PROGRESS REPORT OF RESEARCH SUPPORTED BY THE MORAN FOUNDATION

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PROGRESS REPORT:

Calnexin functions in a lectin-like capacity. Sucrose velocity sedimentation and immunoabsorption analyses indicate that all human alpha1-antitrypsin (AAT) variants form a 7.6 S complex as they bind the molecular chaperones BiP/grp78 and calnexin mmediately following synthesis. Both molecular chaperones dissociate from newly synthesized AAT within eight minutes. Within 30 minutes for the Z variant, or 60 minutes for variant null(Hong Kong), BiP has completely dissociated from this ternary complex, leaving a 6.1 S AAT-calnexin binary complex. The existence of a small fraction (10-20%) of AAT monomers during their intracellular retention suggests that they probably dissociate from calnexin prior to their degradation and it may also reflect that they undergo cycles of dissociation and reassociation with calnexin during that period.

Trimming of glucose residues from oligosaccharides of newly synthesized AAT is required for association with calnexin. Inhibition of asparagine-linked glycosylation (with tunicamycin) or trimming of terminal glucose residues from oligosaccharides (with castanospermine) prevents the interaction of calnexin with all AAT variants examined. At these conditions, only BiP is associated with AAT. BiP is specifically dissociated from AAT in response to incubation with ATP. Calnexin specifically dissociates in the presence of deoxycholate detergent micelles. *Thus, calnexin is the lectin-like protein that we have sought during the funding period.* Aalthough an appropriate oligosaccharide structure is apparently necessary to initiate calnexin binding, its stable association likely involves binding to the actual polypeptide.

Association with calnexin delays intracellular degradation of retained variants. In the absence of calnexin association, retained AAT variants are degraded at a faster rate. Thus, calnexin association delays the onset of intracellular degradation of partially-folded variants. Thus, Various factors, including the presence of ATP and trimming of mannose is somehow necessary for degradation to proceed.

Intracellular retention pathway. Glucose-trimmed, asparagine-linked oligosaccharides attached to unfolded glycoproteins in the ER undergo cycles of reglucosylation as the enzyme UDP-glucose glycoprotein: glucosyltransferase (UGTr) catalyzes the transfer of a single glucose residue from UDP-glucose to the Man8-9GlcNAc2 oligosaccharide'acceptor'. Monoglucosylated oligosaccharides are bound to transport-impaired glycoproteins. Helenius and coworkers have proposed a model in which UGTr, calnexin, and alpha-glucosidase II function in a combinatorial manner to prevent intracellular transport of partially-folded glycoproteins in the exocytic pathway. In this model, UGTr is a folding sensor that reglucosylates oligosaccharides of unfolded glycoproteins. Calnexin is a retention protein that recognizes this structure to initiate binding to the polypeptide. Finally, alpha-glucosidase II functions as a signal modifier that hydrolyzes the terminal glucose residue at some point following calnexin binding. Sequential cycles of glucose transfer and subsequent calnexin association should continue until complete folding is achieved or until endogenous alpha-mannosidases in the ER modify the oligosaccharide to a structure no longer suitable for glucose transfer. Unfolded proteins would then be susceptible to degradation by proteases in the ER. *It is now evident that components of this model are used in the intracellular retention of secretion-incompetent AAT variants.*

PRESENTATIONS:
(none during this funding period)

PUBLICATIONS:
Sifers, R. N. (1995). Defective protein folding as a cause of disease. Nature Structural Biology 2:355-357.

Liu, Y., Cabral, C., and Sifers, R. Sequential interaction of alpha1-antitrypsin with molecular chaperones BiP and calnexin. A role of oligosaccharides in calnexin binding. (*In preparation for the Journal of Biological Chemistry*).

Liu, Y. and Sifers, R. The secretion-impaired Z variant of human of alpha1-antitrypsin is stably bound to the molecular chaperone BiP. (*In preparation for the Journal of Biological Chemistry*).

AWARDS:
Recipient of a Research Career Investigator Award from the American Lung Association (Results generated from this project allowed me to submit an outstanding proposal to the American Lung Association).

Defective protein folding as a cause of disease

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A defined, spontaneous protein folding defect can act as a common link between lung and liver diseases associated with the Z type of plasma α1-antitrypsin deficiency.

α1-antitrypsin (α1-AT) is a member of the serine proteinase inhibitor (serpin) superfamily[1], mutations of which are the cause of a number of disease conditions[2]. It is synthesized in hepatocytes of the liver[3,4] and secreted into the circulation where it exists as a component of human plasma[3]. The major physiological role of the inhibitor is to protect lung elastin fibers from proteolytic attack by neutrophil elastase released into the local intercellular medium[5].

The structural gene for human α1-AT is extremely polymorphic[6] and several alleles encode a polypeptide that is prevented, to some degree, from being secreted into the circulation[7]. An inherited deficiency of α1-AT in human plasma can perturb the elastase-antielastase balance in the lower respiratory tract compromizing the protection of lung connective tissues[8], a condition which can result in the develoment of lung emphysema[5]. Intracellular retention of some α1-AT variants can result in their accumulation as insoluble aggregates in the endoplasmic reticulum of hepatocytes[6,7], resulting in liver damage.

