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Review

Molecular bases of neuroserpin function and pathology

Sonia Caccia[1], Stefano Ricagno[2–4] and Martino Bolognesi[4,*]

[1] Department of Biomedical Sciences and Technology, Università di Milano, Via F.lli Cervi 93, 20090 Segrate (MI), Italy
[2] Department of Biochemistry, Università di Pavia, Via Taramelli 3/b, 27100 Pavia, Italy
[3] Biotechnology Research Laboratories, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy
[4] Department of Biomolecular Sciences and Biotechnology, CNR-INFM and CIMAINA, Università di Milano, Via Celoria 26, 20133 Milan, Italy

* Corresponding author
e-mail: martino.bolognesi@unimi.it

Abstract

Serpins build a large and evolutionary widespread protein superfamily, hosting members that are mainly Ser-protease inhibitors. Typically, serpins display a conserved core domain composed of three main β-sheets and 9–10 α-helices, for a total of approximately 350 amino acids. Neuroserpin (NS) is mostly expressed in neurons and in the central and peripheral nervous systems, where it targets tissue-type plasminogen activator. NS activity is relevant for axogenesis, synaptogenesis and synaptic plasticity. Five (single amino acid) NS mutations are associated with severe neurodegenerative disease in man, leading to early onset dementia, epilepsy and neuronal death. The functional aspects of NS protease inhibition are linked to the presence of a long exposed loop (reactive center loop, RCL) that acts as bait for the incoming partner protease. Large NS conformational changes, associated with the cleavage of the RCL, trap the protease in an acyl-enzyme complex. Contrary to other serpins, this complex has a half-life of approximately 10 min. Conformational flexibility is held to be at the bases of NS polymerization leading to Collins bodies intracellular deposition and neuronal damage in the pathological NS variants. Two main general mechanisms of serpin polymerization are currently discussed. Both models require the swapping of the RCL among neighboring serpin molecules. Specific differences in the size of swapped regions, as well as differences in the folding stage at which polymerization can occur, distinguish the two models. The results provided by recent crystallographic and biophysical studies allow rationalization of the functional and pathological roles played by NS based on the analysis of four three-dimensional structures.

Keywords: α1-antitrypsin; crystal structure; dementia; familial encephalopathy with NS inclusion bodies (FENIB); neuroserpin (NS); polymer; protease inhibitor; serine protease; serpinopathies; suicide inhibitor.

Neuroserpin and the serpin superfamily

Neuroserpin (NS) is a 55-kDa secretory protein mainly expressed in neurons of the central and peripheral nervous systems (1); from the neuron it is secreted in a function-related manner, after being stored in regulated secretory granules (2). NS seems to have various physiological and physiopathological functions. It participates in axonogenesis and synaptogenesis during development, and it contributes to synaptic plasticity in the adult (3). NS is also involved in the regulation of permeability between the vascular and nervous system compartments, where, under pathological conditions such as cerebral ischemia, seizures and stroke, it has been shown to play a protective role, preventing neuronal cell death (4–6). NS exerts its recognized roles mainly as an inhibitor of tissue-type plasminogen activator (tPA). NS mutants are responsible for a severe neurodegenerative disease leading to neuronal death and dementia, known as familial encephalopathy with NS inclusion bodies (FENIB) (7). Finally, NS has been found to interact with the β-amyloid (Aβ) peptide in Alzheimer's disease (8).

NS belongs to the serpin (serine protease inhibitor) family, thus sharing the general fold, structural properties and mechanism of action of several known family members; indeed, the serpin superfamily comprises proteins from every kind of organisms, from archaea to man (9, 10). Most serpins act as serine-protease inhibitors; generally, each serpin recognizes a target protease, which is specifically and efficiently inhibited. However, members of the family have been identified as cysteine proteinase inhibitors (11). In addition, there are few but notable exceptions: some selected serpins perform other roles, such as hormone transport, protein folding and chromatin condensation (12–14).

Despite displaying a low sequence conservation within the whole family (below 25%), serpins share a core domain of similar size (~350 residues) and, in particular, all three-dimensional structures available to date show that the serpin fold is well conserved through the phyla (15, 16). Such a fold consists of a domain characterized by three β-sheets (named A, B and C), eight or nine α-helices (named A to H/I) and a 20-residue exposed loop, known as the reactive center loop (RCL) (17) (Figure 1). The main β-sheet, sheet A, is composed of five or six β-strands depending on the serpin conformation (explained below): the A-sheet is mainly

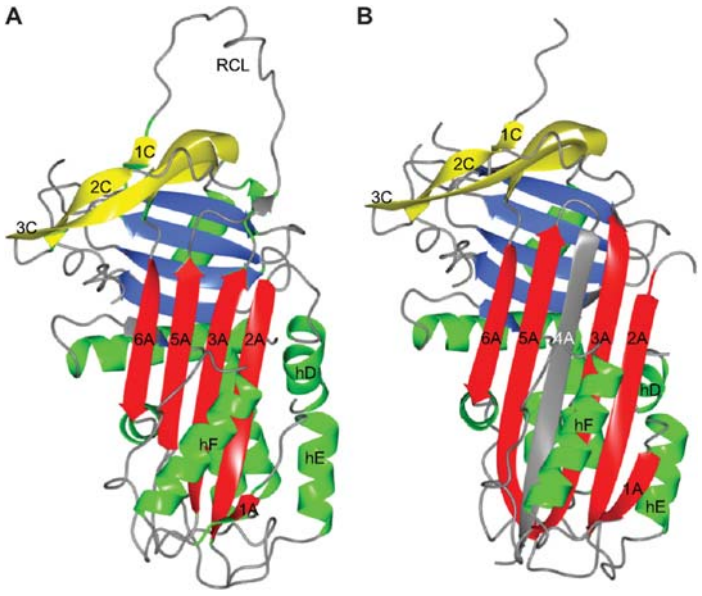


Figure 1 Sketch representation of (A) native and (B) cleaved hNS.
The A-, B- and C-sheets are colored red, blue and yellow, respectively; the helices are green and coil regions including the RCL are colored gray (see also Figure 3). β-Strands and α-helices are labeled according to the standard serpin nomenclature.

solvent exposed and it is a major player in the mechanism through which serpins exploit their function. The antiparallel six-stranded B-sheet is almost completely buried in the serpin fold, sandwiched between the A-sheet and helices hA, hG and hH. The C-sheet is on the apex of the serpin fold, it is solvent exposed and it is contiguous to the RCL. As explained below, this sheet consists of three or four strands, in the native or the cleaved forms, whereas in the latent conformation the first β-strand of the C-sheet (s1C) becomes unfolded (18).

