Structure and Function of HAMLET: Epitopes, Membrane Interactions and Molecular Recognition

Ho, Chin Shing

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The Structure and Function of HAMLET

Epitopes, Membrane Interactions and Molecular Recognition

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Abstract

HAMLET (Human Alpha-lactalbumin Made LEthal to Tumor cells) is a complex of partially unfolded human alpha-lactalbumin and oleic acid that kills many different types of tumor cells and shows therapeutic efficacy in animal models and clinical studies. This thesis aims to (1) elucidate the structure of HAMLET and the exposure of biologically active domains, (2) define the contribution of lipids to the tumoricidal effect of HAMLET, (3) characterize the membranes response to HAMLET and the perturbation of membrane associated signaling cascades, (4) use proteomic screens to identify conserved features of HAMLET targets in tumor cells.

Elucidating the structure of HAMLET is important to understand its tumoricidal activity. Paper I present the first low-resolution solution structure of HAMLET, derived from small angle X-ray scattering data. In HAMLET, α-lactalbumin is partially unfolded, with an enlarged globular domain and an extended C-terminal conformation from L105 to L123. Synthetic globular or extended domain peptides triggered rapid ion fluxes in the presence of oleate, were internalized by tumor cells and caused rapid changes in cell morphology and tumor cell death with comparable efficiency as HAMLET. These findings demonstrate that the gain of tumoricidal activity in HAMLET is due to a loss of tertiary structure definition compared to native α-lactalbumin, which lacks such activity.

The contribution of the lipid to HAMLET's tumoricidal activity has been debated. Paper II investigates the contribution of lipids to the tumoricidal effect of HAMLET. Deprotonated oleic acid (oleate) is identified as the functional cofactor in HAMLET and shown to contribute to some but not all of HAMLET's cellular interactions. Partial effects on ion fluxes were observed in tumor cells but unlike HAMLET, oleate did not cause metabolic paralysis or cell death at concentrations relevant to HAMLET. Furthermore, oleate did not trigger cancer related gene expression. Cellular responses to oleic acid were weak or absent, suggesting that fatty acids exert some of their essential effects on host cells when in the deprotonated state. The results highlight the unique properties of the HAMLET complex compared to the lipid alone and suggest that the cellular effects of lipids may be modified in the context of a partially unfolded protein.

Membrane perturbations by HAMLET initiate cellular attack and death. Paper III identifies three critical molecular-level features for the conserved tumoricidal response. I. Rapid membrane perturbations in receptor-free model vesicles and tumor cells suggested that HAMLET-membrane interactions are receptor-independent. II. Formation of HAMLET-Ras membrane clusters in tumor cells and Ras inhibition provided a mechanism to activate a conserved cell death programs. III. Membrane responses were absent in differentiated cells, indicating tumor selectivity. The membrane perturbations might thus provide a physical means for HAMLET to excite membrane conformations serving as surrogate receptors for subsequent signal transduction, leading to cell death.

Paper IV examined the hypothesis that the apparent multitude of cellular targets reflects structural homology and that HAMLET targets epitopes shared by molecules critical for cell survival. By protoarray, HAMLET targets represent protein families critically involved in energy metabolism and cellular homeostasis including ATPases, kinases and small GTPases. In an *in vitro* kinase activity assay, about 70 % of kinases were inhibited by HAMLET. Broad kinase inhibition in HAMLET treated cells was confirmed by a phosphorylation antibody microarray, which identified kinases involved in cancer pathways. The results identify nucleotide-binding proteins as HAMLET targets and suggest that dysregulation of the ATPase/kinase/GTPase machinery contributes to cell death, following the initial, selective recognition of HAMLET by tumor cells.

Key words
HAMLET, alpha-lactalbumin, peptide, oleate, oleic acid, lipid, SAXS, membrane , kinase, nucleotide-binding protein, ion flux, cancer, cell death, protein unfolding

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Submitted, *Equal contribution

Paper IV
Broad recognition of nucleotide-binding proteins by HAMLET.

James Ho Chin Shing, Aftab Nadeem, Catharina Svanborg.

Manuscript.

ABBREVIATIONS

AFM	Atomic force microscopy
ANS	8-Anilino-1-naphthalenesulfonic acid
$BaCl_2$	Barium chloride
BAMLET	Bovine Alpha-lactalbumin Made LEthal to Tumor cells
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
CD	Circular dichroism
CIS	Crystalline insoluble substance
CNG	Cyclic-nucleotide-gated
DEAD	Diethylaminoethyl
DUSP1/10	Dual-specificity phosphatases 1/10
ELOA	Equine Lysozyme-Oleic Acid
ENaC	Epithelial Na^+ channel
ERK1/2	Extracellular-signal-regulated kinases 1/2
EYPC	Egg york phosphatidylcholine
GC/MS	Gas chromatography-mass spectrometry
GdnHCl	Guanidine hydrochloride
HAMLET	Human Alpha-lactalbumin Made LEthal to Tumor cells
HEWL	Hen egg white lysozyme
HIF-1α	Hypoxia-inducible factor 1α
HK1	Hexokinase I
MKK3	Mitogen-activated protein kinase kinase 3
NaCl	Sodium chloride
NAG	UDP-galactose:N-acetylglucosamine
NMR	Nuclear magnetic resonance
PBPS	Porcine brain phosphatidylserine
PFKFB1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1
photo-CIDNP	Photochemically induced dynamic nuclear polarization
SAXS	Small angle X-ray scattering
siRNA	Small interfering RNA
TRP	Transient receptor potential
UV	Ultraviolet

SUMMARY

HAMLET (Human Alpha-lactalbumin Made LEthal to Tumor cells) is a complex of partially unfolded human alpha-lactalbumin and oleic acid that kills many different types of tumor cells and shows therapeutic efficacy in animal models and clinical studies. This thesis aims to

I. elucidate the structure of HAMLET and the exposure of biologically active domains
II. define the contribution of lipids to the tumoricidal effect of HAMLET
III. characterize the membranes response to HAMLET and the perturbation of membrane associated signaling cascades
IV. use proteomic screens to identify conserved features of HAMLET targets in tumor cells

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The Discovery

"All things are ready if our minds be so."
William Shakespeare

"In the fields of observation chance favours only the prepared mind."
Louis Pasteur

The serendipitous finding of HAMLET (**H**uman **A**lpha-lactalbumin **M**ade **LE**thal to **T**umor cells)[1] started a now nearly two decade long period of research[2-11], leading to successful clinical trials[2,12-17]. The HAMLET discovery triggered intriguing questions about protein folding and structural biology, conserved mechanisms of tumor cell death, therapeutic and prophylactic benefits, as well as more general, philosophical scientific questions about the nature of molecular recognition.

HAMLET consists of a partially unfolded human alpha-lactalbumin and multiple oleic acid molecules. The experiment in which HAMLET was discovered investigated the molecular mechanism of *Streptococcus pneumoniae* attachment to host cells, using fractions from human milk to prevent bacteria from binding to A549 lung carcinoma cell line. A casein fraction was shown to inhibit bacterial attachment but in addition, dramatic cell death was observed, suggesting that casein components were able to kill tumor cells[1]. To identify the active constituent of the casein fraction, ion exchange chromatography was used, first without success as no active fraction could be eluted from the matrix using the normal protocol. After it was realized that the active component might be retained

on the column, it was successfully eluted as ''Fraction VI'' with high salt (1M NaCl). This fraction contained human alpha-lactalbumin, as shown by N-terminal amino acid sequencing[7].

Interestingly, purified human alpha-lactalbumin did not cause cell death, suggesting that a structural difference must exist between the tumoricidal component and the native protein but mass spectrometry ruled out post-translational modifications[6]. As casein is produced by low pH precipitation of milk and low pH has been shown to unfold alpha-lactalbumin, we investigated if the 3D structure of the protein might be changed in the active fraction. Near-UV CD spectroscopy showed decreased intensity for the 270 nm minimum and 294 nm maximum, indicating increased flexibility of aromatic residues, while higher exposure of solvent accessible hydrophobic surface was shown by increased ANS fluorescence and a blue-shifted emission spectrum.

Furthermore, the active fraction was shown to contain oleic acid, which is the main fatty acid of human milk and present in human casein. The requirement of this lipid cofactor was demonstrated by producing the complex from the purified constituents[18]. First, alpha-lactalbumin was partially unfolded by removal of the calcium ion and then a protein lipid complex was generated on an ion exchange matrix, which had been preconditioned with oleic acid. The protein-lipid complex was eluted with a NaCl gradient, yielding a single sharp peak. Tumoricidal activity of the eluted complex was demonstrated and the complex was named HAMLET. By screening of 14 fatty acids of different chain length, degree of saturation and cis/trans isomerism, oleic acid (C18:1:9cis) was identified as the optimal cofactor in the HAMLET complex[11].

Alpha-lactalbumin

General properties

The structurally conserved alpha-lactalbumins consist of 123 amino acids, with the exception of rat alpha-lactalbumin, which contains a C-terminal extension of 17 residues. Native human alpha-lactalbumin is defined by a large alpha domain, comprising three major alpha-helices (residues 5-11, 23-34 and 86-98) and two 3_{10} helices (residues 18-20 and 115-118) and the smaller beta domain, composed of a small triple-stranded anti-parallel beta-pleated sheet (41-44, 47-50 and 55-56), a series of loops and a short 3_{10} helix (residues 77-80)[19]. A deep cleft separates the two domains and four disulfide bridges stabilize the overall structure (6-120, 61-77, 73-91 and 28-111). In particular, residues 73-91 connect the two domains. Two hydrophobic cores of alpha-lactalbumin are important for protein folding.