Although many human α1-AT alleles have been cloned and sequenced[6], the Z variant has been studied most extensively because of its relatively high frequency in the population, the severity of its secretory defect, its extensive intracellular accumulation and its association with disease. Studies in transgenic mice have verified that intra-hepatic accumulation of the insoluble Z variant can act, in part, as a causative agent of liver disease[9].

During the last several years extensive work in elucidating the structural biology of serpins has led to an understanding of how mutations within this class of proteins can result in natural, but inappropriate, conformational changes which can give rise to diverse diseases[2]. In this issue of *Nature Structural Biology*, Yu and colleagues[10] provide direct experimental evidence that a defect in the folding of a protein rather than in the properties of the fully folded, mutant protein can function as the underlying cause of both lung and liver disease phenotypes associated with the Z type of plasma α1-AT deficiency.

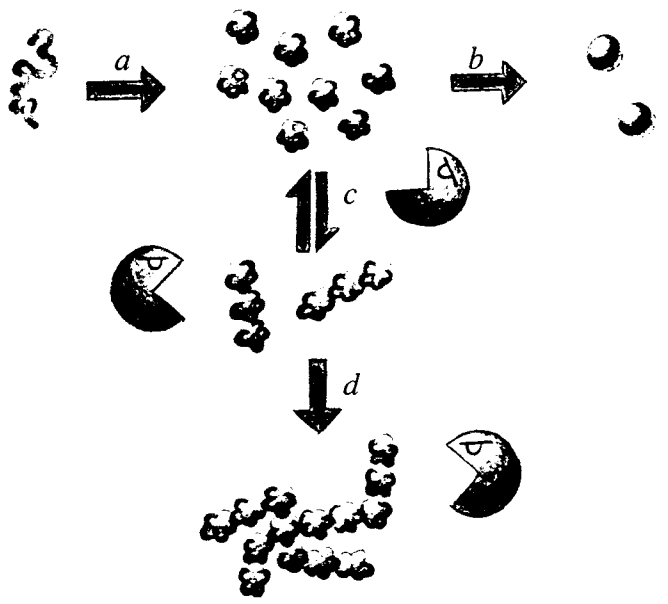

Fig. 1 Potential intracellular fates of the newly synthesized Z variant. Following biosynthesis, the newly synthesized Z variant polypeptides fold into a stable intermediate state (step a). While others attain the native conformation (step b) and are secreted from the cell, the accumulating intermediate can oligomerize as loop-sheet polymers (step c) which are deposited as large entangled polymers in the lumen of the endolasmic reticulum (step d). Endogenous proteinases degrade the accumulating protein, probably at multiple stages. Finally, interaction with molecular chaperones might play a role in the overall intracellular retention mechanism.

Unique structural mobility of serpins

The serpin superfamily consists of a wide variety of both inhibitory and non-inhibitory proteins which fold into a similar highly-ordered tertiary structure composed of three β-sheets and several α-helices[1,11,12]. Target proteinases interact with a reactive, inhibitory site centred around two amino acids, unique to each inhibitor, located in a peptide loop exposed to the surface of the folded molecule[11,13,14]. For α1-AT, this involves a Met–Ser bond at residues 358 and 359 (ref. 15). Subsequent formation of a tight, equimolar complex between inhibitor and proteinase renders both molecules inactive.

A unique structural feature of inhibitory serpins is that the reactive-centre loop is quite mobile, allowing the molecule to adopt various con-

formations to modulate its inhibitory activity[1,12]. This is quite different from members of other proteinase inhibitor families which exhibit a fixed reactive-centre loop with a 'canonical' conformation. Three serpin conformations have been described[2] that involve partial or total insertion of the mobile reactive-centre loop into a gap between strands 3 and 5 in sheet-A. For example, in the 'quiescent' conformation the loop is fully exposed for interaction with proteinases. After docking,stabilization of the proteinase–inhibitor complex requires transition to a stressed, 'locking' conformation characterized by partial loop insertion into sheet-A. Finally, a 'latent' conformation has been identified where the entire reactive centre loop has inserted into sheet-A, thereby making the reactive-centre inaccessible to proteinases.

Analysis of a wide variety of normal and mutant serpin molecules by X-ray crystallography, molecular modelling, and biochemical methods has led to the identification of structural domains within the folded polypeptide which control reactive-centre loop mobility. Intramolecular mobility of serpins is expectionally vulnerable to the effects of mutations which can remove structural constraints and may result in spontaneous, but inappropriate, conformational changes (reviewed in ref. 2).

