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Structure-function analysis of HAMLET

(human alpha-lactalbumin made lethal to tumor cells)

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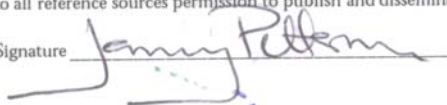
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Abstract <p>The human genome sequence encodes fewer proteins than expected, suggesting that one protein can have several functions and adjust their structure to meet different structural demands. Changes in tertiary structure have mostly been associated with disease and the most striking example is the prion protein, which changes from a mixed α-helical and β-sheets to a β-sheet rich, disease-causing isoform. Protein folding is becoming recognized as a mechanism to generate beneficial functional diversity, however. One example is α-lactalbumin, which by unfolding forms a tumoricidal complex with oleic acid called human alpha-lactalbumin made lethal to tumor cells (HAMLET).</p> <p>We investigated how protein sequence variation influences the formation of HAMLET by comparing human, bovine, equine, caprine and porcine α-lactalbumin (paper I). The fatty acid specificity in HAMLET was studied using fatty acids differing in chain length, saturation and orientation of the double bond (paper II). We have also used stably unfolded α-lactalbumin mutants (paper III) to exclude effects of the native protein in tumor cells. In an attempt to determine the three-dimensional structure of HAMLET (paper IV), crystals were characterized by X-ray diffraction to a 1.8 Å resolution. The unit cell differed significantly from native α-lactalbumin, confirming that the protein had undergone major structural alterations to form HAMLET.</p> <p>The studies have added significantly to our understanding of HAMLET as a tumoricidal molecular complex. It is possible that HAMLET is only one of many beneficial protein folding variants waiting to be discovered.</p>			
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2007

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LIST OF PAPERS

This thesis is based on the following papers that will be referred to by their Roman numerals.

- I **J. Pettersson**, A.-K. Mossberg and Svanborg, C. α -Lactalbumin species variation, HAMLET formation and tumor cell death.
Biochemical and Biophysical Research Communications (2006), 345:260-270.

- II M. Svensson, A.-K. Mossberg, **J. Pettersson**, S. Linse and C. Svanborg. Lipids as cofactors in protein folding: Stereo-specific lipid-protein interactions are required to form HAMLET (human α -lactalbumin made lethal to tumor cells).
Protein Science (2003), 12:2805-2814.

- III **J. Pettersson**, A.-K. Mossberg, M. Trulsson, K. Hun Mok and C. Svanborg. α -Lactalbumin, engineered to be non-native, kills tumor cells in complex with oleic acid.
Submitted to *Journal of Molecular Biology*.

- IV **J. Pettersson**, D. Thompson, L. C. Serpell and C. Svanborg. Crystallization of HAMLET (human α -lactalbumin made lethal to tumor cells), a partially unfolded protein in complex with oleic acid.
Manuscript.

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ABBREVIATIONS

A β	amyloid- β
AFM	atomic force microscopy
ANS	8-anilinonaphtalene-1-sulfonic acid
APP	amyloid precursor protein
β -GT	β -1,4-galactosyltransferase
BAMLET	bovine alpha-lactalbumin made lethal to tumor cells
BSE	bovine spongiform encephalopathy
C18:1:9 <i>cis</i>	oleic acid
CD	circular dichroism
CJD	Creutzfeldt-Jakob disease
ER	endoplasmic reticulum
EM	electron microscopy
ERAD	ER-associated degradation
FABP	fatty acid binding protein
HAMLET	human alpha-lactalbumin made lethal to tumor cells
HDAC	histone deacetylase
HDI	histone deacetylase inhibitor
IUP	intrinsically unstructured protein
MAL	multimeric α -lactalbumin
NMR	nuclear magnetic resonance
PBS	phosphate-buffered saline
Photo-CIDNP	photochemically induced dynamic nuclear polarization
PrP	prion protein
PS	phosphatidylserine
TSE	transmissible spongiform encephalopathy
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
UPR	unfolded protein response
UV	ultraviolet

One and three letter codes for amino acids

A=Ala=alanine, C=Cys=cysteine, D=Asp=aspartate, E=Glu=glutamate, F=Phe=phenylalanine, G=Gly=glycine, H=His=histidine, I=Ile=isoleucine, K=Lys=lysine, L=Leu=leucine, M=Met=methionine, N=Asn=asparagine P=Pro=proline Q=Glu=glutamine, R=Arg=arginine, S=Ser=serine, T=Thr=threonine, V=Val=valine, W=Trp=tryptophane and Y=Tyr=tyrosine.

Mutant notation

One-letter codes are used for naming mutants whereas three code letters are used when referring to amino acids or substitutions. For example, the mutant K99E has Lys99 substituted for Glu.

SUMMARY

The human body harbors about 100,000 proteins, which control the many intricate processes of life. To solve the diverse functional demands, the proteins must fold properly and in most cases, the native conformation defines a specific function. For many years, the prevailing dogma has been “one sequence – one protein – one function”, but more recent observations from several different areas of science have modified this view. The human genome sequence contains fewer distinct genes than previously expected, and encodes fewer proteins, suggesting that proteins must adjust their structure and function through post-translational events. Indeed, changes in tertiary structure are becoming recognized as a basis of functional diversity. The most striking example is the prion protein, which changes from a mixed α -helical and β -sheet conformation to the β -sheet rich, disease-causing isoform, PrP^{sc}. Amyloid forming proteins exemplify how a change in secondary structure may lead to fibril deposits, tissue damage and disease.

Protein folding is also becoming recognized as a mechanism to generate beneficial functional diversity, however. One example is α -lactalbumin, which by unfolding can form a tumoricidal complex with oleic acid called human α -lactalbumin made lethal to tumor cells (HAMLET). The native protein functions as a coenzyme in the lactose synthesis, but after partial unfolding, the protein binds oleic acid and forms HAMLET, which kills tumor cells but not healthy differentiated cells.

We have investigated how protein sequence variation influences the formation of HAMLET-like complexes by comparing human, bovine, equine, caprine and porcine α -lactalbumin (**paper I**). The proteins all formed tumoricidal complexes on oleic acid-conditioned column, suggesting that the sequence variation does not impair this activity. Active complexes were not found in casein from species other than human, however. This was tentatively explained by the difference in fatty acid content. Human milk contains large amounts of oleic acid, which is a necessary cofactor for HAMLET formation.

The fatty acid specificity in HAMLET was studied using fatty acids differing in chain length, saturation and orientation of the double bond (**paper II**). All *cis* fatty acids tested formed complex with partially unfolded α -lactalbumin, whereas saturated fatty acids and unsaturated *trans* fatty acids were unable to form complexes with the protein. Only α -lactalbumin in complex with oleic acid (HAMLET) killed the tumor cells efficiently and other *cis* fatty acids in complex with α -lactalbumin showed intermediate activity.

α -Lactalbumin needs to be partially unfolded to form HAMLET, but it has been unclear if a return to the native state is required to obtain activity. An α -lactalbumin mutant, which persists in a partially unfolded state, was used to answer this question (**paper III**). The mutant formed a HAMLET-like complex with high yield and the complex killed tumor cells in a dose-dependent manner. The new complex was taken up by tumor cells and accumulated in the nucleus where it caused DNA damage. The

unfolded protein alone did not change the cells, showing that the fatty acid and the protein are both needed for the tumoricidal activity.

In an attempt to determine the three-dimensional structure of HAMLET, crystallization experiments were set up (**paper IV**). Crystals were rapidly and reproducibly obtained and their shape resembled a bowtie, consisting of multiple thin plates. Diffraction data at 1.8 Å resolution suggested a twinned crystal with high mosaicity. The size of the unit cell differed significantly from native α -lactalbumin, suggesting that α -lactalbumin in HAMLET had undergone major structural alterations. These studies have added significantly to our understanding of HAMLET as a tumoricidal molecular complex.

HAMLET exemplifies how proteins or protein domains respond to specific environments, and how the function in different tissues may be adjusted. In case of HAMLET, the protein needs to change fold and to bind a fatty acid cofactor, in order to become tumoricidal. Little is known about protein folding in physiological environments, but it is possible that HAMLET is only one of many beneficial functions of protein folding variants waiting to be discovered. The properties of HAMLET suggest that it would be of great value to use such molecules for therapeutic purposes.

SVENSK SAMMANFATTNING

Ett optimalt cancerläkemedel slår endast på tumörceller, men lämnar frisk vävnad opåverkad. HAMLET (human α-lactalbumin made lethal to tumor cells) är en intressant molekyl med sådana egenskaper. HAMLET består av ett protein (α -lactalbumin) och en fettsyra (oleinsyra) och båda komponenterna finns i bröstmjölk. För att det aktiva komplexet ska bildas måste proteinet ändra tredimensionell struktur och binda till oleinsyra. Detta sker när man avlägsnar en starkt bunden kalciumjon från proteinet, antingen med kemikalier eller genom att sänka pH. HAMLET finns alltså inte i mjölken, men kan bildas då mjölken fälls med syra och kasein erhålls. För att förstå hur HAMLET kan döda tumörceller behövs mer kunskap om komplexets utseende och funktion. Denna doktorsavhandlingen fokuserar på HAMLETs struktur och hur den påverkar funktionen.

I **arbete 1** studerades sekvensvarianter av α -lactalbumin och förmågan att bilda ett HAMLET-liknande komplex med oleinsyra. Proteinets struktur varierar mellan arter och denna variation användes. Renat α -lactalbumin från ko-, häst-, gris- och getmjölk visades interagera med oleinsyra och bildade HAMLET-liknande komplex, som dödade tumörceller med samma effektivitet som HAMLET. Däremot bildades komplexet inte i mjölk från andra arter vid lågt pH. Detta förklaras troligen av att mjölk från de andra testade arterna har lägre oleinsyrainnehåll än human mjölk.

I **arbete 2** testades olika fettsyrors förmåga att bilda HAMLET med α -lactalbumin. Fettsyrorna skiljde sig åt i kolkedjelängd samt om de var mättade, omättade eller fleromättade. Endast omättade fettsyror med lång kolkedja bildade komplex som kunde döda tumörcellerna. Interaktionen mellan α -lactalbumin och fettsyran föreslås därför vara mycket specifik för proteinet och för tumörcellerna.

Tidigare har nativ struktur ofta betraktats som en förutsättning för funktion. För att bevisa att α -lactalbumin inte behöver sin nativa konformation för att bilda HAMLET användes en α -lactalbumin mutant som saknar disulfidbryggor. Därmed är mutanten låst i en oveckad form som ej kan återta nativ konformation (**arbete 3**). Mutanten bildade ett komplex med oleinsyra med lika hög effektivitet som α -lactalbumin och komplexet dödade tumörceller med samma effektivitet som vanligt HAMLET. Detta tyder på att den nativa strukturen inte är nödvändig för att kunna döda tumörceller.

En tredimensionell bild av HAMLET skulle visa hur konformationsförändringen påverkar strukturen samt var fettsyran binder till proteinet. I **arbete 4** användes kristallisering följt av röntgendiffraktion som metod för att bestämma HAMLETs tredimensionella struktur. Kristaller av HAMLET erhöles vid förhållande där α -lactalbumin ej kristalliserar. Resultatet tyder på att HAMLET har genomgått stora strukturella förändringar jämfört med α -lactalbumin. En optimering av kristallerna pågår för att lösa strukturen i mer detalj.

Dessa resultat har utökat vår förståelse om HAMLETs molekylära egenskaper. Lipidinteraktionen visade sig vara mycket specifik, medan proteinkomponenten kan varieras mer eftersom art- och veckningsvarianter kunde användas. Den tredimensionella strukturen är ännu inte bestämd, men preliminära resultat tyder på att proteinet i HAMLET har genomgått stora strukturella förändringar. Dessa fynd är också viktiga för att kunna utveckla HAMLET till ett läkemedel.

INTRODUCTION

“ONE GENE – ONE PROTEIN – ONE FUNCTION”

The paradigm “one gene – one protein – one function” was proposed by Tatum and Beadle in the early 1940s. They received the Noble Prize in Medicine 1958 for their work on *Neurospora* (bread mold). Point mutations were introduced in *Neurospora* strains by X-ray irradiation and the mutant strains were characterized by their inability to carry out specific biochemical processes. One mutant strain required ρ -aminobenzoic acid for growth and Tatum and Beadle showed that a single gene discriminated the parent from the mutant and that this gene was essential for the synthesis of ρ -aminobenzoic acid [1, 2]. They concluded that a gene encodes a single protein and the “one gene – one protein – one function paradigm” was born.