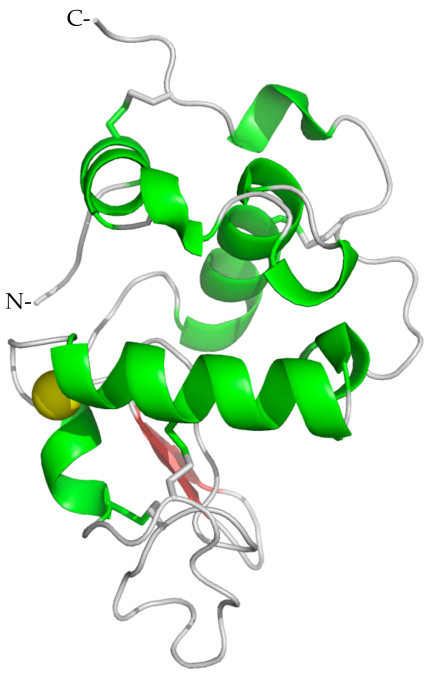

Figure 1 Crystallographic structure of human alpha-lactalbumin (PDB id: 1B9O)

The native structure of alpha-lactalbumin is defined by a high-affinity calcium-binding site, coordinated by three Aspartate residues (82, 87 and 88) and two peptide-carbonyl oxygens (79 and 84). One or two water molecules are commonly found to participate in the coordination. This calcium-binding site differs from the EF-hand motif in the majority of calcium-binding proteins[20] as it is defined by a 10-residue stretch of amino acids (residues 79-88) rather than

a 12-residue stretch with six coordinating residues in the canonical EF-hand motif.

Molten globule states

In addition to the native state (N), alpha-lactalbumin populates several stable intermediately folded states (25) which include Acidic (A), Partly denatured (P), Temperature denatured (T), Apo and Unfolded (U) forms[21]. These molten globules of alpha-lactalbumin are characterized by a **native-like secondary structure, a slowly fluctuating tertiary structure,** a **lack of a cooperative thermal unfolding transition** and **retained compactness**. In these molten globules, the predominant hydrophobic core is the $A/B/3_{10}$ subdomain but the hydrophobic box is poorly formed. The molten globules produced under different conditions often exhibit broadly similar overall characteristics[22,23]. Using pulse-labeled photochemically induced dynamic nuclear polarization (photo-CIDNP), different alpha-lactalbumin molten globules were shown to have different patterns of hydrophobic-core surface accessibilities, likely representing different local minima of the folding landscape[24].

Structural characterization of HAMLET

Against the 'one gene – one protein – one function' paradigm[25,26], we proposed that a protein may respond to different environments by changing their fold and that this process allows a single polypeptide chain to exert vastly different and beneficial biologic functions in different tissue compartments[18].

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HAMLET exemplifies how a loss of three-dimensional structure definition may allow a protein to alter its function. To form HAMLET, alpha-lactalbumin must undergo partial unfolding and bind the fatty acid cofactor[10,18,27]. In complex with oleic acid, alpha-lactalbumin retains its partially unfolded characteristics even at physiological solvent conditions, unlike alpha-lactalbumin molten globules. In the absence of the fatty acid the unfolded state is unstable, and at physiological solvent conditions the protein binds calcium and reverts to the native state.

The loss of tertiary structural packing but a retention of secondary structural content was demonstrated by near- and far- UV CD spectroscopy and the flexibility of the proteins was confirmed by ^{1}H-NMR[18]. Enhanced ANS fluorescence indicated increased exposure of hydrophobic domains and differences in surface topology were detected by limited proteolysis and amide hydrogen/deuterium exchange experiments coupled to mass spectrometry, compared to the native protein[28]. In addition, HAMLET differed from the apo-alpha-lactalbumin, suggesting that the structural characteristics of the oleic acid bound form differ from the molten globule. Different sites in HAMLET, such as those in the beta sheet domain, were less accessible for enzymatic digestion as compared to the apo-alpha-lactalbumin[28].

In addition, the formation of HAMLET also tolerated a certain extent of sequence variation, as alpha-lactalbumin from different species, including bovine, caprine, porcine and equine were shown to form tumoricidal complexes[29]. A lower conversion yield was evident for alpha-lactalbumin derived from other species, however. Naturally occurring, active complex was found only in acid-precipitated human casein, showing that HAMLET formation is unique to human milk. Importantly, unfolding alone is not sufficient to make alpha-lactalbumin cytotoxic. The high affinity calcium-

binding site mutant (D87A)[10] and the fully reduced cysteine-free mutant ($rHLA^{All\text{-}Ala}$)[27], in which all cysteines are substituted for alanines, show no tumoricidal activity, but could be converted to become active when bound to oleic acid.

These structural studies show that a loss of tertiary structure definition can be a mechanism for a protein to attain a new physiological function. This gain of function and loss of 3D structural definition may appear paradoxical, as the functional state has mostly been equated with the lowest free energy state or the native state. Using bovine pancreatic ribonuclease in a system, Christian Anfinsen showed that the protein could refold completely from its fully denatured and reduced form[30]. Thus, the folding was driven entirely by the free energy of conformation.

Protein folding is a dynamic process, through which the primary sequence assembles into the functional three-dimensional configuration. The loss of 3D structure often is equated with ''misfolding'' and related to the creation of harmful protein species[31,32], rather than gain of beneficial biological properties.

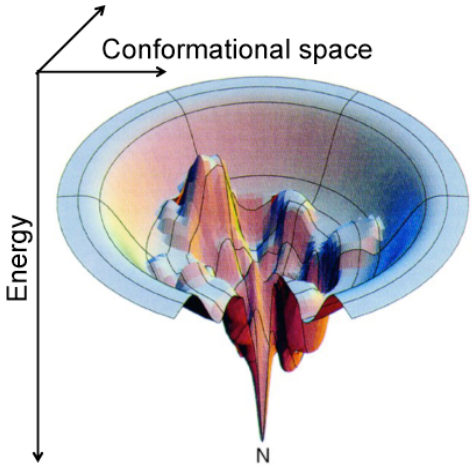

Figure 2 Protein folding energy landscape

The folding process is illustrated by the energy landscape theory[33,34]. The folding funnel, which depicts the folding landscape, assumes a polypeptide chain to adopt a continuum of folding states on the way down the folding funnel through an increasing number of intra-molecular contacts. Various local free energy minima exist on the folding landscape,

depicting folding intermediates. The properties of HAMLET suggest that the binding of oleic acids help to stabilize a kinetic trap[35], a local energy minimum, and keep the protein from reaching the native state. Remarkably, in this state a new biological function is obtained.

Cellular targets of HAMLET

In early studies, HAMLET was shown to invade tumor cells and to interact with different cellular compartments.

Mitochondria and apoptosis

HAMLET interacts with mitochondria, causing mitochondrial swelling and loss of mitochondrial membrane potential[36]. As a consequence, HAMLET-treated cells show responses typical of apoptosis, including cytochrome c release, proapoptotic caspase activation and exposure of phosphatidylserine on the cell surface[37]. Importantly, the apoptosis response is not the cause of cell death, as tumor cells die in the presence of pan-caspase inhibitor, overexpression of anti-apoptotic BCL-2 and BCL-XL proteins, and in Caspase-3 knockout cells. Furthermore, death is independent of the cellular p53 status.

Nuclear uptake and histone interactions

HAMLET crosses the cytoplasmic membrane and rapidly reaches the nuclei of tumor cells[6]. By confocal microscopy, biotinylated HAMLET was detected in the nuclei and >70 % of radiolabeled HAMLET was recovered from the nuclei after 1 hour. High-affinity interactions identified histones H3, H4 or H2B as nuclear targets for HAMLET and *in vitro* experiments demonstrated that HAMLET perturbed the formation of nucleosomes by

binding to these histones[9]. Furthermore, HAMLET acts in synergy with histone deacetylase inhibitors[38] by enhancing histone hyperacetylation, leading to cell death. We have suggested that the interactions of HAMLET with histones and chromatin may 'lock' the cell into an irreversible death pathway.

Proteasome

Proteasomes controls the levels of endogenous misfolded proteins by degrading them in the proteolytic core. In view of HAMLET content of partially unfolded alpha-lactalbumin, the interaction with proteasomes was investigated. HAMLET was shown to target 20S proteasomes in tumor cells and to bind *in vitro* to intact proteasomes and proteasome subunits[39]. Interestingly, HAMLET was less efficiently degraded by proteasomal enzymes than the partially unfolded, fatty acid-free protein. Using intact proteasomes, *in vitro,* HAMET was shown to inhibit proteasome activity and to perturb the proteasome structure and to act as a proteasome inhibitor in intact cells. Thus, the interaction of HAMLET with 20S proteasomes leads to structural changes and inhibition of proteasome activity, which may contribute to the tumoricidal effects of HAMLET.

Prerequisites for the HAMLET sensitivity of tumor cells

Cancer represents a large group of diseases manifested by uncontrolled cell growth. An organizing principle for oncogenesis has been summarized as the 'hallmarks of cancer'[40,41]. These include sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, reprogramming of energy metabolism and evading immune destruction. Underlying these hallmarks is genome instability, which

accounts also for issues stemming from the intervention and treatment of cancer.

