Structural and Functional Studies on Chimeric Proteins and Mutants of alpha-Hemolysin.

α-Hemolysin of *Staphyloccus aureus* has attracted a lot of attention from structural biologists and biotechnologists for its potential applications in biotechnology and therapeutics. Structural information as provided by the crystal structure has helped to inspire new applications. Engineered αHL molecules with altered functional properties and with built in triggers and switches have been prepared. Hence, basic studies on the folding of molecules like αHL are always welcome, since they reveal information that helps in exploiting their functions.

The crystal structure of its heptamer stage has been a tremendous help in assigning functions to various regions of the molecule. As per the crystal structure, the carboxyl terminus is well exposed to the solvent and is not critically involved in inter-protomer interactions. Hence, it is unable to explain why deletion of residues from the carboxyl terminus cripples the protein to such an extent that it is unable to undergo even the oligomerisation step on the target membranes. In the present study, we have delineated the role of the carboxyl terminus of αHL in greater detail by examining the physical state of two model mutants of αHL: αHL(1-289), a carboxyl terminal deletion mutant (CDM) and αHL(1-331), a carboxyl terminal extension mutant (CEM). Denatured αHL can be refolded efficiently with near total recovery of its activity upon restoration of non-denaturing conditions. Various biophysical and biochemical studies on the three proteins have revealed the importance of an intact carboxyl terminal in the folding of αHL. The CDM exhibits a marked increase in susceptibility to proteases as compared to αHL. αHL and CEM exhibit similar fluorescence emission maxima and that of the CDM is red shifted by 9nm, which indicates a greater solvent exposure of the tryptophan residues of the CDM. In addition, the CDM binds 8-anilino-1-naphthalene sulfonic acid (ANS) and increases its fluorescence intensity significantly unlike αHL and CEM, which show marginal binding. The circular dichroism studies point that the CDM possesses significant secondary structure, but its tertiary structure is greatly diminished as compared to αHL. These data suggest that the CDM has several of the features that characterise a molten globule state. Experiments with freshly translated mutants, using coupled *in vitro* transcription and translation, have further supported our observations that deletion at the carboxy terminus leads to major structural perturbations in the water soluble form of αHL. Hence, while the

amino terminus is important for the functional pore formation, the carboxyl terminus is crucial for correct folding and maintenance of the water-soluble form of αHL. Removal of just four residues from the carboxy terminus makes the protein unable to proceed beyond a molten globule-like stage. This information would be valuable to understand how membrane-binding toxins like αHL carry out their folding and function. Since single amino acid substitutions at the carboxyl terminus do not affect the function, the length (*i.e.* backbone) of the protein appears to be vital instead of the actual sequence at the carboxyl terminus. In addition, we have shown that the addition of an extra sequence at the carboxyl terminus does not affect the folding or function of αHL. Such molecules could form the basis for construction of new molecules in the future.

The channel forming property of αHL is well known and well exploited. One observation that has not been pursued in detail is the ability of αHL to inhibit the autophosphorylation of the EGFR. This is a significant observation because inhibitors of EGFR signaling pathway are in great demand at present for their potential against disorders caused due to the unregulated EGFR signal transduction pathway. In the present study, we have analysed this phenomenon in detail by employing full length αHL as well as its chimeras with TGFα. Two chimeras were constructed in which the gene for TGFα was fused to the 5' end of the gene coding for αHL. The two fusion proteins, designated THL and TCHL, varied in the number of residues between the two protein moieties (3 and 7 respectively). They were found to be similar in all the biological properties tested which indicated that the length of the linker peptide does not influence the folding and function of the two moieties TGFα and αHL.

The chimeras were expressed in *E. coli* and a protocol for the purification and refolding of the chimeras from inclusion bodies was devised. This involved purification of the inclusion bodies, their solublisation in urea, equilibration in a redox buffer in order to allow formation of the disulfide bonds of TGFα, renaturation of the proteins by dialysis and purification by ion-exchange chromatography. The devised protocol yielded good quality proteins for deeper study. Both the moieties, *i.e.*, TGFα and αHL, have retained their individual

functions. TGFα was able to phosphorylate the EGFR, as demonstrated by phosphorylation experiments with detergent extracts of A431 cells. The αHL moiety of the fusion proteins THL and TCHL was able to bind, oligomerise and lyse the rRBCs. In solution proteinase K digestion pattern showed that the folding of the αHL moiety in fusion proteins is similar to that of αHL. Hemolytic activity studies revealed that the chimeras could lyse the cells, but were about four times poorer in their activity than αHL. Kinetics of lysis studies suggests that this is due to a delay in the prepore to pore transition. This is in agreement with the knowledge that the amino terminus has role to play in undergoing the prepore to pore transition, as has been shown with an amino terminal deletion mutant which is considerably slow in undergoing this transition step. Hence, extension of the amino terminus in the form of TGFα has most likely resulted in a similar effect. The fusion protein oligomers have been observed by electron microscopy and appear to be similar to the oligomers formed by αHL. Hence, it is possible to construct fusion proteins by attaching moieties at the amino terminus of αHL without significantly affecting its folding and function.