Since the human genome sequence was completed in 2003, this paradigm has been questioned. The human genome was shown to contain 30,000-40,000 genes [3, 4] coding for 20,000-25,000 proteins [5]. This number is inconsistent with function-based estimates, which propose that the protein-dependent functions in the human body would require at least 1,000,000 proteins including post-translational modifications. Furthermore, there is a remarkably small difference in gene number between species of vastly different complexity. The genome of *Caenorhabditis elegans* contains 20,000 genes [6], which is at most 30% fewer than the human genome, and only five times higher than bacteria like *Pseudomonas Aeruginosa* [7]. Provided that biological complexity would reflect the number of genes, the difference in the number of protein coding genes is not sufficient to explain the difference in functional complexity between humans, *C. elegans* and bacteria. Thus, even though alternative splicing of mRNA transcripts may generate additional diversity, it is likely that each polypeptide chain can possess more than one function.

Protein “moonlighting”

There are numerous examples where a single polypeptide may give rise to functional variants. The term “moonlighting” refers to proteins that serve one main and several additional functions, while retaining the same amino acid sequence [8]. Implicit is the need for structural modifications to distinguish the functional variants. In case of moonlighting proteins, the alternative biological function is mostly made possible by a structural change.

Known structural modifications that allow proteins to change their function include glycosylation, phosphorylation and oligomerisation. Such modifications may occur in response to change of cellular localization, cell type, substrate availability or ligand binding. As discussed by C.J. Jeffery, moonlighting proteins are well-folded proteins [8, 9], but more recently, intrinsically unstructured proteins (IUPs) were proposed to be included in the moonlighting group [10]. These proteins lack well-defined tertiary structure, but can fulfill specific biological functions. α -Lactalbumin from human milk can obtain new tumoricidal activity after partial unfolding and binding to oleic

acid and the resulting complex called HAMLET (human alpha-lactalbumin made lethal to tumor cells) might represent an early example of an IUP [11, 12].

Protein folding

The folding of polypeptide chains to correct three-dimensional structures is critical to define their function. During protein synthesis, mRNA is translated in the ribosomes and the nascent chain contains the structural information needed to fold to the native state. The nascent chain is mainly random coil or α -helical, to allow passage through the narrow ribosome exit channel. Tertiary structure formation is prevented until the entire polypeptide has been synthesized and thus, the nascent polypeptides are protected from misfolding or aggregation by chaperones, such as Hsp70 and trigger factor. After release of the polypeptide chain from the ribosomes, the fold is completed in large chaperonin complexes [13]. The majority of this folding occurs in the cytosol, but for example secreted proteins are folded by chaperones inside the endoplasmic reticulum (ER) [14].

The rate of protein synthesis varies, as does the accuracy of protein folding. About 30% of newly synthesized peptides have errors, which prevent them from folding to the native state, and are transported to the proteasomes for degradation [15]. The proportion of misfolded proteins increases during stress or in metabolically active cells like tumor cells [16]. The ER employs two main mechanisms to deal with misfolded proteins, the unfolded protein response (UPR) and ER-associated degradation (ERAD). The UPR is a stress response, which acts to remodel the ER capacity, while ERAD translocates the misfolded proteins to the cytosol for degradation by the proteasome [14]. If these degradation systems fail due to for example overload of unfolded or misfolded proteins, a stress response might be initiated, which could lead to cell death. Misfolded proteins may also arise as a result of mutations that hinder the normal folding of the polypeptide chain.

In vitro folding

Chaperones are not crucial for protein folding as a correct protein fold can be obtained *in vitro* in absence of chaperones [17]. *In vitro* refolding experiments differ from *in vivo* folding as refolding *in vitro* often requires concentrations and conditions different from within the cell. Also *in vivo* folding occurs much faster compared to refolding *in vitro*, especially the formation of disulfide bonds [18]. Protein misfolding and aggregation is often a problem during *in vitro* refolding for example when proteins are expressed in *E. coli* in non-physiological amounts. Folding aggregates are then formed and deposited in inclusion bodies. The inclusion bodies are clearly separated from the rest of the cell, but are not surrounded by a membrane. The bodies are dense and can easily be isolated by centrifugation of broken cells. The expressed protein can be purified from denatured inclusion bodies and the correct folding of the protein can be achieved by protein refolding *in vitro* [19].

The energy landscape

Each polypeptide chain could in theory fold to a large number of possible conformations, and yet, an unstructured polypeptide can fold to the correct fold in a short period of time [20]. This Levinthal paradox led to the search of folding pathway models. The “classical view” of protein folding emerged and suggested linear folding events including the on-pathway model, the off-pathway model and the sequential model [21].

In the mid 90s, the energy landscape was introduced as a new metaphor for protein folding [22]. The energy landscape is thought to resemble a U-shaped valley, frequently called “folding funnel” [23]. The shape of the folding funnel is unique for every protein as it is defined by the amino acid sequence. The polypeptide chain is thought to adopt a continuum of folding states on the way down the folding funnel. The energy is lowered with an increasing number of intra-molecular contacts, and the lowest free energy is obtained at the bottom of the funnel. The native fold of the protein is often the most thermodynamically stable structure with the lowest free energy

The folding process can be more complex if the folding funnel contains kinetic traps, where folding intermediates can accumulate (figure 1) [21]. To escape a trap, energy must be added, but subsequently folding may continue down to the lowest free energy state. The example of HAMLET suggests that the binding of a cofactor may help to stabilize a kinetic trap and keep the protein from reaching the valley and the lowest energy state. HAMLET consists of partially unfolded α -lactalbumin and oleic acid. The secondary structure of the protein is retained, but the tertiary structure is less defined than the native state. The protein remains in this energetically unfavorable state by incorporating the fatty acid and in addition, a new biological function is obtained. There is preliminary data from other groups suggesting that other proteins can obtain new functions by maintaining the structure of their folding intermediates. This could be an alternative mechanism to generate functional diversity of proteins, despite the low number of genes.

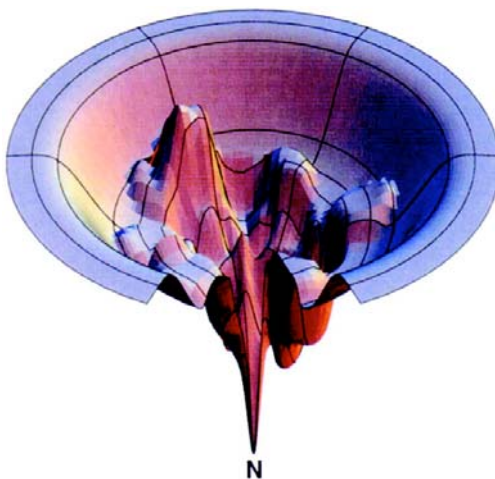


Figure 1. An energy landscape with kinetic traps where folding intermediates can accumulate [21]. The figure has been printed with permission from copyright owners.

It is a great challenge to study protein folding with experimental methods due to the high rate of conformational changes. However, biophysical methods such as circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy can be used [24]. Another approach is to study changes in folding upon mutating individual residues in a protein [25]. The computer simulation technique has made it possible to perform simulations and to study energy landscapes of individual proteins including α -lactalbumin [26].

Protein misfolding and disease

The term “misfolding” refers to a change in secondary and/or tertiary structure, which abolishes the functional integrity of the native, folded protein. Furthermore, the term infers that misfolding may generate harmful protein species, which damage the cells that produce them thereby causing disease. Folding changes may promote disease by gain of toxic activity or by loss of the native biological function [27]. Furthermore, the folding change may cause aggregation and formation of insoluble fibrils and plaques [28]. The native protein usually contains a mixture of α -helices and β -sheets, but the misfolded protein aggregates show mainly β -sheet conformation [27, 28]. The biophysical laws that rule fibril formation have been extensively studied *in vitro* but so far, results have mainly been obtained under extreme, denaturing conditions using mutated proteins [28].

Several human diseases are caused by protein misfolding and aggregate formation [27, 29]. Alzheimer’s disease, transmissible spongiform encephalopathies (TSEs) and Parkinson’s disease are neurodegenerative disorders included in the conformational diseases [30]. In each of the diseases a particular protein is misfolded, leading to aggregate formation called amyloid, which deposits in organs and may result in tissue breakdown.

Amyloid- β

Alzheimer’s disease (AD) is the leading cause of dementia and involves the formation of amyloid plaques in the brain. The amyloid- β (A β) peptide is derived from the amyloid precursor protein (APP) upon cleavage by secretases. APP is a 120kDa membrane-associated protein, which is present in both neural and non-neural tissue [31]. The plaques consist of A β , a 39-42 residues peptide. The hydrophobic C-terminal domain of A β adopts β -strand conformation and the N-terminal domain sequence permits formation of both α -helices and β -sheets [32]. Fibril formation involves a change in secondary structure to mainly β -sheets [33]. By mutating hydrophobic residues in the C-terminal into hydrophilic residues, the β -sheet content is reduced, resulting in decreased ability to form fibrils [34].

After fibrillogenesis, the fibrils eventually aggregate and form plaques, but it is believed that the fibrils themselves are cytotoxic. Geula *et al* injected A β fibrils into brains of monkeys and discovered that the fibrils caused neuronal loss. The cytotoxic effect was only seen in old monkeys and not in young monkeys and in much lower extent in rats, suggesting that this process is stronger in aging brains in higher order primates [35]. The fibrillogenesis is inhibited in rats by short 5-residue peptides,

known as β -sheet breakers [36]. A combination of the latter two studies is of great interest for the development of future therapy against Alzheimer's disease.

Prions

The prion proteins (PrPs) cause mammalian spongiform encephalopathies such as Creutzfeldt-Jakob disease (CJD) in humans and bovine spongiform encephalopathy (BSE) in cattle [37-39]. The prion protein (PrP^c) is a monomeric, non-essential, protease-sensitive glycosylphosphatidylinositol-anchored cell-surface protein [37, 39]. PrP^c is present in neurons, but the function of the native protein is not fully understood. PrP^c knock out mice show alterations in for example circadian rhythm, brain copper levels and neural-stem-cell differentiation. Known PrP^c binding partners include copper, laminin and laminin receptors [37]. PrP^c , which contains 40% α -helices and little β -sheets, can be converted into the cytotoxic form called PrP Scrapie (PrP^{sc}) with 30% α -helix and 45% β -sheet secondary structure [40]. PrP^c is monomeric and protease-sensitive, whereas PrP^{sc} is multimeric and more protease-resistant [38]. The characteristics of PrP^{sc} as well as its ability to form amyloid fibrils associates TSEs with other conformational diseases [37].

The principal target of prion pathology is the brain, but still most TSEs display prion replication at several extra-cellular locations. Orally ingested prions are absorbed in the intestines and transported to the blood and lymphoid fluids. After replication in for example the spleen, muscles, appendix or tonsils, the prions are transported by peripheral nerves to the brain. The prions causes pathology with loss of neurons, causing the brain to look like a sponge by histology. The cytotoxicity and tissue disorder results in impairment of brain function, including memory loss and locomotory problems [41].

α -LACTALBUMIN

The molecular structure

α -Lactalbumin [42] was identified already in the late 19th century [43], but the first crystals were not obtained until about 65 years later [44]. The 14 kDa protein consists of 123 amino acids and is present in high amounts in human breast milk [45, 46]. Many research groups have determined the structure of native human α -lactalbumin with similar results [47-51]. The crystal structure reveals two domains, the α - and the β -domain [47]. The large α -helical domain contains three, major α -helices (amino acids 5-11, 23-34, 86-98) and two short 3_{10} -helices (amino acids 18-20, 115-118). The small β -domain consists of a triple-stranded anti-parallel β -sheet (amino acids 40-50) and a short 3_{10} -helix (amino acids 76-82) [47]. The protein is stabilized by four disulfide bonds [52] and binding of calcium to a high affinity binding site [53]. The disulfide bonds are spread throughout the protein (C6-120, C28-111, C61-77, C73-91) and connect the two domains (figure 2).

