7.1 Domain orientation and inter-domain interaction in t-PA

Human tissue plasminogen activator (t-PA) activates plasminogen (PG) to plasmin (PN) on the surface of fibrin clot and hence used as therapeutic agent in cardiovascular diseases. To date, there is no high resolution structural information available for the full-length t-PA. Crystallization of intact t-PA molecule is difficult and 17 intra-domain disulfide bonds maintain correct folding and activity of t-PA in physiological conditions. Although atomic-resolution crystal and NMR structures of individual domains except Kringle1 are available, structural information on how these domains and glycosylations are oriented in space relative to each other is missing; thereby failing to explain physiological functions. Therefore, to develop the bonsai versions of this protein with judicially altered activity, availability of a full length structure would be helpful. Relative orientation of the individual domain to obtain the selective activity against fibrin is the quest of this work.

SAXS data analysis followed by constraint-based modeling of intact t-PA showed that the predominant conformation exhibited by this protein has a R_G and D_{max} of 33 and 110 Å respectively. Interestingly, computational model generated within SAXS based shape constraints revealed that the global shape of t-PA is bi-lobal in solution where F-domain orients towards the K2 domain.

SAXS intensity profile acquired from samples of t-PA was used to 'steer' structures of individual domains and the homology model of the first kringle domain to generate a structural model of the protein part of t-PA. Differences in the shape profiles of SAXS data-based dummy atom and proteinogenic models aided in grafting glycosylated moieties on the coordinates of t-PA. Normal-mode analysis (NMA) of our model revealed that the fibrin binding F/E domains 'communicate' with the active-site in the P domain via Kringle2, while Kringle1 is positioned away from these long-distance interactions. NMA of the

composite model also showed that the molecule has large extent of inherent motion relative to each domain oriented in the space. SAXS study also brought forth the role of a catalytic domain solvent exposed exosite involving Asn^{184}.

We developed the first composite highly reliable first working model of full length t-PA which would be of help in rational designing of engineered activity in this protease. This model is physiologically relevant and correlates well with the biochemical analysis published earlier. In absence of high resolution structure for full length t-PA, this composite model of t-PA will be helpful, particularly, in engineering the functions of this fibrin specific enzyme. For pictorial summary of this part of thesis, please see figure 7.1 A.

7.2 t-PA potentiation in the presence of fibrin mimic peptide

To date, no small molecule potentiator of thrombolytic protein t-PA is known either due to lack of any leads in the activity based screening assays and/or due to paucity of reliable structural model of t-PA. Extending SAXS based study of t-PA, we screened for molecules which may induce fibrin-like functioning of t-PA. Through trials, we found that penta-L-lysine (P5) has ability to enhance t-PA activity *albeit* much lower than fibrin does. P5 could increase t-PA activity in dose dependent manner, averaging to almost three fold more than in absence of P5 or any other peptide. SAXS data analysis confirmed that there is no P5 induced cross linking or association of t-PA molecules, and no change in the dimensions of t-PA. SAXS profile of t-PA+P5 remained similar to t-PA but showed differences in the wide-angle zone corresponding to dimensions in the range of 2-3 nm. Surprising result was seen in the Kratky plots which revealed that P5 quenches the inherent disorder in t-PA in a dose dependent manner. Model building supported the observation that the 'gap' between two gross

