogen: 26-28 kDa)

In protocols for insertion of a target protein into nanodiscs it was proposed that the Histidine tag on the MSP used for its purification should be cleaved off before reconstitution (6). However, several attempts were made to use cleaved MSP but no reconstitution was observed and hence the protein yield was investigated. Figure 9a shows a 12 % SDS-PAGE of $MSP_1 E_3 D_1$, before and after cleavage but also the AcTEV protease is shown: Lane A is the chymotrypsinogen marker (25 kDa), Lane B is the AcTEV protease from Invitrogen (26-28 kDa), Lane C is the crude uncleaved fraction of $MSP_1 E_3 D_1$ (32 kDa), Lane D is the $MSP_1 E_3 D_1$ obtained after cleavage and purification and expected to be at 29 kDa. Severe loss of protein seemed to occur due to the cleavage and purification procedure (fig 9a Lane C and D). Perhaps this depended on problems with this enzyme batch not cleaving the His-tag as expected. It is furthermore not so good that the molecular weights of the TEV protease and $MSP_1 E_3 D_1$ are similar as it could be unclear what the band in lane D (fig 9a) actually shows (AcTEV protease or $MSP_1 E_3 D_1$).

This lack of reconstitution together with the similarity in size of the TEV protease and $MSP_1 E_3 D_1$ made us to try two things: 1) The cleavage of His-tag from $MSP_1 E_3 D_1$ was performed after reconstitution 2) Another TEV protease (Sigma Aldrich) was used with molecular weight 54 kDa (fig 9b).

Result from reconstituted FLAP nanodiscs with TEV protease (Invitrogen)

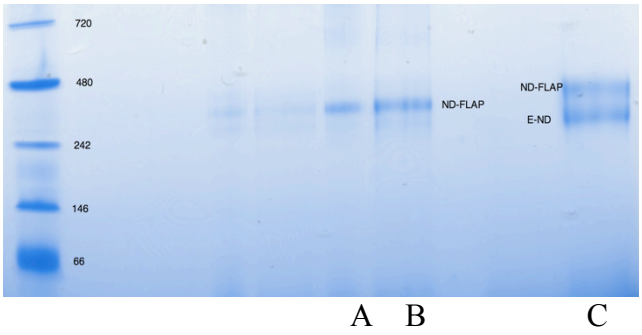


Figure 10: Blue native page result of reconstitution of FLAP with MSP_1 E_3 D_1

Here, the cleavage of the His-tag from MSP_1 E_3 D_1 was performed after reconstitution but still with the AcTEV protease (fig 10). FLAP protein remains uncleaved inside the nanodisc since it does not contain the TEV protease cleavage site and can be used for purification to remove the empty cleaved nanodiscs since the crude containing both empty and reconstituted FLAP discs could not be used directly for structural or complex formation studies. Fig 10 shows the result from the Blue native page gel (4-10 % Bis-tris gel). Lane C shows the crude uncleaved sample which contains both FLAP nanodiscs (ND-FLAP) and empty discs (E-ND), whereas both lane A and B shows the cleaved and purified FLAP nanodiscs (ND-FLAP). The molecular weight of the reconstituted FLAP nanodisc (and His-cleaved) was calculated to be 290 kDa at LPR 115 (Lane A). Lane A (and B) contains an extremely weak band of empty discs (calculated to 242 kDa) indicating that the AcTEV protease worked well. The clear single band in lane A and lane B showed that the reconstitution of FLAP into nanodiscs was successful. Hence it was expected that the EM pictures should show no stacking (cf fig 8) and only show separated discs. However, we could see some stacking and aggregation of discs (fig 11) despite the purity of the FLAP nanodiscs (fig 10, Lane A and B).

