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SHORT COMMUNICATION

On the molecular structure of human neuroserpin polymers

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ABSTRACT

The polymerization of serpins is at the root of a large class of diseases; the molecular structure of serpin polymers has been recently debated. In this work, we study the polymerization kinetics of human neuroserpin by Fourier Transform Infra Red spectroscopy and by time-lapse Size Exclusion Chromatography. First, we show that two distinct neuroserpin polymers, formed at 45 and 85°C, display the same isosbestic points in the Amide I′ band, and therefore share common secondary structure features. We also find a concentration independent polymerization rate at 45°C suggesting that the polymerization rate-limiting step is the formation of an activated monomeric species. The polymer structures are consistent with a model that predicts the bare insertion of portions of the reactive center loop into the A β-sheet of neighboring serpin molecule, although with different extents at 45 and 85°C.

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Key words: protein aggregation; serpins; serpinopathies; serpin polymerization; FTIR.

INTRODUCTION

Human neuroserpin is an inhibitor of tissue-type plasminogen activator, involved in regulating synaptic plasticity,[1–3] and with a role in Alzheimer disease.[4,5] It belongs to the serpin superfamily, a large class of proteins with different functions, and is involved in many physiological processes.[6] As the acronym says, most serpins are serine protease inhibitors. Point mutations in a serpin may cause misfolding or polymerization, resulting in a lack of inhibitory activity or tissue damage due to protein deposition.[7] This is the origin of a class of conformational diseases known as serpinopathies. Among these, most common is the deficiency of α_1-antitrypsin;[6,7] in the case of human neuroserpin, the associated disease is known as familial encephalopathy with neuroserpin inclusion bodies.[1,8–10]

Serpinopathies are rare diseases, but share a common molecular basis, because serpins share high structural homology. The main structural features of serpins are a 5-stranded β-sheet (sheet A) and a solvent exposed reactive center loop (RCL) that acts as bait for the target protease, which binds to the loop and cleaves it. The cleavage triggers the insertion of the loop into the A β-sheet causing the disruptive translocation of the protease.[11] This efficient and fast responsive mechanism is due to the thermodynamic stabilization of the serpin–protease complex occurring during the conformational change, which involves the loop insertion.[12] An analogous stabilization is achieved by the insertion of the RCL into the A β-sheet of another serpin, thus forming dimers, trimers, and eventually polymeric aggregates. We refer to this scheme, proposed by Lomas and

Abbreviations: FTIR, Fourier transform infrared; RCL, reactive center loop.
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collaborators, as loop/β-sheet model, since the main conformational changes occur in the RCL and in the A β-sheet.[7] Recently, the issue of the serpin polymerization has been re-opened by Huntington and coworkers, who put forward a new model for serpin polymerization, based on the crystal structure of an antithrombin dimer. This model implies the domain swapping of both the RCL and the neighboring strand of the A β-sheet.[13]

In a recent study, we have shown that human neuroserpin forms two different types of polymeric structures upon incubation at 45 or 85°C.[14] These two polymers exhibit a different thermal and chemical stability, and a distinct structure at both the molecular and supramolecular length scale, as evident also from EM images.[14] In this study, by Fourier transform infrared spectroscopy (FTIR) kinetic experiments, we reveal the molecular details of neuroserpin polymers formed at 85 and 45°C. Our results show that both polymers share the same molecular features, consisting mainly in an increase of multi-strand β-sheet structure; however, the amount of intermolecular β-sheet structures is larger at 85°C than at 45°C. These simultaneous analogy and dissimilarity suggest a close molecular structure for the two types of polymers. We argue that the classical loop/β-sheet model holds in both cases with an amount of loop insertion, which is larger at 85°C than at 45°C.

MATERIALS AND METHODS

Recombinant monomeric neuroserpin, obtained as previously reported,[15] was diluted with deuterated buffer (10 m*M* Tris, 50 mM KCl, pH* 7.4, uncorrected for isotopic effect), and then concentrated by ultra-filtration with a cut-off of 30 kDa (Amicon-30, Millipore). A 10-μL sample was placed between two CaF_2 windows separated by a 46-μm Teflon spacer and put in a Jasco J-400 spectrometer (Jasco, Tokyo, Japan), equipped with a temperature-control system and purged with a continuous flow of dry nitrogen gas. FTIR spectra were recorded upon incubation at 45 and 85°C of samples with a final concentration of 1.1 and 1.3 m*M*, respectively. The temperature equilibration time of the apparatus, measured in separate experiments, was lower than 3 min. For comparison, a sample of monomeric neuroserpin was also measured at 25°C. Each spectrum is the average of 16 interferograms with a spectral resolution of 2 cm^{-1}. FTIR spectra of the buffer solution taken under identical conditions were used as a reference to calculate the absorption spectrum of each sample.