Oncogene

The behavior of tumor cells is defined by complex genetic alterations. Still, single oncogenes may be essential and according to the oncogene addition concept[42], tumor cells become heavily reliant on a single oncogene, whose inhibition results in cell death. Dependency of at least two genes is described by the concept of synthetic lethality[43], when inhibition of the inter-dependent components is required for cell death. An extension of the two concepts is the non-oncogene addiction[42], where a wild type gene is as essential as an oncogene.

HAMLET identifies conserved features in cancer cells, as shown by the diversity of tumor cell types that are killed by HAMLET[1]. To identify molecular determinants of HAMLET sensitivity, we used a combination of small-hairpin RNA inhibition, proteomic and metabolomics approaches[44]. The c-Myc- and Ras oncogenes were identified as essential determinants of HAMLET sensitivity in tumor cells. Furthermore, HAMLET sensitivity was influenced by the glycolytic status of tumor cells, with hexokinase 1 (HK1), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 (PFKFB1) and hypoxia-inducible factor 1α (HIF-1α) as significant targets. Binding to HK1 was confirmed *in vitro* and HAMLET caused a rapid metabolic paralysis, characterized by a reduction in about 70 % of the altered metabolites. These studies demonstrated that the HAMLET sensitive phenotype is defined by classical, conserved oncogene defined features in cancer cells.

Membrane responses of tumor cells to HAMLET

The effect on tumor cells is initiated at the cytoplasmic membrane, but distinct molecular changes to the membrane have been difficult to define. To address if the rapid change in cell morphology and the internalization of HAMLET might reflect direct effects on the cell membrane, lipid bilayer models were used *in vitro*[4]. HAMLET was shown to perturb the integrity of egg yolk and soybean membranes, causing membrane elongation and changes in fluidity. In addition, HAMLET caused leakage of vesicular contents, suggesting membrane permeabilisation. Native alpha-lactalbumin or oleic acid had no effect, indicating that a concerted action by the partially unfolded protein and oleic acid for these membrane changes to occur.

HAMLET triggers ion fluxes

In early studies, HAMLET was shown to trigger calcium fluxes in tumor cells[1]. The dramatic membrane responses[4] to HAMLET further suggested that ion fluxes across these perturbed membranes might be a general mechanism to alert tumor cells to the presence of HAMLET and to trigger the death response. Using fluorometry, real time imaging and patch-clamp measurement, HAMLET was shown to trigger K^+, Na^+ and Ca^{2+} fluxes in tumor cells[45]. The characteristics of HAMLET-induced current differ from the biophysical characteristics of widely expressed cation channels such as the transient receptor potential (TRP), epithelial Na^+ channel (ENaC), cyclic-nucleotide-gated (CNG) channels as well as various K^+ channels. Importantly, Amiloride and barium chloride ($BaCl_2$), which inhibit Na^+-carrying channels and transporters or K^+ fluxes, blocked the effects of HAMLET on tumor cells, including morphological changes, global

transcription, MAPK signaling, and cell death.

Ion fluxes activate an early, p38 dependent death response

Transcriptomic analysis of HAMLET-treated tumor cells showed that the p38 MAPK signaling pathway is activated by HAMLET after 1 hour[45]. The activated components included MKK3, a direct upstream activator of p38 MAPK, two dual-specificity phosphatases (DUSP1 and DUSP10) and eight other members in the pathway. The levels of phosphorylated p38 MAPK were increased. In parallel, a loss of ERK1/2 phosphorylation was observed, indicating a shift from cell proliferation to cell death. Inhibition using pharmacological p38 inhibitors or siRNA specific for p38α and p38β delayed cell death by at least 6 hours. Importantly, the p38 activation and the loss of ERK1/2 were reversed by amiloride or $BaCl_2$, suggesting an ion flux dependent activation of p38 MAPK pathway in response to HAMLET.

Normal, differentiated cells showed a weaker ion flux response to HAMLET as well as a major difference in transcribed genes and signaling pathway activation.

Therapeutic and prophylactic efficacies of HAMLET

The mechanism of tumor cell death in response to HAMLET is complex, as HAMLET acts on multiple pathways and organelles concurrently. This complexity, however, does not reduce the importance of therapeutic and prophylactic efficacies of HAMLET, which have been demonstrated in two human studies and several animal models.

(1) HAMLET treatment delayed the progression of human glioblastoma xenografts in nude rats and increased survival, triggering apoptotic changes in the tumor without evidence of cell death in healthy tissue[12].

(2) In a placebo-controlled clinical study, topical administration of HAMLET removed skin papillomas, with no adverse effects[13].

(3) In patients with bladder cancer, local instillations of HAMLET reduced tumor size[14]. Biopsy specimens showed apoptotic response in tumor tissue but not in the surrounding healthy tissue. In addition, HAMLET triggered rapid shedding of tumor cells into the urine.

(4) In mouse MB49 bladder carcinoma model, topical application of HAMLET reduced tumor development. Similar to the patient study, an accumulation of HAMLET was detected in tumor tissue but not in the surrounding healthy tissues[2].

(5) Peroral HAMLET administration reduced tumor progression and mortality in $Apc^{Min/+}$ mice[15]. Moreover, in a prophylactic regimen, HAMLET significantly prevented tumor development.

PRESENT INVESTIGATIONS

AIMS

I. **To elucidate the structure of HAMLET and the exposure of biologically active domains**
II. **To define the contribution of lipids to the tumoricidal effect of HAMLET**
III. **To characterize the membranes response to HAMLET and the perturbation of membrane associated signaling cascades**
IV. **To use proteomic screens to identify conserved features of HAMLET targets in tumor cells**

Paper I

Low Resolution Solution Structure of HAMLET and the Importance of its Alpha-Domains in Tumoricidal Activity

Background

HAMLET is the first member of a new family of protein-lipid complexes with broad tumoricidal activity. Differences in tertiary structural characteristics and solvent exposure of alpha-lactalbumin protein in HAMLET have been documented by CD spectroscopy, ANS spectroscopy, proteolysis and support for the integration of oleate into the complex has been obtained by NMR spectroscopy. However, the structure of HAMLET and the exposure of functional domains have not been determined. Elucidating the structure of HAMLET and the domains interacting with tumor cells is essential, to understand the tumoricidal activity.

Results

In this study, we used small angle X-ray scattering (SAXS) to obtain the low-resolution solution structure of the HAMLET complex. HAMLET exists as a monomer in solution and shows a two-domain conformation with a large globular domain and an extended part of about 2.22 nm in length and 1.29 nm width. HAMLET has a radius of gyration (R_g) of 1.78±0.05 nm and a maximum dimension (D_{max}) of 5.69±0.1 nm. Comparison of the forward scattering of HAMLET with that of a reference solution yielded a molecular mass of 15±2 kDa, further supporting a monomeric complex. The increase in higher angles in the Kratky plot indicates that the protein is slightly flexible.

Superimposition of the crystallographic structure of native human alpha-lactalbumin onto the SAXS model revealed that the major part of alpha-lactalbumin accommodates well in the shape of HAMLET, yielding a good fit with χ^2 of 1.531. The globular domain showed an increase in size, however, consistent with a less defined tertiary structure. An extended conformation of the C-terminal residues from L105 to L123 in HAMLET was not present in the crystal structure of the human alpha-lactalbumin, suggesting that this alteration might be resulted from partial unfolding and oleic acid binding.

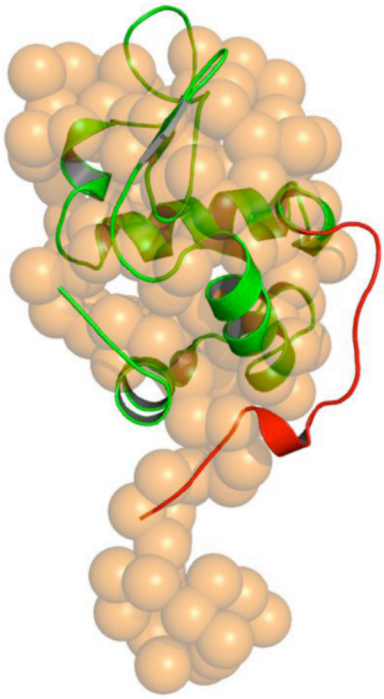

Figure 3 Superposition of the SAXS structure of HAMLET with human alpha-lactalbumin (PDB id: 1B9O)

To identify molecular motifs that become exposed in HAMLET, we further obtained synthetic peptides covering the N- (alpha1) and C- terminal (alpha2), as well as the beta domain (beta) of alpha-lactalbumin. The alpha1 or alpha2 peptides formed complexes with oleate, triggered rapid ion fluxes, were internalized by tumor cells, and caused rapid and sustained changes in cell morphology. The alpha1- and alpha2- oleate complexes also triggered tumor cell death with comparable efficiency as HAMLET. In contrast, the beta peptide was functionally negative in these assays. Further detail was obtained using a library of shorter 15-residue peptides covering the alpha-lactalbumin sequence. The most N-terminal

peptide of alpha1 and two peptides in alpha2 formed complexes with oleate, triggered weak ion fluxes and were taken up by tumor cells.

Conclusion

These findings provide novel insights into the structural properties of HAMLET and the contribution of peptide motifs to the effects of HAMLET on tumor cells. The low resolution SAXS structure supports the notion of HAMLET as a largely monomeric molecular entity with alteration of its tertiary structure in the globular domain and gain of a tail domain due to an extended conformation of the C-terminal portion of the molecule. The alpha-helical domains of HAMLET are identified as functional domains, triggering many of the cellular responses seen in HAMLET-treated cells.

