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Quality Control of Protein Folding in Extracellular Space

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Abstract

The pathologies of many serious human diseases are thought to arise from the effects of intra- or extracellular aggregates of non-native proteins. Inside cells, chaperone and protease systems regulate protein folding; however, little is currently known about any corresponding mechanisms that operate extracellularly. Identification of these mechanisms is important because this knowledge may lead to the development of new disease therapies. This review briefly discusses the consequences of protein misfolding, and intracellular mechanisms that control protein folding, before examining potential corresponding extracellular control processes. Finally, a new speculative model is described which proposes that newly discovered extracellular chaperones bind to exposed regions of hydrophobicity on non-native, extracellular proteins to target them for receptor-mediated endocytosis and intracellular, lysosomal degradation.

It has been estimated that about 400 grams of protein are synthesized and degraded each day in the human body. Individual proteins are degraded at extremely varied rates, with half-lives ranging from several minutes to many hours. Intracellularly, this variation in half-life has been attributed to differences in the intrinsic stability of proteins and the recognition of non-native structures by highly selective and precisely regulated protein quality control (QC) systems.

Protein misfolding and disease

Correct protein folding is essential to provide the functional machinery for living organisms. Mistakes in transcription or translation that result in errors as small as an amino acid substitution can destabilize, and thus prevent, normal folding. In addition, environmental conditions such as macromolecular crowding, inappropriate ionic strength, oxidative stress and extremes of pH and temperature are known to promote the (i) formation of misfolded states or slowly reacting intermediates that are trapped on the folding pathway, and (ii) partial unfolding of fully folded native proteins. Exposed hydrophobic regions on non-native proteins bind to similar regions on nearby proteins by highly specific self-association (Rajan *et al,* 2001). The aggregating species can continue to bind exposed hydrophobic regions of neighbouring proteins and thus has the potential to form various stable structures, including soluble aggregates and insoluble deposits (Fig 1). All of these protein structures have been found associated with disease states. For example, soluble oligomeric protein aggregates have been implicated as cytotoxic species in a variety of neurodegenerative disorders (Demuro *et al,* 2005). Insoluble protein deposits contribute to the pathology of a variety of serious human diseases (Table 1), including

the amyloidoses that are characterized by insoluble, fibrillar protein aggregates with a β-sheet-rich structure.

Intracellular defences against non-native proteins

Prokaryotic and eukaryotic cells utilize post-translational QC systems, including molecular chaperones and proteases, which are designed to repair or remove damaged proteins. Consequently, misfolded intracellular proteins have three possible fates: rescue by chaperones, destruction by proteases, or aggregation. Chaperones are a diverse group of proteins that selectively recognize and bind exposed hydrophobic surfaces of non-native proteins in a stable non-covalent interaction. Several families of chaperones, defined by size, cellular compartment and function, work together to prevent protein aggregation and facilitate the correct folding of non-native proteins. By regulated binding and release, chaperones may directly facilitate the folding of proteins by a mechanism that is ATP-dependent (for example, heat shock protein 90 (Hsp90), Hsp70, and Hsp60 in the cytosol) or independent (for example, calnexin and calreticulin in the endoplasmic reticulum) (Fink, 1999; Hartl & Hayer-Hartl, 2002). Other types of chaperones (for example, the small heat shock protein Hsp25 and α-crystallin) bind reversibly to exposed hydrophobic regions on unfolded polypeptides but are generally unable to independently facilitate refolding. They are thought to protect non-native proteins from aggregation until "folding-helper" chaperones are available (Bross *et al,* 1999).