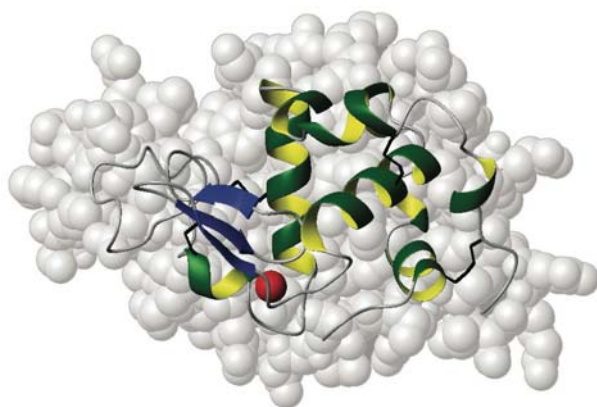


Figure 2. The crystal structure of α -lactalbumin (pdbID 1HML [54]) after modification in MOLMOL [55]. The calcium ion and the disulfide bonds are shown in red and black, respectively.

The molten globule

Outside the crystallization field, α -lactalbumin has been studied as a model of protein folding [56-59]. The protein forms a molten globule [57], which is a partially unfolded state characterized by a native-like secondary structure with a loss of well-defined tertiary packing [60, 61]. The α -lactalbumin molten globule has been studied by several techniques including hydrogen exchange combined with NMR [62, 63], photochemically induced dynamic nuclear polarization (photo-CIDNP) NMR [58], limited proteolysis [64] and mutational studies [65]. The results indicate that the α -lactalbumin molten globule is characterized by a native-like structured α -domain and a less ordered β -domain and the α -domain has been shown to be stabilized by a hydrophobic core [66].

The α -lactalbumin molten globule state is obtained at low pH (A-form), at low concentration of denaturant (P-form), at high temperature (T-form) or by removal of the calcium by chelators such as EDTA (apo-form), where the A-form is the most studied form. The molten globule state consists of a mixture of various unfolded conformations, from native-like to nearly totally unfolded [67]. The tertiary structure differs between the A-, P-, T- and apo-form as shown by near-UV CD spectroscopy [68]. The human apo-form spectrum shows the most distinct signals in contrast to the spectrum of the A-form. Near-UV CD spectra similar to the A-form are obtained when α -lactalbumin is subjected to heat, oleic acid and trifluorethanol [64], suggesting that these conditions induce a molten globule state of α -lactalbumin. Photo-CIDNP NMR examines the solvent accessibility of tryptophans, histidines and tyrosines and has been used to detect structural differences between the A-, P- and apo-form of α -lactalbumin [58]. Seven residues can be monitored in human α -lactalbumin and four are exposed in the α - and β -domains of the native protein. After partially unfolding by low pH, two residues are buried (Y103 and W118) and two residues are exposed (Y36, W60). The two exposed residues are located in or close to the β -domain, supporting previous suggestions that the β -domain is less structured in

the molten globule than in the native protein. By using denaturant only Y36 is exposed while Y103 and W118 are buried. In the apo form, only Y36 is exposed and Y103 is buried. The results confirm that small structural differences exist between the native protein and the molten globule.

Differences between the native and apo bovine α -lactalbumin have also been observed by NMR spectroscopy [69]. Major structural changes were limited to the calcium-binding region. The results were confirmed by hydrogen exchange experiments, showing that this area and the C-helix are more solvent-exposed in the apo compared to the native protein.

Because of the flexible state of the molten globule, the crystal structure has been difficult to determine. Only the bovine apo protein has been crystallized and even then only as a tetramer [70].

Biological function and binding partners

α -Lactalbumin is the major protein in human milk whey and is secreted by the mammary gland epithelium. In the absence of α -lactalbumin, the milk cannot be secreted due to high viscosity, and the deletion of the α -lactalbumin gene in mice is not compatible with survival of the offspring [71, 72].

α -Lactalbumin functions as a coenzyme in lactose synthesis [73]. Lactose synthase, which catalyses the formation of lactose from galactose and glucose, consists of two proteins, A and B [74]. The proteins were identified as β -1,4-galactosyltransferase (β -GT) and α -lactalbumin, respectively [73, 75]. Ramakrishnan and colleagues have studied the structure of lactose synthase comprehensively, including the structure of recombinant mouse α -lactalbumin in complex with wild type β -GT [76] and mutated variants [77-80]. They show that conformational changes occur in β -GT upon interaction with α -lactalbumin, which promotes the binding of glucose to β -GT. Mutational studies performed by Grobler *et al* have shown that the affinity for β -GT is reduced by mutating residue 117 or 118 in α -lactalbumin, [81]. The critical residues in α -lactalbumin have also been identified. Phe31 and His32 are to be important in glucose binding [82] and Ala106 and Leu110 interact with β -GT [83].

HAMLET – A TUMORICIDAL PROTEIN/LIPID COMPLEX

Introduction

HAMLET (human α -lactalbumin made lethal to tumor cells) is a complex of partially unfolded α -lactalbumin and oleic acid (C18:1:9 cis) [12], which kills tumor cells but not healthy differentiated cells. The tumoricidal activity was discovered in casein, obtained after low pH precipitation of human milk [11]. To identify the active component, casein was fractionated by ion exchange chromatography. Five casein peaks eluted with increasing salt, but did not show tumoricidal activity. The active component was subsequently shown to remain on the column due to high affinity for the matrix and was only eluted after raising the salt concentration in the elution buffer to 1 M NaCl. The major component of the eluate was α -lactalbumin and the fraction was named MAL (multimeric α -lactalbumin) due to the oligomeric nature on SDS-PAGE [11, 84].

Native α -lactalbumin was shown to lack tumoricidal activity, however, suggesting that MAL might be structurally modified. As no post-translational modifications were detected, we examined the conformation of α -lactalbumin in the complex using CD spectroscopy and the fluorescent hydrophobic dye 8-anilinonaphtalene-1-sulfonic acid (ANS). MAL contained partially unfolded protein, as expected from the low pH that was used to precipitate MAL from milk. The MAL fraction was tumoricidal at neutral pH and in the presence of Calcium. This was unexpected, as partially unfolded α -lactalbumin is known to return to the native fold at these conditions. We therefore investigated if MAL was stabilized by a cofactor, which bound to the protein and prevented refolding. The cofactor was identified as oleic acid, and the conditions required for complex formation were defined by deliberate conversion of native α -lactalbumin to HAMLET in the presence of oleic acid [12].

Molecular properties

HAMLET is formed from α -lactalbumin and oleic acid in a two-step procedure (figure 3). First, the protein is partially unfolded by removal of calcium, which results in a conformational change to the apo state. Unfolding increases the affinity for oleic acid and enables the fatty acid to form a complex with the protein.

The HAMLET structure has been examined by CD, fluorescence and NMR spectroscopy [12]. In the near-UV range, the HAMLET CD spectrum resembled apo α -lactalbumin, but with a decrease in signal suggesting a partially unfolded state [12]. Increased exposure of hydrophobic domains was detected using the fluorescent hydrophobic dye ANS. The HAMLET ANS spectrum showed a blue shift compared to the native protein indicating exposure of hydrophobic surfaces enabling ANS to bind. The ^1H -NMR spectrum of HAMLET showed broader peaks indicating a less-ordered protein, confirming the near-UV CD result. Oleic acid was detected in the spectrum and the signal was broader than oleic acid alone suggesting that oleic acid was integrated in the protein [12]. The results suggested that α -lactalbumin is a partially unfolded in the HAMLET complex.

When comparing HAMLET and native α -lactalbumin, HAMLET is shown to be less stable [85]. Differential scanning calorimetry and CD and fluorescence spectroscopy show that the difference becomes smaller when calcium concentration is increased.

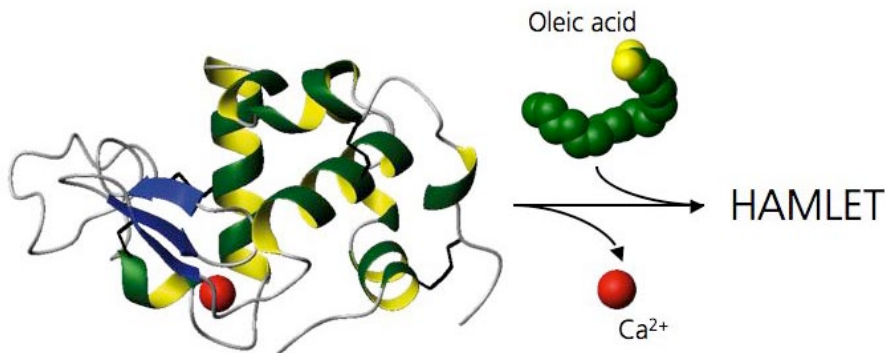


Figure 3. Schematic figure of HAMLET formation. HAMLET consists of α -lactalbumin and oleic acid. The protein is partially unfolded by removal of the calcium ion, which enables oleic acid binding. Structures from pdbID 1HML [54] and 1LFO [86] were modified in MOLMOL [55].

Recombinant α -lactalbumin and α -lactalbumin mutants form HAMLET

Recombinant wild type α -lactalbumin, expressed in *E. coli*, shows identical CD and ANS spectra as the native protein and is readily converted to HAMLET on a oleic acid-conditioned column [12]. Like milk-derived HAMLET, this recombinant, HAMLET-like complex eluted at high salt concentration and was shown to be as biologically active as HAMLET [12]. It is known that recombinantly produced α -lactalbumin contains an N-methionine, which destabilizes the protein [87-89], but this did not affect the HAMLET formation, suggesting that the fluctuating N-terminal of α -lactalbumin is not crucial for HAMLET formation. Since an active recombinant HAMLET-complex can be formed from recombinantly expressed α -lactalbumin, structure-function relationships can now be analyzed using mutant proteins expressed in *E. coli*.

To test if unfolded α -lactalbumin alone is cytotoxic, a calcium mutant (D87A), where aspartic acid at position 87 was substituted for alanine, was used [90]. The mutant was unable to bind calcium and persisted in a partially unfolded state even at physiological solvent conditions. The partially unfolded protein alone did not kill the tumor cells, suggesting that oleic acid is crucial for tumoricidal activity. After conversion of the mutant with oleic acid in absence of EDTA, a tumoricidal HAMLET-like complex was formed.

Mechanism of tumor cell death in response to HAMLET

Apoptosis and macroautophagy in response to HAMLET.

More than 40 different tumor cell lines have been exposed to HAMLET *in vitro* and all were sensitive, regardless of tumor type, species and tissue origin [91]. We initially observed that HAMLET-treated cells show characteristics of apoptosis with typical changes in morphology and DNA fragmentation [11]. Subsequently, HAMLET was

shown to interact with mitochondria, causing mitochondrial swelling and loss of mitochondrial membrane potential [92, 93]. This response was accompanied by rapid cytochrome c release, an activation of proapoptotic caspases and exposure of phosphatidylserine on the cell surface [93]. Apoptosis was not the only cause of cell death, however, as caspase inhibitors did not rescue HAMLET-treated cells from dying [92-94].

This conclusion was further supported by genetic studies, focusing on the Bcl-2 family of proteins and the *p53* tumor suppressor. Both gene families are involved in apoptosis and the altered death response of tumor cells has been explained by mutations or other changes in the expression levels of those genes. Overexpression of Bcl-2 contributes to chemotherapy resistance in tumors while *p53* mutations disrupt the control of cell cycle and DNA repair in tumor cells. Using stably transfected or mutant cell lines, HAMLET was shown to kill tumor cells independently of their Bcl-2 and *p53* status [94]. This is consistent with apoptosis being a cellular response, but not the cause of death.

More recently, HAMLET was shown to cause macroautophagy. Macroautophagy is a mechanism used to degrade long-lived proteins and organelles. The cytoplasm and organelles are enwrapped in membrane sacs, resulting in formation of the characteristic double-membrane-enclosed autophagosomes. After fusion with lysosomes, the contents are degraded for reutilization [95]. Macroautophagy occurs at basal levels in most cells, but increases in response to cellular stress such as starvation [96]. Extensive macroautophagy has been proposed as a form of programmed cell death, called autophagic/type II cell death. However, the role of macroautophagy in cell death is still debated [97, 98]. Double-membrane vesicles, LC3 translocation and accumulation, typical of macroautophagy, are observed in tumor cells after HAMLET treatment (Sonja Aits, unpublished data).

Autophagosome formation involves a battery of proteins, including Beclin-1, LC3 and Atg5. Reduction of Beclin-1 and Atg5 by siRNA is known to reduce macroautophagy. We have shown that HAMLET treatment results in increased levels of Beclin 1, Atg5 and Atg7 mRNA and the formation of Atg5-Atg12 conjugates, which might activate macroautophagy. Inhibition of macroautophagy by Beclin 1 and Atg5 siRNAs significantly reduced HAMLET-induced cell death, suggesting that HAMLET causes macroautophagy in tumor cells and that macroautophagy is a pathway of cell death in response to HAMLET (S. Aits *et al*, unpublished data).