The Z variant and spontaneous conformational changes

Spontaneous polymerization[16] of the Z variant of α1-AT is an example of this phenomenon and involves a single Glu 342 to Lys substitution[17] located at the amino-terminal base of the reactive-centre loop[11]. Normally, Glu 342 forms a crucial salt bridge with Lys 290 located on an adjacent strand of sheet A[10]. Substitution with lysine is predicted to destabilize the sheet and enhance the opening of sheet A for insertion of the reactive-centre loop. Lomas and colleagues[16] demonstrated that spontaneous polymerization of the Z variant occurred *in vitro* at high concentration during incubation at 37 °C, and coined this mechanism 'loop-sheet' polymerization. A similar phenomenon was observed for the normal protein under mild denaturing conditions. The initial explanation for this molecular event was that the reactive-centre loop of one molecule inserted into the opened sheet A of another, thereby initiating noncovalent polymerization of the proteins. This model has since been modified to include overinsertion of the reactive-centre loop into sheet A and subsequent release of a strand of sheet C to accept the loop from another molecule[18].

In their original report[16], Lomas and coworkers provided compelling evidence that the mechanism of loop-sheet polymerization is responsible for accumulation of the Z variant in the endoplasmic reticulum. That is, by electron microscopic analysis, the accumulated protein from liver inclusion bodies was very similar to that of the Z variant polymerized *in vitro*. Because this evidence was of a circumstantial nature, however, direct proof of the mechanism has awaited further experimentation.

Direct evidence for a folding defect

Yu and coworkers[10] have now compared folding kinetics of the Z variant and normal polypeptide by analysis of their electrophoretic migration in transverse urea gradient gels at various times following their *in vitro* translation. In this technique more highly-folded structures exhibit a greater resistance to denaturation and migrate further in the gel. Using this methodology, the authors observed that while folding of normal α1-AT was rapid, most of the Z variant polypeptides were arrested at an intermediate stage identified as a slower migrating species. Most Z variant polypeptides were able to eventually fold into a native, active conformation during an extended incubation at 30 °C which prevented formation of aggregates. Folding into the native conformation was verified by electrophoretic migration of the polypeptides and ability to form a complex with elastase. Although these data do not indicate the exact nature of the intermediate in terms of its role in the normal folding pathway, it is apparent that a late step, essential for transition into the native conformation, is hindered.

Protein aggregates were observed during subsequent incubation at 37 °C which should induce loop-sheet polymerization of the molecules. Furthermore, the data suggest that it is the folding intermediate, rather than the native polypeptide, that is required for aggregate formation. *In vitro* refolding of recombinant Z variant polypeptides from *Escherichia coli* inclusion bodies was used to prepare soluble aggregates which exhibited physical characteristics expected of loop-sheet polymers. Although these results are less than convincing evidence that aggregates formed from the *in vitro* translation products actually consisted of loop-sheet polymers, it does support the idea.

Additional cellular factors

Considering the evidence that most Z polypeptides are capable of folding into the native conformation, it is reasonable to ask why only 10-20 % of the newly synthesized molecules are secreted[19]. One would predict that the folding intermediate observed in this study would be present in the endoplasmic reticulum of hepatocytes and accumulate to substantial levels. Because the Z variant undergoes spontaneous polymerization at high concentration and normal physiologic temperature[16] it might be expected that a shift in the equilibrium in favour of polymers woulc lower the intracellular concentration of the intermediate thereby preventing further folding (Fig. 1). Also, a 'quality control' mechanism is known to exist in the secretory pathway that ensures delivery of only correctly folded polypeptides to the cell surface[20]. In addition to the formation of loop-sheet polymers, loss of the folding intermediate may result from its intracellular degradation, a phenomenon that has been observed for the Z variant[19].

Because this quality control pathway involves interaction with molecular chaperones thought to function in the prevention of protein aggregation[21], mechanisms in addition to loop-sheet polymerization might participate in the actual intracellular retention of the Z variant. In support of this idea is the reported

interaction between normal[22] and variant[23,24] human α1-ATs with calnexin[25], a resident membrane-associated protein of the endoplasmic reticulum. Thus, multiple factors might function in the overall retention of the Z variant which is responsible for lowering circulating inhibitor levels resulting in elastolytic attack of lung elastin fibers.

In terms of liver disease, it is the intracellular accumulation of the insoluble Z variant[26] which is sufficient to induce liver injury in transgenic mice[9]. Conceivably, formation of loop-sheet polymers, insolubility of larger oligomers, and the rate at which both soluble and insoluble polymers are degraded are all likely to contribute to the net rate and extent of Z protein accumulation in hepatocytes. The fact that only 12–15 % of ZZ homozygotes in the population develop liver injury[27] points to a role for additional genetic or environmental factors in the development of this phenotype. In this regard, Wu and colleagues[25] have reported evidence that susceptibility to liver disease in these individuals might involve differences in the protein degradation machinery of the endoplasmic reticulum. This observation will undoubtedly prompt further investigation.

Overall, the folding defect of the Z variant translates into a protein mis-localization problem. Such is the case for cystic fibrosis[28]. But development of tissue injury involves several additional environmental and genetic factors. However, the novelty of Z-type plasma α1-AT deficiency exists in the ability of a single point mutation to cause a defined, spontaneous protein folding defect that can act as a common link between diverse diseases[2].

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