Misfolded proteins may be degraded by a variety of proteases in different cell compartments, however, the major intracellular site of degradation is the proteasome,

a barrel-shaped cytosolic protein complex that selectively degrades proteins tagged with ubiquitin (Hohfeld *et al*, 2001). Polyubiquitin chains are attached to misfolded proteins by ubiquitin ligases and cofactors in the cytosol (Welchman *et al*, 2005); ubiquitination is also known to occur in the ER lumen (Tsai *et al*, 2002). Proteins destined for secretion into the extracellular environment undergo a rigorous QC process within the ER lumen, which includes interaction with a battery of ER-resident chaperones. Some of the ER chaperones, such as calnexin and calreticulin, are lectins that have a specialized role in the QC of glycoprotein folding. Many QC substrates cycle between the ER and the Golgi during their maturation (Trombetta & Parodi, 2003). When misfolded proteins originally destined for secretion cannot be refolded, they are usually either degraded by ER proteinases, directed to the lysosome or, more commonly, retro-translocated into the cytosol for proteasomal degradation (Fig 2). Under circumstances when the chaperone/protease machinery is overwhelmed, misfolded proteins may form insoluble aggregates inside the ER (Trombetta & Parodi, 2003) or in the cytosol (an aggresome). Once proteins are secreted, they escape characterised intracellular QC processes and enter an environment in which the mechanisms of folding control (if they exist) have yet to be discovered.

Are extracellular proteins subject to quality control?

There is, on average, 15 litres of extracellular fluids in a 70 kg human, including five litres of blood. Compared with intracellular fluid, extracellular fluids have a lower protein concentration (6% in plasma and 2% in interstitial fluid, 30% in cytosol) but are more oxidizing (Sitia & Braakman, 2003). Unlike intracellular fluids, extracellular fluids are continuously subjected to shear stress (for example, the pumping of plasma around the body) which is known to induce protein unfolding and

aggregation (Ker & Chen, 1998). Intuitively, the relatively harsh extracellular conditions suggest that active protein QC mechanisms are required. Studies from past decades provide substantive support for this hypothesis. For example, in the 1970's and 80's it was shown that denatured plasma proteins were catabolized *in vivo* more rapidly than their native counterparts (Margineanu & Ghetie, 1981) and that the catabolic rates of plasma proteins correlate with their *in vitro* susceptibility to proteases (Dice & Goldberg, 1976). In addition, liposomes with greater surface-exposed hydrophobicity were cleared from circulation more quickly than those coated with a hydrophilic polymer; it was proposed that clearance was enhanced by plasma factors that could bind to exposed hydrophobic surfaces on the liposomes (Senior *et al,* 1991). These data are consistent with the hypothesis that, as occurs intracellularly, exposed hydrophobicity may be the structural change that identifies individual extracellular proteins as needing QC intervention. Further support for this hypothesis comes from the demonstration that a recently discovered extracellular chaperone, clusterin (see below), binds to its substrates by hydrophobic interactions (Poon *et al*, 2002).

What protein QC mechanism(s) actually operate in extracellular space? Substantial recent advances in understanding intracellular protein QC mechanisms highlight our near-complete lack of understanding of corresponding extracellular processes. While not excluding other possibilities, the potential involvement of chaperones and selective protease systems are clearly worth considering.

Extracellular chaperones and proteasome-like systems

Intracellular folding-helper chaperones (for example, Hsp70 & Hsp90) may be released from necrotic cells or during viral cell lysis; they have been discovered in

human plasma and associated with cell surfaces, in particular cancer cells. Numerous extracellular roles have been postulated for these chaperones, such as cancer cell invasiveness (Eustace *et al,* 2004) and immune presentation (Becker *et al,* 2002). If established, these specialized functions are clearly important. However, in the context of generic mechanisms for extracellular protein QC, it is pertinent to note that these "normally intracellular" chaperones are present extracellularly at very low (ng/ml) levels and require ATP to effect protein refolding. The abundance of extracellular ATP is at least 1000 times lower than inside cells (Farias *et al,* 2005). In the event of a large-scale presentation of extracellular non-native protein(s), such as might occur during a chronic infection, the capacity of the low levels of Hsp70 and similar chaperones present extracellularly would be quickly exceeded. Thus, although a role for these chaperones in extracellular protein QC cannot be excluded, it is far more likely that large-scale "handling" of non-native proteins is dealt with by much more abundant extracellular chaperones (ECs). Two such ECs have recently been identified.