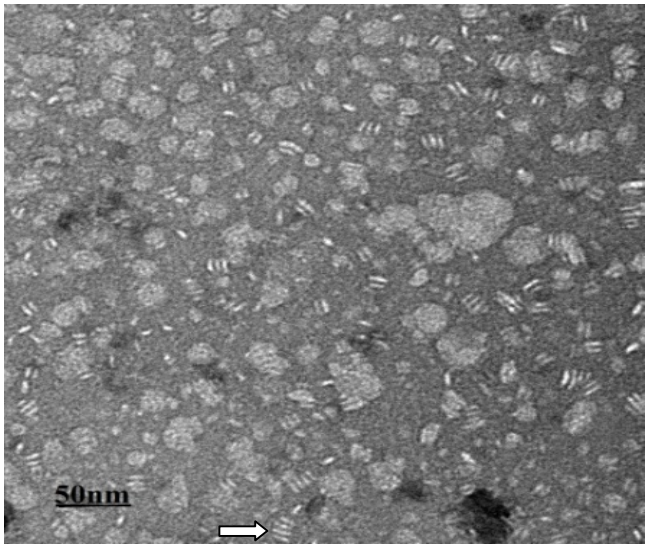


Figure 11: EM image of FLAP reconstituted into $MSP_1 E_3 D_1$ nanodiscs (arrow indicates a stack of four discs seen from the side) (1 % PTA (55.6 kX)

Result from cleaved reconstituted FLAP with TEV protease (Sigma)

Since AcTEV protease (Invitrogen) used in the previous results seemed a problem at first, another engineered TEV protease of molecular weight (54 kDa) was used from Sigma. Also here, reconstitution of FLAP into $MSP_1 E_3 D_1$nanodiscs was performed first and cleavage of the $MSP_1 E_3 D_1$ - His-tags secondly that leaves the His-tag on the FLAP (lacking a TEV cleavage site). Finally, separation was performed using a His-column (GE Healthcare).

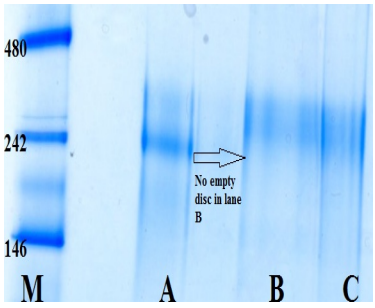


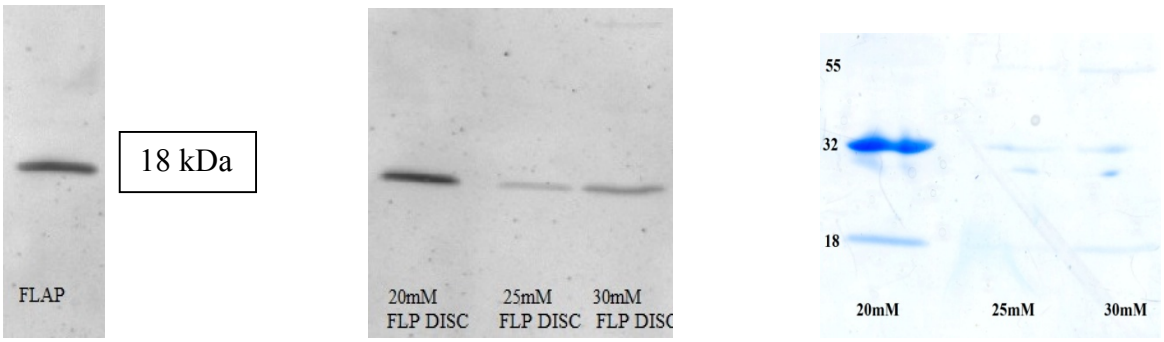
Fig 12: Blue native PAGE analysis of empty and cleaved reconstituted FLAP into $MSP_1 E_3 D_1$ nanodiscs

The main difference from Figure 11 is that Figure 12 shows the cleaved crude (lane A). In Figure 12, lane A sample shows the crude containing the TEV protease (not visible as a band), empty discs (at 242 kDa) and reconstituted discs (faint band above 242 kDa). Lane B (fig 12) shows that after purification on a His column, there was no presence of empty discs but only reconstituted FLAP nanodiscs expected at 290 kDa. This confirms that there is

successful cleavage activity. Lane C is the pure reconstituted disc which is eluted after buffer exchange using desalting column (ZEBA spin desalting column (40 MWCO)).