To inhibit their target protease serpins exploit a unique mechanism: the RCL is specifically recognized by the target protease, which cleaves it triggering a fascinating structural rearrangement. Such conformational change results in the insertion of the cleaved RCL in the A-sheet as strand 4 (s4A) (Figure 2). In cases of protease inhibition, the protease remains acylated and thus covalently bound to the RCL. Conversely, when the serpin acts as a substrate, the RCL is cleaved and inserted as s4A, but the acyl-enzyme complex is promptly hydrolyzed. Such conformational change is energetically driven owing to a transition from a 'stressed' (S) to 'relaxed' (R) serpin conformation. Indeed, the higher stability of the cleaved relaxed conformation versus the stressed native conformation has been well established (17, 19). In fact, unlike most globular proteins, serpins spontaneously fold to a metastable native conformation (20). Although the serpin folding mechanism is yet to be completely uncovered, it has been observed that serpins always fold to the native metastable but physiologically active conformation, which is a local minimum, and do not reach the most stable conformations (latent and polymer, described below).

The metastability of native state renders serpins extremely susceptible of misfolding and prone to transition to non-functional conformations. In fact, native (uncleaved) serpins can assume a second conformation known as 'latent', which is characterized by the insertion of the RCL in the A-sheet in the absence of a proteolytic cleavage (21). As a consequence of the insertion of the RCL, the first strand of the three-stranded C-sheet (s1C) is unfolded and converted to a coil (18). The serpin latent form very closely resembles the RCL-cleaved form in several ways: (i) structurally, both forms have the RCL inserted into the A-sheet; (ii) functionally, both are inactive as protease inhibitors; and (iii) thermodynamically, both have similar fold stability (17). In a few cases the latent form has been proven to act as a serpin reservoir, which upon ligand binding can readily revert to native and inhibit the target protease (22).

In addition to the latent form, both *in vitro* and *in vivo*, serpins can also aggregate to form quasi-native elongated polymers that can be depicted in electron microscopy images as beads-on-a-string filaments (17, 23). Although the molecular details of serpin monomeric forms are well characterized owing to several crystal structures, different models of serpin polymerization that are rooted in structural modifications of the A-sheet or of the C-sheet, have been proposed, and are still openly debated (24–28).

In the present review, the physiological and pathological roles of human NS will be examined in the frame of the wider knowledge acquired on the entire serpin superfamily. First, the NS gene expression and cellular localization will be reviewed. Then, the somewhat unclear NS role in brain development and pathology will be analyzed together with its peculiar inhibitory properties. Human NS (hNS) point mutations are at the base of FENIB, an autosomal dominant dementia, and the effects of such pathological point mutations on hNS function, fold and aggregation state will be evaluated. Finally, structural and biophysical data on hNS will be reviewed. Despite a substantial ongoing scientific

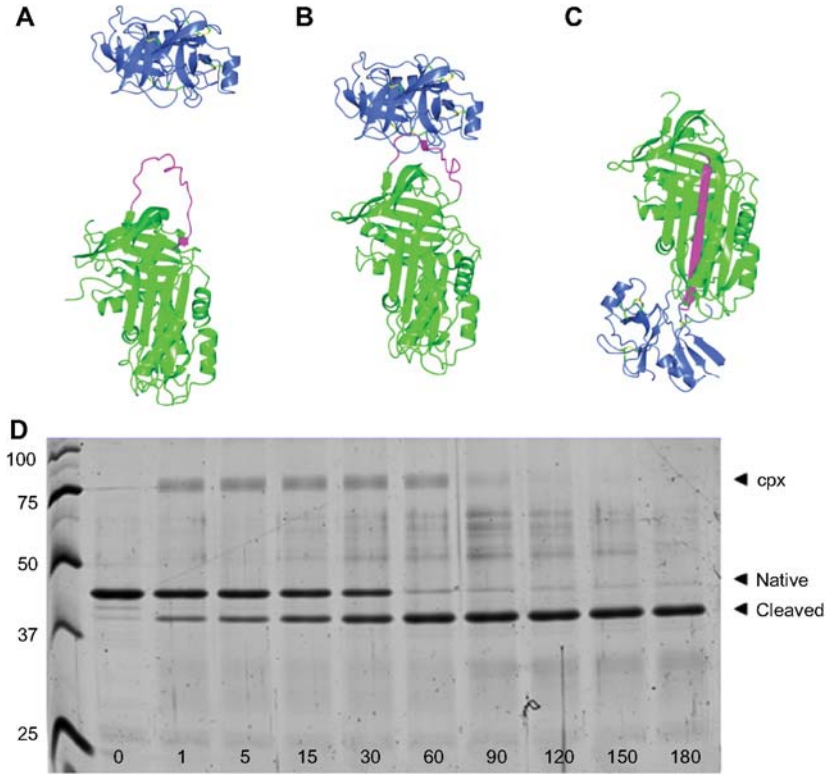


Figure 2 (A–C) Sketch representation of the three steps of serpin-protease complex formation, leading to protease inhibition. The serpin molecule is shown in green, the protease in blue; the RCL is colored magenta. (A) A serpin molecule and a serine protease are shown separately; (B) the Michaelis complex, where the RCL interacts tightly with the protease active site; and (C) once the RCL is cleaved, it is inserted in the A-sheet and the protease is dragged on the opposite side of the serpin molecule. To produce the Figure the following pdb codes were used: (i) 1OPH and 3F5N, (ii) 1OPH, and (iii) 1EZX. (D) SDS-PAGE shows the reaction between tPA and hNS over time (from 0 to 180 min): the bands corresponding to native and cleaved hNS as well as the covalent NS-tPA complex (cpx) are visible. Adapted, with permission, from Elsevier Ltd., J Mol Biol (2009; 388: 109–21), © (2009).

effort to reveal the intriguing cellular and molecular aspects of hNS function in healthy individuals and in disease, a full picture is far from being depicted. The present review will show that NS can be considered a most interesting member of the serpin superfamily, both for its role in human health and as a molecular model for serpin physiological and pathological processes.