Clusterin, a ubiquitous and highly conserved secreted protein, is an efficient EC (Humphreys et al, 1999; Wilson & Easterbrook-Smith, 2000). Clusterin potently inhibits stress-induced protein aggregation by ATP-independent binding to non-native proteins to form soluble, high molecular weight complexes (Humphreys *et al,* 1999; Poon *et al,* 2000). More recently, it was established that haptoglobin (Hp; previously best known for its high-affinity binding to haemoglobin) is also an abundant EC with a chaperone action similar to clusterin (Yerbury *et al,* 2005). Both ECs are highly conserved, widely distributed glycoproteins that are present in most physiological fluids, including plasma and cerebrospinal fluid (Bowman & Kurosky, 1982; Jenne &

Tschopp, 1992). They are composed of variably sized oligomers made up of different numbers of disulfide-linked α and β subunits, bind to a broad range of ligands, and have been detected in association with clinical amyloid deposits *in vivo* (Calero *et al,* 2000; Powers *et al,* 1981). In human plasma, clusterin is present at about 100 μg/ml (Humphreys *et al,* 1999) and Hp at 0.3-1.9 mg/ml (Bowman & Kurosky, 1982). Immunoaffinity depletion of either clusterin or Hp from human serum renders proteins in whole serum more susceptible to aggregation and precipitation (Poon *et al,* 2000; Yerbury *et al,* 2005).

Neither clusterin nor Hp has the ability to independently refold heat-stressed, non-native enzymes (Poon *et al,* 2000; Yerbury *et al,* 2005). However, like the small heat shock proteins, clusterin is able to preserve heat-inactivated enzymes in a state competent for subsequent ATP-dependent refolding by Hsc70 (Poon *et al,* 2000). Although it is possible that specifically extracellular folding-helper chaperones exist, they remain to be convincingly demonstrated. It was reported that the plasma pentraxin serum amyloid P component (SAP) had some ATP-independent refolding activity *in vitro*. Currently, the physiological relevance of this is uncertain because, to effect significant refolding, SAP was required at a 10-fold molar excess to the only substrate tested (lactate dehydrogenase) (Coker *et al,* 2000). Further studies of the chaperone action of SAP, using other substrates, and investigation of potential interactions between SAP and other ECs would be enlightening. SAP is a major plasma acute phase protein in mice but this role is played in humans by the related pentraxin C-reactive protein (CRP) (Mold *et al*, 2001); it is tempting to speculate that CRP may also be an EC. It is likely that other abundant ECs will be discovered in the future.

Previous studies have indicated that 50-90% of plasma proteins are degraded in the liver and reticuloendothelial system (Bouma, 1982). This suggests that a major component of any extracellular protein QC system comprises mechanism(s) for cellular uptake and intracellular degradation of aged/non-native proteins. However, this does not exclude the existence of selective mechanism(s) for the proteolysis of extracellular non-native proteins. Both ubiquitin and the proteasome are found in human plasma and their concentrations increase during disease, possibly reflecting their release from dead and dying cells. The plasma concentrations reported range from 7.7-200 ng/ml (Akarsu *et al,* 2001; Savas *et al,* 2003) and 2.1-2.4 μg/ml (Lavabre-Bertrand *et al,* 2001; Stoebner *et al,* 2005), respectively. At these levels, ubiquitin and the proteasome are probably at least 300 times less abundant in human plasma than inside cells (Born *et al,* 1996; Lightcap *et al,* 2000). In addition, extracellular proteasome-mediated protein degradation would require extracellular ATP which, as noted above, is scarce. Therefore, current evidence does not support a major role for the mammalian ubiquitin/proteasome system in extracellular protein QC. Nevertheless, the existence of other extracellular protease systems that might perform a similar function, perhaps independently of ATP, remains a possibility.

A control mechanism for extracellular protein folding

There are four, not mutually exclusive, theoretical models for extracellular QC. Non-native extracellular proteins may be:

(1) Assisted to refold in the extracellular space. As discussed above, there is currently no evidence to substantiate this.