Samples of 5 μ*M* neuroserpin solution were incubated at 45°C and transferred via a 50-μL sample loop to a Phenomenex BioSep (S 3000) column (Amersham) connected to a Shimadzu HPLC system (Kyoto, Japan). The samples were eluted at a flow-rate of 0.8 ml min^{-1}, and absorption was measured at 280 nm.

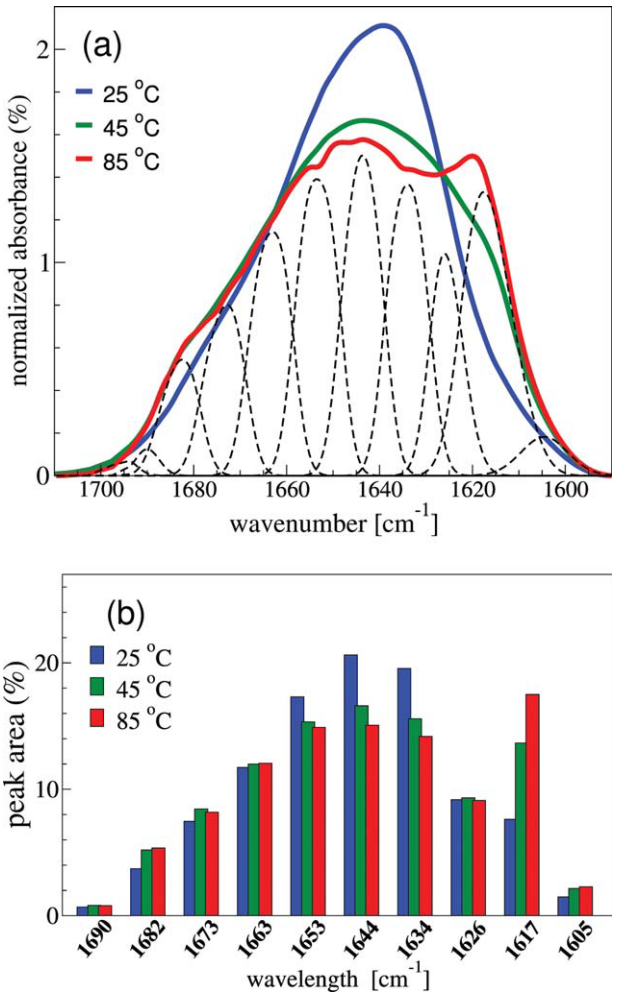

Figure 1

(a) Amide I' FTIR spectra: normalized absorbance of native monomeric neuroserpin at 25°C (blue curve) and neuroserpin polymers upon incubation at 45°C (green curve) and 85°C (red curve) for 24 and 3 h, respectively. Dashed lines outline the different band components used to fit spectra at 85°C. **(b)** Percent amount of each component of the spectra in the upper panel (with matching colors).

RESULTS AND DISCUSSION

Figure 1a reports the Amide I′ bands measured for native monomeric neuroserpin at 25°C and for neuroserpin polymers at the end of polymerization kinetics at 45 and 85°C (the prime in I′ denotes a deuterated buffer solution). The main feature observed in the polymerized samples is an absorbance increase at frequencies below ~1620 cm^{-1} and above ~1670 cm^{-1} (characteristic of multi-strand β-sheets), with a parallel decrease in the 1625/1665 cm^{-1} region. The presence of two isosbestic points at ~1626 and 1668 cm^{-1} is also clearly observed. Note also that the results shown in Figure 1a refer to completely polymerized samples since, in view of the long incubation times at high temperatures of highly

