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Serpins show structural basis for oligomer toxicity and amyloid ubiquity

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Abstract Many disorders, including Alzheimer's, the prion encephalopathies and other neurodegenerative diseases, result from aberrant protein aggregation. Surprisingly, cellular toxicity is often due not to the highly-ordered aggregates but to the oligomers that precede their formation. Using serpins as a paradigm, we show how the active and infective interface of oligomers is inherently toxic and can promiscuously bind to unrelated peptides, including neurotransmitters. Extension of the oligomer and its eventual sequestration as amyloid can thus be seen as a protective response to block the toxic interface. We illustrate how the preferential self-association that gives this protection has been selectively favoured.

Keywords: Oligomers; Amyloid; Serpin; Alzheimers; Prions

1. Introduction

The protein aggregation that underlies conformational diseases such as the prevalent Alzheimer's, Parkinson's and prion encephalopathies [1–4], is due not to random addition but to sequential β-interlinkages resulting in progressive fibral elongation [5,6]. The end-point of these progressive β-linkages is the formation of highly-ordered fibrillar and amyloid deposits. The presence in many of the conformational diseases of readily recognisable amyloid deposits led to the presumption that the diseases themselves were due to the formation of amyloid. The massive deposition of amyloid can indeed result in organ failure but for the most part and particularly with the neurodegenerative diseases, the appearance of amyloid deposits is a late and inconsistent feature. Similarly the accumulation of long-chain protein polymers can directly affect cellular viability [7] but there is now increasing evidence that cellular damage and specifically neurotoxicity often arises much earlier in the disease process, at the stage of initial oligomer formation [8–12]. The generality of this unexpected finding [13]. indicates a shared mechanism. This has focused our attention on the one mechanistic feature common to all the conformational diseases, the formation of the intermolecular β-bonding responsible for the protein aggregation.

To determine why early oligomers formed by such linkages are consistently toxic we have examined the β-interlinkages formed by members of a widely distributed family of serine protease inhibitors, the serpins [6,14]. The advantage of using the serpins as a model is that the mechanism of their fibrillar aggregation is known in crystallographic detail [15,16]. The inhibitory efficiency of the serpins is dependent on the ability of their cleaved reactive–centre peptide loop to insert into the middle-strand position of the 5-stranded β-sheet A of the molecule. This ability of the A β-sheet to undergo a transition from a 5- to a 6-stranded form makes the serpins susceptible to intermolecular linkages, as an aberrant opening of the A-sheet allows the insertion into it of the reactive loop of another molecule (Fig. 1). The sequential formation of similar domain exchanges in a number of other proteins likewise results in oligomer and polymer formation in a process descriptively summarised as 'runaway domain swapping' [17]. This notably occurs with the plasma serpin α1-antitrypsin that protects the lungs against the proteases released by inflammatory cells. People of European descent commonly carry the unstable Z-variant of α1-antitrypsin that readily forms loop-sheet polymers. The polymerisation of Z-antitrypsin [18] principally takes place at its site of synthesis in the hepatocyte and its accumulation there, as intracellular inclusions, is in itself damaging and leads to the eventual development of liver cirrhosis. By a precisely similar process, mutations in a neurone-specific serpin can result in its intracellular polymerisation in neurones leading to an Alzheimer-like late-onset encephalopathy and dementia [7,19].

We have recently shown [20] that the initiating step in serpin polymerisation occurs when two molecules with coincidentally perturbed conformations link to form an initial dimer with two active interfaces (Figs. 1 and 2a). In one unit of the dimer the A-sheet is stabilised in a partially opened form, making it an activated acceptor for β-strand linkages, whereas the other unit has an exposed reactive centre peptide loop held in a constrained conformation optimal for β-strand donation. Once it is formed this nucleating and infective oligomer can then recruit further molecules resulting in a sequential re-formation, molecule by molecule, of each of the active interfaces [20]. We show here, with the serpins α1-antitrypsin and antithrombin, how the exposed β-acceptor interface of such oligomers is potentially toxic in that it can promiscuously link to neurotransmitter and other vital peptides. This toxicity will be limited however by the competitive blocking of the site by auto-linkages resulting in the progressive extension of the oligomer and its eventual sequestration. An indication as to why self-association is so competitively successful comes from the demonstration here of a β-bonding interface in antithrombin that is constantly being exposed in blood but is immediately and preferentially blocked by a specific auto-linkage.

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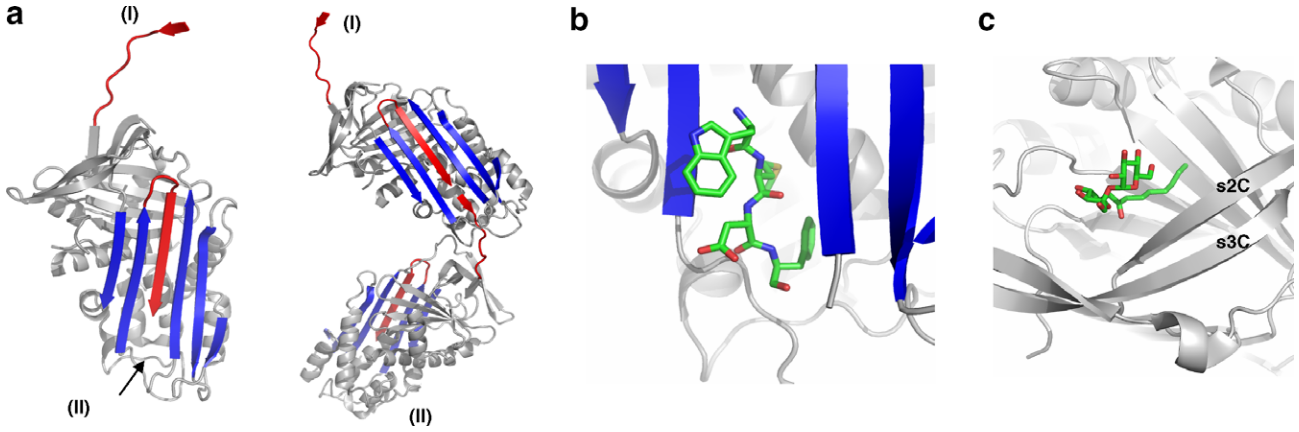

