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Mini review

HAMLET, protein folding, and tumor cell death

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HAMLET (Human α-lactalbumin made lethal to tumor cells) was discovered by serendipity. The first publication in 1995 described the discovery and the unusual properties of what later became the HAMLET complex [1]. A fraction of human milk was able to kill tumor cells by a mechanism resembling apoptosis, and many different types of tumor cells were susceptible to this effect while healthy differentiated cells were resistant. The activity was shown to reside in a complex between the human milk protein α-lactalbumin and oleic acid, which is the most abundant fatty acid in human milk [2]. Since then, the HAMLET complex has been characterized in detail in order to determine the structural basis and mechanism(s) of the tumoricidal activity, and HAMLET has been used to treat tumors in animals and patients [3,4]. In 2006, researchers working on HAMLET and related topics met in Lund in Sweden for the First International HAMLET Symposium. This review summarizes the current state of knowledge in this area based on the presentations made at the conference.

Protein folding and the structural properties of HAMLET

Many unfolded proteins represent a threat to tissue homeostasis [5,6] and protein unfolding has often been associated with tissue destruction and disease. Misfolding accompanies normal protein synthesis, and a portion of newly synthesized peptides does not fold properly and must be removed by the proteasomes. Protein misfolding is also caused by mutations that permanently disturb the conformation, and such misfolded species accumulate in the tissues of patients with amyloid disorders [7a,b]. Alpha-lactalbumin is the major protein constituent of human milk. The three dimensional structure of this globular 14 kDa protein has been elucidated, revealing four α-helices, a triple-stranded β-sheet, and a Ca^{2+}-binding site [8,9]. The native protein serves as a co-enzyme in lactose synthesis, but does not cause tumor cell death. To become tumoricidal, the protein must undergo partial unfolding and bind the fatty acid cofactor (Fig. 1A), which allows α-lactalbumin to remain partially unfolded under physiological conditions (Fig. 1B). In the absence of the fatty acid the unfolded state is unstable, and at physiological solvent conditions the protein reverts to the native state. HAMLET exemplifies how a change in three-dimensional structure may allow a protein to alter its function in response to environmental signals. In addition, HAMLET is of potential interest as a model of unfolded protein cytotoxicity, particularly in view of its relative selectivity for tumor cells.

C.M. Dobson (University of Cambridge, UK) reviewed current knowledge of protein folding and amyloidogenesis. In 1998, Dobson and colleagues first discovered and characterized amyloid fibrils in proteins that do not have any association with disease, subsequently leading to his hypothesis about "the formation amyloid fibrils as a

Abbreviations: AFM, atomic force microscopy; ATP, adenosine triphosphate; BAMLET, bovine α-lactalbumin made lethal to tumor cells; CIDNP, chemically induced dynamic nuclear polarization; ER, endoplasmic reticulum; H/D, hydrogen/deuterium; HAMLET, human α-lactalbumin made lethal to tumor cells; HDAC, histone deacetylase; kDa, kilo Dalton; LAMPA, α-lactalbumin modified by poly-amino acid; MAL, multimeric α-lactalbumin; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PrP, prion protein; TEM, transmission electron microscopy; TUNEL, TdT-mediated dUTP nick end labelling.

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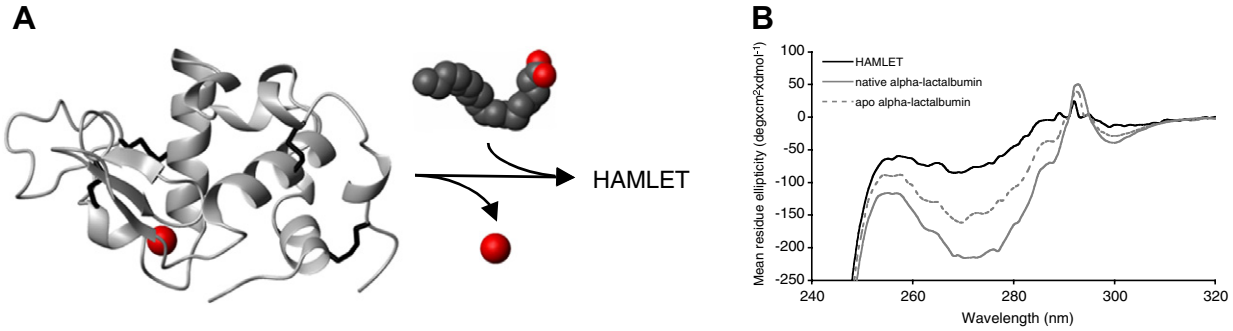