Proteasomes interact with HAMLET

HAMLET binds to the surface of tumor cells, enters the cytoplasm and accumulates in the nuclei (figure 4) [12, 99-101]. Native α -lactalbumin, in contrast, is only taken up in no or small amounts and does not reach the nuclei (figure 4) [12, 84, 100], suggesting that unfolding of α -lactalbumin and oleic acid binding are both required for uptake into tumor cells. Healthy cells only take up small amounts of HAMLET and there is no evidence of nuclear translocation of HAMLET in healthy cells [99, 100].

The massive invasion of a partially unfolded protein into tumor cells triggers a 20S proteasome response in tumor cells, as the 20S proteasomes are responsible for degradation of endogenous, unfolded proteins. Furthermore, 20S proteasomes have been proposed to degrade unfolded α -lactalbumin *in vitro*. In tumor cells, we

detected strong co-localization of HAMLET and proteasomes in the cell periphery and throughout the cytoplasm of the adherent cells. Unfolded proteins are degraded by proteasomal enzymes, but the native species are usually resistant unless they have been equipped with specific tags like ubiquitin. Native α -lactalbumin was resistant to degradation, in contrast to partially unfolded α -lactalbumin, which was degraded within minutes. HAMLET was intermediately resistant to proteolytic degradation. HAMLET was also shown to bind directly to isolated 20S proteasome subunits *in vitro* and to cause a rapid structural change of the proteasome subunits, possibly as a result of the resistance to proteolytic degradation. Blocking of proteasome activity was shown to reduce cell death response and the change in chromatin structure, suggesting that the recognition of unfolded α -lactalbumin by the 20S proteasome and the failure to degrade α -lactalbumin are essential for tumor cell death [102], Gustafsson *et al* unpublished data].

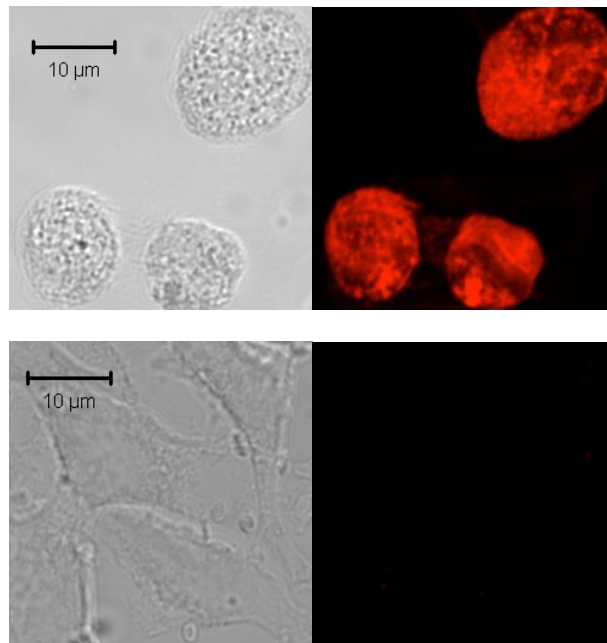


Figure 4. Light microscopy (left) and confocal microscopy (right) images of lung carcinoma cells after 3 h treatment with Alexa-labeled HAMLET (top panel) or Alexa-labeled α -lactalbumin (bottom panel). HAMLET is rapidly taken up by tumor cells, while α -lactalbumin enters the cells in very small amounts and leaves the cells unaffected.

Nuclear receptors and chromatin interactions of HAMLET

HAMLET accumulates in the nuclei of tumor cells and histones have been identified as nuclear receptors for HAMLET [99]. High affinity interactions with histone H3 and weaker interactions with H4, H2A and H2B have been documented with isolated histones, in nuclear extracts and by confocal microscopy. Furthermore, histones and HAMLET have been shown to co-localize in the nuclei of tumor cells. HAMLET, histones and DNA form virtually insoluble complexes and this interaction disrupts transcription.

The core histones consist of two main structural regions, the histone tails and the common conserved histone fold region. Like α -lactalbumin, the histone proteins are highly conserved during evolution. The fold mediates both histone-histone and histone-DNA interactions in the nucleosome core. The histone tails are N-terminal to the histone-fold region and contain sites for post-translational modifications, which are important for nucleosome assembly, transcriptional regulation, chromatin structure and activation. Newly synthesised histones and histones in active chromatin are marked by acetylation, histones in heterochromatin are methylated and phosphorylation of histone H3 is associated with mitotic chromatin condensation. Other modifications include ubiquitination and poly-ADP ribosylation [103, 104]. It has been hypothesised that the histone modifications constitute a histone code that is read by structural chromatin proteins and transcription factors [105].

The accessibility of the chromatin for HAMLET is controlled by acetylation and deacetylation of the histone tail. Histone deacetylases (HDACs), which close the chromatin, are often over-expressed in tumor cells and HDAC inhibitors (HDIs) are therefore used to treat malignancies. Recently, HAMLET was shown to act in synergy with HDIs by enhancing the hyperacetylation response to the HDIs and by promoting cell death (Brest *et al*, *Cancer Research*, in press). It was also shown that HAMLET binds to tailless histone proteins, in contrast to HDIs, which modify the histone tail. Future studies in tumor models should investigate if the combination of HAMLET and HDIs may be used to increase the therapeutic effect.

The multi-faceted cell death response to HAMLET is illustrated below (figure 5). We have proposed that this complex response is advantageous, as it ensures that HAMLET may kill a broad spectrum of tumor cells, despite varying defects in their cell death program.

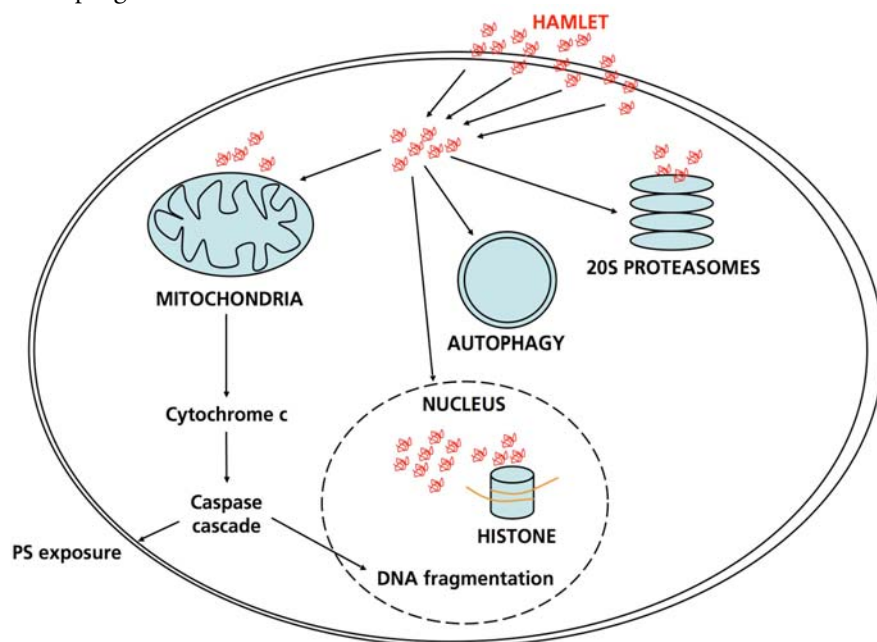


Figure 5. Summary of known cellular targets involved in HAMLET-induced tumor cell death.

CANCER BIOLOGY AND THERAPY

Cancer is a group of diseases characterized by uncontrolled cell division, tissue infiltration, spread and metastasis. Tumor cells show unrestricted replication, loss of contact inhibition by surrounding cells or matrix, and fail to differentiate to specialized cells [106, 107]. Normal cells control their replication and cell cycle and differentiate to acquire specialized functions. Extracellular stress like lack of oxygen or nutrients and intracellular stress like DNA damage can trigger transformation, but most cells manage to repair the damage or to escape tumorigenesis for example by activating an apoptosis pathway.

Typical characteristics of cancer cells have been summarized by Gibbs [108]. Tumor cells possess several unique functions such as their ability to bypass the cell cycle control passages and divide in an unlimited manner. Normal cells can only divide a limited number of times, which is worked around in the tumor cells by interfering with telomeres. The tumor cells also keep on growing despite “stop” signals from squeezed surrounding tissues and subsequently invade tissues and spread to other organs. The tumors support themselves with oxygen by forming new blood vessels and can also bypass the programmed cell death mechanism, which is activated in healthy cells in response to DNA damage.

A number of anti-cancer therapies are available for clinical use, but the majority lack tumor cell specificity and cause severe side effects. A combination of therapies is often used to achieve the best outcome and to prevent induction of resistance to one of the substances. Surgery, chemotherapy and radiation therapy are often used in cancer treatment. Removal of the tumor by surgery is the most common treatment, but there is also the risk of damage to surrounding tissue and organs. Chemotherapy and radiation therapy interfere with DNA replication in rapidly dividing cells, but cause side effects due to damage of other rapidly dividing cells such as cells in hair follicles and epithelial cells in the gastrointestinal tract. Recently, targeted therapy has emerged, which acts specifically on a defined target or a biological pathway. The ideal target should be expressed differently in tumor and healthy cells or be non-essential in normal tissue. Examples of targeted therapies are anti-angiogenesis substances and pro-apoptotic drugs.

When comparing HAMLET to present cancer treatment, we believe that HAMLET has a great potential of becoming a future cancer therapeutic. One step in this development is to improve the knowledge about HAMLET structure and function. This thesis focuses on elucidating the molecular properties of HAMLET.

Cancer therapeutics often lack selectivity for tumor cells and cause severe side effects. A major aim of new treatments is to increase the specificity of different agents for tumor cells and to kill the cells without affecting surrounding tissue.

HAMLET as a therapeutic agent

HAMLET is an interesting candidate drug, as the complex shows selectivity for tumor cells *in vitro* and kills the cells by several mechanisms including apoptosis. The tumoricidal effect of HAMLET *in vivo* has been tested both in animals and in patients [100, 109, 110].

Human glioblastoma xenografts

A rat glioblastoma model, which reproduces the invasive growth of the human tumors, was used [100]. Nude rats were xenotransplanted with glioblastoma cells obtained from surgical specimens of human tumors. The tumor cells were injected into the striatum and allowed to grow for one week. The tumor area was subsequently infused with HAMLET or α -lactalbumin for 24 hours by intra-cerebral convection-enhanced delivery. By magnetic resonance imaging scan, HAMLET was shown to reduce the tumor size and to delay the development of pressure-related symptoms. HAMLET caused apoptosis in the tumor, as determined by TUNEL staining on biopsies, but there was no apoptotic response to α -lactalbumin. No toxic side effects were observed.

Placebo-controlled study of human skin papillomas

The effect of HAMLET was further studied in patients with skin papillomas [109]. Patients with severe, therapy-resistant papillomas on hands and feet were enrolled in a placebo-controlled and double-blind study. HAMLET or saline solution was applied daily for three weeks and the effect on lesion volume was recorded. This phase was followed by an open 3-week trial where all the patients received HAMLET. Finally, most of the patients were examined 2 years after the end of the study.

After the placebo-controlled study, all the HAMLET-treated patients showed a decrease in lesion volume by at least 75 % and after 2 years, all lesions had resolved in 83 % of the patients. We conclude that HAMLET has beneficial effects on skin papillomas without side effects.

Human bladder cancer

The placebo-controlled study of topical HAMLET administration in papilloma patients suggested that HAMLET administration might be useful also in cancer patients. We selected to study bladder cancers as a variety of topical treatments are used for intravesical instillation to prevent or delay cystectomy [110]. Bladder cancers are common and remain a challenge, despite significant therapeutic advances. The prevalence is about 1/4000 [111]. Surgery is often successful alone or in combination with cytostatic drugs, but therapy-resistant tumors still cause significant morbidity and mortality [111]. In addition, successfully treated patients need life-long follow up due to a high recurrence rate and a risk for dedifferentiation [112]. Invasive tumors are eventually treated by radical cystectomy and some patients receive adjuvant systemic chemotherapy but these tumors have a poor prognosis with 5-year survival rates of 40% or less [113].