Fig. 1. Serpin β-interfaces and linkages. Crystallographic depictions: (a) Cleavage of the reactive centre loop (red) of α1-antitrypsin creates a β-donor strand I, and an accompanying opening of the A-sheet (blue) creates a β-acceptor site II. Subsequent interlinkage, as shown with the dimer, re-forms each of these sites as the oligomer extends (PDB 1QMB) [15]. (b) The hexapeptide acceptor site II seen here in a polymerogenic form of antithrombin [25], can promiscuously bind other peptides including as shown the neurotransmitter peptide CCK4 (PDB 1JVQ). But even the peptide that naturally occupies this site, TAVVIA, has to be in a 100-fold excess to compete with the preferential linkage to the site of another molecule of antithrombin. (c) The potential promiscuity and hence toxicity of a vacated β-acceptor site is seen here with exposure of the s1C strand position in α1-antitrypsin (see also Fig. 3a) and the binding to it of a liposaccharide from Im et al (PDB 1IZ2) [30].

2. Materials and methods

2.1. Materials

Human α-antithrombin (ATIII) and α1-antitrypsin (α1AT) were purified from frozen plasma as previously described [18,21]. Polymers of ATIII or α1AT were prepared by heating the protein at 60 °C for 15 min at 1 mg/ml at pH7.4 or by PP4 protease cleavage [22]. Human α1AT mutants with P7-P3 of the reactive loop substituted to AVVIA (P7-P3 of the reactive loop of ATIII) or VTFKA (strand 1 of C-sheet of ATIII) were prepared from *E. coli* and purified using nickel-chelating and ionic exchange HiTrap Q columns (GE Healthcare) as previously described [23]. Peptides CCK4 (WMDF), CCK6 (DYMGWM) and C.NP, the cardioexcitatory neuropeptide (FLRF),were from Sigma–Aldrich Ltd., Dorset, England. Latent antithrombin and its dimeric derivatives were prepared and characterised as previously described [21,24].

2.2. Biotin labelling

To label the only cysteine residue of α1AT with biotin, plasma α1AT was first treated with 10 mM DTT, pH 7.4, at room temperature for 15 min and purified from free DTT by a NAP10 desalting column. A freshly-made solution of *N*-(3-maleimidylpropiony)biocytin (Molecular Probes) was added to the protein solution (~1 mg/ml) at 10 molar excess and the mixture was kept at room temperature for 2 h. The reaction was stopped by adding 10 mM DTT and the labelled α1AT was purified by a Hitrap Q column. To detect the biotin labelled protein, samples were analysed by native gel electrophoresis, and then transferred to a nitrocellulose membrane, blotted with streptavidin-peroxidase polymer (Sigma) and visualised using the ECL Western Blotting Detection Regents (GE healthcare). Native gel electrophoresis was performed as previously described using an 8% polyacrylamide gel [24].

3. Results and discussion

3.1. Toxic interface

The potential toxicity of the activated β-interface of early oligomers, is demonstrated in Fig. 2b with the polymerisation of α1-antitrypsin induced by cleavage of its reactive loop by the protease PP4 [15,16,22]. This shows the relatively non-specific amino acid sequence requirement for β-strand linkage to the acceptor interface, with polymerisation being blocked not only by the annealing of the β-strand peptide that normally occupies the acceptor interface in α1-antitrypsin (FLEAI) but also, with even greater efficiency, by the equivalent but quite different peptide from antithrombin (TAVVIA) [25]. As shown the interface can also bind with equal efficiency to a range of other small peptides, including the cholecystokinin neuropeptides *CCK6* (DYMGWM) and *CCK4* (WMDF) and the cardioexcitatory neuropeptide *C.NP* (FLRF). The ability to bind to heterogeneous peptide sequences in this way is not confined to the polymers of α1-antitrypsin induced by loop cleavage but also occurs with the heat-induced oligomers of polymerogenic forms of antithrombin and with mutant Z α1-antitrypsin [25]. This inherent ability of oligomers to promiscuously form β-linkages with a range of peptides, explains their potential toxicity. Although this potential toxicity is demonstrated here with the ready ability to bind neurotransmitter and other peptide messengers, lethal damage is more likely to occur within the milieu of the cell due to a similar binding to the peptide loops of receptors [26] or of other key cellular and membrane components [27]. But protection against such promiscuous and potentially lethal linkages will be provided by the much more competitively avid formation of auto-linkages.

3.2. Specificity of auto-linkages

Although we show in Fig. 2b the blockage of polymer extension by various peptides, unless the competing peptide is present in greater than 50:1 molar ratio with respect to the monomer[25,28] serpin oligomers will preferentially link with further serpin monomers to give oligomeric extension and polymerisation. Moreover the auto-linkage is relatively specific for each serpin. As shown in Fig. 2c, monomers of α1-antitrypsin readily link to pre-formed polymers of α1-antitrypsin but only detectably so to polymers of the closely related serpin, antithrombin. Such preferential auto-linkages are readily explicable if they involve major domain exchanges but the surprising finding is that preferential auto-linkage also takes place even when the linkage involves only a small 6-residue peptide sequence, as with the polymerogenic serpins depicted in Fig. 1a and b. We conclude that the interlinkage of serpins is dependent not only on strand sequences but also requires a confor-