Paper II

Lipids as Tumoricidal Components of Human Alpha-lactalbumin Made Lethal to Tumor Cells (HAMLET); Unique and Shared Effects on Signaling and Death

Background

Long-chain fatty acids are internalized by receptor-mediated mechanisms or receptor-independent diffusion across cytoplasmic membranes and are utilized as nutrients, building blocks, and signaling intermediates. While fatty acids, specifically oleic acid, are integral components of the HAMLET complex, their structural and functional contribution has remained unclear and debated. Using techniques different from those defining HAMLET, other protein-lipid complexes have been produced and used to kill tumor cells, suggesting to some, that the tumoricidal response is triggered by oleic acid alone. Furthermore, effects of lipids on host cells depend on the protonation state.

Results

This study first determined the chemical shifts of the carboxyl carbons of oleic acid (177 ppm) and oleate (182 ppm) by natural abundance ^{13}C NMR. In the HAMLET spectrum, a prominent 182 ppm oleate peak was observed. A second, small and broad 130 ppm peak, corresponding to the olefinic carbon of bound oleate, suggested that oleate was bound to the protein in HAMLET. The function of oleate as a cofactor in HAMLET was further demonstrated by producing oleate-HAMLET, with similar tumoricidal activity as HAMLET. By CD spectroscopy, the oleate-HAMLET complex was structurally identical to HAMLET but the melting temperature, T_m of oleate-HAMLET was higher, suggesting more stable complex that with oleic acid. The lipid concentration in the complexes was determined as 1:4 or 1:5 by acid hydrolysis and GC/MS.

We next compared the tumoricidal effect of HAMLET, oleate-HAMLET, oleate and oleic acid. HAMLET and oleate-HAMLET showed no difference in dose-dependent cytotoxicity (> 80 % dead cells after 3 hour at 35 μM). Oleate or oleic acid did not alter cell viability at the concentration present in HAMLET (175 μM oleate and 35 μM protein). At higher concentrations (15 times), oleate was more cytotoxic than oleic acid and at 25 times, all cells were killed, as expected from the unspecific cytotoxic effects of high lipid concentrations. By real time holography imaging, HAMLET and oleate-HAMLET caused rounding up of cells with similar kinetics. The effect of oleate alone was less pronounced.

HAMLET and oleate-HAMLET triggered rapid K^+, Na^+, and Ca^{2+} fluxes across cell membrane but oleate was a weak inducer of Na^+ and K^+ fluxes. High oleate concentrations did not reproduce the ion flux pattern of HAMLET.

HAMLET causes a metabolic paralysis in tumor cells, with > 60 % reduction in metabolite abundance after 1 hour. By non-targeted metabolite profiling by GC/MS, the effect of oleate was weaker and cellular responses to oleic acid were marginal, suggesting that deprotonation favors cellular interactions of fatty acids. To further characterize the metabolic response, we subjected the samples to targeted metabolite profiling of fatty acids, amino acids and citric acid cycle constituents. HAMLET caused an accumulation of fatty acid metabolites, a loss of early metabolites in the citric acid cycle but an accumulation of late metabolites, and a reduced level of metabolites feeding into the citric acid cycle. In comparison, oleate caused less pronounced change in the fatty acid metabolites, a similar loss of early and accumulation of late citric acid cycle metabolites and a more apparent change in amino acid metabolism with an additional nine showed decreased level.

Furthermore, genome wide transcriptomic analysis was used to compare the cellular effects of HAMLET and oleate (175 μM). A pronounced effect of HAMLET (74 up- and 128 down- regulated) was observed compared with oleate (19 up- and 2 down- regulated). Increased transcription of genes associated with cell death and transcriptional regulation was detected while DNA damage and repair and cell cycle regulation genes were suppressed. Most cancer-related genes that were regulated by HAMLET were not regulated by oleate. More overlap was observed in the high oleate samples, but with important qualitative differences compared to HAMLET and equimolar oleate concentrations.

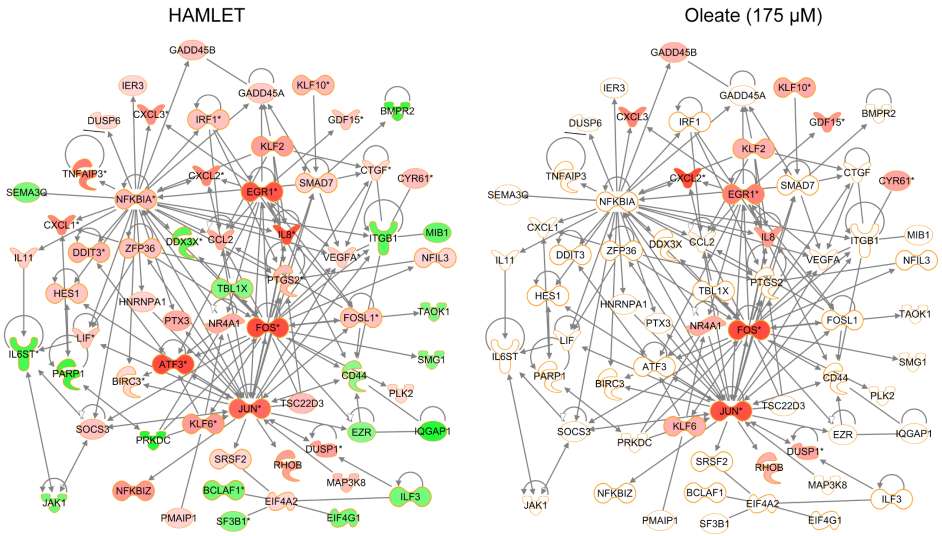

Figure 4 A network of cancer-related genes differentially regulated by HAMLET (left panel) is shown. Down-regulated genes were colored in green, and up-regulated genes are in red. Expression values from the oleate sample (right panel) were overlaid onto the same network, showing that most genes were not regulated.

Conclusion

In this study, oleate is identified as a relevant HAMLET constituent but oleate alone did not reproduce the effects of HAMLET on tumor cells, arguing against oleate as the sole tumoricidal constituent of the HAMLET complex. The results suggest that fatty acids may exert some of their essential effects on host cells when in the deprotonated state and when presented in the context of a partially unfolded protein, like alpha-lactalbumin.

Paper III

HAMLET Drives Plasma Membrane Remodeling and Tumor Cell Death by Receptor-independent Mechanisms

Background

The dynamic membrane bilayer is more than merely a barrier, separating the external environment from the cell interior. Membranes play an active role in virtually all cellular functions, guiding molecular recognition and signaling, membrane trafficking and endocytosis, the generation of energy in the mitochondria, ribosomal localization to the endoplasmic reticulum and nuclear integrity. Since the seminal work on the fluid mosaic model of the plasma membrane by Singer and Nicolson[46], the use of simplified model membrane systems have advanced the understanding of physical properties and responses to stimuli that alter the membrane structure. Such properties include curvature, rigidity, tension and elastic moduli[47]. The effects of HAMLET on tumor cells and lipid vesicles suggested that HAMLET perturbs the membrane, *per se*, but mechanisms and effects have not been characterized.

Results

This study addressed how HAMLET modifies cell membranes and if these modifications may be propagated into oncogene-specific intracellular signals. We identify three critical effects, proposed to characterize the conserved tumoricidal response. **I.** HAMLET triggered rapid membrane perturbations in receptor-free artificial vesicles, converting the well-defined structures into a dense tangle of HAMLET-integrating protrusions. The massive membrane remodeling was reproduced in tumor cells, where HAMLET induced the formation of membrane blebs. **II.** Clusters of HAMLET and Ras were formed in tumor cell membranes, leading to the

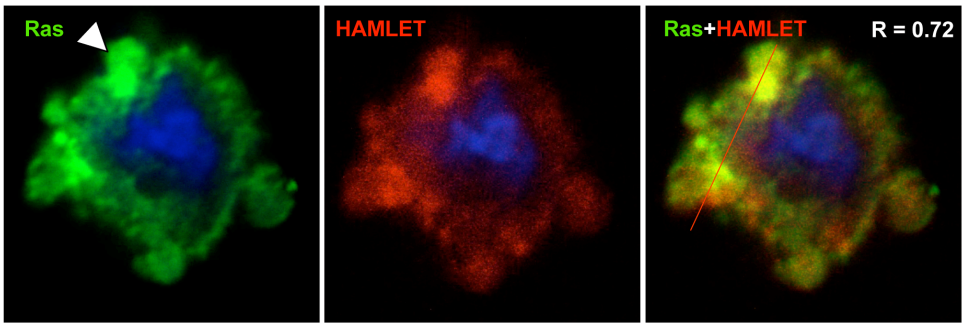

Figure 5 Membrane co-localization (yellow) of HAMLET (red) with Ras (green). R = Pearson coefficient for co-localization.

inactivation of Ras. The interactions of different Ras family members with HAMLET were supported by protein microarray. **III.** These responses were absent in differentiated cells, suggesting that HAMLET-membrane interactions provide a receptor-independent mechanism to activate a conserved cell death program in cancer cells, otherwise conditioned to outlive healthy cells.

Conclusions

This study suggest that membrane perturbations provide a physical means to excite membrane conformations, serving as surrogate receptors for downstream signal transduction, ultimately leading to cell death. These findings also propose a mechanistic basis for tumor-specific cell death. Physical principles underlying this mechanism might explain how HAMLET initiates a universal cell death program conserved in cancer cells, otherwise conditioned to outlive healthy cells.