The LPR used in this preparation was 108. Since the LPR 108 was observed to be too low, LPR 115 was experimented (fig 10). Fig 10 shows equal amount of empty discs and reconstituted FLAP nanodiscs.

Western-blot results from a denaturing gel



A B C

Figure 13 (A) -Western blot of isolated FLAP, (B) -Western blot of FLAP reconstituted in nanodiscs, (C) - 15 % SDS-PAGE denaturing gel, Coomassie stained, after transfer of protein to the membrane for antibody detection as shown in B

In order to confirm that the purified protein was FLAP and reconstituted discs contain the FLAP protein (cf. figs 10, 11 and 12), a western blot was performed. The primary antibody used in this experiment was anti ALOX 5AP/FLAP from rabbit IgG. One hour western blot kit purchased from Genscript Inc was used in this method since it was easier to use and time consumed for the entire experiment was less than that of the classical western blot method which usually takes 2-3 days. The process was initiated by running an SDS PAGE (15 %) denaturing gel followed by Western Blotting. Figure 13A shows the antibody detection of purified FLAP monomeric protein (18 kDa), fig 13B shows the FLAP monomer from reconstituted FLAP nanodiscs prepared from various concentrations of sodium cholate (i.e 20 mM, 25 mM and 30 mM). In fig 13C it is observed that in lanes A, B and C, even after transfer from SDS-PAGE gel to the nitrocellulose membrane, protein is left in the gel corresponding to the FLAP monomer seen in fig 13B. It is also interesting to see the thick band of MSP protein in lane A (fig 13C) after denaturing of the reconstituted FLAP nanodisc.

Western-blot result from a native gel

The main change was that the initial gel run takes place in a native environment (fig 14). The remaining step till development remains the same (15).

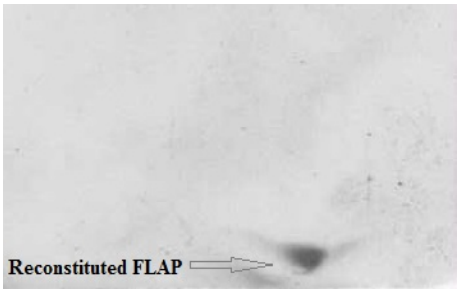


Figure 14 –Western blot analysis of purified FLAP nanodiscs prepared using $MSP_1 E_3 D_1$

Western blot confirmation of 5-LO

Though, this project focuses on the reconstitution of FLAP into nanodiscs, it is the interaction with the 5-Lipoxygenase enzyme we are interested in considering the longer perspective of the project in the group. 5-LO was expressed and purified (as described in Methods section pg 16, 17). Apart from Western Blot tests of FLAP nanodisc formation, western blot was performed on 5-LO after purification. A 12 % SDS PAGE shows 5-LO at 78 kDa (fig 7, 15a).

In figure 15b, both lane A and lane B contains 5-LO protein detected by the one hour western blot technique. The primary antibody used was rabbit IgG five lipoxygenase polyclonal antibody.