Neuroserpin: from gene to secretion

The very first evidence for the existence of NS (originally called axonin-2, because its most prominent feature was its axonal secretion) dates to the late 1980s, when it was identified as a protein secreted by cultured chicken dorsal-root-ganglia neurons (29). Subsequently, when its cDNA was cloned and the deduced amino acid sequence qualified the protein as a novel member of the serpin family, it was named neuroserpin (30). One year later, hNS cDNA was identified from a fetal retina cDNA library (31), with the Human Gene Mapping Workshop (HGMW)-approved symbol PI12 (protease inhibitor 12). In 2001, Silverman et al. revised the serpin nomenclature classifying it as SERPINI1 (10). Mouse and rat NS cDNAs have also been cloned (32, 33). Either cloned or predicted, NS cDNA has been recognized from mammals to non-mammalian vertebrates (http://www.ncbi.nlm.nih.gov/homologene), with a high level of sequence identity in mammals (from 85% to 99%), where the cDNA encodes a 410-residue protein. It soon appeared evident that NS is mainly secreted from neurons of the central and peripheral nervous systems (1). Later NS was also detected in cells of the myeloid lineage (34) and of endocrine tissues (32, 35, 36), where it is stored in regulated secretory granules (2).

The molecular mass of the mature protein is ~55 kDa, as seen in SDS-PAGE (1, 3, 7, 30, 32, 37), in accordance with the presence of potential N-glycosylation sites (three in mammalian NS, located at Asn157, Asn321 and Asn401; two in chicken NS, where residue 321 is Asp) (37) (Figure 3); nevertheless, the actual glycosylation pattern has never been analyzed in depth. With some exceptions (1, 38, 39), the majority of biochemical studies on NS have been performed with a protein expressed in *Escherichia coli*, thus lacking glycosylation. It should be recalled that all available evidence indicates that basic serpin inhibitory mechanism does not depend on NS glycosylation. Recently, a eukaryotic

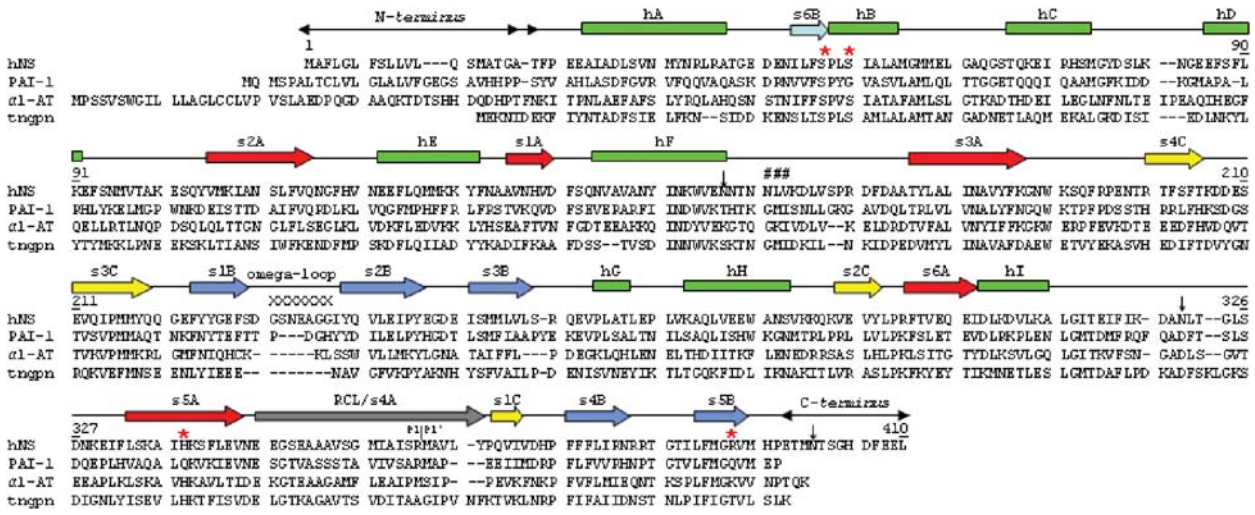


Figure 3 Sequence alignments of human neuroserpin (hNS), plasminogen activator inhibitor-1 (PAI-1), α1-antitrypsin (α1-AT) and tengpin (tngpn) from the extremophile *Thermoanaerobacter tengcongensis*. Sequence numbering is based on hNS (15). The following residues are highlighted: N- and C-terminal peptides are marked with a two-headed arrow above the sequence; the 'omega loop' (from Gly231 to Ala237) is highlighted by a black X; the putative N-glycosylation sites (Gln157, Gln321, Gln401) by black arrows; residues 161–163 (NLV) by a black #; residues whose mutation cause FENIB (Ser49, Ser52, His338, Arg392) by red stars. 'h' and 's' represent helices and strands, respectively. The A-, B- and C-sheets are colored red, blue and yellow, respectively; the helices are green and coil regions including the RCL are colored gray (secondary structures are color-coded as in Figure 1).

high-level expression system in *Drosophila melanogaster* has been developed (40).

Some doubts remain on the primary structure of hNS concerning the length of the N-terminal signal peptide: both from *ex vivo* and cell culture experiments, hNS seems to present a heterogeneous *N-terminus*, with the majority of the molecules starting at Thr20, and the rest at Ala19 (37, 40, 41) (Figure 3). The chicken, rat and mouse proteins, instead, present a more obvious signal sequence of 16 residues.

At the other end of the protein molecule, NS *C-terminus* is peculiar among serpins, because a novel C-terminal 13-amino acids sorting signal has been identified that is both necessary and sufficient to target NS to the regulated secretion pathway (2) (Figure 3). Unlike NS, most serpins are either constitutively secreted or localized to the cytoplasm (42). NS, pancpin (a pancreas specific serpin) and the ER-localized serpins are exceptions in this general pattern, and both NS and the ER-localized serpins appear to contain their targeting sequences at their *C-termini*. The results on the only available crystal structures comprising the 10 residues at the *C-terminus* (43) confirmed that this loop is extremely flexible and exposed to the solvent, potentially allowing interaction with other ligands. The levels of secreted NS could thus be regulated post-translationally. As detailed below, NS levels are also regulated transcriptionally and post-transcriptionally, indicating that NS activity is finely tuned.

By fluorescence *in situ* hybridization the hNS gene was mapped to region q26.1 of chromosome 3 (31). It spans 1907 bp and includes nine exons. Cell culture studies indicated that its transcription is enhanced by neuronal depolarization (44), and it is post-transcriptionally regulated by thyroid hormone (T3), through a stabilizing effect on mRNA (45); the T3 effect was also verified in a mouse model. A regulation of NS expression both at the transcriptional and translational level was confirmed in a physiological contest in *Xenopus laevis* (46).