The response to HAMLET in bladder cancer patients was studied by intra-vesical application. Nine patients received five daily HAMLET instillations prior to scheduled surgery and tumor size and shedding of cells into the urine was monitored. HAMLET caused a rapid cell shedding. The shed cells were dead as determined by Trypan blue exclusion and showed signs of apoptosis, as determined by positive TUNEL staining. NaCl, PBS and α -lactalbumin did not trigger cell shedding. At surgery, a reduction in tumor size was observed in 6 patients and 4 of the patients had positive TUNEL staining in biopsies from the remaining tumor. The results thus show that HAMLET has a direct effect on bladder cancer tissue *in vivo* [110].

AIMS OF THE STUDY

This thesis aimed to study the structure of HAMLET and the structural basis of the tumoricidal activity of the complex. The specific aims were

- To study α -lactalbumin species variation, using naturally occurring species variants and to compare their ability to form HAMLET-like complexes with oleic acid.
- To determine the fatty acid specificity of partially unfolded α -lactalbumin by studying different fatty acids ability to form HAMLET-like complexes with apo α -lactalbumin.
- To study if the native state of α -lactalbumin is required to form HAMLET and to kill tumor cells, using a mutant that fails to revert to the native state due to lack of disulfide bonds.
- To determine the three-dimensional structure of HAMLET by X-ray crystallography.

PRESENT INVESTIGATIONS

α -LACTALBUMIN SPECIES VARIATION, HAMLET FORMATION AND TUMOR CELL DEATH (PAPER I)

The aim of this study was to use the sequence variation in naturally occurring α -lactalbumins from different species to understand the contribution of different regions of the molecule to the formation of HAMLET-like complexes with oleic acid.

Background

α -Lactalbumins are structurally conserved among species and in a previous study, bovine α -lactalbumin was shown to bind oleic acid and form BAMLET (bovine α -lactalbumin made lethal to tumor cells) with anti-tumor activity [90], showing that non-human α -lactalbumin variants form functional complexes. The findings also suggested that the sequence variation of α -lactalbumin species might be used as a tool to understand how sequence variation influences the formation and function of HAMLET.

Methods

The tumoricidal effect of HAMLET was first discovered in human milk casein [11]. The casein fraction was isolated by acid precipitation and the active component was identified as partially unfolded α -lactalbumin in complex with oleic acid (HAMLET). Casein was isolated from human, bovine, equine and porcine milk and fractionated by ion exchange chromatography in the search of an active component. The elution profile was compared between the different species and HAMLET-like complexes were collected and tested for tumoricidal activity. To study if α -lactalbumin from different species could form HAMLET-like complexes, the protein was purified from human, bovine, equine, caprine and porcine milk and applied to the oleic acid-conditioned matrix. In addition, isolated casein fractions were applied to the same matrix and conversion to HAMLET-like complexes was examined. The tertiary structure of the native proteins and the HAMLET-like complexes was examined by near-UV CD spectroscopy. The biological activity of the caseins and the HAMLET-like complexes was tested in a tumor cell death assay (0.5 and 0.2 mg/ml, respectively) and the cell viability was determined after 5 hours by Trypan blue exclusion.

Results

Are HAMLET-like complexes formed in milk from all species?

Since the HAMLET complex was discovered in human casein, we examined if casein from other species might be tumoricidal and if oleic acid or different fatty acids could function as cofactors in an active complex.

In the search of active components, caseins from different species were fractionated by ion exchange chromatography and fractions corresponding to the active, human milk fraction were eluted with high salt. About 16 % of the applied human casein, but only five percent or less of the bovine, equine and porcine casein eluted after high salt. Because of these low amounts, we tested the biological activity of the whole casein on mouse lymphoma cells. Only human casein decreased the cell viability to about 50 % after 5 hours (paper I, figure 1B) and DNA fragmentation was detected (paper I, figure 1C). The other casein variants tested were inactive.

Sequence variation

α -Lactalbumin sequence variation was examined as an explanation of the difference in tumoricidal activity. Human, bovine, equine, caprine and porcine α -lactalbumin show 71 % sequence homology and the calcium binding site is 100 % conserved (paper I, figure 2). The human sequence is 76-79 % identical to the species studied in this paper (figure 6).

		human	porcine	equine	caprine	bovine
% identity	human	100.0	77.2	78.0	78.9	75.6
	pig	77.2	100.0	83.7	73.2	73.2
	horse	78.0	83.7	100.0	73.2	73.2
	goat	78.9	73.2	73.2	100.0	94.3
	cow	75.6	73.2	73.2	94.3	100.0
% homology	human	100.0	84.6	85.4	84.6	85.4
	pig	84.6	100.0	88.6	79.7	78.9
	horse	85.4	88.6	100.0	80.5	80.5
	goat	84.6	79.7	80.5	100.0	95.1
	cow	85.4	78.9	80.5	95.1	100.0

Figure 6. Species variation in the α -lactalbumin sequence.

To study if the difference in activity was due to sequence variation, α -lactalbumin was purified from human, bovine, equine, caprine and porcine milk and subjected to the conversion protocol on oleic acid-conditioned matrices. The apo proteins were retained on the column and eluted as HAMLET-like complexes after high salt (paper I, figure 3). The human protein showed a higher yield (62 %) than the other species (24 to 46 %).

The biological activity of isolated α -lactalbumins and the HAMLET-like complexes was tested using L1210 cells and cell viability was determined by Trypan blue exclusion. There was no significant difference in activity between the complexes and HAMLET. α -Lactalbumin from the different species did not affect the cell viability. The results showed that α -lactalbumin from the tested species can form active HAMLET-like complexes with oleic acid and suggested that the difference in casein activity was not due to α -lactalbumin sequence variation.

The tertiary structure of the HAMLET-like complexes

The tertiary structure of the proteins and complexes was examined by near-UV CD spectroscopy. The spectra of native human, bovine and caprine α -lactalbumin confirmed previous reports [64, 68, 90, 114-117]. Equine α -lactalbumin protein was the least folded protein according to the CD spectra and the porcine protein was

slightly more folded. All the HAMLET-like complexes lost near-UV CD signals compared to the native protein and the equine complex was the least folded.

Fatty acid content in milk

The fatty acid content in milk differs between the species [118-123]. Human and porcine milk contain a majority of unsaturated fatty acids in contrast to bovine, equine and caprine milk with a majority of saturated fatty acids. Human milk contains the highest amount of oleic acid (38 %), followed by porcine (33 %), caprine (25 %), bovine (19 %) and equine milk (17 %).

To test if the inability to form tumoricidal casein complexes was due to a lack of oleic acid, casein was applied on an oleic acid-conditioned matrix (paper I, figure 5A). About 27 % of the human casein eluted as a HAMLET-like complex, which is twice as much as in the absence of oleic acid. The conversion yield also increased for the other caseins. Porcine casein showed the highest yield (50 %), compared to 5 % on a clean matrix. Bovine and equine casein showed yields of 43 and 25 %. The casein-oleic acid complexes were tested in the cell death assay. The cell viability decreased to about 15 % after 5 hours (paper I, figure 5B) and no significant difference between the complexes was obtained.

The results suggest that partially unfolded α -lactalbumin in casein forms HAMLET-like tumoricidal complexes when oleic acid is provided.

LIPIDS AS COFACTORS IN PROTEIN FOLDING: STEREO-SPECIFIC LIPID-PROTEIN INTERACTIONS ARE REQUIRED TO FORM HAMLET (PAPER II)

The aim of this paper was to determine the fatty acid specificity of partially unfolded α -lactalbumin by studying the ability of different fatty acids to form HAMLET-like complexes with apo α -lactalbumin.

Background

The specificity of the interaction between apo α -lactalbumin and fatty acids was determined, using fatty acids differing in carbon chain length, degree of saturation and double bond conformation (figure 7).

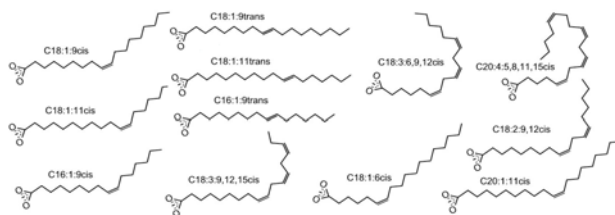


Figure 7. Fatty acids, differing in saturation, chain length and double bond conformation, were tested for their ability to form active HAMLET-like complexes together with α -lactalbumin.

Methods

Clean ion exchange matrix was conditioned with the individual fatty acids. After extensive washing to remove unbound fatty acids, EDTA-treated α -lactalbumin was added to the column, and complexes were eluted with a NaCl gradient. The structure of the complexes was examined by CD, ANS fluorescence and NMR spectroscopy and the tumoricidal activity was tested on mouse lymphoma cells. In addition, the protein/lipid stoichiometry in HAMLET was determined by acid hydrolysis and GC/MS.

Results

Formation of HAMLET-like complexes

The C18 *cis* fatty acids and other fatty acids with *cis* double bonds readily formed complexes with apo α -lactalbumin on the ion exchange matrix. The highest yield was obtained for oleic acid (C18:1:9*cis*) and a variant (C18:1:11*cis*) (paper II, figure 2). In contrast, fatty acids of similar carbon chain length, but with the double bond in the *trans* orientation were unable to form complexes. Saturated fatty acids did not form complexes with apo α -lactalbumin either. The results show that the protein-fatty acid interaction is specific as only flexible *cis* fatty acids can form complexes with apo α -lactalbumin.

The tertiary structure of HAMLET-like complexes and the stoichiometry of HAMLET

The near-UV CD spectrum of native α -lactalbumin showed distinct signals of a well-folded protein. These signals were less distinct in the HAMLET spectrum, suggesting a less defined tertiary structure. The spectra of the other HAMLET-like complexes resembled that of HAMLET. By ANS fluorescence spectroscopy, native α -lactalbumin showed low binding with a maximum at about 500 nm, as expected from the lack of exposed hydrophobic regions. HAMLET showed an increase in hydrophobicity compared to native α -lactalbumin and the spectra of apo α -lactalbumin and HAMLET showed increased and blue shifted maxima. Similar characteristics were obtained for the HAMLET-like complexes.

By NMR spectroscopy, native α -lactalbumin showed characteristics of a folded and well-defined protein with narrow lines and significant shift dispersion (paper II, figure 5). The spectrum of HAMLET and the C18:1:11*cis* complex differed from the native spectrum and showed broad lines and the lack of out-shifted methyl signals suggesting a partially unfolded state. A fatty acid signal was detected and was shown to be broader than the fatty acid control, suggesting that the fatty acid is in complex with the protein.

The protein and oleic acid concentration in HAMLET was determined by acid hydrolysis and GC/MS, respectively. The results gave an approximate stoichiometry of one fatty acid molecule per protein molecule. Subsequent stoichiometric analyses have shown that 4-8 oleic acid molecules per protein molecule is more accurate.

The tumoricidal activity of the HAMLET-like complexes

The tumoricidal activity was tested using mouse lymphoma cells. Loss of cell viability and DNA fragmentation were examined (paper II, figure 4). Only α -lactalbumin in complex with C18:1:9*cis* (HAMLET) and C18:1:11*cis* killed the cells efficiently and

caused DNA fragmentation. Other *cis* fatty acids in complex with α -lactalbumin showed intermediate activity. As no complex with *trans* and saturated fatty acids was obtained, the activity could not be tested. The fatty acids alone did not have any effect on the cells. The results suggest that the oleic acid complexes are tumoricidal due to their affinity for apo α -lactalbumin and to specificity at the cellular level.

α -LACTALBUMIN, ENGINEERED TO BE NON-NATIVE, KILLS TUMOR CELLS IN COMPLEX WITH OLEIC ACID (PAPER III)

The aim of this study was to determine if the native state of α -lactalbumin is involved in the formation of HAMLET or in the attack on tumor cells, using a mutant that fails to revert to the native state due to the lack of disulfide bonds.