Paper IV

Broad recognition of nucleotide-binding proteins by HAMLET

Background

HAMLET interacts with multiple organelles and molecular targets. It is not clear if these broad and apparently unrelated interactions may represent molecular motifs, which are conserved among the different HAMLET targets. This study examined the hypothesis that the apparent multitude of cellular HAMLET targets reflects some degree of structural homology among these targets.

Results

Using a proteomic screening approach, nucleotide-binding proteins were identified as HAMLET targets, including ATPases, kinases and GTPases. Remarkably, nucleotide-binding proteins accounted for about 50 % of all HAMLET targets in a protoarray comprising 8000 human proteins. The interaction with HAMLET was verified using purified ATPase/ATP synthase and protein kinases. As mentioned in Paper III, GTPases have previously been shown to interact with HAMLET.

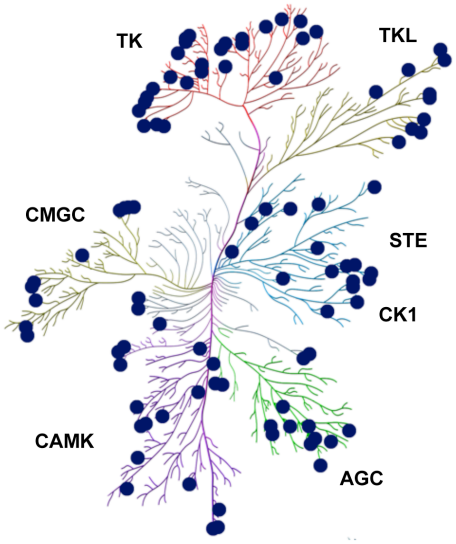

Figure 6 Kinases targeted by HAMLET are mapped onto the human Kinome (blue dots).

HAMLET was shown to bind *in vitro* to the ATPase/ATP synthase and by confocal microscopy, colocalization with HAMLET was detected throughout the cells. Furthermore,

HAMLET inhibited ATPase/ATP synthase activity, *in vitro*, and in tumor cells, a reduction in cellular ATP was observed, consistent with an inhibitory effect.

HAMLET targets were present in all branches of the Kinome map, including Tyrosine kinases, Tyrosine kinase-like and Casein kinase. By cellular functions, 24 belonged to the MAPK signaling pathway, 17 were receptors and 22 were kinases involved in the regulation of mitosis. Inhibition of protein kinases from all branches of the Kinome tree was observed *in vitro*. Phosphorylation array and examination of phosphorylated targets in HAMLET-treated cells confirmed inhibition for a small number of targets. A pathways in cancer analysis demonstrated that the majority of cancer-related kinases are inhibited.

Conclusions

Protein kinases are crucial for signal transduction and over-activity of kinase-dependent cellular functions in tumor cells contributes to increased proliferation and other ''Hallmarks'' of cancer. According to the ''oncogene addiction'' concept, the interference with individual oncogene-driven functions can cause tumor cell death. This study suggests that HAMLET reverses this phenotype by broadly inhibiting nucleotide-binding proteins.

GENERAL DISCUSSION

This thesis addresses the structure-function relationship of HAMLET and the molecules that it perturbs. Specific areas of study include (1) the structure of HAMLET and the function of individual domains in HAMLET, (2) how the lipid influences HAMLET's structure and effects on tumor cells (3) HAMLET's membrane perturbation mechanisms (4) conserved molecular targets of HAMLET in tumor cells.

The structure of HAMLET

To form HAMLET, alpha-lactalbumin undergoes a loss of tertiary structural packing, resulting in increased surface hydrophobicity, as well as differences in surface topology compared to native alpha-lactalbumin. Biophysical characterizations have suggested that HAMLET represents a kinetically trapped intermediate in the folding pathway of alpha-lactalbumin[35].

Paper I provided the first 3D structural model of HAMLET. Based on the resolved two-domain structure and the retained structural similarity to the compact precursor protein alpha-lactalbumin, we demonstrated that alpha-lactalbumin in HAMLET is non-native with an expanded volume of the globular domain and an extended conformation of the C-terminal end of the protein. This structural alteration is predicted to allow the binding of multiple oleic acids, leading to the formation of the functional non-native complex, which is stable at physiological conditions.

Two alpha domain peptides functionally reproduced the effects of HAMLET. This was expected for the alpha2 domain, which corresponded to the C-terminal extension in HAMLET. The SAXS structure did not

resolve the changes in the alpha1 domain structure, but the activity of the alpha1 peptide suggested that N-terminal modifications also help to create the properties of tumoricidal complex. The findings also enabled us to formulate a step-wise unfolding sequence from native alpha-lactalbumin, to a kinetically trapped intermediate, stabilized by binding to oleate. Initial unfolding of the alpha2 domain is proposed to lead to the exposure of a hydrophobic core, which might serve as a primary oleate-binding site. By destabilizing intra-molecular interactions, these events may initiate the unfolding of the alpha1 domain and the resulting exposure of the hydrophobic core in alpha1 would provide a secondary oleate-binding site.

Proposing that unfolding can generate functional diversity challenges the dogma that a rigid three-dimensional structure is needed for a protein to perform its proper function. Since this was suggested by the HAMLET studies[1,18], it has been reported that more than 50 % of total eukaryotic proteins and 75 % of signaling proteins in mammals contain at least one disordered region[48,49]. Even though functionality of these regions has not been confirmed, this immense structural complexity suggests an alternative paradigm of structure-function relationship of proteins in general[50,51] and HAMLET in particular. These effects are not to be confused with ''Moonlighting'' proteins[52-54], used as a term to describe proteins with one main function but able to adopt several additional functions without loosing native structure. Intrinsically unstructured proteins lack a native state and can alternate between different folded states with different functions. A classical example is p53, which can interact with a large number of targets[55,56]. Distinct from this group of proteins, native alpha-lactalbumin unfolds to form HAMLET and remains as such when binding to oleic acid and executing the new biological function.

Paper I shows that the tumoricidal activity of HAMLET is reproduced by alpha-domain peptides bound to oleate, demonstrating that certain amino acid sequences are favorable for host cell interactions. Furthermore, support for independent membrane associations of specific alpha-lactalbumin peptides has been obtained with higher membrane affinity for lipid vesicles[57]. The functional differences between the alpha1- and alpha2-oleate complexes and native alpha-lactalbumin mixed with oleate confirm the importance of *de novo* exposure of these peptide epitopes, which are protected in the native protein structure. These include ion flux induction, membrane remodeling, active transcriptional regulation and tumoricidal activity. Further functional and structural studies are under way, using shorter peptides from the exposed domains. Interestingly, antibodies raised, against these epitopes inhibited HAMLET-induced cell death, confirming that these epitopes are exposed and involved in the cell death response.

Contribution of oleic acid/oleate to HAMLET's tumoricidal activity

In early studies, we demonstrated that both oleic acid alone and partially unfolded alpha-lactalbumin alone lacks tumoricidal activity and that oleic acid alone does not reproduce HAMLET's effects, suggesting that the complex possesses unique novel properties not found in its constituents. The extent to which the lipid contributes to the tumor cell death in response to HAMLET has been extensively debated, however. A number of investigators have emphatically claimed that the effects of HAMLET are nothing but lipid toxicity[58-65]. As a consequence, the HAMLET literature is becoming increasingly fragmented, with inconsistencies and contradictory views. These contradictions reflect the use of production methods to generate protein-lipid complexes that are different from HAMLET and less well characterized, structurally and functionally.

The chromatographic method used to produce HAMLET yields reproducible and stable complexes with a defined lipid stoichiometry and the effects summarized in **Paper II** exemplify the detailed understanding of their cellular effects. Other protein-lipid complexes have been made, by heating at neutral pH, under acidic[66] or alkaline[64] conditions, or in the presence of EDTA, using bovine alpha-lactalbumin[61,64,65,67], lysozyme[68], beta-lactoglobulin[69], pike parvalbumin[65] or alpha-lactalbumin fragments[60]. The more characterized cytotoxic complexes are ELOA (a complex between equine lysozyme and oleic acid)[68] and BAMLET (Bovine Alpha-lactalbumin Made LEthal to Tumor cells)[70-72].

These complexes carry a large number of oleic acid molecules. For example, ELOA exists as an oligomer with up to 48 oleic acid molecules[68] while BAMLET may contain about twice the amount of oleic acid in ELOA, due to oligomer formation[59,67]. The high lipid content changes the mechanism of cellular attack and the high lipid complexes trigger a different mode of cell death, compared to HAMLET. ELOA causes membrane rupture, which lead to cell death. Cytotoxic effects for different cancer cell lines have been reported but detailed mechanistic characterization of the pathways leading to death have not been studied, making comparisons with HAMLET difficult.