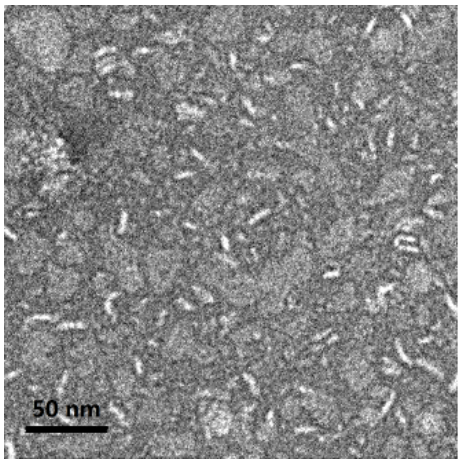
Figure 15a: 12 % SDS –PAGE of purified 5-lipoxygenase

Figure 15b: Western blot detection of 5-lipoxygenase

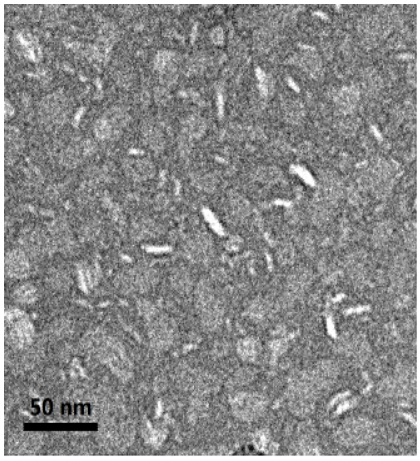
EM pictures of pure reconstituted FLAP nanodisc

EM pictures of FLAP nanodiscs (0.45 mg/ml) were taken with our JEOL 2100F 200 kV transmission electron microscope. Since the protein concentration was high, several dilutions were done and finally 0.15 mg/ml was found to be optimum for visualisation of discs in negative stain. PTA, UrAc and UrFo were used in the experiments. Each stain has distinct properties.

Figures 16a and 16b show EM pictures of FLAP nanodiscs stained with 1 % PTA. This stain induces a stacking-like formation in the case of empty discs (see fig 8 a, b) (24) and fewer (fig 11) or almost no stacks in the reconstituted FLAP as in figure 16a and b. With PTA the side views of the nanodiscs are clearly seen as white band-like objects. The low number of stacks in fig 16a and 16b could indicate that the presence of FLAP prevent stacking, however, there was a lot of stacking in fig 11. It is difficult to say, however, what the medium dark areas in figure 16 represent (darkest is background).



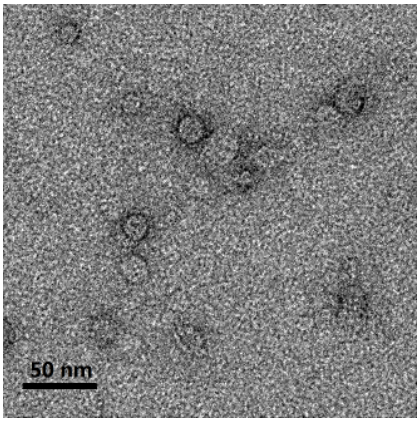
16a



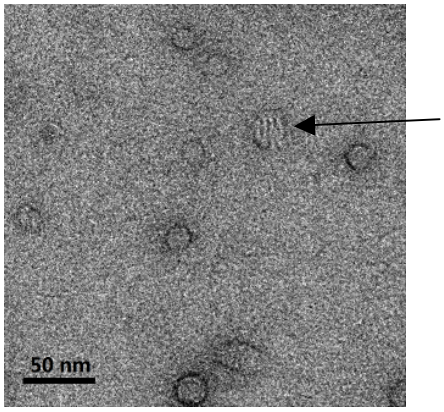
16b

Figure 16 a, b – EM pictures of reconstituted FLAP in $MSP_1 E_3 D_1$ nanodiscs stained with 1 % PTA (69.5 kX)

In UrAc stained samples (fig 17a and b), pictures were of a surprise. There were top views of the discs as expected (24) as well as some stacking formation which was not expected for this stain (24 and C. Jegerschold personal communication). This raises questions and open discussions about the FLAP inside the disc.