Fig. 1. HAMLET consists of partially unfolded α-lactalbumin and oleic acid. (A) HAMLET is formed by a two-step procedure. First, α-lactalbumin is partially unfolded by removing the calcium ion (red) with EDTA or acid. Second, oleic acid is bound to the protein and HAMLET is formed. Protein and oleic acid structure are from pdbID 1HML [51] and 1LID [52], respectively, and modified with MOLMOL [53]. (B) Native α-lactalbumin shows distinct signals in near UV CD spectroscopy, characterizing a well folded protein. The HAMLET spectrum shows a decrease in signal compared to the native and apo protein suggesting a partially unfolded state.

generic property of protein folding" [10,11]. Currently, more than 60 proteins have been shown to form amyloid-like fibrils by a variety of techniques. The simple principles extracted from aggregation rates for proteins based on extensive mutational analyses are now being implemented in *in vivo* studies, in which the expression of human amyloid β1-42 peptide variants in *Drosophila melanogaster* was shown to cause major behavioural changes (unpublished observation). Dobson also presented computational data from M. Vendruscolo et al., suggesting that highly accurate three-dimensional structures can be determined with sole information from heteronuclear chemical shifts. Obtaining high-resolution details of amyloid fibrils has been challenging, as protein aggregates in general do not usually form well-diffracting crystals. L.C. Serpell from the University of Sussex, UK described an elegant model system, where the atomic level structure of a designed 12-mer peptide has been solved with cryo-electron microscopy and electron and X-ray diffraction [12]. The 12-mer peptide sequence (KFFEAAAKKFFE) is notable for its high content of Phe side chains, and there is extensive π–π stacking of the aromatic rings for the purpose of inter-sheet stabilization (a "Phe zipper"). The results agree with the high occurrence of aromatic side chains in proteins involved in amyloidogenesis, such as PrP (the prion protein), Sup35p (yeast prion protein), amyloid β, human calcitonin, and others. Owing to these and many other significant developments, the field is now at the point of actively designing drug candidates to intervene with the formation of pre-fibrils and mature fibrils *in vivo*.

The formation of HAMLET from its constituent components (calcium-depleted α-lactalbumin and oleic acid) through chromatographic methods has always intrigued workers in this area. HAMLET is formed during ion-exchange chromatography, when the unfolded protein interacts with a matrix, preconditioned with oleic acid, and is eluted with high salt [2,13,14]. The structural prerequisites for complex formation were discussed by A.-K. Mossberg and J. Pettersson (Lund University, Sweden). An experiment using an α-lactalbumin mutant showed that unfolding of the protein alone does not cause cytotoxicity; rather, it was the resulting three-dimensional structure of the protein that was responsible for the activity. The calcium binding site mutant was stable in a molten globule like state but was not cytotoxic, unless in complex with oleic acid [15]. When a large range of different fatty acids were tested for the generation of the active complex, it was found that fatty acid stereospecificity may be a significant factor in the conversion of HAMLET and for its biological activity [13].

A structural description of HAMLET and BAMLET (the bovine α-lactalbumin complex analogue) was presented by K.H. Mok (University of Oxford, UK, and presently Trinity College, Dublin, Ireland). By probing the various classical molten globular forms of α-lactalbumin using a battery of biophysical techniques including NMR, TEM (transmission electron microscopy), AFM (atomic force microscopy), and pulse-labelled CIDNP (chemically-induced dynamic nuclear polarization) NMR [16], it was shown that the partially denatured form of the protein could be created and stabilized by many different combinations of internalized amino acid side chains, and that the hydrophobic cores of HAMLET and BAMLET appear to be distinct from these classical forms. In addition, diffusion NMR and small angle X-ray scattering results were presented to characterize the hydrodynamic properties, and Mok showed fascinating AFM images of the surface-deposited complex, suggesting, perhaps, a new structural paradigm where protein is encapsulating lipid molecules and not *vice versa*—as would be the case for micelles or vesicles. These structural features may account for the *in vivo* activity of the complex.

The conformational differences between HAMLET, apo-α-lactalbumin, and holo-(native)-α-lactalbumin have also been characterized by mass spectrometry, H/D exchange, and limited proteolysis by L. Birolo (University of Naples, Italy) [17]. Whereas the number of exchanged deuterated amide NH-sites was greater in HAMLET than in the apo-protein, analysis of proteolytic fragments suggested that the oleic acid moiety may be altering the con-

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