Background

The native state of α -lactalbumin is stabilized by four disulfide bonds, which are located throughout the protein (residue 6-120, 28-111, 61-77 and 73-91) [52, 54]. The importance of the disulfide bonds for folding has been characterized using mutant proteins lacking individual or all of the cysteines needed for disulfide bond formation [65, 117, 124, 125]. The α -lactalbumin^{All-Ala} mutant with all cysteines substituted for alanines showed a native-like secondary structure by far-UV CD spectroscopy, but a loss in side chain packing was detected by near-UV CD spectroscopy [125]. NMR spectroscopy confirmed these results, suggesting that the conformation of α -lactalbumin^{All-Ala} is similar to the α -lactalbumin molten globule state [126]. While α -lactalbumin was shown to undergo a molten globule-like conformational change when forming HAMLET [12], it is unclear if a return to the native state occurs upon interaction with certain tumor cell compartments, or if the α -lactalbumin molten globule might be tumoricidal *per se*. As the α -lactalbumin^{All-Ala} mutant persists as a molten globule even at physiological solvent conditions, we used this mutant to address these questions.

Methods

α -Lactalbumin^{All-Ala} was expressed in *E. coli* with high yield and purified from inclusion bodies using ion exchange chromatography. The mutant was applied to the oleic acid-conditioned matrix and the formation of HAMLET^{All-Ala} was studied. The structure of α -lactalbumin^{All-Ala} and HAMLET^{All-Ala} was examined with far- and near-UV CD spectroscopy. The biological activity of HAMLET^{All-Ala} was determined using four cancer cell lines; mouse lymphoma (L1210), human lymphoma (Jurkat), human lung carcinoma (A549) and human cervical carcinoma cells (HeLa). The cells were subjected to HAMLET^{All-Ala} for 6 h at 7, 14 and 21 μ M (lymphoma cells) or 14, 28 and 42 μ M (carcinoma cells) and the cell viability was determined by Trypan blue exclusion and decrease in ATP levels (paper III, figure 3). Cellular uptake of α -lactalbumin^{All-Ala} and HAMLET^{All-Ala} was studied using Alexa-labeled protein and confocal microscopy. DNA damage in response to HAMLET^{All-Ala} (14 μ M for

lymphoma cells and 28 μ M for carcinoma cells) was examined using TUNEL staining.

Results

The mutant was readily converted to HAMLET^{All-Ala} on the oleic acid-conditioned matrix and eluted as a sharp peak at the same position as HAMLET (paper III, figure 1B). The conversion of α -lactalbumin^{All-Ala} to HAMLET^{All-Ala} was more efficient than that of EDTA-treated α -lactalbumin, with a yield of 99% of applied protein compared to 71% (paper III, figure 1C). This may reflect the structural homogeneity of the mutant protein, and suggests that a greater proportion of the α -lactalbumin^{All-Ala} molecules are in the molten globule state under the conditions of the chromatographic conversion to HAMLET.

Substantial weakening of the tertiary interactions in α -lactalbumin^{All-Ala} and HAMLET^{All-Ala} was detected by near-UV CD spectroscopy, as described for the molten globule cases of α -lactalbumin (paper III, figure 2A). The expressed mutant and the mutant-oleic acid complex retained a secondary structural content similar to the wild type protein as shown by far-UV CD spectroscopy (paper III, figure 2B). Thus, the binding of oleic acid did not increase tertiary structure content as detected by this methodology.

The biological activity of HAMLET^{All-Ala} was tested on four cancer cell lines. The complex was shown to kill the tumor cells in a dose-dependent manner and there was no significant difference in tumoricidal activity between HAMLET^{All-Ala} and HAMLET. α -lactalbumin^{All-Ala} and native α -lactalbumin did not reduce cell viability, confirming previous observations [127].

Fatty acid bound complexes were internalized by the tumor cells and translocated to their nuclei with similar, but not identical kinetics. Significant amounts of HAMLET were taken up after 15 minutes, but uptake of HAMLET^{All-Ala} was delayed. After 3 hours, a larger number of cells had taken up HAMLET^{All-Ala} than HAMLET. The HAMLET^{All-Ala} complex caused DNA damage to similar extent as HAMLET (paper III, figure 5). The oleic acid-free α -lactalbumin^{All-Ala} mutant was shown to bind to the surface of the tumor cells, but was not internalized and did not translocate to the nuclei. Neither α -lactalbumin^{All-Ala} nor α -lactalbumin influenced DNA integrity.

CRYSTALLIZATION OF HAMLET, A PARTIALLY UNFOLDED PROTEIN IN COMPLEX WITH OLEIC ACID (PAPER IV)

The aim of this study was to determine the three-dimensional structure of HAMLET by X-ray crystallography.

Background

HAMLET is a novel protein-lipid complex where the protein is partially unfolded and bound to oleic acid. There are no prior examples where the structural properties of such complexes have been characterized. It is known, however, that molten globules or proteins in the apo state are usually flexible, and this lack of native or native-like structure in the protein makes it difficult to predict the structure and to obtain crystals. The three-dimensional structure of native α -lactalbumin has previously been determined by X-ray crystallography, but crystals of apo α -lactalbumin have not been obtained, except for the bovine protein tetramer [70].

In a previous study, limited proteolysis suggested that HAMLET may represent a conformational subset of the apo α -lactalbumin population with a more defined structure. This could be due to the fatty acid, which might help lock the protein in a three-dimensional complex, which is structurally and functionally more defined than the unfolded protein alone. In this study, we have attempted to determine the three-dimensional structure of HAMLET by X-ray crystallography.

Methods

Excess fatty acid in HAMLET was removed by gel filtration and tumoricidal activity was confirmed prior to crystallization. Decrease in cell viability of HAMLET-treated human lung carcinoma cells was examined by ATP measurements and trypan blue staining. DNA damage in HAMLET-treated cells was examined using TUNEL staining.

For crystallization, lyophilized HAMLET was dissolved in water to 10, 15 or 20 mg/ml and experiments were set up at room temperature using the hanging drop method. Commercial screening kits were used and in addition, the known conditions for crystallization of bovine apo α -lactalbumin were tested [70]. X-ray diffraction patterns were collected on obtained crystals and a unit cell size was estimated.

Results

The starting material for crystallization was tested for tumoricidal activity and was shown to kill tumor cells. The decrease in ATP levels and an increase in trypan blue staining is shown in paper IV, figure 1A. HAMLET caused DNA damage as shown by TUNEL staining (paper IV, figure 1B). As expected, α -lactalbumin had no lethal effect and did not cause DNA damage.

Crystals were reproducibly obtained after 1-3 days when using 10, 15 and 20 mg/ml HAMLET and a reservoir solution of 0.1 M bicine, pH 9.0, 2% dioxan and 10% PEG 20,000 (paper IV, figure 2). The crystal shape resembled a bowtie and the

crystals consisted of multiple thin plates. In some cases, several plates were combined to form a star-like aggregate. No crystals were obtained at the conditions where bovine apo α -lactalbumin crystallizes, and native human α -lactalbumin did not crystallize at the conditions used to obtain the HAMLET crystals.

The X-ray diffraction pattern was indicative of protein crystals, but the quality of diffraction differed between crystals. Diffraction data from a crystal at 1.8 Å resolution suggested a twinned crystal with high mosaicity, and manual inspection of the diffraction patterns gave a possible monoclinic unit cell of $a=80$ Å, $b=9.6$ Å, $c=110$ Å, $\alpha=90^\circ$, $\beta=\text{non}90^\circ$, $\gamma=90^\circ$. Further processing was not possible due to the significant twinning and mosaicity.

The estimated unit cell differs significantly from the unit cell for native α -lactalbumin (*e.g.* pdbID 1A4V, $35 \times 70 \times 45$ Å). The diffraction data suggested that the normally globular α -lactalbumin in HAMLET had undergone major structural alterations and formed an almost planar unit cell. Near-UV CD spectroscopy of HAMLET confirmed the predicted loss of tertiary structure compared to native α -lactalbumin [127]. A loss of secondary structure might explain the shape of the HAMLET unit cell, but far-UV CD spectroscopy showed no major differences between HAMLET and α -lactalbumin indicating that HAMLET still retains its secondary structure (Paper III).

The smallest unit cell dimension estimated from the X-ray diffraction pattern is around 9.6 Å. The diffraction patterns show a “striped” appearance (paper III, figure 3) that arises from this small, regular repeat. This is reminiscent of diffraction patterns of amyloid crystals [128, 129]. Amyloid fibrils are composed of a highly repeating arrangement of β -strands arranged into β -sheets in which the strands are hydrogen bonded 4.7 Å apart. In an antiparallel arrangement [128], the repeat distance is 9.6 Å and diffraction patterns from highly oriented amyloid fibril samples show layer lines at 9.6 Å [128, 130]. It may be that the α -lactalbumin-oleic acid complex forms a compact arrangement in which the molecules are associated via β -sheet type interactions and share some structural similarities with amyloid fibrils. However, this remains to be discovered upon structure determination from non-twinned, diffracting crystals.

CONCLUSIONS

Paper I

Human casein kills tumor cells, but bovine, equine or porcine caseins are inactive. If oleic acid is applied to the inactive caseins, tumoricidal complexes are obtained. Purified bovine, equine, porcine and caprine α -lactalbumin can form active HAMLET-like complexes together with oleic acid.

Paper II

C18:1:9*cis* fatty acids (oleic acid) and C18:1:11*cis* fatty acids form an fully active HAMLET-complex with apo α -lactalbumin. Other *cis* fatty acids form complexes with intermediate activity, but saturated and *trans* fatty acids do not form complexes with apo α -lactalbumin. The specificity for oleic acid may thus reflect both the molecular fitness of protein and fatty acid and the role of oleic acid in tumor cell death.

Paper III

A contribution of the native state to the tumoricidal activity was excluded, since the cystein mutant α -lactalbumin^{All-Ala} can form fully active HAMLET-like complexes. The mutant cannot revert to the native state due to the lack of disulfide bonds. The unfolded mutant protein alone was not toxic and was unable to invade tumor cells, demonstrating that the lipid cofactor is an essential component of the tumoricidal complex.

Paper IV

HAMLET forms crystals at conditions where native α -lactalbumin does not crystallize. The crystal unit cell has the shape of a flat planar box, which suggests major changes of the tertiary structure of α -lactalbumin in HAMLET compared to native α -lactalbumin.

GENERAL DISCUSSION

HAMLET is formed from partially unfolded α -lactalbumin and oleic acid and the complex kills tumor cells with high efficiency. This thesis concerns the structural properties of HAMLET and the basis of the tumoricidal activity. A summary of the information is shown in figure 8. HAMLET or complexes with similar activity can be formed by α -lactalbumins from different species, despite minor sequence variation. In HAMLET, the α -lactalbumin fold resembles the molten globule state. The secondary structure is retained, but the molecule lacks tertiary packing. Using mutant α -lactalbumin, we could exclude that the native protein fold is required for HAMLET formation or activity. The α -lactalbumin^{All-Ala} mutant, which persists in a partially unfolded state due to the lack of disulfide bonds, readily formed a tumoricidal complex with oleic acid. A calcium-site α -lactalbumin mutant behaved in a similar manner [90]. The X-ray diffraction pattern of HAMLET showed some resemblance to amyloid fibrils, which consist of anti-parallel β -sheets, but the far-UV CD spectrum of HAMLET indicates a mixture of α -helices and β -sheets. By electron microscopy (EM) and AFM, HAMLET was shown to mainly be monomeric and fibril formation was not detected with either of the techniques, despite long incubation times. A unique, three-dimensional structure was suggested by X-ray crystallography, with a flattened, collapsed shape. The results predict that α -lactalbumin must undergo major tertiary structure changes when forming HAMLET, to fit into the estimated unit cell.

The specificity of the fatty acid for partially unfolded α -lactalbumin was defined by (1) the ability to condition ion exchange columns for the formation of HAMLET-like complexes with α -lactalbumin and (2) the tumoricidal activity of these complexes. While several unsaturated, long-chained fatty acids formed complexes, only complexes with oleic acid and a variant with the double bond at carbon 11 were fully active. We conclude that oleic acid fits both partially unfolded α -lactalbumin and the tumor cell.

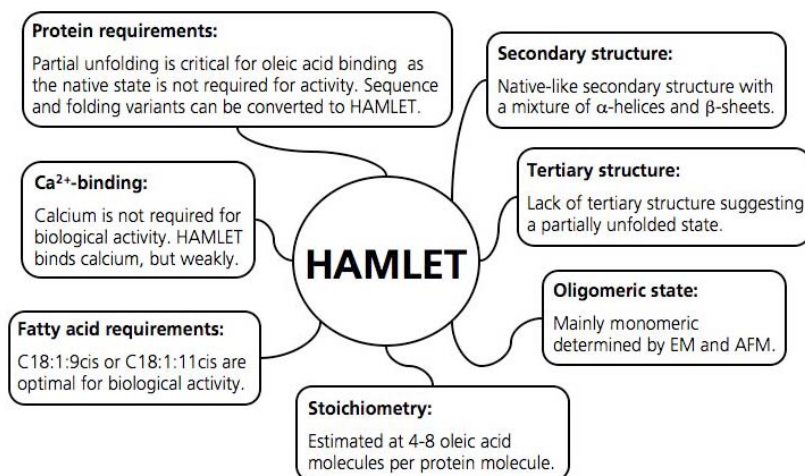


Figure 8. Summary of the molecular properties of HAMLET.