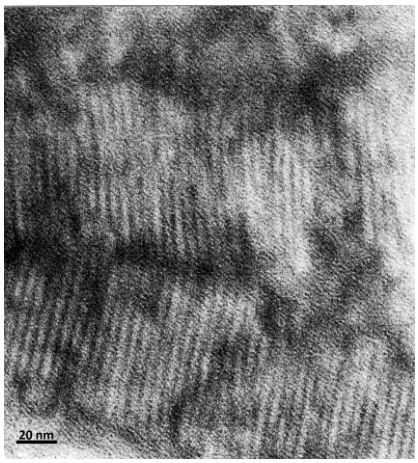


17a

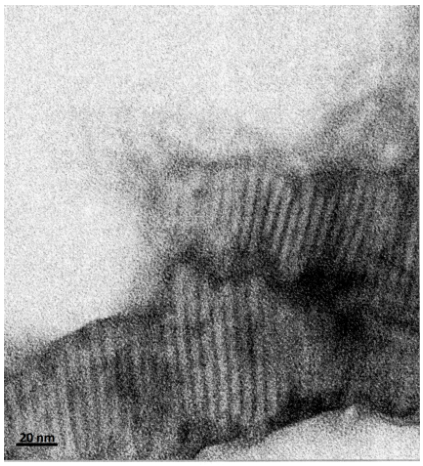


17b

Figure 17 a, b – EM images of Reconstituted nanodisc stained with UrAc (69.5 kX), Arrow from fig 17 b shows the presence of stacked pattern of discs even with UrAc



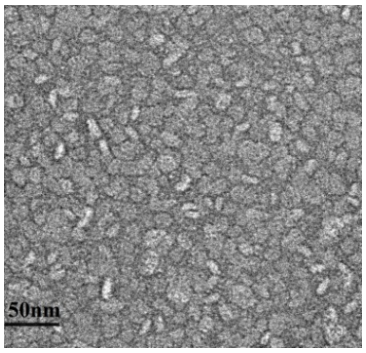
18a



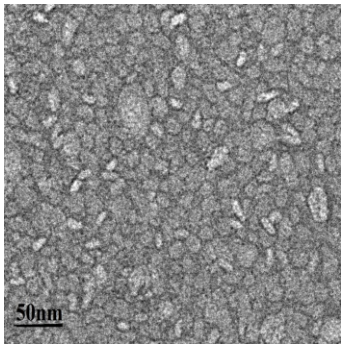
18b

Figure 18 a, b: EM images of FLAP protein in vesicles stained with UrAc (102 kX, CM120 microscope at 120kV) (with permission from C. Jegerschold, unpublished results)

However, EM images of two-dimensional crystallisation trials of FLAP stained with UrAc shows a stacking formation (fig 18a and b, made by C. Jegerschold, unpublished results). In the so called two-dimensional crystallisation of FLAP, purified FLAP in detergent micelles and phospholipids solubilised in detergent were mixed followed by removal of the detergent by dialysis. A natural phosphocholine enriched extract from AVANTI co, USA was the lipid used during the experiment (C. Jegerschold personal communication). Fig 18a and 18b shows flattened stacked vesicles containing FLAP. This shows that the stacking formation of FLAP protein was seen earlier when it was stained with UrAc. Hence, it is observed that the stacking pattern may be the property of FLAP protein itself, irrespective of the negative stain UrAc.



19a



19b

Figure 19a, b: FLAP reconstituted in nanodisc stained with 1 % Uranylformate (69.5 kX)

Figure 19 a, b shows the reconstituted FLAP nanodisc sample stained with 1 % Uranyl Formate. As mentioned earlier, different negative stains have distinct properties. With Uranyl formate, in fig 19a, b it was noticed that there were very few stacking formation seen from the side (cf. arrow in fig 8a on empty disc stack and fig 11 on stack in a FLAP nanodisc sample). While comparing to the other stains, in UrFo the sample looks more homogenous.