(2) Selectively endocytosed via receptors and degraded intracellularly. This appears feasible and will be discussed in more detail below.

(3) Randomly endocytosed and selected at the level of (for example) sorting endosomes, for degradation, while recycling native extracellular proteins back to the extracellular space. This model cannot be excluded but currently there is no evidence to support it.

(4) Randomly endocytosed and non-selectively degraded intracellularly. This model can be excluded because it cannot account for the more rapid catabolism of denatured versus native plasma proteins observed *in vivo* (described above).

While not excluding models (1) or (3), by providing a framework for how it might work in practice, recent advances support model (2) . If a QC mechanism "senses" increased hydrophobicity on non-native proteins to select them for "processing", obvious candidates for the sensing role are the recently identified ECs clusterin and Hp. Other yet to be identified ECs may also be involved. The formation of non-covalent complexes between ECs and non-native extracellular proteins would effectively label the latter for QC processing. The suggestion that ECs complex with misfolded proteins *in vivo* is strongly supported by (i) *in vitro*, ECs inhibit the aggregation of purified proteins subjected to chemical and physical stresses by forming stable non-covalent complexes with them (Humphreys *et al*, 1999; Yerbury *et al*, 2005), and (ii) the same (endogenous) ECs in unfractionated human serum inhibit heat-induced aggregation of serum proteins (Poon *et al,* 2000; Yerbury *et al,* 2005). It will be important to experimentally verify the formation of complexes between ECs and non-native proteins *in vivo*. There are precedents for the idea that by complexing with proteins, ECs can mediate uptake of their protein ligands via cell surface receptors and direct them towards intracellular degradation. For example, complexes formed *in vivo* between clusterin and Alzheimer's beta peptide (Aβ) are

taken up by neural epithelial cells via megalin (LRP2)-mediated endocytosis and degraded in lysosomes (Hammad *et al,* 1997). Furthermore, Hp-haemoglobin complexes are quickly bound and internalized by CD163 expressed on human macrophages and are subsequently degraded (Kristiansen *et al,* 2001). We propose that by virtue of their interactions with both extracellular non-native proteins and cell surface receptors, ECs identify and deliver non-native proteins for cell uptake and degradation (Fig 3).

If this model is correct, then it would be expected that manipulation of EC expression might affect the clearance and/or aggregation of proteins in animal models. Recent work with clusterin "knock-out" (KO) mice supports the hypothesis that clusterin exerts important effects on protein clearance and aggregation *in vivo*. For example, a long-term study of clusterin KO mice showed that aging mice developed a progressive glomerulopathy characterized by the accumulation of insoluble protein (containing immunoglobulins) in the kidney mesangium (Rosenberg *et al*, 2002). Another study of double KO mice with ablated expression of both clusterin and apolipoprotein E genes concluded that these two proteins worked together to inhibit the deposition of fibrillar Aβ in a model for Alzheimer's disease (DeMattos *et al*, 2004). Published studies of Hp KO mice have not examined the effects of Hp on the aggregation/clearance of non-native extracellular proteins; it is however interesting to note that ablation of Hp expression caused a small but significant reduction in post-natal viability (Lim *et al*, 1998).

The literature reveals a number of cell surface molecules that might be involved in receptor-mediated endocytosis of EC-(non-native)protein complexes. For example,

clusterin interacts with several members of the low density lipoprotein (LDL) receptor family. Clusterin binds to chicken LR8 and an LDLR-related protein (Mahon *et al,* 1999), and uptake of clusterin-leptin complexes by apoER2 and VLDLR has been proposed to facilitate leptin clearance (Bajari *et al,* 2003). Furthermore, clusterin and LRP1/megalin have been implicated in the clearance of cellular debris by non-professional phagocytes (Bartl *et al,* 2001). Aside from CD163, another potential candidate receptor for Hp-(non-native)protein complexes is the CD11b/CD18 integrin (Mac-1/CR3), which binds to Hp, denatured proteins and the iC3b fragment of complement (Ross, 2000). SAP, and C-reactive protein, are also known to bind and be internalized by Fc receptors (Mold *et al*, 2001). These observations provide leads to pursue in future studies of extracellular protein QC.