An unusual feature of the NS gene is that it is closely adjacent in a head-to-head configuration to a non-homologous gene, the *PDCD10* programmed cell death gene (47). These two genes are separated by an exceptionally short intergenic region of 851 bp that tightly links them together through an asymmetric bidirectional promoter of 175 bp. In cooperation with other *cis*-acting elements, this promoter is responsible for the coregulation of the genes expression pattern as well as their tissue specificity, being essential for transcriptional activation of both genes. The oncogenic c-Myc transcription factor was identified to drive the expression of the two genes, suggesting that it plays an important role in regulating their coordinated transcription, and it is possibly involved in their differential expressions in central nervous system diseases such as brain cancer (48). Finally, the structure of the *PDCD10-SERPINI1* bidirectional gene pair is conserved in mammals, indicating an evolutionary requirement to maintain their cotranscriptional status and functional association (47).

Physiological and pathophysiological function of neuroserpin

Although all physiological functions of NS are not completely understood, it is recognized that NS participates in axonogenesis and synaptogenesis during development, while it contributes to synaptic plasticity (learning and memory) and control of emotional behavior in the adult (3). The main

evidence supporting NS involvement in these processes include: (i) its spatial and temporal expression in tissue concerned with neural plasticity (3, 30), together with the observation of an activity-regulated transcription, meeting a criterion generally associated to 'plasticity-related' proteins (44, 46, 49–51); (ii) impaired explorative behavior and neophobia in mice lacking or overexpressing the protein (52); (iii) cell culture studies showing that NS regulates processes such as neurite outgrowth (32, 36); (iv) and finally the fact that it is generally accepted that NS exerts its recognized physiological role mainly as an inhibitor of tPA; indeed, the involvement of tPA in synaptic plasticity is well proven (53), although a tPA-independent action has also been demonstrated (52, 54).

The indication that tPA is the target protease inhibited by NS comes primarily from spatiotemporal tissue distribution analysis, that demonstrates that expression of tPA and NS in the central nervous system (CNS) overlaps (55, 56), pointing to NS as the natural counterbalance to tPA, during both normal brain function and disease. *In vitro* enzyme assays revealed that NS inhibits tPA, and only to a lesser extent urokinase-type plasminogen activator and plasmin (1, 3, 38, 43), although the NS-target protease complex has not thus far been characterized *in vivo*.

The mechanism that regulates NS/tPA balance is now emerging via the characterization of the regulation of NS secretion. As detailed above, new findings indicate that NS is targeted to a regulated secretory pathway in neurons via a novel C-terminal sorting sequence (2). Ishigami et al. (2) suggest that the mobilization and secretion of NS from a reserve pool could be mediated by retrograde signaling from a synaptic site that is triggered in response to increased levels of tPA activity at the synapse. Alternatively, tPA and NS could be extensively copackaged in dense core granules (57). NS could thus act as inhibitor of the residual tPA activity intracellulary. In favor of this hypothesis, a recent study by Belorgey et al. (58) has shown that NS is stabilized at low pH and maintains its inhibitory activity, in accordance with the acidic environment (pH 5–6) of the storage vesicles.

NS and NS-tPA complex cell internalization and degradation is mediated by the low-density lipoprotein receptor-related protein (LRP) on the cell surface (59); in fact, LRP is the principal clearance receptor for serpins and serpin-proteinase complexes.

The role of NS has also been proven under CNS pathological conditions. tPA has different functions in the CNS (53); other than its physiological participation in synaptic plasticity, it is also involved in the regulation of permeability between the vascular and nervous system compartments. An unbalanced tPA activity could thus be the cause of pathological outcomes, such as cerebral ischemia, seizures and stroke. In these situations, NS has been shown to play a neuroprotective role, slowing the progression of injury throughout the CNS and preventing neuronal cell death (4–6). Moreover, NS is thought to participate in disparate pathological states such as motoneuron degeneration and brain cancer (48, 60, 61).

Point mutations of NS are responsible for FENIB, a severe neurodegenerative disease leading to neuronal death and dementia (Figure 5). FENIB is an autosomal dominantly inherited disease, characterized clinically as a spectrum of phenotypes, from dementia to epilepsy with variable electrical status (23). Histologically, FENIB is associated with unique neuronal inclusion bodies distributed throughout the cerebral hemispheres, but is significantly more abundant in the cortex and in the substantia nigra (7). At the biochemical level NS polymers are a hallmark of the disease (62). The NS inclusion bodies, known as Collins bodies, after Dr. George H. Collins, who initiated the first study on FENIB, are distinctly different from any other protein form previously linked to neurodegeneration (7). NS polymers are based on an entanglement of ordered polymeric chains that display a morphology identical to the polymers (intracellularly) formed by other serpins (41). For this reason, FENIB is included among the diseases imputable to serpin polymerization, collectively known as serpinopathies (63).

To date, five hNS mutations (each in a single family, except for the S52R mutation that has been found in two unrelated families) have been found associated with FENIB: S49P, S52R, H338R, G392E and G392R (64, 65) (Figure 4). Evidence has been accumulated that shows a direct correlation between severity of disease, amount of intraneuronal NS inclusions and predicted degree of conformational instability of the NS mutants, according to the following order of severity/protein instability: G392E/R>H338R>S52R>S49P (23). The main phenotypical and biochemical features of FENIB deduced, respectively, from its clinical manifestations and from the postmortem analysis of affected brains were confirmed through the expression of mutants in mice (52, 66), in *Drosophila melanogaster* (62) and in diverse cellular systems (62, 67). The animal models confirmed the clinical phenotype, whereas the cellular systems were essential to disclose the molecular details of intracellular protein accumulation. As seen from *ex vivo* studies, NS mutants accumulate as polymers within the endoplasmic reticulum (ER) (67), given that the quasi-folded nature of the component moieties fails to activate the unfolded protein response (UPR) (68). In contrast, the accumulation of polymers activates a UPR-independent ER-to-nucleus signal transduction pathway that has been called the ordered protein response, mediated by the activation of the transcription factor NF-κB. Because this pathway might regulate key processes induced during cellular stress, it could contribute to the cell death that is triggered by the accumulation of hNS polymers leading to dementia (69). Such studies point out that intracellular polymerization could be an important source of neurodegeneration, but do not exclude that the loss of hNS inhibitory activity due to mutations, with the consequent increase of active tPA concentration at the synapses, could aggravate the neuropsychiatric and epileptic aspects of FENIB (70–72). Mainly based on studies on α1-antitrypsin (α1-AT) polymerization, therapies to block polymerization and treat the serpinopathies are under investigation (73–75). Although some achievement has been reached, none of the proposed remedies has yet been proven to be effective in man.