Conclusion

From the results, the confirmation tests such as blue native page and western blot provide evidence of the presence of FLAP in the nanodiscs. Though TEM pictures did not provide concrete evidence of presence of FLAP in the nanodiscs, images obtained were equally interesting for further structural analysis. TEM images of FLAP nanodisc bound with FLAP antibody will also provide additional evidence of the presence of FLAP inside the nanodisc. FLAP reconstituted in nanodiscs will be used to make the whole functional complex with 5LO in vitro for studies of the structure using so called single particle cryoEM analysis. Next step is to determine the activity of this complex. The results from activity and structure measurements could together provide information on the mechanism for the involvement of FLAP in the production of leukotrienes from arachidonic acid. Also other proteins in the leukotriene pathway are believed to associate with FLAP or 5-LO and such complexes could also be investigated based on the results in this thesis. On a longer term, this nanodisc approach to study complex-formation between transmembrane proteins and soluble proteins may provide information concerning membrane dependent reactions and might bring vital breakthrough to identify drugs, thereby alleviating many inflammatory diseases such as asthma and atherosclerosis.

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Popular science abstract

Title

Insertion of transmembrane 5-lipoxygenase activating protein (FLAP) into nanodiscs: towards structure function studies of complex formation with soluble proteins.

Background

Leukotrienes are specialized lipid molecules found abundant in white blood cells, so called leukocytes important in our immune system. It is important to remember that inflammation is a vital process in the body (normally) in the combat in cases of infection or trauma or some kind of irritation. After the inflammation phase with a lot of specific reactions comes the "resolution phase" to heal completely. Sometimes inflammation is chronic in certain diseases and then needs to be treated. For example, cardiovascular diseases such as asthma and atherosclerosis are chronic and needs to be treated on a long term basis. The origin of such diseases initiates with the above said leukotriene molecules and their formation can be prevented by leukotriene inhibitors.

Biosynthetic pathway

To prevent the diseases, it is important to know about the biological pathway of leukotrienes. Leukocytes have the capability for the complete bio-synthesis of leukotrienes from arachidonic acid, an unsaturated fatty acid found abundant in liver and brain. The leukocytes can be activated by a variety of external stimuli such as antigens, oxidants and immune complexes that trigger an increase in the intracellular concentration of calcium. This promotes the transfer of several proteins to the surface of the nuclear membranes like 5-LO (5-lipoxygenase) where a membrane embedded protein FLAP (Five lipoxygenase activating protein) is located. Ultimately, arachidonic acid is converted to the leukotriene derivative LTA_4 with the assistance from 5-LO and FLAP. LTA_4 is then converted to other leukotrienes that act as chemo attractants for specific white blood cells to the site of inflammation or cause mucus secretion in the airways.

Function of FLAP (Five lipoxygenase activating protein)

In the leukotriene synthesis pathway, the only function of FLAP is to assist in the transfer of arachidonic acid from the membrane to 5-LO. In fact, FLAP does not have any enzymatic activity on its own. It is a protein embedded in the nuclear membrane whereas 5-LO is recruited to the membrane by calcium but it is unclear if they physically interact or how arachidonic acid presented to 5-LO.

Nanodiscs

Since the complex formation between the integral membrane protein FLAP and soluble protein 5-LO depends on the presence of a membrane, we thought that the novel nanodiscs would be suitable for this study. Nanodiscs are circular fragments of phospholipid bilayer

encircled by two copies of membrane scaffold proteins (MSPs). The nanodiscs are small soluble pieces of membranes of defined sizes and have lot of advantages for structural and functional studies. To make it simple, nanodiscs resembles a compact disc.

Aim

The main aim of this project is to develop a protocol for the generation of nanodiscs containing five lipoxygenase activating protein (FLAP) and to verify the presence of FLAP protein in the nanodiscs.