In **Paper II** structural studies and extensive functional studies were used to define the protonation state of the lipid in HAMLET and the cellular responses to the lipid alone, as compared to HAMLET. The production method for HAMLET has been assumed to favor the deprotonated state of oleic acid, but this has not been documented previously. HAMLET is produced at pH 8.5, at which the protonated as well as the deprotonated state exist in equilibrium shifted towards the deprotonated state with

increasing pH. In **Paper II**, we demonstrated by NMR that the deprotonated form of oleic acid is the functional cofactor in HAMLET as oleate-HAMLET was structurally and functionally identical to HAMLET. Furthermore, we detected protein-bound oleate in HAMLET, reflected by the broad olefinic carbon peak, suggesting that oleate binds to specific sites in human alpha-lactalbumin, and not just by weak, non-specific attachments to hydrophobic surfaces. The functional comparison of oleate to HAMLET also suggested that tumor cells respond differently to a deprotonated versus a protonated lipid and that the recognition of the lipid is modified when presented in the context of a partially unfolded protein, such as human alpha-lactalbumin.

In contrast to HAMLET, a recently obtained SAXS structure of BAMLET[73] showed a coiled and elongated globular conformation. Further, in an NMR study comparing lipid binding sites of human- or goat- alpha-lactalbumin the lipid was shown to bind to several protein sites, which differed between the proteins[74], suggesting that the formation of the protein-lipid complexes may reflect sequence specificity. As a consequence, these protein-lipid complexes may have different cellular effects, confirming the notion that the lipid function varies with the protein to which it binds. This argues against the view that the lipid is the sole cytotoxic component of HAMLET and HAMLET-like complexes.

Membrane perturbations by HAMLET initiate tumor cell death

Membranes act as fluid matrices for lateral- and axial- signal propagation along or across the membrane. Equally important is the membrane association of transmembrane and cytosolic proteins, often in their active state. For lateral propagation, the membrane involves in domain partitioning to facilitate oligomerization-induced trapping[75-77], cellular polarisation for transcytosolic vectorial transport[78] and reaction-diffusion to generate a high local concentration of substrates[79,80]. Immediate axial signaling often involves a cascade of coupled reaction cycles, which bridge multiple local phosphorylation gradients, to ensure global signal propagation, exemplified by Ras pathway. Extended axial signaling involves endocytic receptor recycling and signaling endosomes, which impact on gene expression[81-83]. Given that many of the proteins involved in oncogenesis are membrane associated, it is not surprising that membrane alterations may perturb cellular homeostasis[84].

HAMLET alters the shape of tumor cells, crosses the plasma membrane and trigger ion fluxes[45], suggesting that the membrane is an essential first point of the attack that leads to tumor cell death. In **Paper III**, we identify three critical molecular-level features, which characterize the conserved tumoricidal response. **I.** Membrane perturbations in receptor-free model vesicles and in tumor cells, forming HAMLET-integrating protrusions. **II.** Formation of HAMLET-Ras membrane foci in tumor cells, also detected *in vivo* in intestinal tumors. **III.** The absence of these responses in differentiated cells.

There are multiple ways, by which these membrane shape changes could be generated by HAMLET, including asymmetric insertion of amphipathic molecules like oleate, adsorption of the protein through a crescent-shaped

domain[85], self-assembly of the complex into specialized domains[86] and protein-protein crowding[87,88]. Membrane perturbation was found to be a unique property of the HAMLET, not reproduced by native human alpha-lactalbumin or oleate. Native human alpha-lactalbumin retains membrane-binding capacity, suggesting that the protein crowding and adsorption is not sufficient to cause tubulation. Similarly, as insertion of oleate did not induce similar tubulation, the amphifile mechanism should not be responsible for the observed membrane perturbations. We suggest that the membrane-perturbing event may involve a new mechanism defined jointly by the lipid and partially unfolded protein.

The translation of membrane perturbations into cellular responses involve ion fluxes, which are specifically activated by the HAMLET complex, but not the native protein or oleic acid. Inhibition of potassium fluxes even blocked membrane blebbing in response to HAMLET in tumor cells. Based on these observations, we propose that HAMLET interacts with and perturbs the tumor cell membrane by an ion flux-dependent mechanism that requires the insertion of both the partially unfolded protein and oleic acid. Furthermore, the membrane insertion of HAMLET from the extracellular space also distinguishes it from endogenous scaffolding proteins such as COPII and BAR domain proteins[89,90].

HAMLET perturbs membrane-associated GTPases

The membrane acts as a fluid matrix for transmembrane and also cytosolic proteins that become membrane associated, often in their active states. A key mediator in this form of signal propagation is the anchorage of proteins on the membrane via lipidation, which is exemplified by the small GTP-binding proteins of the RAS family. The GTP/GDP binding states of RAS dependent on the GEFs and GAPs are facilitated by a reduction in

dimensionality when GEFs and GAPs are located at proximity to the plasma membrane. In the active state, RAS proteins become lipidated and associate with the cytoplasmic leaflet of the plasma membrane[91]. Irreversible post-translational farnesylation of a cysteine residue at the *CAAX* motif followed by the cleavage of AAX sequence and carboxymethylation of the terminal cysteine establishes membrane affinity for RAS. Subsequent reversible palmitoylation on cysteine residues, immediately upstream of the *CAAX* motif[92,93] or at a series of upstream positively charged Lysine residues (K-RAS-4B), confer specificity and higher affinity for different membrane compartments. Computational simulations revealed that a dynamic acylation cycle of RAS is needed for maintaining an asymmetric spatial organization of RAS between the plasma membrane and Golgi[84]. In fact, fully palmitoylated RAS has been shown to redistribute equally between membrane compartments leading to lower oncogenic signaling[94].

Ras[95,96] is a classical oncogene implicated in 20-30 % of all cancers. Ras protein family has shown direct influence on oncogenesis, as Ras acts as master regulator of cell proliferation. When constitutively active, Ras enhances the survival and proliferation of tumor cells. We identified membrane-anchored GTPases as HAMLET targets in a protein microarray screen. HAMLET modified the cellular distribution of Ras as drastic relocalization and accumulation to the membrane blebs was observed, resulting in Ras colocalized with HAMLET. The specificity of the Ras response to HAMLET in tumor cells was also extended to additional Ras family members, suggesting the HAMLET targets GTPases, likely leading to a functional perturbation of these proteins.

The effect of HAMLET on Ras activity is biphasic, with an early activation phase followed by inactivation. Direct binding of HAMLET to membrane

associated Ras probably results in inactivation of Ras as shown by co-immunoprecipitation, suggesting that HAMLET acts as a Ras inhibitor. Structural studies in **Paper IV** further suggested that HAMLET may bind to Ras via a novel mechanism, involving a larger binding surface on Ras than the GTP binding site, which has been so inaccessible for small molecule inhibitors, due to the picomolar affinity of Ras for GTP[97]. To enhance the effect of this inhibition, HAMLET also interacts with targets downstream of Ras. Direct binding of HAMLET to BRAF *in vitro* and cellular colocalization of HAMLET with Braf support that HAMLET causes a global inhibition of this pathway, which commonly shows enhanced activation in cancer cells.

In contrast, membranes of normal cells were refractory to HAMLET, exemplified by intact cell morphology, weak ion fluxes, an absence of Ras redistribution and HAMLET-Ras membrane foci. Furthermore, tissue sections from $APC^{Min/-}$ mice with intestinal tumors, showed colocalization of HAMLET with Ras exclusively in individual tumor cells and not in surrounding healthy tissue. These contrasting responses may be a consequence of differences in tumor cell membrane composition, including increased fluidity due to decreased cholesterol content. In addition, tumor cells have more short-chain, unsaturated fatty acids as compared to long-chain, unsaturated fatty acid, as well as a more disordered asymmetrical distribution of phospholipids, in comparison to those in normal cells.

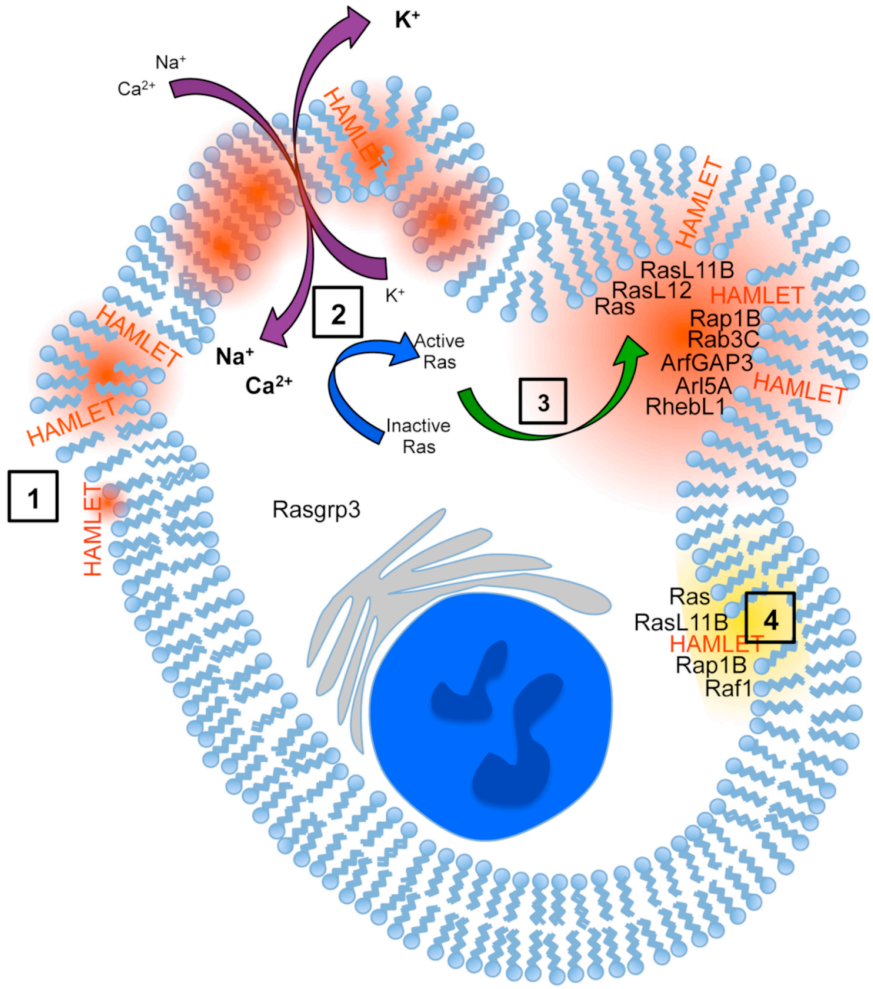

Figure 7. A model depicting how physical remodeling of the membrane by HAMLET drives the activation of transmembrane signaling in the absence of cell-surface receptors. The interaction of tumor cells with HAMLET (1) induces major changes in membrane curvature and integrity; (2) triggers ion fluxes, which activate RAS family proteins; (3) translocates activated RAS family members to the membrane of tumor cells and into membrane blebs; and (4) produces colocalized clusters within the plasma membrane that include HAMLET and several activated RAS family members. This mechanism might explain how HAMLET initiates a universal cell death program conserved in cancer cells, otherwise conditioned to outlive healthy cells.