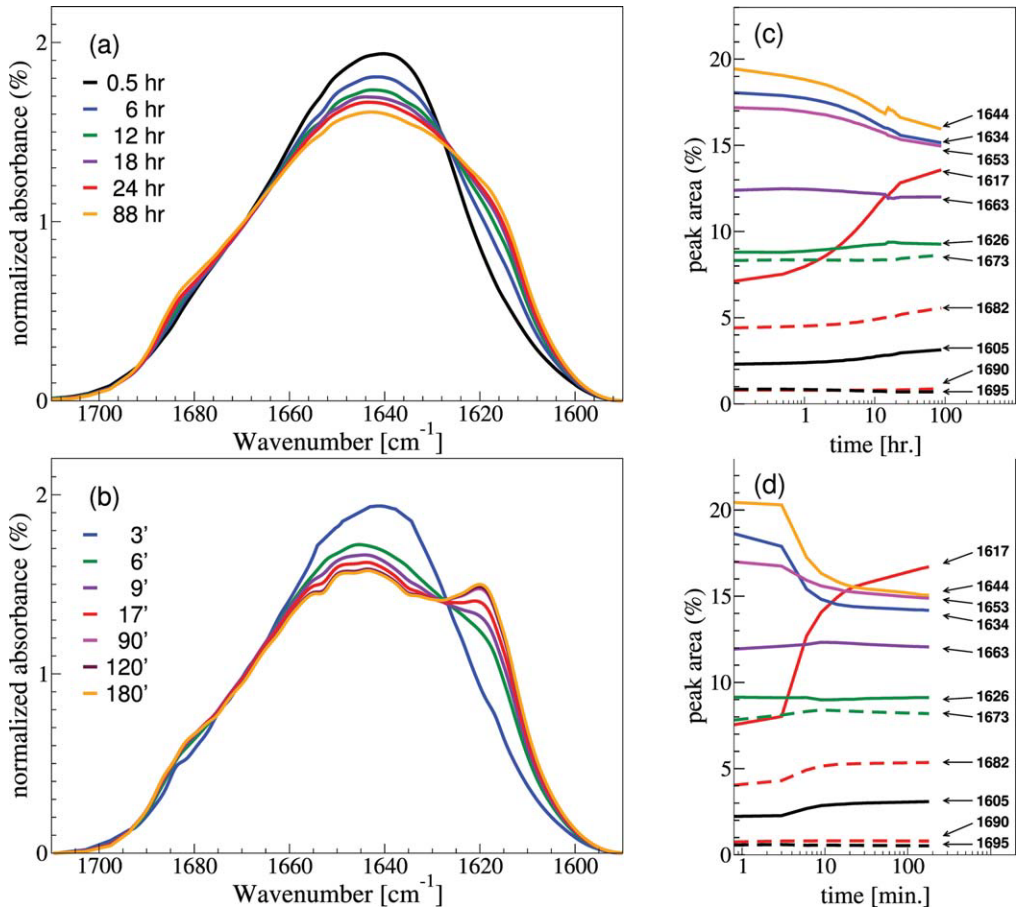

Figure 2

(Left): Amide I' FTIR spectra during neuroserpin polymerization at 45°C (panel **a**) and 85°C (panel **b**). (Right): Kinetics of the spectral components of the Amide I' band at 45°C (panel **c**) and 85°C (panel **d**). The half width of at half maximum (HWHM) of each component was 5 cm^{-1}, except at 1626 and 1617 cm^{-1} where it was 4 and 6 cm^{-1}, respectively.

concentrated samples (>1 m*M*), the monomer fraction is completely consumed.

We deconvoluted the highly structured Amide I′ band of human neuroserpin in terms of single components. We assigned each component to a specific backbone conformation, neglecting any possible contribution from residue side chains,[16] following an approach well established in the literature to interpret the Amide I′ band of proteins.[16–23] In the deconvolutions, the peak positions and widths of the bands were kept fixed, and only the intensities were varied. The bands at 1644, 1653, and 1663 cm^{-1} are assigned to disordered structures (or loops), α-helices, and turns, respectively,[16] while the band at 1605 cm^{-1} can be ascribed to side chain contributions. The bands below 1640 cm^{-1} can be assigned to anti-parallel or parallel β-sheets.[17] Note that the band associated with β-sheet, typically centered at 1632 cm^{-1}, is shifted toward lower wavenumbers when the numbers of strands in the β-sheet increases,[19] or when the amount of inter-strand interactions increases.[20] For example, the appearance of band components below 1620 cm^{-1} has been observed in the formation of unfolding intermediates,[21] or in the formation of amyloid fibrils, which are characterized by the stacking of several β strands.[20,22,23] Anti-parallel β-sheets also cause the appearance of bands above 1670 cm^{-1}, because of the exciton splitting of the energy levels associated with the CO in the β-sheet conformation.[18]

The percent amount of the different components at the end of the kinetics is displayed in Figure 1b and shows that the main difference among the three spectra in Figure 1a is observed in the amount of the 1617 cm^{-1} band.