THE PROPERTIES OF α -LACTALBUMIN IN HAMLET

Primary structure and species variation

α -Lactalbumin is an essential milk protein, which functions as a coenzyme in lactose synthesis. Consequently, α -lactalbumin is found in milk from many species including dolphins, whales, marsupials, rodents, cattle and humans. The protein is structurally conserved and most of the species variants contain 123 amino acids. Exceptions include the rabbit and pig proteins that contain 122 amino acids and the possum and wallaby proteins that have one extra residue, 98b, and totally 121 amino acids. The calcium-binding site defines the native state and the coenzyme function and this part of α -lactalbumin is 100 % conserved.

We used species variants in an attempt to identify the domains of α -lactalbumin that are crucial for HAMLET formation and for the tumoricidal activity. Sequence variation influenced the efficiency of conversion, but not the tumoricidal activity of the complexes, once they were formed. For example, bovine and human α -lactalbumin share 76 % sequence identity with amino acid differences throughout the sequence. EDTA-treated bovine α -lactalbumin formed a tumoricidal HAMLET-like complex called BAMLET (bovine alpha-lactalbumin made lethal to tumor cells) [90]. The conversion yield differed between the α -lactalbumin variants, where the human protein gave the highest yield (62 %) followed by the bovine (46 %), caprine (36 %), equine (35 %) and porcine protein (24 %). However, the tumoricidal activity was comparable between the complexes, suggesting that the primary structure can be varied without affecting HAMLET formation. If additional species variants should be examined, the rabbit protein would be of interest as it shares a lower sequence identity with human α -lactalbumin (66 %).

As different α -lactalbumins are structurally conserved and form HAMLET-like complexes *in vitro*, we examined if active complexes are formed in milk from those species. The milk samples were precipitated at low pH and the casein fractions were isolated. To our surprise, only human casein had tumoricidal activity. This was tentatively explained by the difference in milk oleic acid content between species, since tumoricidal complexes were formed after application of the casein fractions on the oleic acid-conditioned matrices and human milk contains the highest amount of oleic acid among the five species tested. There was thus no evidence that species-specific fatty acids can form HAMLET-like complexes with α -lactalbumin species variants.

Structural variants obtained by introduction of point mutations

Three sets of α -lactalbumin mutants have been examined.

Calcium site mutants

α -Lactalbumin unfolds when the protein is exposed to conditions that remove the strongly bound calcium ion and after EDTA-treatment the protein adopts the apo state [131]. Unfolding with EDTA is reversible, however, as α -lactalbumin reverts to the native state if calcium is present. By mutating the calcium binding site, both calcium binding and reversion may be prevented, however. The D87 mutant cannot bind calcium and thus remains partially unfolded in the presence of calcium and at natural solvent conditions. We have shown that the D87 mutant forms a tumoricidal complex with oleic acid. The experiments with the D87A mutant showed that calcium in α -lactalbumin is not involved in the tumoricidal effect of the complex. Furthermore, the results suggested that the native fold is not required for the formation of the tumoricidal complex or for the tumoricidal activity.

Cysteine mutants

To examine if the tumoricidal activity is independent of the native fold, the α -lactalbumin^{All-Ala} mutant was used. This mutant cannot form disulfide bonds and is incapable of folding to the native state, due to the substitution of all eight cysteines with alanines. The α -lactalbumin^{All-Ala} mutant protein formed HAMLET-like complexes with high efficiency, and killed tumor cells as rapidly as HAMLET. The study showed that the native state is not crucial for HAMLET formation and activity. The calcium binding properties of α -lactalbumin^{All-Ala} are not known, but a similar mutant with cysteine residues 6, 28, 111 and 120 substituted for alanines has been shown not to bind calcium [117].

Tentative fatty acid binding site mutants

Partial unfolding of α -lactalbumin increases the exposure of the interface between the α -helical and the β -sheet domains of the protein. Structural analysis of this domain has suggested that unfolding exposes hydrophobic amino acids in this region of the molecule. We and other groups have therefore speculated that the interface may constitute a new fatty acid binding site, which becomes exposed in the apo state [132, 133]. This hypothesis was tested by substituting the basic amino acids, which tentatively coordinate the fatty acid head group (Pettersson *et al*, unpublished data). A single (K99E), double (R70D, K99E) and triple mutant (R70D, K94E, K99E) was made and expressed in *E. coli* (figure 9). The mutant proteins were difficult to express and to fold after purification from inclusion bodies. Initially, we were concerned that the mutants had to reach the native fold prior to conversion with oleic acid. The results on α -lactalbumin^{All-Ala} described above showed that the tumoricidal activity is independent of the native fold, however, and thus the results of the mutants may be considered. The point mutations were expected to decrease fatty acid binding and thus the efficiency of conversion and the biological activity of the complexes formed.

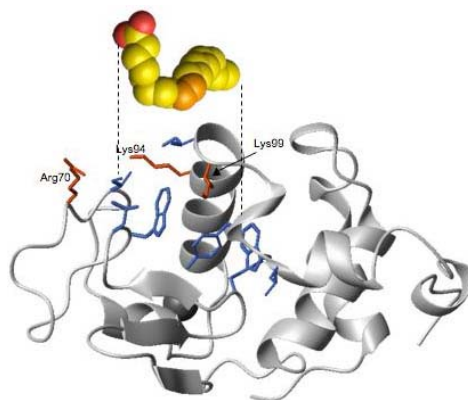


Figure 9. The tentative position of oleic acid is modelled. Basic amino acids tentatively coordinating the fatty acid head group are marked in red and hydrophobic amino acids suggested to coordinate the hydrophobic tail are blue. The calcium ion is shown in grey. Crystal coordinates are from pdb 1HML [54] and pdb 1LID [134] and the structure was modified in MOLMOL [55].

The single, double and triple mutant was expressed in *E. coli*, purified and subjected to conditions known to fold recombinant native α -lactalbumin. The tertiary structure of the mutants was examined by near-UV CD spectroscopy. The mutants showed a reduced tendency to fold to a native-like conformation, but could still be converted to active HAMLET-like complexes. This is consistent with previous studies using the D87A mutant, which does not fold to the native state due to deficient calcium binding, but can be converted to a HAMLET-like complex [90]. The efficiency of conversion and cell death varied between batches, and more work will be needed to evaluate if the cleft between the two domains may be of importance for oleic acid binding. Another way to address the binding site issue would be to mutate hydrophobic residues in the cleft between the two domains. It has been shown that mutating hydrophobic amino acids in α -lactalbumin affect the folding [81], but maybe HAMLET still can be formed. The question is how unfolded or denatured α -lactalbumin can be and still bind oleic acid, forming the HAMLET complex.

In the future, it would be interesting to study the importance of secondary structure for HAMLET formation and activity, by chemical modification or by designing and expressing new α -lactalbumin mutants. The individual α -lactalbumin domains can be isolated after cyanogen bromide cleavage of the C-terminal methionine residues. The digestion should result in three fragments roughly corresponding to the β -domain (residues 31-90) and the α -domain (residues 1-30 and 91-123). Their ability to bind oleic acid and form HAMLET and their tumoricidal activity should be tested. In parallel, a second α -lactalbumin α -domain mutant constructed by Kim *et al* might be used [59]. In this mutant, the β -domain has been deleted and the remaining two fragments have been linked with three glycine residues. Finally, deletion mutations starting from the N-terminal may be useful to understand if smaller fragments of the protein may be sufficient for the tumoricidal activity.

Tertiary structure

Limited proteolysis

A combination of hydrogen/deuterium exchange and limited proteolysis coupled to mass spectrometry was used to study the conformation of HAMLET in solution [135]. Proteolysis experiments were performed using trypsin, chymotrypsin, V8 and AspN endoproteases, subtilisin, and endoprotease K as proteolytic probes. Protein samples were incubated with each protease using an appropriate enzyme-to-substrate ratio, and the extent of the enzymatic hydrolysis was monitored on a time course basis by sampling the incubation mixture at different time intervals, followed by HPLC fractionation. Fragments were identified by mass spectrometry (ES- or MALDI-MS) leading to the assignment of cleavage sites. Proteolytic conditions were carefully selected in order to both ensure maximum stability of the protein conformation and to address proteases activity towards a few sites since the distribution of proteolytic sites within the three conformers had to be compared to detect possible differences in the surface topology.

Although near- and far-UV CD and fluorescence did not discriminate between HAMLET and apo α -lactalbumin, the hydrogen/deuterium exchange clearly showed that HAMLET and apo correspond to two distinct conformational states. HAMLET incorporated a greater number of deuterium atoms compared to the apo and native forms. Moreover, the apo α -lactalbumin appeared to consist of a mixture of at least two populations of conformers with distinct exchange properties. Finally, complementary proteolysis experiments revealed that HAMLET and apo α -lactalbumin are both accessible to proteases in the β -domain, but showed substantial differences in the kinetics of enzymatic digestion. On the basis of these data, a putative binding site of the C18:1 fatty acid, which stabilizes HAMLET conformation was proposed to involve the β -sheet domain.

Crystal structure

Crystals of human α -lactalbumin molten globules have not been reported, but the bovine apo α -lactalbumin has been crystallized and compared with the native protein [70]. The overall fold of the two variants is almost identical. The largest structural difference between the native and apo protein is around Tyr103. The slight expansion of the calcium-binding loop tilts the 3_{10} helix towards the C helix resulting in the disruption of the aromatic cluster (Trp26, Trp60, Phe53, Tyr103 and Trp104). The perturbation of Tyr103 results in an opening of the cleft and the loss of the channel water molecules associated with the calcium-binding site. The crystallization conditions included 2.0 M ammonium sulfate in 0.1 M Tris-HCl, pH 6.0. In contrast, HAMLET did not crystallize under those conditions.

The crystal structure has previously been determined for a number of fatty acid binding protein (FABP) [86, 136, 137]. These proteins consist of ten anti-parallel β -strands that form an internal cavity with affinity for unsaturated fatty acids including oleic acid [86]. Human muscle FABP and P2 myelin protein binding sites can only bind one fatty acid molecule whereas liver FABP possesses a larger cavity with room for two fatty acid molecules. All these cavities contain water molecules in addition to the fatty acids. Apo α -lactalbumin differs from the oleic acid-binding proteins in

structure and fatty acid specificity in that α -lactalbumin contains both α -helical and β -sheet domains and binds *cis* fatty acids, preferably with 18 carbons [133]. Furthermore, the structural information does not support the existence of preformed, fatty acid specific cavities in the structure of α -lactalbumin. There is evidence that unfolding exposes new hydrophobic fatty acids in the interface between the α -helical and the β -sheet domain, but their involvement in fatty acid interactions has not been defined. We have proposed that the role of the lipid cofactor in the conversion of α -lactalbumin to HAMLET differs both structurally and functionally from these previously known protein-lipid interactions [133] and the HAMLET crystals presented here support this notion.

FATTY ACID INTERACTIONS WITH α -LACTALBUMIN AND HAMLET

Fatty acid binding to α -lactalbumin

Bovine α -lactalbumin has been shown to interact with lipids, including saturated C18:0 (stearic acid) and its spin-labeled (doxyl) analog [138]. Native α -lactalbumin also interacted with stearic acid, but with a lower affinity. By intrinsic protein fluorescence and electron spin resonance methods, the apo protein was shown to have a stronger affinity for the fatty acid than the native protein and it was suggested that apo α -lactalbumin possesses two fatty acid binding sites. This is logical, as the exposed hydrophobic surfaces in the apo protein should enable fatty acid binding that does not exist in the native state. According to Barbana *et al*, native bovine α -lactalbumin is unable to bind to oleic (C18:1:9*cis*) and palmitic acid (C16:0), shown by partition equilibrium and fluorescence spectroscopy [132]. In contrast, the calcium-free protein was shown to have one fatty acid binding site with affinity for oleic and palmitic acid with a higher affinity for oleic acid. The stoichiometry was determined to be less than one ligand molecule per protein molecule. By removing the calcium ion a conformational change is obtained, which enables the protein to interact with fatty acids [132]. Yang *et al* has studied the interaction between bovine apo α -lactalbumin and oleic acid at different pH. At pH 4.0 and 7.0, oleic acid induces a dimeric intermediate but at pH 3.0 the molten globule content is increased remarkably [139]. These studies support our studies on the requirements for HAMLET formation. The results also explain why HAMLET cannot be formed efficiently simply by mixing the protein with oleic acid.