Nucleotide binding proteins as conserved molecular targets of HAMLET in tumor cells.

Ships that pass in the night and speak
Each other in passing
Only a signal shown and distant voice
in the darkness

- Henry Wadsworth Longfellow,
"The Theologian's Tale" (1863)

Protein phosphorylation is the most common post-translational modification and protein kinases are key regulators and catalysts of signal transduction and cellular homeostasis[98]. To date, 538 human protein kinases have been described[99,100], making up almost 2 % of the human genome. The majority of kinases phosphorylate serine/threonine residues but 90 kinases phosphorylate tyrosine residue, first observed by Hunter and colleagues[101]. More than half of the known tyrosine kinases have a causal relationship with disease, and mutated or overexpressed kinases are common in cancer [102]. Kinase inhibitors are important novel therapeutics, the most notable example being Imatinib, which inhibits BCR-Abl in the targeted therapy for chronic myelogenous leukemia (CML) and other tumors[103].

In **Paper IV**, we showed that HAMLET binds to a large number of ATP/GTP-binding proteins, of which the majority belongs to the kinase family. All subfamilies in the kinome were included, suggesting that HAMLET targets molecular motifs shared by the different kinase families. The direct binding of HAMLET to kinases causes a loss of kinase activity and as a result, HAMLET acts as a broad-spectrum kinase inhibitor for about 50 % of the kinases *in vitro*. Interestingly, a small group of kinases

show enhanced activity in the presence of HAMLET but no specific structural characteristics were detected.

Kinases are identified by the conserved sequence in the catalytic domain. The typical kinase structure consists of an N-terminal lobe, composed of a beta-sheet and a single C helix as well as a large globular C-terminal lobe domain connected by a hinge. The activation segment in the inactive conformation is often partially disordered for substrate recognition[104], in contrast to the structured, active conformation[105,106]. Preliminary results show weak binding of HAMLET to synthetic activation segment peptides, defined by the conserved DFG motif and a less conserved APE motif, supporting the binding of HAMLET to this region. In view of the difference in binding to wild type or mutant kinases and the flexible conformation of alpha-lactalbumin in HAMLET, we propose that HAMLET interacts with the activation segment of wild-type kinases and restricts the flexibility of this domain, leading to a reduction in kinase activity.

Broad-spectrum kinase inhibitors such as staurosporine, induce non-selective cell death in a wide range of cells, including tumor cells, lymphocytes, neurons, primary- and transformed cells[107-110]. HAMLET, in contrast, showed higher selectivity for tumor cells and acted as a broad-spectrum kinase inhibitor. HAMLET thus appears to act differently as compared to other kinase inhibitors. Unlike peptide inhibitors[111], however, HAMLET is not a structural derivative of a substrate protein, engineered to target specific kinases with high affinity. The inhibitory micromolar IC_{50} values of HAMLET, based on seven kinases from different families, were similar to other peptide inhibitors. On the other hand, the inhibitory effect of HAMLET is also distinct as compared to small molecule inhibitors such as staurosporine, which targets specifically the ATP-binding pocket with

nanomolar IC_{50} values. In HAMLET-treated tumor cells, multiple signaling cascades involved in cancer were inhibited, including the PI3K/Ras/Raf/MEK/ERK- and p38 MAPK- signaling cascades. This set of changes compatible with a cell death phenotype, is consistent with the response of tumor cells treated with HAMLET, suggesting that the functional perturbation of the large number of nucleotide-binding proteins contributes, in part, to the tumor cell death response.

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Appendix

Tracing the History of Alpha-lactalbumin

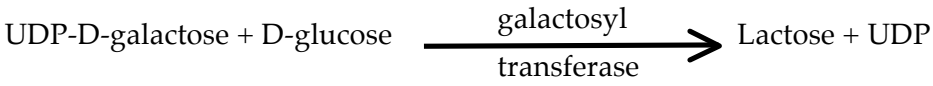
(i) The biosynthesis of lactose

The biosynthesis of lactose has been dubbed one of the most exciting natural transformations in carbohydrate metabolism[112]. The observation that led to the eventual proof of the monosaccharide constituents of lactose could be traced back to as early as 1880s. Physiological evidence in the early days suggested a link between blood glucose level and the formation of lactose. In animals with the mammary gland removed followed by pregnancy, hyperglycaemia was observed and glucose was found in the urine[113,114]. Glucose in blood was found to be the source of lactose formation in the mammary gland[115]. Moreover, observation showed that the degree of hypoglycaemia corresponded to the amount of milk produced[116].

Direct *in vitro* evidence of the biosynthesis of lactose was first shown approaching the 1920s[117] in an extract of the mammary gland upon added sucrose. In 1935, Grant demonstrated quantitatively, using sliced mammary gland tissue from guinea pig, that glucose allowed complete conversion into lactose, but not other hexoses tested, including fructose, mannose and galactose[112].

The 'chase' for elucidating the lactose synthesis pathway intensified in the 1950s. With the knowledge that various enzymatic activities, which included hexokinase, phosphoglucomutase, uridyl transferase and UDP-D-galatose 4-epimerase, were present in mammary tissue, as well as the evidence for a galactosyl transferase, which transfers D-galactose from

UDP-D-galactose to alpha-D-glucose 1-phosphate[118,119], a 5-step synthesis process was proposed. However, several contradictory results mainly concerning the unclear identity of lactose 1-phosphate, an intermediate in the proposed synthesis process, prompted a reinvestigation. Eventually, it led to a confirmation by Watkins and Hassid that the final stage in the formation of lactose involves the following reaction[120].



Using homogenized mammary tissue from guinea pigs and cows, lactose formation was shown chromatographically when incubated with ^{14}C-labeled UDP-galactose and glucose. Fractionation experiment isolating nuclear, mitochondrial and microsomal fractions revealed that only the latter two fractions were active. Importantly, critical concentration of D-glucose permissive for lactose synthesis was demonstrated. Babad and Hassid, in 1964, successfully obtained a soluble form of the lactose-synthesizing enzyme from bovine milk[121].

(ii) The origin of alpha-lactalbumin

Milk proteins were once thought to be identical with the corresponding serum proteins, with parallels shown between different components, such as colostrum globulin with serum globulin. Hence, in the early 1910s, physiologists assumed a simple transfer of proteins from the blood stream to mammary gland secretion[122-124]. In the following years, chemical methods were used to demonstrate the difference between milk and blood serum proteins. By using electrometric titration method, it was confirmed that lactalbumin was indeed different from serum albumin[122].

The lactalbumin fraction was considered a homogenous fraction until Palmer[125] isolated and crystalized a protein that shared albumin- and globulin-like properties. It was called beta-lactalbumin (which was later renamed beta-lactoglobulin), following its identity as the beta-peak in free-boundary electrophoretograms of milk proteins. Identification of alpha-lactalbumin originally derived from what was called a "crystalline insoluble substance" (CIS) from the mother liquor of beta-lactoglobulin crystallization[126,127]. Subsequent works on the preparation of CIS[128,129] showed that the electrophoretic mobility and sedimentation coefficient of CIS were identical to those of the alpha-peak in the electrophoretogram. CIS was then renamed alpha-lactalbumin.

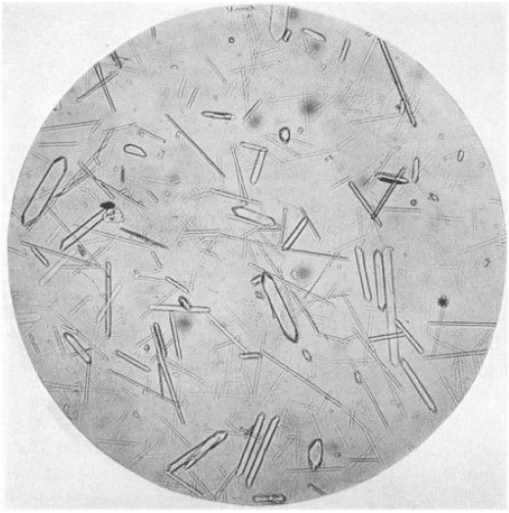


Figure 8 Crystalline insoluble substance

(iii) Alpha-lactalbumin as Protein B of lactose synthase and acts as a 'glucose specifier'

Following the successful isolation of the solution form of lactose-synthesizing enzyme, Brodbeck and Ebner identified two components of lactose synthase from bovine milk[130]. Gel filtration of ammonium sulphate-precipitated bovine skim milk revealed a two unsymmetrical protein peaks – fraction A, of higher molecular weight, and fraction B. Individually, they appeared as inactive protein. When they were combined, however, lactose synthase activity was obtained. The possibility of a dissociation of the lactose synthase complex by the purification procedure was ruled out as

the two fractions were readily obtained when acid and ammonium sulphate precipitation was replaced with centrifugation. Similar observations were obtained from sheep, goat and human milk.