The time evolution of the molecular secondary structure changes during thermal incubation were monitored by the kinetics of the Amide I′ band [Fig. 2(a,b)]. The kinetics of each component [Fig. 2(c,d)] confirm that the main structural changes are related to an increase of the band at 1617 cm^{-1}, and the corresponding bands above 1680 cm^{-1}, assigned to the formation of

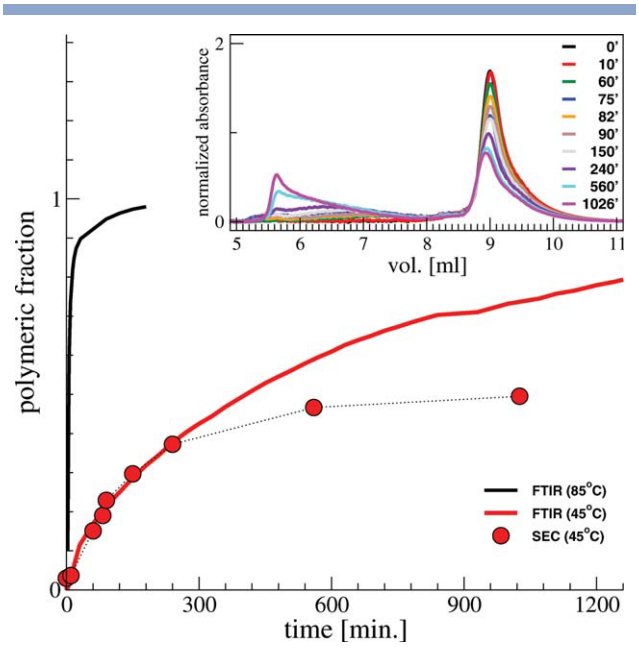

Figure 3

Kinetics of polymerization: fraction of β-sheet "polymeric" species measured by FTIR at 85°C (black curve) and 45°C (red curve); fraction of nonmonomeric, "polymeric" species measured by SEC at 45°C (red circles). Inset: SEC chromatograms upon incubation at 45°C for different time, as shown in the legend. Absorbance at 280 nm is normalized with respect to the total area. The polymer and monomer peaks are taken within a range of elution volume of 5–8.6 and 8.6–12 mL, respectively. The FTIR polymer fractions are measured by the area of Gaussian bands centered below 1626 and above 1668 cm^{-1} (as in Fig. 1) and are rescaled to assign 0 and 1 value to the first and the last data point, respectively. The SEC polymer fractions are measured by the area of the polymer peaks in SEC chromatograms.

multiple-stranded structures, and a decrease of the band related to native β, α, and loop structures. This result can be ascribed to the insertion of the RCL as a strand of the A β-sheet, during the polymerization process.

Most interestingly, both kinetics exhibit two isosbestic points at about 1626 and 1668 cm^{-1} [Fig. 2(a,b)]. The presence of isosbestic points implies the existence of two secondary structure populations, a native and an aggregated one. The kinetics of the polymeric fraction can be highlighted by the amount of the bands related to the multi-strand β-sheet structures, namely the bands below 1626 and above 1668 cm^{-1} (Fig. 3). One may estimate the rate of polymerization (k_P) by considering the time when the polymeric fraction has reached half of the total ($t_{1/2}$, with $k_P = 1/t_{1/2}$). While at 85°C, aggregation is extremely rapid ($t_{1/2} < 10$ min and $k_P > 2 \times 10^{-3}$ s^{-1}), at 45°C one obtains $t_{1/2} = 420$ min and $k_P = 4 \times 10^{-5}$ s^{-1}. Such value is very close to the rate of activation (k_A) measured under similar conditions but at much lower concentration by Chiou *et al.*[5] ($k_A = 7 \times 10^{-5}$ s^{-1}). In that study, the activation process was found to represent the initial conformational change required in a neuroserpin monomer to initiate polymerization. Due to the close values between the rates of activation (Chiou et al.)[5] and polymerization (present study), one may argue that at the high concentration used in our experiments, polymerization is rate-limited by activation. Indeed, this is quite reasonable if one considers that our experiments are run above 1 mM neuroserpin concentration and that the overlapping concentration for neuroserpin is 11 mM. To confirm this suggestion, We performed time-lapse SEC experiments with samples of 5 μ*M* neuroserpin solution incubated at 45°C. The monomer and polymer fractions were derived from the area of the related peaks in the chromatograms at different times (inset of Fig. 3). At such low concentration, monomer consumption is not reached due to the concurrent formation of latent monomeric conformers, which are not able to participate to the polymerization.[4] Nevertheless, the kinetics of polymer fraction has the same initial rate of that obtained by FTIR experiments (Fig. 3), notwithstanding a gap of two orders of magnitude in the initial concentrations. This certainly confirms that the rate-liming step of polymerization in such a high concentration range is a first-order process, such as the activation of an intermediate conformer.