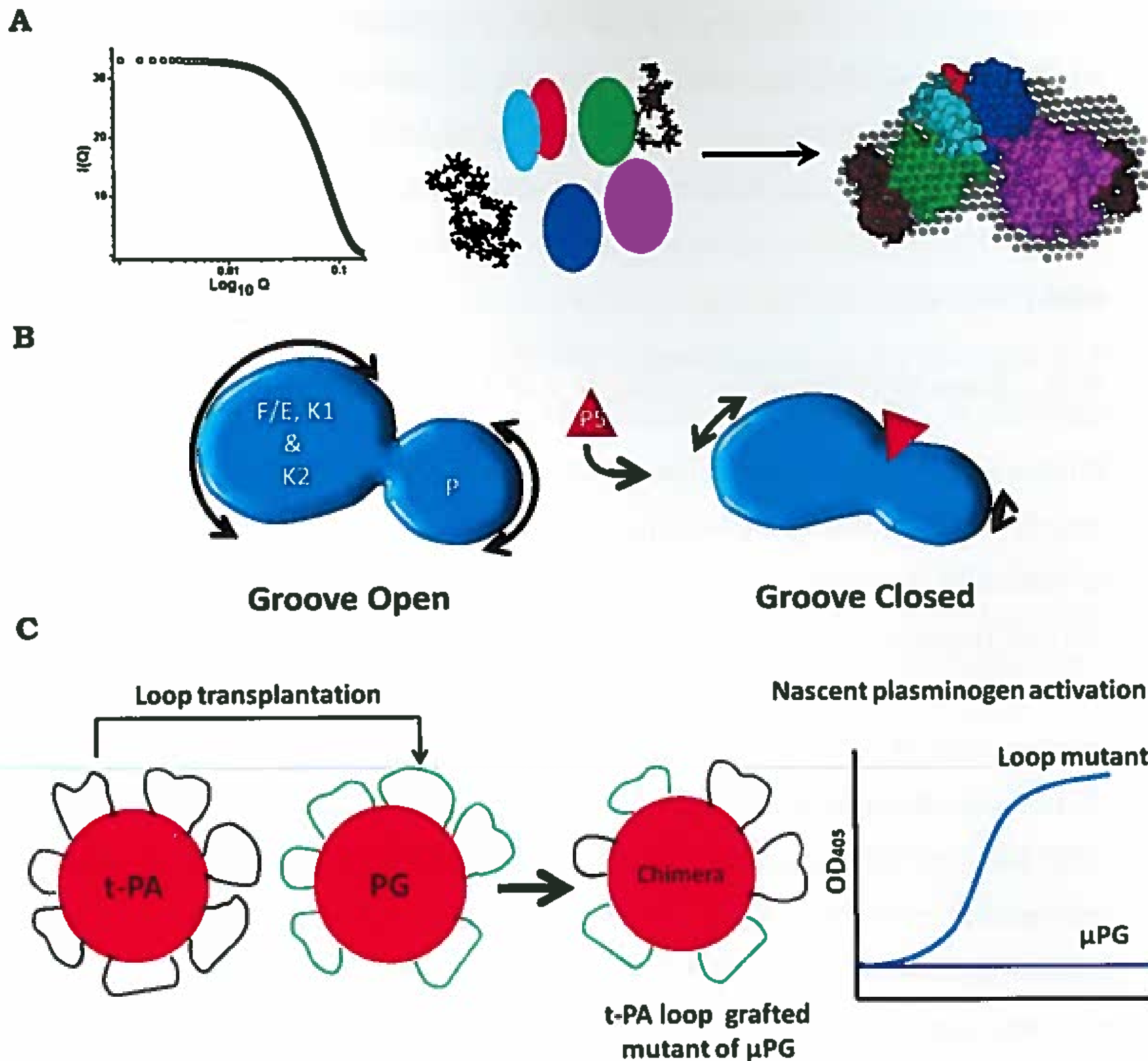

Fig 7.1 Overall summary of thesis. A) Data of the unaggregated protein sample was collected and SAXAS data based dummy model was compared with the full length t-PA domains joined by SASREF program. The remaining gap was filled by the sugar moieties which fitted well in remaining gap and the interactome analysis of the inter-domain and long range interactions brought forth the role of the exosites in communicating the information from F/E domain to t-PA catalytic domain. This figure has been 'Reprinted (adapted) with permission from (Rathore, Y. S., M. Rehan, *et al.* (2012). 'First structural model of full-length human t-PA: a SAXS data-based modeling study.' J Phys Chem B 116(1): 496-502.) © Copyright (2015) American Chemical Society. **B)** Poly-L-lysine peptides of 2-21 amino acid length were synthesized and check for their potentiation. Penta-L-lysine enhanced the t-PA activity and structural studies brought forth that P5 specifically 'slips into' the groove between the K2 and P domain. This peptide enhances the exosite mediated interactions/communications between F/E-K2-P domains resulting in increased catalytic efficiency. **C)** Reported exosites and loops of t-PA that interact with PG were grafted on the scaffold of µPG. Transplantation of the loops of t-PA on µPG resulted in the activation of PG. As a proof of concept exercise, we transferred the enzyme activity of t-PA onto the related conserved scaffold.

domains of t-PA disappeared with P5. Molecular docking showed that while other peptides docked mainly on surface of different t-PA domains, P5 'slipped into' a groove formed by K2 and P-domain in the model of t-PA. Long distance interaction analysis of P5-docked t-PA revealed a substantially enhanced number of interactions between K2 and P domains *via* P5 (Figure 7.1 B).