Project

The functional unit of FLAP is a homotrimer of 54 kDa that was purified in the detergent DDM. Two derivatives of membrane scaffold protein such as MSP_1 E_3 D_1 and MSP_2 N_2 were expressed and purified. MSP_2 N_2 should give 17 nm diameter discs, however it turned out to be unstable in our hands and the smaller version of MSP named MSP_1 E_3 D_1 was used for the project. This gives 12.8 nm diameter discs and provides just enough space for one FLAP and one 5-LO. So called reconstitution of FLAP into MSP_1 E_3 D_1 nanodiscs was done by solubilization of the lipid POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) with detergent (sodium cholate) followed by addition of MSP_1 E_3 D_1 and FLAP in various ratios to optimize the protocol. The reason for choosing the lipid POPC is that 5-LO binds to phospholipids that specifically contain an unsaturated acyl chain at the sn-2 position like the oleoyl in POPC. Cleavage of His-tag from MSP_1 E_3 D_1 was performed with AcTEV protease after reconstitution of FLAP in MSP_1 E_3 D_1 nanodisc. FLAP protein remains uncleaved inside the nanodisc since it does not contain the TEV protease cleavage site. Purification was done by His-column and purified reconstituted FLAP nanodiscs were stored at 4 °C.

Results

Gel electrophoresis both in denaturing and native conditions followed by Western Blot gave clear evidence that FLAP was present in the nanodiscs. Negative stain EM (Electron Microscopy) was also used to visualize but was less conclusive.

Conclusions

This project aim, the protocol for production of nanodiscs containing FLAP was achieved. This experiment provides the first step in the attempt to answer the biological question if 5-LO and FLAP interact physically and how arachidonic acid is presented to 5-LO using nanodiscs as a membrane model.

Future

Many protein complexes in the cells carry out their function on a membrane surface or within the membrane but very little is still known about these reactions. The simple way to prepare model membranes, the nanodiscs, by just mixing a membrane scaffold protein (MSP) and lipids and for some projects also a target membrane protein, makes the study of membrane proteins in some ways easier. Both sides of the nanodisc membrane surfaces are accessible and thus also both sides of a membrane protein inside the disc. This project contributes to the studies of complex formation between integral membrane proteins and soluble proteins. Finally, the research project not only teaches us about FLAP at structural and functional level, but could contribute in pharmaceutica research for many cardio vascular diseases which possess a threat to the society.

Appendices

Appendix 1

$MSP_1E_3D_1$ sequence

MGHHHHHHHHDYDIPTTENLYFQGSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMS
KDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLS
PLGEEMRDRARAHVDALRTHLAPYLDDFQKKWQEEMELYRQKVEPLRAELQEGAR
QKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGA
RLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKKLNTQ.

MSP_2N_2 sequence

MGHHHHHHHHDYDIPTTENLYFQGSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMS
KDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLS
PLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKAT
EHLSTLSEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKKLNTQGTPVTQEFWDN
LEKETEGLRQMSKDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGA
RQKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGG
ARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKKLNTQ

Appendix 2

Buffers and chemicals preparation

- LB medium:

  LB broth base powder (Invitrogen) and distilled water.

- TB medium:

  Terrific broth powder (Invitrogen), 4 mL glycerol, and distilled water.

- MSP buffer preparation:

- Wash buffer : 40 mM Tris HCl pH 7.8 0.3 M NaCl 1 % triton pH 8.0

- Wash buffer 2: 40 mM Tris HCl pH 7.8 0.3 M NaCl 50 mM cholate pH 8.0, 20mM Imidazole

- Wash buffer 3: 40 mM Tris HCl pH 7.8 0.3 M NaCl 50 mM Imidazole pH 8.0

- Elution buffer: 40 mM Tris HCl pH 7.8 0.3 M NaCl 0.5 M Imidazole.

- MSP standard buffer: 20 mM Tris HCl pH 7.8 0.1 M NaCl 0.5 mM EDTA pH 7.4.