Finally, NS has been found to interact with the Aβ peptide (8, 76) with remarkable effects. First the interaction with Aβ

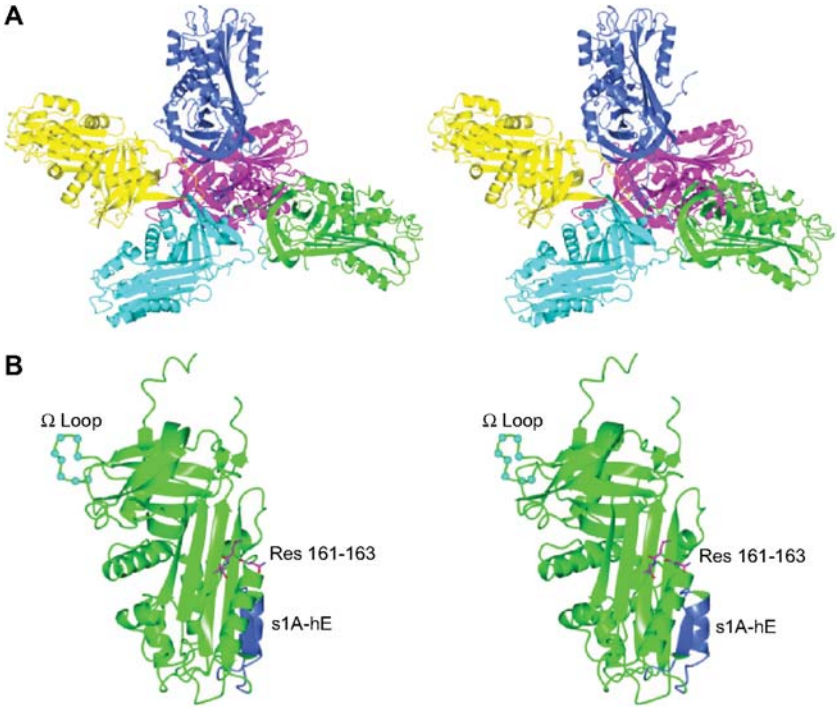


Figure 4 (A) Stereo sketch representation of the crystal packing in the asymmetric unit of full length hNS (pdb code 3F5N). It should be noted that the five RCLs are at the core of the chain-to-chain interaction region. (B) Stereo representation of hNS (pdb code 3FGQ): the omega loop is highlighted as cyan spheres (residues 231–237), residues 161–163 are shown as magenta sticks, and the region s1A-hE is in blue.

decreases the NS protease inhibitory activity, and second it promotes Aβ amyloid aggregation (8). The same study revealed that in cell lines and in a *Drosophila* model, NS exerts a protective role against the toxicity of Aβ peptide aggregates. Furthermore, it has been proven that NS is upregulated in brain of patients affected by Alzheimer's disease (77), and higher cerebrospinal fluid levels of NS in Alzheimer's patients were associated with the clinical diagnosis of the disease, facilitating its diagnostic classification (78). Notably, another serpin, α1-antichymotrypsin, is always found associated with Aβ plaques in Alzheimer's patients (79). The role of α1-antichymotrypsin, however, is controversial because it has been proposed to either inhibit (80) or accelerate amyloid aggregation (81). The latter studies, supporting an α1-antichymotrypsin role in fibrillogenesis acceleration, indicate a specific role for accessory proteins in the formation of amyloid deposits.

Inhibitory properties of neuroserpin in relation to general serpinology

Serpins are slow-binding inhibitors (82); the molecular mechanism they exploit to inhibit serine proteases has been established in fascinating detail. In the serpin active conformation, the RCL is a solvent exposed and flexible loop, bearing the P1 residue (Figures 1 and 3), a recognition site for the target protease, which usually binds the serpin with very high affinity. Thus, first the protease binds to the serpin forming a non-covalent Michaelis-like complex mainly through recognition of the RCL (83, 84). Then, the protease cleaves the RCL at the P1-P1′ bond by a conventional Ser-protease nucleophilic attack on the carbonyl C of the scissile peptide bond. Such cleavage triggers a profound conformational change, which is the beauty and the Achilles' heel of the serpin molecule. Upon cleavage the RCL moves from its exposed site to a location nestled within the A-sheet, as s4A (Figure 2). The A-sheet, thus, opens up to accommodate the additional β-strand. The region of A-sheet which moves to make room for the RCL is known as the shutter region and mainly consists of strands s3A, s5A and the neighboring secondary structures (s1A, s2A, s3A, hF) (17). Such dramatic conformational changes occur before hydrolysis of the acyl-enzyme complex, and therefore the protease is dragged by the RCL for approximately 70 Å, from one side to the opposite of the serpin structure (Figure 2). Crystal structures have elegantly shown that the protease active site is inactivated by stereochemical deformation, preventing hydrolysis of the complex, which is generally stable for weeks (85, 86). Two X-ray structures of serpin/protease complexes available to date show a distorted protease active site, but the protease moieties appear differently affected and significantly rotated relative to one another. Thus, the extent of the serpin-induced protease distortion, although substantial, remains unclear and could well vary in different serpin-protease complexes. The serpin conformational changes triggered by the protease cleavage are thermodynamically driven; the cleaved species where the RCL is inserted in the A-sheet is energetically

much more stable than the native (uncleaved) form, which in turn is considered a metastable conformation (19, 87, 88).

The P1-P1′ bond in hNS links residues R362–M363 (Figure 3). The P1 residue is the primary determinant of serpin specificity for the target protease; not surprisingly, the P1 specific residue for tPA is an arginine. Nevertheless, the kinetic behavior of hNS clearly differs from the virtually irreversible inhibitory processes paradigmatically related to other serpins. Despite a relatively efficient rate of inhibition, corresponding to a second order rate constant of the order of 10^5 $M^{-1}s^{-1}$, *in vitro* the hNS/tPA complex is short-lived (with a half-life of 10 min) (Figure 2), more reminiscent of a delayed intermediate of a substrate hydrolysis process (39, 43, 70). An immediate physiological consequence is that recognition of the NS-tPA complex by receptors must occur in a matter of minutes for cellular internalization to take place. No direct evidence of the hNS-tPA complex has been thus far reported *in vivo*, although cellular internalization, a process mediated by LRP receptor recognition, has been observed in cell cultures, both for active hNS and for the hNS-tPA complex (59). It should, however, be mentioned that an undefined complex of the size of hNS-tPA has been identified in murine brain extract (1). To date, no cofactors have been found to bind hNS or tPA in a physiological environment that could stabilize the complex (39).