Concluding remarks

If the hypothesis is correct that a QC system exists to oversee the folding of extracellular proteins, then it follows that dysfunctions of this system may contribute to the development of diseases associated with inappropriate extracellular protein aggregation and deposition. Therefore, elucidation of the mechanisms controlling extracellular protein folding may lead to the development of new treatments for serious human diseases.

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Figure Legends

Figure 1 Schematic diagram of pathways of protein aggregation. For each protein, there is thought to be an ensemble of possible structures: native (N) and non-native (NN), the latter including partly unfolded intermediates (I_1 and I_2) and unfolded (U) structures. The non-native structures are prone to associate with each other to form aggregates, although native proteins may sometimes also aggregate to form fibres. Non-fibrillar (amorphous) aggregates result from non-specific interactions between many different conformations, whereas highly structured amyloid fibrils can be formed by β-sheet-rich intermediates. Soluble structures are shown in the internal white rectangle, insoluble structures in the outer yellow region.

Figure 2 Outline of major intracellular controls of protein folding. In the cytosol, non-native proteins are recognized by chaperones and are targeted for refolding or proteolytic degradation by the proteasome or lysosome (Hohfeld *et al*, 2001). In the endoplasmic reticulum (ER), newly synthesized proteins or other non-native proteins are assisted to fold by chaperones. ER-Golgi cycling may occur at this stage. If a native conformation is achieved, the protein may be secreted from the cell. If a non-native conformation persists, it can be proteolytically degraded either within the ER, by the proteasome following retro-translocation into the cytosol, or following transport to the lysosome (Trombetta & Parodi, 2003). Under some circumstances, aggregated non-native proteins can accumulate as insoluble deposits inside the ER or in the cytosol as an aggresome. For simplicity, cofactors involved in many of these processes are not shown. See key for other details.

Figure 3 Speculative model for extracellular chaperone-mediated clearance of non-native proteins. Non-native extracellular proteins are bound by extracellular chaperones that mediate their uptake into cells by receptor-mediated endocytosis. EC-NN complexes are internalized and moved by vesicular transport to lysosomes, where they are degraded. Receptors are recycled back to the cell surface (not shown). The primary sites of action of this process are likely to be the liver and the reticuloendothelial system. See key for other details.

Table 1 Proteins forming insoluble deposits associated with disease

Protein involved	Disease(s)	Deposit structure
Amyloid–β *	Alzheimer's disease	F, A
Tau	Alzheimer's disease	F
α-Synuclein	Parkinson's disease, Lewy body dementia	F, A
Amylin*	Diabetes type 2	F, A
SOD1	Amyotrophic Lateral Sclerosis	F
β_2-Microglobulin *	Haemodialysis-related amyloidosis	F, A
Amyloid-A *	Reactive amyloidosis	F, A
Haemoglobin	Sickle cell anaemia	F
Huntingtin	Huntington's disease	F
PrP *	Creutzfeldt-Jakob disease	F, A
Androgen receptor	Spinobulbar muscular atrophy	F
Ataxins 1, 2 & 3	Spinocerebellar ataxia's 1, 2 & 3	F
IgG *	Non-amyloid monoclonal IgG deposition disease	NF
Kerato-epithelin *	Certain corneal dystrophies	NF
Ten other proteins*	Systemic and cerebral hereditary amyloidosis	F, A

* indicates the protein is deposited extracellularly. F = fibrillar, A = amyloid, NF = non-fibrillar

Data sourced from (Korvatska et al, 1999; Lin et al, 2001; Sherman & Goldberg, 2001; Soto, 2001)