FLAP buffer preparation:

- FLAP equilibration buffer: 25 mM Tris HCl pH 7.8, 10 % glycerol, 0.1 % DDM 5 mM β-Mercaptothanol.

- FLAP wash buffers: (all buffers contains 0.03 % DDM, and 5 mM β - ME)

- Wash buffer 1: 25 mM Tris HCl pH 7.8, 10 % glycerol, 0.1 M NaCl, 20 mM Imidazole.

- Wash buffer 2: 25 mM Tris HCl pH 7.8, 10 % glycerol, 0.5 M NaCl, 50 mM Imidazole, 0.5 M NaCl.

- FLAP Elution buffer : 25 mM Tris HCl pH 7.8, 10 % glycerol, 0.5 M NaCl, 300 mM Imidazole.

- PD-10 column : 25 mM Tris HCl pH 7.8, 10 % glycerol, 0.1 M NaCl, 0.03 % DDM, 5 mM β - ME)

- NI-IMAC column regeneration: 50 mM Tris HCl pH 7.8, 50 mM EDTA.

5-LO buffer preparation:

- Lysis buffer : 50 mM Tris pH 8 2 mM EDTA 2 mM β - ME
- Wash buffer 2 : 50 mM Tris pH 8 2 mM EDTA 2 mM β - ME, 0.5 M NaCl
- Wash buffer 1 : 50 mM Tris pH 8 2 mM EDTA 2 mM β - ME, 0.1 M NaCl
- AMP elution buffer : 12 mM AMP 50 mM Tris pH: 8.0 2 mM EDTA
  5 mM β – Me 0.1 M NaCl
- ATP elution buffer : 12 mM ATP 50mM Tris pH: 8.0 2mM EDTA 5mM β - Me
  0.1 M NaCl

Western blot transfer buffer:

- 1 X Tris glycine (with SDS): 0.05 M Tris, 0.19 M Glycine 0.1 % SDS)

Phosphate buffer:

- 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4, 0.24 g of KH2PO4 pH: 7.4 adjusted to 1 L.

Appendix 3

Procedure for reconstitution of FLAP

i. xx µl of POPC (32.9 mM) was dried under a stream of nitrogen gas to give a thin layer of lipid film desiccated in a high vacuum overnight.

ii. Next day, the lipid film was resuspended with 65.8 mM sodiumcholate and then sonicated and vortexed until the solution is transparent.

iii. In an eppendorf tube, xxx µl of FLAP protein at xx µM and xxx µl of $MSP_1 E_3 D_1$ at xx µM was added to the detergent solubilised lipid (xx µl).

iv. xxx µl of sodium cholate from the initial concentration at 232 mM was added to the above recipe to give xx mM final concentration.

v. To attain the total volume of xxx µl, xx µl of MSP standard buffer was added (20 mM Tris-HCL, 0.1 M NaCl, 0.5 mM EDTA, pH 7.4).

vi. Reconstituted FLAP nanodisc sample was incubated in ice for 60 minutes.

vii. Since reconstituted FLAP and nanodiscs were solubilised with detergents (DDM and sodium cholate respectively), Bio-beads SM-2(two-third to the volume of the final mixture, i.e. xxx µl) were added to remove them and incubated at 4 °C overnight.

viii. It is necessary that the final detergent concentration in the reconstituted mixture should be in the range of 12-40 mM. In this protocol, the final sodium cholate concentration was xx mM.

ix. LPR plays a significant role in the reconstitution of membrane proteins. In this project, several titrations were performed with different LPRs such as 108, 112.5, 115,120,125;. Finally, the optimised LPR was xxx.

x. The molar ratio of $MSP_1 E_3 D_1$: FLAP monomer: POPC was x:x:x

xi. The final concentration of $MSP_1 E_3 D_1$ and FLAP in the nanodisc was calculated at xx µM and xx µM respectively.

xii. The prepared reconstituted FLAP nanodisc was stored at 4 °C and used within a month or frozen to -80 °C.