From a structural point of view, transient inhibition of tPA by hNS, resulting in cleaved NS, implies that the deformation of the protease active site in the hNS-tPA complex is not sufficient to prevent the deacylation reaction (85, 86). Calugaru et al. have shown that the inactive protease conformation (in the serpin-protease complex) is in a dynamic equilibrium with active conformation that can complete the catalytic cycle and thereby slowly release a cleaved serpin and its protease partner (89). The equilibrium can be shifted by the preferential binding of protease ligands to one of the two conformations. To date, such a ligand has not been found for the NS-tPA pair.

Recent reports have shown that stable serpin/acyl-enzyme complexes require full insertion of the RCL in the A-sheet (42, 90), thus short-lived complexes might be generated by improper RCL length (91). As in NS the cleaved RCL, after release of the protease, adopts a conformation (as s4A) similar to other cleaved serpins (43) (Figure 1), the length of the RCL is unlikely to be responsible for the faster turnover of the NS-tPA complex. RCL full insertion could also be prevented by competing interactions between the serpin and the protease (92). The high affinity displayed by serpins for cognate proteases is modulated by exosites present on the interacting surfaces of both macromolecules (93). A well-known example is the inhibition of tPA by plasminogen activator inhibitor-1 (PAI-1). The tPA variable region-1 (VR-1 or 37-loop) is an exposed loop rich in positively charged residues, mapping near one edge of tPA active site, which is crucial for enhancing the rate of inhibition of tPA by PAI-1. The region of PAI-1 interacting with tPA VR-1 has been identified as the negatively charged residues in the RCL region C-terminal to the cleavage site (proximal hinge) (94, 95). Differently from PAI-1, NS does not exhibit charged residues at the proximal hinge; instead, a peculiar negatively charged patch is present on one side of the A-sheet, which, on the contrary, is absent in PAI-1. Because, upon cleavage, the bound protease is translocated toward the opposite pole of the serpin molecule, electrostatic interactions between the positive tPA VR-1 loop and the strongly negative NS surface might lead to a reduction in the rate of tPA translocation (43). This would allow trapping of the NS-tPA acyl-enzyme complex into a relative energy minimum before the RCL is fully inserted into the A-sheet. Such an intermediate step might affect tPA migration on the NS surface and the achievement of protease active site deformation, leading to hydrolysis of the complex and enzyme dissociation (92).

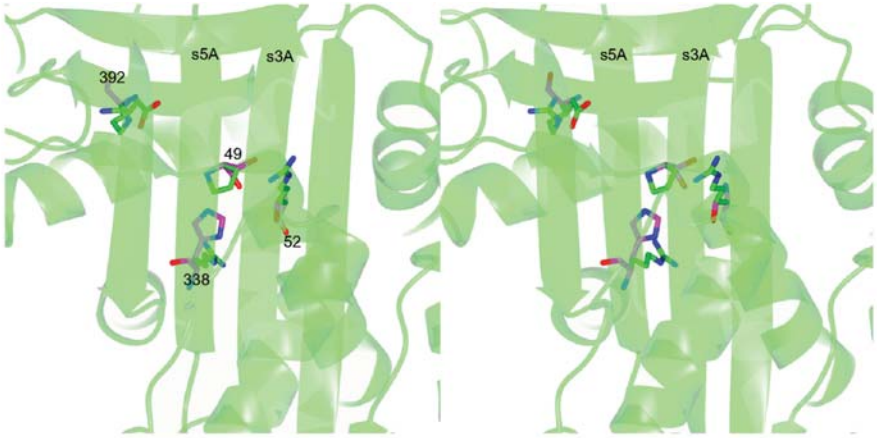
Neuroserpin crystal structures

To date, four NS crystal structures have been reported. The structure of the RCL-cleaved mouse NS (mNS) was first reported at 3.06-Å resolution (pdb code 1JJO) (96); more recently, the structure of cleaved hNS has been reported at 1.85-Å resolution (3F02) (43) (Figure 1B). Moreover, two native hNS structures have been published: a full length hNS crystal structure has been reported at a resolution of 3.15 Å (3F5N) (43) (Figure 1B) and a hNS construct bearing a 10-residue truncation at the C-*terminus* is available at 2.1 Å resolution (3FGQ) (97) (Figure 4B).

As expected, mNS and hNS share the common serpin fold, consisting of three β-sheets, nine α-helices and a 20-residues exposed RCL. Both cleaved structures clearly have their RCL completely inserted in the A-sheet as s4A, displaying a r.m.s.d. (root mean square deviation) of 1.1 Å over the whole molecule, after structure superposition. A long stretch of residues is not visible in the mNS crystal structure (residues 69–106, comprising α-helices hC and hD and the connecting loop), suggesting that either mNS has been further cleaved by trypsin (used to cleave the RCL) or that such a region is highly flexible (96). Conversely, in the crystal structure of cleaved hNS such a region is defined by electron density; however, the hD helix has high B-factors and it is isolated from the rest of the molecule by two small electron density gaps at the N- and C-*termini*. In the full length hNS native structure, the hD helix is partially or completely visible but with high B factors and solvent exposed (3F5N and 3F02) (43).

The two native hNS structures are very similar and superpose with a r.m.s.d. of 1.30 Å, calculated over 353 Cα pairs. In both cases, the RCL is completely exposed to solvent and it does not interact with the A-sheet, but the strands 3 and 5 of the A-sheet are tightly bound to each other. The crystal structure of full length hNS displays five molecules per asymmetric unit (43) (Figure 5A). An internal structural comparison between the five hNS copies shows that the serpin core is mainly rigid, whereas just a few regions of the molecule can adopt different conformations depending on the environment. Above all, the five RCLs show five different conformations, where part of the RCL is often organized in β-structure (43). The RCL conformational freedom allows

A



B

Mutation	Rate of polymerization	Age of onset of symptoms	Clinical manifestations	Histology of inclusions at postmortem
Ser49Pro	+	48	Dementia, tremor, seizures in terminal stages	
Ser52Arg	++	24	Myoclonus, status epilepticus, dementia	
His338Arg	+++	15	Myoclonic seizures, dementia, tremor, dysarthria	N/A
Gly392Glu	++++	13	Myoclonus, status epilepticus, dementia, chorea	
Gly392Arg	++++	8	Dementia, epilepticus of slow-wave sleep	N/A