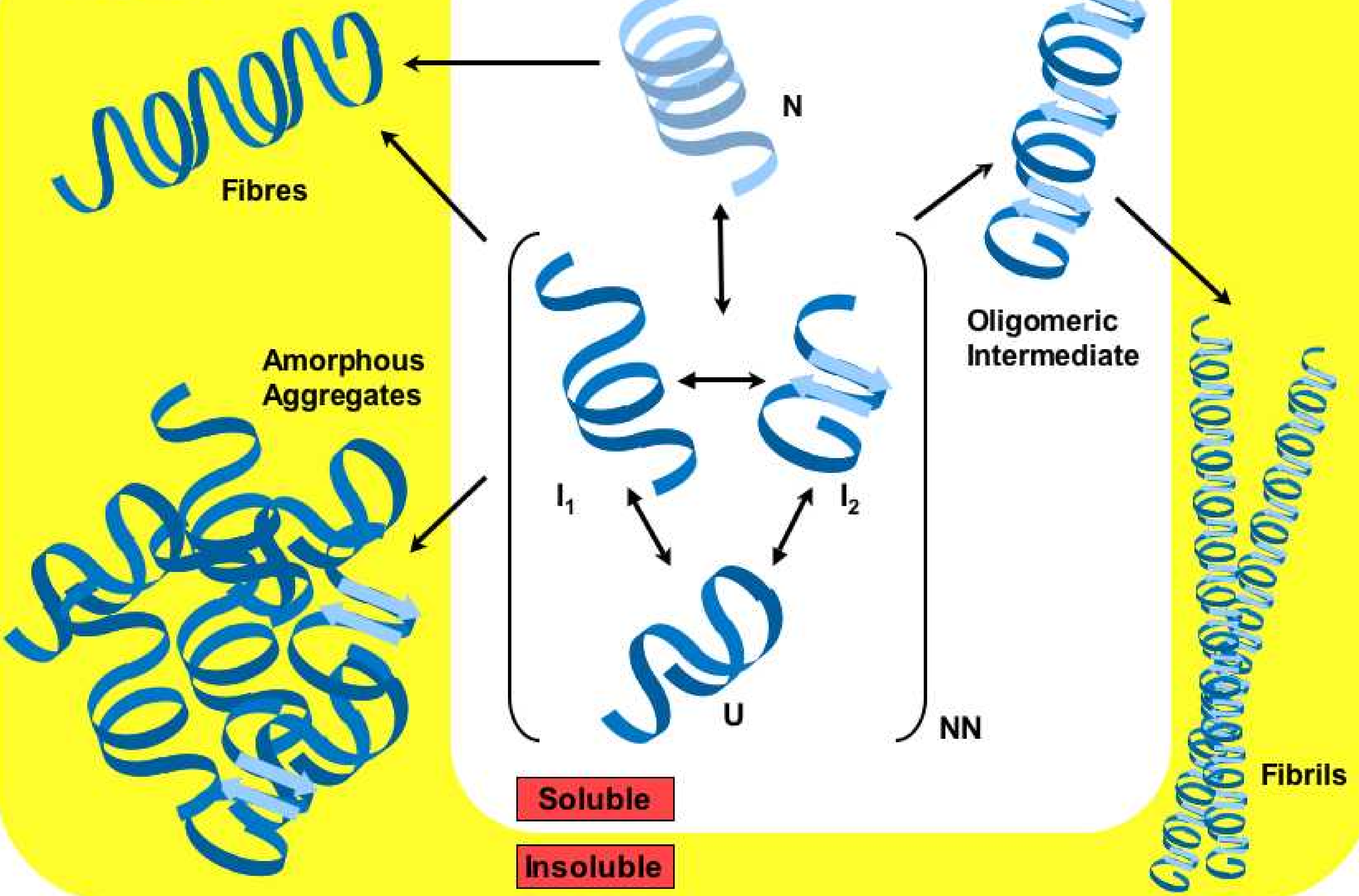
Figure 1
Fibres
N
Amorphous
Aggregates
I1
I2
U
NN
Oligomeric
Intermediate
Fibrils
Soluble
Insoluble