In paper II, the protein/lipid stoichiometry was determined to be 1:1, but after extensively repeating the experiment, we now estimate that HAMLET contains 4-8 oleic acid molecules per protein molecule. It is however unsure if all the oleic acid molecules are required for activity. By NMR spectroscopy, oleic acid was suggested to be in a compact form where the two ends are close both to each other and to the double bond [140]. The stoichiometry suggests that several domains of apo α -lactalbumin may interact with fatty acids.

Molecular modelling of the fatty acid binding sites

In a preliminary study, we have used a computational method to identify candidate domains in α -lactalbumin that may interact favorably with oleic acid. The Energy Minimization program AutoDock3.0 was chosen for its extensive free energy function and its refined optimization procedure based on a genetic algorithm. The program was evaluated by simulation of two protein-oleic acid complexes in which the binding sites have been identified. AutoDock reproduced the protein-ligand complexes and identified the fatty acid binding site internal to these proteins. The fit for α -lactalbumin was confirmed by modeling of the lactose synthase complex where α -lactalbumin acts as a substrate specifier. The simulations of HAMLET were based on the crystal structure of bovine apo α -lactalbumin and AutoDock identified three oleic acid-attracting areas on the surface of the protein and one in the interface between the α - and the β -domain, as described above. Recent NMR studies and Molecular Dynamics simulations with α -lactalbumin have shown that this region of the protein is highly disordered when it enters the molten globule state while the α -domain and the β -domain keep a native-like structure [26]. The model thus predicts that oleic acid may become associated with the surface of apo α -lactalbumin and with the interface between the α -helical and the β -sheet domains after partial unfolding and exposure of hydrophobic residues.

Simulations with other oleic acid binding proteins verified that AutoDock identified known oleic acid binding sites in a protein complex. The FABP proteins form a family of fatty acid transporters that are involved in lipid uptake and in the regulation of lipid metabolism [86, 136, 137]. The proteins share a three-dimensional fold with ten anti-parallel strands, forming a clam-like structure with a well-defined oleic acid binding site in the hollow. The sequence homology is significantly higher at the binding site as shown for P2 and muscle FABP with 79 % sequences identity at the binding site and 59% overall identity. The binding site is geometrically suitable for a U-shaped ligand, which fits the *cis* conformation of oleic acid. In P2, muscle-FABP and liver-FABP, the oleic acid carboxyle head group interacts favorably with three hydrophilic residues while the non-polar oleic acid tail wriggles through a region dominated by hydrophobic residues. Myelin protein has a cavity surface area of 420\AA^2 and a cavity volume of 330\AA^3 , as estimated with the GRASP program. Corresponding values are 391\AA^2 and 323\AA^3 for heart FABP and 610\AA^2 and 440\AA^3 for liver FABP, respectively. The FABP structure thus offers a combination of hydrophobic, hydrophilic and geometric characteristics that optimizes the fit for oleic acid. The simulations of oleic acid binding to apo α -lactalbumin gave no evidence of a clam-like structure with a hollow for fatty acid binding. The three-dimensional structure of bovine apo, bovine and human native α -lactalbumin are quite compact in comparison with the oleic acid binding proteins. We conclude that the FABPs and apo α -lactalbumin share the propensity for oleic acid binding, but show little structural similarity.

STRUCTURAL DETERMINANTS OF THE CELLULAR DEATH RESPONSE TO HAMLET

The HAMLET complex binds to cell surfaces, is internalized and translocates to the nuclei in tumor cells. In healthy cells, smaller amounts of HAMLET are internalized, and there is no evidence that the complex moves to the nuclei. This has suggested that both tumor cells and healthy cells can interact with α -lactalbumin and oleic acid, but that this interaction has vastly different consequences in sensitive and resistant cells.

Membrane interactions

It is well established that α -lactalbumin interacts with cell membranes and the mechanisms of interaction have been studied using artificial membranes. A conformational change occurs when α -lactalbumin interacts with membranes. The protein changes conformation to a molten globule-like state when interacting with vesicles, indicated by a loss of tertiary structure and by a combination of hydrophobic and electrostatic interactions. The structural changes are restricted to the α -helical domain of α -lactalbumin [141]. Halskau *et al* obtained similar results, suggesting that residue 10, 12, 77, 94, 98, 99 and 104 were involved in the interaction as they were buried in the membrane-bound protein [142]. In addition, apo α -lactalbumin has been shown to permeabilize loosely packed vesicles consisting of unsaturated fatty acids. A tighter vesicle counteracted these interactions [143]. Similar studies are presently being performed with apo α -lactalbumin and with the HAMLET complex.

Oleic acid (C18:1:9*cis*) and other *cis* fatty acids formed HAMLET-like complexes with apo α -lactalbumin, but only the oleic acid complex showed high biological activity (paper II). We concluded that oleic acid must be important for the uptake of HAMLET into tumor cells and/or for intra-cellular interactions. Tumor cells have increased expression of fatty acid receptors, and use oleic acid as one of their main energy sources. Healthy cells, in contrast, rely on glycolysis through aerobic pathways. It is thus possible that some of the tumor cell specificity may reflect the propensity of cells to take up oleic acid, and that the unfolded protein is brought into the cells as a consequence of the fatty acid uptake.

Nuclear translocation and histone interactions

HAMLET translocates to the nuclei of tumor cells and binds to histones and chromatin with high affinity [99]. Recent studies using labeled oleic acid have shown that the protein-lipid complex reaches the nuclei (Mossberg *et al*, unpublished data). Oleic acid is not required for interaction of HAMLET with the histones, as α -lactalbumin has been shown to bind histones *in vitro*, under non-native conditions [144]. Binding of histones to the native protein cause destabilization, but the apo protein was stabilized by histone binding. Binding studies of α -lactalbumin and basic polypeptides, resembling histone proteins, were also performed [145]. α -Lactalbumin was shown to undergo structural changes and to become less stable when interacting with basic polypeptides. The conformational state resembled the molten globule state but with a decreased affinity for calcium.

PARALLELS BETWEEN HAMLET AND AMYLOID FIBRILS

The X-ray diffraction pattern of the HAMLET crystals was shown to resemble the diffraction pattern of amyloid crystals and α -lactalbumin shares about 40% sequence identity with lysozyme, which is known to form amyloid at various conditions [146, 147]. Amyloid fibrils may be characterized by EM and amyloid fibrils stain positive for Congo red and Thioflavin-T, which can be visualized by fluorescence microscopy [148]. Fibrils, visualized by EM, show uniform, straight and unbranched fibrils with a diameter of about 100 Å. The structure of amyloid fibrils has also been determined by X-ray fibre diffraction [129, 149, 150]. Amyloid fibrils are composed of a highly repeating arrangement of β -strands arranged into β -sheets in which the strands are hydrogen bonded 4.7 Å apart. In an antiparallel arrangement [128] the repeat distance is 9.6 Å and diffraction patterns from highly oriented amyloid fibril samples show layer lines at 9.6 Å [128, 130].

It is not clear how prions and amyloid fibers exert their cytotoxic effects. Silveira *et al* have identified aggregates with 14-28 PrP molecules as the most efficient disease inducers, but neither the small oligomers nor the larger aggregates are toxic [151]. The lysozyme amyloids were proposed to be cytotoxic as the oligomers killed neuroblastoma cells by an apoptosis-like mechanism and the fibrils lead to a necrosis-like death [152]. In this case, the amyloid was obtained after 12 days at pH 2.2 and 57 °C. Bovine α -lactalbumin forms fibrils at pH 2.0 and 37 °C [153]. The same results was obtained with *S*-(Carboxymethyl)- α -lactalbumin, a variant with three out of four disulfide bridges reduced. Yang *et al* obtained fibril formation of bovine α -lactalbumin from pH 4.5 and below. The fibrillogenesis was completely inhibited by oleic acid (640 μ M) at pH 4.0-4.5 and insoluble aggregates were obtained [154]. This did not occur at lower pH. The apo protein at neutral pH did not form fibrils, however.

These results suggest that HAMLET, obtained by EDTA-treatment at neutral pH followed by oleic acid binding, should not be able to form fibrils. By EM, we obtained no evidence of fibril formation after incubating HAMLET (1 mg/ml in Tris buffer) in 37°C for four months. Still the X-ray diffraction pattern indicated major structural changes. These changes must occur at the tertiary level as the secondary structure is retained. With a stoichiometry of 4-8 oleic acids per protein molecule in mind, we speculate that this number may be sufficient to form a “mini-bilayer”, allowing the α -lactalbumin molten globule to integrate in a more linear fashion and as described previously, α -lactalbumin structure changes to a molten globule-like state when interacting with lipid bilayers. It remains to be shown if the amino acids 86 to 99 are involved, as these amino acids are located in one of the helices in the α -domain and have been identified as the amino acids interacting with artificial membranes [142, 155].

Are cofactors involved in fibril formation and disease pathogenesis?

Prion aggregates contain different prion protein species varying from non-fibrillar oligomers to large amyloid plaques [37]. These different molecular forms have been produced *in vitro*, but have mostly not been toxic, and thus, the molecular basis of toxicity is not fully understood. The aggregates are formed by specific misfolded protein species, but other components, including other proteins or carbohydrates maybe incorporated [156]. The contribution of tissue cofactors has been extensively discussed, as disease is readily transferred by injection of brain extracts from disease bearing animals, but not through injection of the *in vitro* produced prion variants. There are no reports claiming that prion aggregates contain exclusively protein but extensive studies were performed to exclude that the infectious prion preparations were contaminated with nucleic acids. While it is accepted that the change in fold is required for disease, the possible involvement of other proteins or nucleic acids is still being discussed [37]. This unknown factor has been called Protein X [38, 157] and three different classes of possible cofactors have been suggested [157]. The first class includes cellular receptors, which might facilitate conversion of PrP^c to PrP^{sc} and the uptake by endosomes. The known interactions with for example the laminin receptor and heparan sulfate support this hypothesis. A second possibility involves nucleic acids. The prion protein is known to bind to nucleic acids and the binding results in a structural modification. The third possibility includes lipids, which could act as chaperones. This theory is supported by the fact that different lipids can stabilize α -helix and β -sheet-enriched structures [157]. This may be a parallel to the stabilization of partially unfolded α -lactalbumin by oleic acid, as this form of the protein contains both α -helical and β -sheet structure.

Fibrillogenesis has been shown to be a membrane-associated process [158-163]. Fibril formation of several proteins, including lysozyme, transthyretin, endostatin and α -lactalbumin, was enhanced by membranes containing phosphatidylserine (PS) and fluorescent lipid tracers indicated presence of lipid in these amyloid fibrils [162, 163]. The rapid fibril formation could be due to the low-pH environment on the membrane surface [163] or the high proportion of incompletely desolved hydrogen bonds in the amyloidogenic proteins [158]. Endostatin, a tumor angiogenesis inhibitor, was shown to only form fibril on acidic PS membranes [162] and since cancer cells have been reported to expose PS on the surface [164, 165], they might be a target for endostatin, which increases membrane permeability [160, 162].



A future cancer therapeutic should ideally be selective for tumor cells and without toxicity for healthy tissue. HAMLET has shown tumoricidal activity, with selectivity for tumor cells both *in vitro* and *in vivo*. Since the complex is derived from human milk, the immune response is thought to be minimal. These observations lend further credibility to the notion that partial unfolding may allow proteins to attain beneficial effects. Folding changes might thus contribute to the functional diversity of proteins, in parallel with post-translational modifications and other mechanisms generating structural diversity.

Breast-feeding has been proposed to protect the newborn child against cancer and the overall incidence of childhood cancer is reduced in breast-fed children [166]. It may be speculated that molecules such as HAMLET can have a protective function and that HAMLET is one of several natural surveillance molecules that purge unwanted cells from local tissues. By inducing tumor cell death, HAMLET may reduce the pool of potentially malignant cells that could serve in future tumor development.



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