One of the most interesting observation in the present experiments is the coincidence of the isosbestic points at 45 and 85°C [Fig. 2(a,b)]. This implies that We are observing an intensity increase of the band at 1617 cm^{-1} in the polymer formed at 85°C with respect to the polymer formed at 45°C, rather than a frequency shift, meaning that the same "native" and "aggregated" states can be considered at the two temperatures. On the other hand, we can exclude that the lower intensity of the 1617 cm^{-1} band observed at 45°C is due to the presence of monomers in the kinetics at 45°C since we have already ruled out this chance in our earlier study.[14,24] Therefore, the "aggregate" state should not be referred to the amount of polymerized proteins but to the amount of protein elements or residues, which acquire a conformation typical of the polymer state. In such a case, the occurrence of the same spectroscopic state, in different amounts at the two temperatures, points out the existence of two different polymeric species composed of similar secondary structure elements.

The FTIR measurements are able to highlight the existence of similarities/differences at the level of secondary structure, but not to elicit the details of polymeric structures. The classical loop/β-sheet model allows to rationalize these findings by suggesting a different amount of loop insertion in the two cases, consistent with the suggestion by Onda *et al.*[9] Indeed, an increased number of amino acid residues participating in the β strand explains the observed absorbance increase of the 1617 cm^{-1} band. Figure 4 depicts this proposal by showing two possible neuroserpin dimers, associated by inserting different portions of the RCL as a new strand into the A β-sheet. The

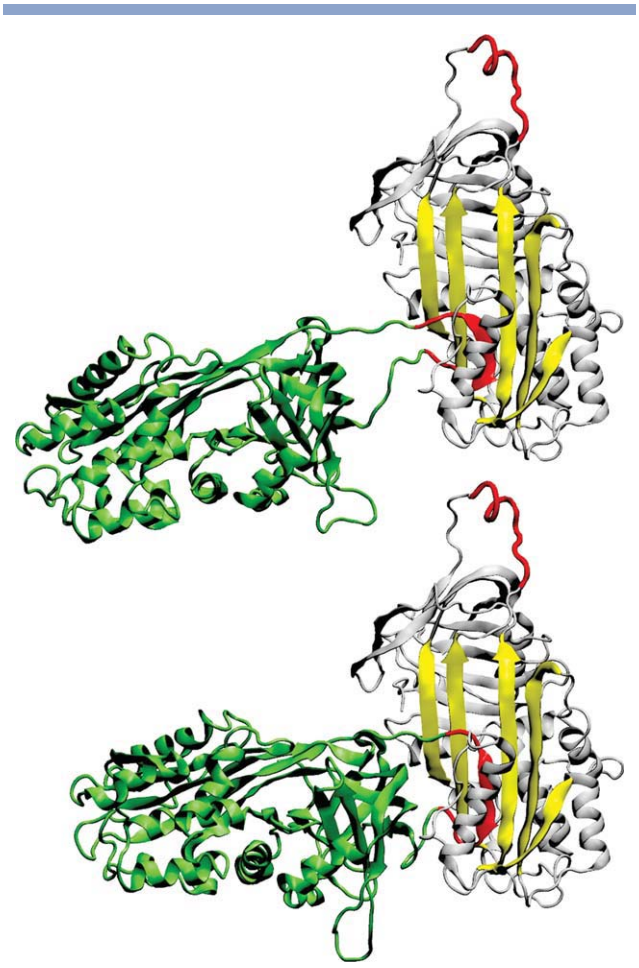

Figure 4

Loop/β-sheet serpin dimerization cartoon model for neuroserpin (RCL and A β-sheet are in red and yellow, respectively). The inserted RCL forms a β-strand with a length of four residues (upper picture) or six residues (lower picture).

insertion of the RCL into A b-sheet is based on the molecular structure of cleaved neuroserpin, solved in ref 15. Moreover, We note a similarity among the spectra measured at 45°C at the end of the kinetics and the first spectrum measured at 85°C (Fig. 2). This observation strengthens the global similarity between the two structures and therefore the proposed model (Fig. 4); also it calls into question, whether the polymeric structure typical of 45°C incubation may be on pathway for the polymeric structure eventually formed at 85°C.

CONCLUSIONS

In summary, we have studied the formation of neuroserpin polymers at 45 and 85°C by FTIR spectroscopy and time-lapse SEC experiments. We have shown that, at the high concentrations studied, the polymerization is rate-limited by the activation of an intermediate monomeric conformer. We have also shown that, although different polymeric structures are formed at 45 and 85°C,[14] the kinetics exhibit the same isosbestic points, thus evidencing an analogous change in the secondary structure features, related to multiple-stranded β-sheets. We believe that these observations strongly support to the classical loop/β-sheet model, with a variable level of loop insertion, consistent with more recent findings.[5,24,25]

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