Figure 5 (A) Stereo view of the hNS shutter region (pdb code 3F5N). The wild-type residues in positions 49, 52, 338 and 392 are shown in magenta sticks; green sticks show the mutated residues responsible for FENIB. (B) Table showing for each of the five known hNS mutants the tendency for polymerization, the corresponding severity of symptoms and the accumulation of Collins bodies in histological samples. Adapted, with permission, from ANNUAL REVIEWS Inc., Annu Rev Biochem (2009; 78: 147–76).

the five molecules to be organized in a star-like arrangement where the RCL establishes most of the intermolecular interactions in the pentameric assembly. By contrast, in the C-terminally truncated hNS structure two identical molecules are present in the asymmetric unit (r.m.s.d. of 0.1 Å/374 Cα pairs). In this case, a stretch of six residues of the RCL adopts a conformation characterized by a left-handed α-helix (97).

All the above observations on the two native hNS crystal structures underline once more the extreme flexibility of the serpin RCLs. However, additional structural elements show evident flexibility. The loop between s1B and s2B (residues

231–237), which protrudes from the molecule surface, is unique among serpins (97). Such a loop is only partially visible in the five molecules of the full length native hNS and shows wide conformational variability. In two cases, the electron density appears to extend the s1B strand, molecules B and C in the crystal asymmetric unit, thus establishing β interactions with the neighboring RCL of molecule D; whereas, in the other cases the loop appears totally flexible (43), as in the cleaved hNS structure (43). In the structure of truncated native hNS (and to a lesser extent in mNS) this region adopts an omega-loop conformation (Figure 5B), establishing interactions with two neighboring molecules. Notably, residues Gly231, Gly236 and Gly237 provide substantial conformational freedom that can result in high flexibility and structural adaptability to interacting partners (97). Indeed, omega loops are known to act as ligand binding site or to regulate enzymatic activity (98). Thus, the above observations seem to suggest a role for the 231–237 loop in protein-protein interaction; in particular, it might be involved in the interaction with tPA, as a deletion mutant lacking this region suggests (97).

A different structural region possibly involved in protein-protein interactions could be found in NS. Recently, two serpins (PAI-1 and tengpin) have been reported to host an allosteric site stabilizing the native versus the latent conformation (22, 99); such a site would comprise the hE and hF helices and strand s1A (residues 122–157) (Figure 5B). In serpins, the hF C-terminal end bears a conserved consensus sequence (GKI), which adopts a type I′ β turn conformation (Figure 5B). hNS is an exception because this sequence is not conserved, being substituted by NLV. The hNS triple mutant restoring the GKI sequence (residues 161–163) shows higher thermal stability (97). In the full length hNS native crystal structure, such a region is involved in protein-protein interactions that stabilize the crystal packing contacts (43). Moreover, three residues in the crevice between hE and s1A, shown to tune tengpin conversion from native to the latent form, are conserved in hNS, suggesting the same controlling role for such a region in hNS (43, 99). All the above observations seem to suggest the presence of a site in the area hF-s1A-hE, which can have a regulatory role in native hNS (43, 97).

In summary, the hNS crystal structures show marked conformational variability in their surface regions, together with a trend towards intermolecular interactions in three specific regions: (i) the RCL; (ii) the loop between s1B and s2B, which is unique for NS and could still play an undisclosed role in the NS protein-protein network *in vivo*; and (iii) a patch on a side of the A-sheet (secondary structure elements: hF, s1A, hE) that, in analogy with other serpins, could tune the stability of native NS (Figure 5B).

Neuroserpin mutants and polymerization

In all serpin polymer models the RCL is responsible for bridging neighboring protein molecules to form long strings. The s7A-insertion model has been observed in PAI-1 and MENT, where the RCL of one molecule interacts with the A-sheet of an adjacent molecule as the seventh β-strand (26–28). The intermolecular interactions provided by s7A-linkage are limited and considered transient and reversible (26–28). The formation of C-sheet based polymers has also been reported. As already mentioned, the s1C β-strand unfolds upon latent formation; in such a case, the RCL interacts *in trans* substituting s1C and bridging neighboring molecules (25, 100). In particular, both latent PAI-1 and the latent conformation of the NS mutant (S49P) are thought to polymerize forming C-sheet polymers (101, 102). Cleaved serpin molecules can also be introduced in this type of polymer, as previously reported (24).

However, most of the pathological polymers *in vivo* are thought to be formed via the intermolecular insertion of the RCL into A-sheet as s4A (Figure 6); therefore, this is the type of polymer considered the most medically relevant. Two somehow contrasting models of serpin polymers are currently available, but their correctness is still a matter of debate. The first model, proposed by Lomas et al., suggests that the RCL is the only structural element inserted (swapped) into the A-sheet of an adjacent molecule during polymerization, in analogy with what happens during the formation of the latent form (where the insertion is, however, intramolecular) (103). The iterative insertion of RCLs would create a long string of molecules (beads-on-a-string). More recently, Yamasaki et al. have proposed a modified model (104). Starting from the crystal structure of a domain swapped antithrombin dimer, they built a model in which not only the RCL but also the neighboring β-strand s5A is intermolecularly swapped and inserted in the A-sheet of a neighboring molecule. The two models differ not only for the number of swapped strands. According to the one-strand insertion model, the protein needs to be completely folded before being able to polymerize; by contrast, for the swap of two strands the polymerization process could start from a serpin folding intermediate (104, 105) (Figure 6).

