The relation between dynamics and activity of phospholipase A/acyltransferase homologs

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Summary

Phospholipase A/acyltransferases (PLAATs) play important roles in living organisms. From being tumor suppressors to regulating obesity to producing important bioactive lipids, new facets of these enzymes are being discovered and much of the inner workings of these enzymes are still unknown.

In **chapter 1**, the PLAAT family is introduced along with the knowledge obtained over the last two decades. PLAATs are a five-membered family of enzymes that share common secondary structure and a highly conserved sequence NCEHFV, which contains the catalytic triad member cysteine that acts as a nucleophile in catalysis. With the exception of PLAAT5, all PLAATs have an N-terminal catalytic domain and a C-terminal membrane anchoring domain. The PLAATs catalyze the first step of reaction, leading to the formation of N-acylethanolamines (NAEs), an important class of bioactive lipids that play a variety of roles, such as anti-inflammation, catabolism of fat, anti-apoptotic activity, ligands for endocannabinoid receptors etc. PLAAT3 and PLAAT4 are homologues but both display marked differences in their activity and specificity. For PLAAT3, the C-terminal domain is crucial for $PLA_{1/2}$ activity, whereas the same domain is not critical for the same in PLAAT4. Furthermore, the N-terminal domain of PLAAT4 is a more active phospholipase, more rapidly hydrolyzing acyl-protein intermediates than PLAAT3. Physiologically, PLAAT3 regulates triglyceride metabolism in adipose tissue, whereas PLAAT4 plays a crucial role in tumor suppression, particularly in metastasis and invasion. Studying these two proteins on a molecular level was the topic of this thesis. The two methods- NMR spectroscopy and MD simulations that were deployed to elucidate the differences, are described in the chapter along with the thesis outline.

In **chapter 2**, the insights obtained from thermostability, NMR dynamics experiments and salt bridge analysis are outlined. PLAAT3 shows higher thermostability than PLAAT4, owing to its compact structure with well dispersed network of salt-bridges throughout the structure which in PLAAT4 are less and are confined to certain regions. Residue specific fast (ps-ns) and slow (ms) dynamics were studied using NMR spectroscopy. Apart from the highly disordered loop L1 in both the enzymes, PLAAT3 largely maintains a rigid structure and its active site does not show significant dynamics. This is however not true in case of PLAAT4, which is overall more flexible than PLAAT3 and its active site is much more mobile. This was

inferred by the low order parameters for residues that immediately precede the catalytic nucleophile C113. The absence of resonances for residues in the active site region indicated broadening due to chemical exchange. Millisecond timescale dynamics experiments also identified a dynamic active site region. Such a dynamic patch is not observed in PLAAT3. We speculated that the difference in dynamics can explain the activity differences observed PLAAT3 and PLAAT4. The NTD of PLAAT3 may show little activity toward its substrate due to lack of active site dynamics, whereas the dynamics of PLAAT4 may enable its activity.

To develop a model of what the dynamics of PLAAT4 would entail, molecular dynamics simulations were used, findings of which are presented in **chapter 3**. The global RMSD of PLAAT4 was observed to be higher than of PLAAT3, suggesting that the former undergoes conformational fluctuations during the simulations, confirmed by higher radius of gyration than for PLAAT3. Principal component analysis to study essential dynamics shows that PLAAT3 maintains a largely rigid conformation, especially an ordered secondary structure. In PLAAT4, more concerted motions were observed, especially in and around the active site. Apart from the flexible loop L1, loop L2(B6) and C113S_γ showed correlated motions. A significant rearrangement was observed in L2(B6) and since the α-helix A3, of which C113 is a part, is connected to this loop, its rearrangement rendered the active site quite mobile. The interaction between the cysteine of the catalytic triad and the histidine rings was also disrupted during each of the two runs.

The significance of the loop L2(B6) in modulating the activity was further studied in **chapter 4**. *In-vitro* and *in-silico* mutagenesis were carried out to swap the loop L2(B6) between PLAAT3 and PLAAT4. $L2(B6)_4$ mutation in PLAAT3 conferred higher phospholipase activity than in wild type PLAAT3 by introducing greater dynamics around the active site region, causing increased substrate accessibility. However, when $L2(B6)_3$ was introduced in PLAAT4, the activity increased as well. By MD simulations on the L2(B6) mutants, the role of these loops in overall dynamics were also studied, which did not yield clear differences in dynamics between the wild-type and mutant proteins. Salt bridge life-time analysis revealed that introduction of PLAAT4 L2(B6) in PLAAT3 caused disappearance of 50% existing salt bridges, enabling mobility. This mobility due to lack of salt bridges might be crucial for greater solvent accessibility. A similar effect was observed in PLAAT4_$L2(B6)_3$, which caused disappearance of 50% existing salt bridges in PLAAT4 suggesting that the swapping of L2(B6) changes the chemical environment and existing salt bridge networks in both PLAAT3 and PLAAT4. This observation, along with the data from phospholipase assay, strongly support

our hypothesis that the L2(B6) mutation in PLAAT3 restructured the salt bridges and introduced greater dynamics around the active site region, causing increased substrate accessibility.

Chapter 5 describes a project done with Dr. Hugo van Ingen aimed at removing slow pulsing artifacts in ^{15}N CPMG relaxation dispersion experiments introduced by using a single ^{1}H decoupling power for all CPMG pulsing rates. The slow-pulsing artifacts were analyzed in detail and it was demonstrated that the artifact can be suppressed through the use of composite pulse decoupling (CPD). The performances of various CPD schemes are reported and it was demonstrated that CPD decoupling based on the 90_x–240_y–90_x element results in high-quality dispersion curves free of artifacts, even for amides with high ^{1}H offset.

Chapter 6 describes general discussion on the outcomes and insights generated in this thesis.