The oligomers formed by αHL and its chimeras on the epidermal cell line A431 appear to be similar to those formed on rRBCs in terms of their stability and kinetics of formation. Moreover, ATP release assay has shown that permissive pores are formed within 5 mins of incubation with αHL, and in about 20 mins in case of TCHL.

Following up the observation that αHL can tamper with the auto-phosphorylation property of the EGFR, a detailed study of the effect of αHL and the chimeras on the EGRF auto-phosphorylation and its downstream signals have been performed. The fusion proteins stimulated the EGFR auto-phosphorylation at low concentrations and initial time points of the higher concentrations, which shows that the TGFα moiety of the fusion protein has bound to the receptor and mediated its function. At later time points of higher concentrations, the αHL moiety brought about the inhibition of the auto-phosphorylation of the receptor. This inhibitory effect of αHL and the fusion protein TCHL was observed on both A431 cells as well as the KB cell line. Inhibition of self-phosphorylation of the

receptor appears to shut off atleast some of the downstream signals of the receptor, because both the association of PLC-γ1 with the receptor as well as its phosphorylation by the tyrosine kinase of the receptor is inhibited.

Various possible reasons for this inhibitory effect of αHL have been explored:

⇒ Direct Binding: Since the toxin inhibits the auto-phosphorylation of both the EGFR and PDGFR, and as there is no sequence similarity between the extracellular domains of the two receptors, the possibility of direct binding to the receptor is very remote.

⇒ The possibility of leakage of ATP and Mg^{2+}/Mn^{2+} ions being responsible for the inhibitory effect of αHL has been ruled out. No difference in the effect of αHL occurred when the phosphorylation experiment was performed in their presence. Although the presence of Ca^{2+} ions in the reaction buffer was found to partially prevent this inhibitory effect of αHL, the involvement of Ca^{2+} ions cannot be concluded in view of the prior studies which have shown that Ca^{2+} ions can modulate the oligomer properties of αHL.

⇒ Involvement of PTPases: PTPase inhibitors orthovanadate and $ZnCl_2$ were able to prevent this inhibitory effect of αHL, which provides compelling evidence for the involvement of a phosphatase in αHL action. The inability of iodoacetamide (acts by alkylating an essential cysteine residue) to prevent the inhibitory effect of αHL might be because not all PTPases get inhibited by all the inhibitors to the same extent. For example, vanadate, which is a strong inhibitor of PTPs, fails to inhibit the nickel-activated calcineurin phosphotyrosine phosphatase.

Hence, the study presented here strongly suggests that αHL is recruiting the help of a PTPase for its action.

Examination of the morphology of the toxin treated A431 cells showed that TCHL caused rounding off and clumping of the cells, which is a characteristic of TGFα action on A431 cells. After 1-2 hrs, vacuolisation of the cytoplasm occurred and a few hrs later, chromatin clumping appears to occur. A more detailed study of these steps would be required to analyse these observations, which, along with the loss of membrane asymmetry as seen by Annexin V binding, are characteristic

features of programmed cell death. Prolonged treatment with both the toxins results in disintegration of a significant cell population. Cell death was also demonstrated by the MTT assay, which provides an indirect measure of cell death by measuring the ability of mitochondria to convert the tetrazolium salt MTT into a colored formazan product. This cell death could have occurred through either or both of the following two possibilities: (a) Inhibition of EGFR signaling, which has been shown to cause apoptosis in a few cases. (b) Perturbation of plasma membrane solute flux due to the formation of permissive pores on the surface of the cells, thereby leading to programmed cell death.

The potential of the fusion protein TCHL was examined in terms of applications in therapeutics. Being an inhibitor of EGFR signal transduction, it has potential against carcinomas that overexpress the EGFR. However, there is the problem of non-specificity of the molecule, which is a characteristic of the αHL moiety. Attempts to remove this non-specificity of αHL may make this molecule a more worthwhile prospect.

The data presented here has opened up many avenues of research. Studies can be carried out in order to explore the mechanism by which αHL causes the activation of PTPase(s) and the type of PTPase involved. Being a membrane binding protein, the involvement of a membrane bound PTPase is a strong possibility. Several receptor like PTPases have been reported, but their functions are not yet known. αHL and its mutants can serve as valuable tools to explore their roles in the cell. Such studies, along with studies on the mechanism by which αHL can trigger cell death, might help in uncovering new molecules involved in cell signaling pathways.