In analogy with what has been observed for other serpins, the pathological mutations (promoting *in vivo* polymerization) found to date for hNS lie in the shutter region: they are S49P, S52R, H338R, G392E and G392R. Models of hNS mutants built on the wild-type protein structure provide explanations for their instability (Figure 4A). The S49P mutation lies in the hinge between strand s6B and the B-helix: as a consequence of this mutation two consecutive prolines are placed at this hinge region, probably resulting in a conformational strain, which affects the shutter region as previously suggested (43). Except for the S49P mutant, in all other cases the mutation substitutes a buried residue with a bigger and charged one. The modeled side chains of Arg52, Arg338, Arg392 and Glu392 all point to strands 3A and 5A, probably affecting native hNS stability (Figure 4A). All these mutations lead to accumulation of polymers *in vivo* and give rise to the typical FENIB symptoms, with different severity levels and onset age for the disease. As already mentioned, the correlation between the expected mutant protein instability, the amount of Collins bodies and the severity of symp-

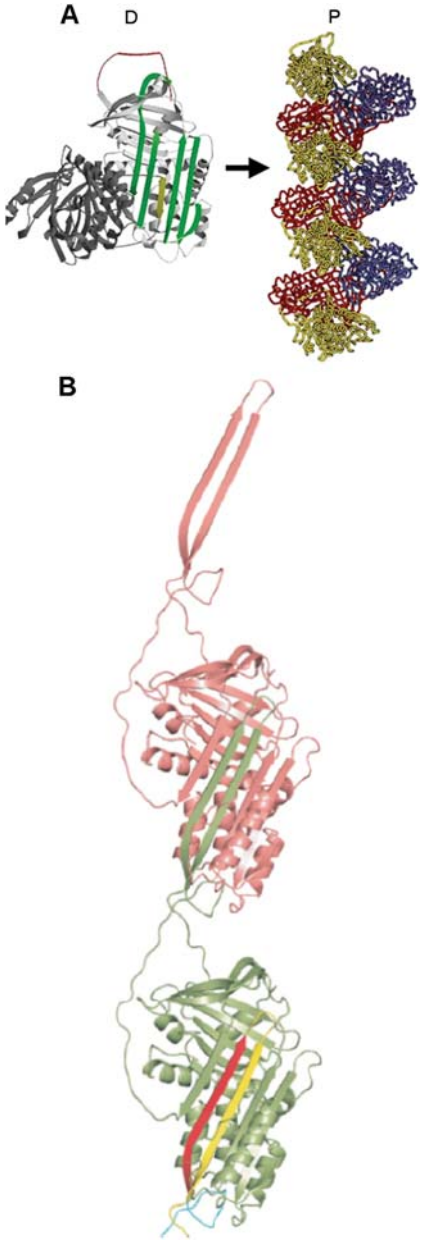


Figure 6 The two models proposed for the assembly of pathological A-sheet serpin polymers. (A) The model proposed by Lomas et al. (103) is based on the swapping of the RCL region alone (D indicates dimer, P denotes polymer). Adapted, with permission, from Birkhäuser Basel, Cell Mol Life Sci (2006; 63: 709–22), © (2006). (B) The model proposed by Yamasaki et al. (104) is based on the swapping of a more extended serpin region, comprising the RCL and the neighboring strand s5A. Adapted, with permission, from Macmillan Publishers Ltd., Nature (2008; 455: 1255–8), © (2008).

toms is striking (Figure 4B). The tendency of polymerization is reported to be S49P>S52R>H338R>G392E>G392R (64, 65). The FENIB onset age is reported to be approximately 48, 24, 15, 13 and 8 years, for the different mutants, respectively. The severity of the symptoms follows a similar correlation pattern (Figure 4B).

Although no crystal structures of the known hNS mutants are available, the S49P and S52R mutants have been biophysically characterized *in vitro*, and structural models have been discussed (43, 70, 71, 101). The S49P mutant is shown to be a much poorer tPA inhibitor, having a lower inhibition constant and a higher complex dissociation rate (70). Moreover, the native S49P mutant is much more readily converted to both latent and polymeric forms compared to wild-type hNS. Intriguingly, in contrast with latent wild-type hNS, latent S49P can polymerize, probably through a C-sheet polymerization mechanism, in a similar way to what was previously observed for the Mmalton mutant of α1-AT (24, 101). S49P hNS was extracted from Collins bodies in latent form, short and long polymers. Thus, not only is S49P a less efficient tPA inhibitor but it is also less stable than wild-type hNS and readily tends to form polymers and inactive monomers (latent) (70, 101).

The S52R mutation leads to a more severe version of FENIB, where this mutant polymerizes more readily than the wild-type hNS and the S49P variant (64, 71). Unexpectedly, the thermal and chemical stabilities of the S52R mutant are higher than those of the S49P and are very similar to those of wild-type hNS. Moreover, tPA cleaves with very low efficiency the S52R mutant, which in turn cannot be considered neither a tPA inhibitor nor a substrate (71). All the evidence seems to suggest that the S52R mutation leads to an aberrant conformation where the RCL is not available for tPA cleavage, but at the same time it is not in the latent conformation, given the strong tendency to polymerize. Such conformation is reminiscent of what has been observed for the δ conformer of α1-antichymotrypsin, where the RCL is only half inserted in the A-sheet together with the unfolded hF-helix (71, 106).

The other three hNS mutants have been studied only *in vivo*. Both the severity of FENIB symptoms and the amount of Collins bodies found in the brain suggest that the mutations H338R and G392E/R lead to even more abnormal hNS variants, more prone to form polymers and with no ability to act as tPA inhibitors (64, 65). Recently, the behavior of wild-type hNS and the pathological mutants have been compared in relation to their ability to be secreted and/or to accumulate in the ER (62). Whereas in cellular models wild-type hNS is eventually completely secreted as a monomeric species, S49P and S52R are secreted as monomers but are also accumulated intracellularly and extracellularly as polymers. Conversely, monomeric H338R and G392E are not observed at all, and only polymeric hNS is present intra/extracellularly, in particular the fraction of intracellularly accumulated polymer is fully increased (62).

Some evidence has been gathered suggesting that the hNS polymers are formed according to the one-strand insertion model (Figure 6). First of all, in cell models the polymers appear with a 30-min delay from the moment in which hNS expression has been triggered. Although this observation does not rule out the possibility that the polymer can be formed from non-completely folded molecules, it shows that it is a post-translational process (107). Moreover, kinetics of hNS polymerization monitored by single molecule fluorescence seem to be better described by a kinetic scheme compatible with an activated molecule characterized by the

opening of the A-sheet and partial loop insertion, therefore according to the one-strand insertion model (76).

Conclusions and perspectives

The availability of suitable cellular and molecular investigation tools is prompting the study of (severe) diseases that are rooted in perturbations of protein stability, protein folds and protein assemblies. The case of serpinopathies, reminiscent of amyloid related disorders but based on entirely different mechanistic models, provides an open example of such a trend. NS shares most of the serpin family structural and functional properties, including the inhibition mechanism against a serine protease (tPA) and the manifestation of different molecular monomeric/polymeric forms. The ensemble of genetic, cellular, molecular, biophysical and structural studies presented here depicts the complexity of the processes that must be understood and controlled to provide clues for human therapies against the growing category of protein misfolding/aggregation diseases.

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