The biological function of alpha-lactalbumin had been known solely for its nutritional value in milk[131]. Based on several similar properties, namely their molecular weight, gel filtration profiles, heat stability, the response to 10% trichloroacetic acid precipitation and the ultraviolet spectra, it was tested if alpha-lactalbumin could replace the B protein. Using spectrophotometric and incorporation rate assays, for the first time, alpha-lactalbumin was recognized for its function as one of the two subunits of lactose synthetase[132].

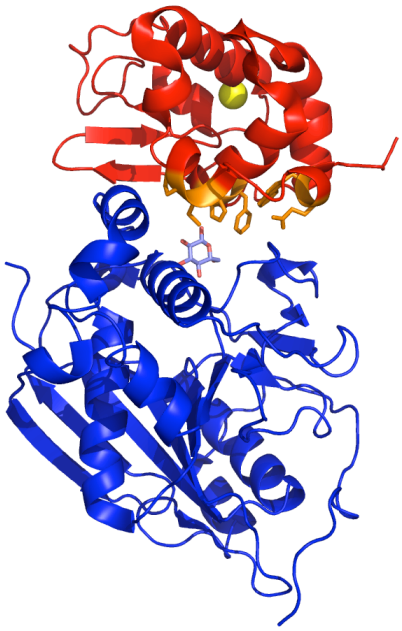


Figure 9 Lactose synthase consists of alpha-lactalbumin (red) and galactosyltransferase (blue). PDB id: 1NF5.

The A protein was identified as UDP-galactose:N-acetylglucosamine [NAG] β1-4 galactosyltransferase (N-acetyllactosamine [NAL] synthase)[133,134], which transfers the galactosyl moiety from UDP-galactose to NAG, the acceptor oligosaccharide in the absence of alpha-lactalbumin. Alpha-lactalbumin alone lacks any enzymatic activity. The acceptor specificity of the galactosyltransferase was modified from NAG to glucose in the presence of alpha-lactalbumin. As such, the biological function as a "specifier" protein was ascribed to alpha-lactalbumin.

Molten globule states of alpha-lactalbumin

One of the most pronounced properties of alpha-lactalbumin is the ability to exist as stable intermediate folding states. Dolgikh and colleagues[21] compared the different states, including the Native (N), Acidic (A), Partly denatured (P), Temperature denatured (T), Apo and Unfolded (U) forms, in terms of native-like characteristics by far- and near- UV spectroscopy for secondary and tertiary structural content, intrinsic viscosities for compactness of molecule, Tryptophan fluorescence for symmetrical environment of aromatic residues, microcalorimetry for cooperative temperature-transition and deuterium exchange for measuring unfoldedness of the molecules. These comparisons resulted in the definitive description of the intermediate state model as a "*compact globule with* ***native-like secondary structure*** *and* ***with slowly fluctuating tertiary structure***." The **lack of a cooperative thermal unfolding transition** and the **retained compactness**, form the four characteristics defining a molten globule.

The protein dissection approach has been used to study protein folding intermediates, whereby one removes regions thought to be unnecessary for the structural properties of interest[135]. For instance, by removing the beta domain, the isolated alpha helical domain of alpha-lactalbumin (α-Domain) was shown to exhibit characteristics of a molten globule, lacking native near-UV CD signal, chemical shift dispersion in ^{1}H NMR and is less compact than the native alpha-lactalbumin, though the overall tertiary fold was similar to the intact alpha-lactalbumin.

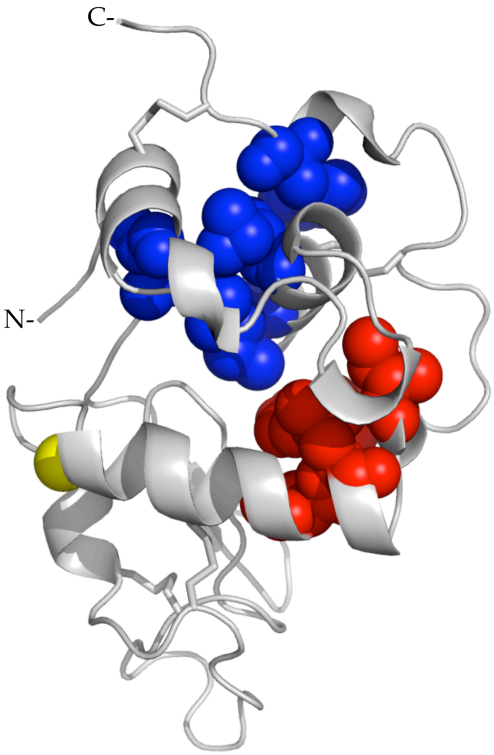
The α-Domain behaves as an autonomous folding unit[136] and resembles early kinetic folding intermediates, emphasizing that the formation of

kinetic intermediates reduce the subsequent conformational space search. Further studies elucidated that alpha-lactalbumin molten globules behave as a bipartiate structure, with a native-like helical alpha domain and an unstructured beta sheet domain. This folding mechanism for specific domains in a protein supports the resemblance of molten globules to expanded native-like proteins, rather than nonspecific collapse of polypeptides[137,138].

Molten globules produced under different conditions often exhibit broadly similar overall characteristics[22,23]. Using pulse-labeled photochemically induced dynamic nuclear polarization (photo-CIDNP), different alpha-lactalbumin molten globules were shown to have different patterns of hydrophobic-core surface accessibilities, likely demonstrating different local minima on the folding landscape[24]. This relates well also to proteins at the native states having side-chain conformation variations[139].

The hydrophobic cores in alpha-lactalbumin molten globules

Are the hydrophobic cores in alpha-lactalbumin molten globules properly formed? The effects of point mutations on the stability of human alpha-lactalbumin molten globules were studied[140]. As known from previous study that 28-111 disulfide bond plays an important stabilizing role (while the 6-120 disulfide bond the weakest), the equilibrium constant for the formation of the 28-111 disulfide bond was used to monitor the effect of the mutations[141].



The subdomain surrounded by A/B/3_{10} helices, was found to form a stabilizing hydrophobic core in alpha-lactalbumin molten globule, reflected by the destabilizing effects of amino acid substitution on L8, I27, M30 or W118 (blue spheres) in contrast to I95 and W104 (red spheres), which show minimal effect on the stability. Taken together the alanine scanning mutagenesis experiment on hydrophobic residues in the helical domain[142] and residue-specific NMR study of the denaturation of wild-type alpha-lactalbumin molten globule[143,144], the predominant hydrophobic core in the molten globule was the A/B/3_{10} subdomain (blue spheres). In contrast, the hydrophobic box (red spheres) was poorly formed.

Protein folding

The number of possible conformations for a polypeptide is astronomically large (10^{300}), suggesting that a folding mechanism must exist for efficient folding of the polypeptide chain[145,146]. Anfinsen addressed the protein folding problem with the 'thermodynamics hypothesis', which describes that a polypeptide has the lowest Gibbs free energy when it reaches the native conformation, at a defined solvent condition. Using bovine

pancreatic ribonuclease, he showed that the protein could refold completely from its fully denatured and reduced form.

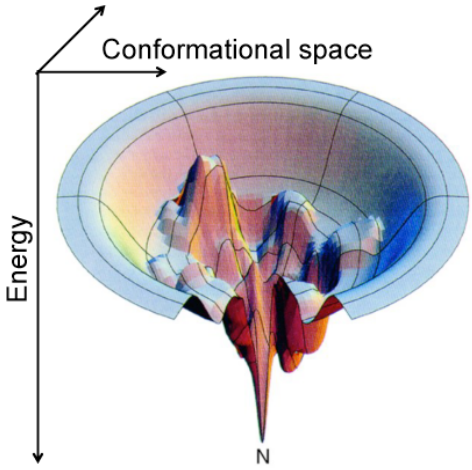


Figure 10 Protein folding energy landscape.

Evolving from the energy landscape theory[33,34], the present folding funnel hypothesis for protein folding assumes that the native state of a protein corresponds to its free energy minimum under the conditions in the cells. The driving force for a protein to reach the free energy minimum is the sequestration of hydrophobic side chains from the aqueous environment to minimize the entropy of the water solvent, described by the hydrophobic collapse hypothesis. To assume the global minimum, further lowering of the free energy of the polypeptide chain is facilitated by the positioning of electrostatically charged side chains on the solvent accessible surface, neutralization of salt bridges within the protein core and the formation of close native contacts.

Other hypotheses have been raised to explain how a protein searches through its vast conformational space so quickly. For example, in the Zipping and Assembly hypothesis (ZA)[147,148], small local fragments of the protein first form local metastable structures and grow into larger, more stable structures, which then assemble into the native structure. Other similar models include the diffusion-collision[149], hierarchical[150] and the foldon model[151].

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