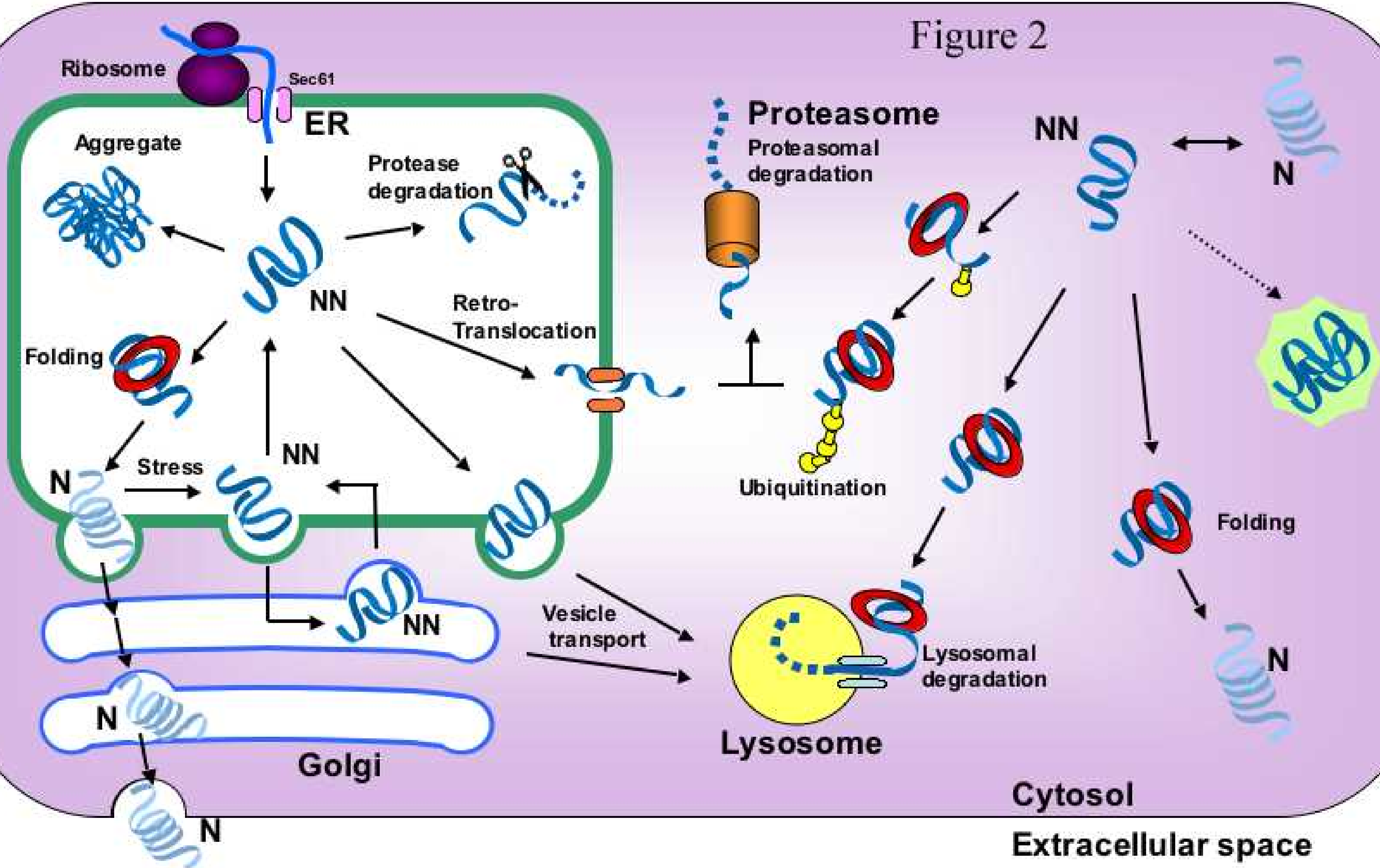

Figure 2

N	Native protein	NN	Non-native protein		Transmembrane channel
	Protease		Chaperone		Ubiquitin

Figure 3

NN
N
L
Macrophage

NN
N
L
Hepatocyte