7.3 New information about t-PA mechanism of action

There was a clear gap between the K2 and P domain of t-PA model which was somewhat connected by long distance interactions, specifically involving Asn^{184}. Possibly, this gap opens and closes as a part of inherent 'breathing' events of the unliganded t-PA maintaining a frequency of communication between one domain composed of tightly packed F/E, K1 and K2 domains and relatively away P domain. In nature, though the LBS resides only on K2 domain, the compact F/E, K1 and K2 domains possibly relay some message to P domain to get 'activated' or increase its catalytic turnover. To date, it remains undeciphered - what is that message? Specifically, in presence of P5 peptide, we found that t-PA gets activated and actually executes function which in nature is triggered by binding to fibrin. SAXS studies showed that P5 induces unique shape changes in t-PA in comparison to other peptides tested, particularly in WAXS zone. Kratky analysis clearly showed that P5 induced loss of inherent motion in t-PA or somehow 'hand-cuffed' the protein. t-PA with 17 S-S linkages is a protein with few orders of motion, and that too was seen to be curtailed by binding of P5. Modeling revealed that the gap between the two shapely domains i.e. one domain composing F/E, K1 and K2, and the other composed of P domain disappeared in presence of P5 peptide. Docking studies suggested that P5 showed high preference to sneak inside the gap formed between the K2 and P domain, and its presence actually enhanced the number of interactions between these two domains, most enabled via this

peptide. Above observations allow us to put forth that either P5 mimics events which occur in t-PA as it binds fibrin or it could be an independent mode of activation of t-PA. This dissection would remain an objective of study in our group, but for now, it is clear that presence of P5 in the communicating groove between K2 and P domain substantially enhances the communication frequency and/or its nature. This communication sends a message from K2 domain to P domain which shifts active-inactive equilibrium of the P domain towards a more active one. For pictorial summary of this part of thesis, please see figure 7.1 B.

7.4 Cross-transplantation of loops of t-PA onto human PG catalytic domain generates detectable PG activation

The most striking part of the protease evolutionary design is the tight control of function in physiological milieu in terms of their specificity, optimized catalytic turnover and tight regulation of their activity. Serine proteases share a great deal of homology in their primary and three dimensional structures. Serine proteases have a similar double barrel β-scaffold and significantly differ in their loops structures. We selected the t-PA and PG proteases as these attracted us given their homology in three dimensional fold yet one protein (PG) acting as substrate of other (t-PA) during fibrin clot dissolution. We were interested to understand basis of differential functions arising from loops. Since the scaffold of these proteases imparts loops optimized required flexibility for natural function, it can be presumed that difference in function arises because of loops and their mobility in some signature movements specific for an enzyme. We docked μPG and t-PA, performed sequence comparison and also studied the data available on their structure –function analysis. These *in silico* exercises helped us filter the t-PA loops which could be grafted on the μPG scaffold for generating t-PA like activity in it, including one loop already reported as hydrophobic exosite in t-PA. We began with three

loops and decorated these loops in PG by replacing loops of PG standalone and in combinations with other loops. Mutant proteins were expressed in the bacteria and protein purified exploiting the ion exchange chromatography. The activity assays of the mutants for activating the PG with proper controls revealed that these mutants activated the PG in loop additive manner. Addition of all the three loops selected resulted in a significant 0.324% of t-PA activity. This shows, as a proof of concept, that enzyme functions can be modified by decorating loops on related/unrelated scaffold. However, some control experiments are required for ruling out any activity that arises due to PG activators present in the plasma membrane. Experiments are underway in our lab to express these mutant proteins in the yeast expression system *Pichia pastoris*. . For pictorial summary of this part of thesis, please see figure 7.1 C.