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Neutron Scattering Reveals Enhanced Protein Dynamics in Concanavalin A Amyloid Fibrils

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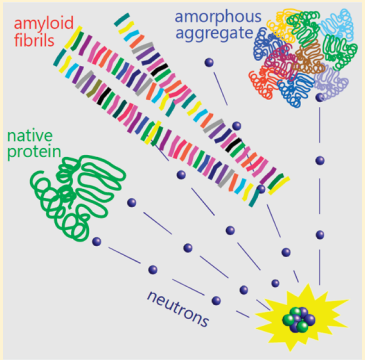
Supporting Information

ABSTRACT: Protein aggregation is one of the most challenging topics in life sciences, and it is implicated in several human pathologies. The nature and the role of toxic species is highly debated, with amyloid fibrils being among the most relevant species for their peculiar structural and functional properties. Protein dynamics and in particular the ability to fluctuate through a large number of conformational substates are closely related to protein function. This Letter focuses on amyloid fibril dynamics, and, to our knowledge, it is the first neutron scattering study on a protein (Concanavalin A) isolated in its fibril state. Our results reveal enhanced atomic fluctuations in amyloid fibrils and indicate that the protein is "softer" in the fibril state with respect to the native and amorphous aggregate states. We discuss this finding in terms of a structural interpretation and suggest that the paradigm ordered structure ↔ lower flexibility can be questioned when considering the local fast side-chain protein dynamics.



SECTION: Biophysical Chemistry and Biomolecules

In suitable physicochemical conditions, proteins can modify their native conformation and associate to form aggregates with different morphologies, such as oligomers, amorphous aggregates, and amyloid fibrils. This phenomenon, one of the most challenging in life sciences, is associated with widely diffused pathologies.[1] Although there is debate on the role of aggregate morphology in toxicity,[1] there is general consensus on the relevance of amyloid fibrils: ordered elongated aggregates with a highly organized H-bond pattern, representing the most stable state that proteins can adopt.[2] Experiments suggest that even non-homologous proteins share analogous fibril structural features, i.e., cross-β structures in which β-strands run perpendicularly to the long axis of the fibril.[3] While many biological and structural pieces of information are now available about amyloids, and in spite of the fundamental paradigm of structure-dynamics-function relation in proteins, much less is known about their dynamics. Here we present the first neutron scattering (NS) study on the nanosecond dynamics of a protein isolated in its amyloid fibril state. In particular, the dynamical behavior of concanavalin A (ConA) in the amyloid fibril state (F-ConA) was compared with that of native (N-ConA) and amorphous aggregate (A-ConA) states. Quite unexpectedly, proteins in the fibril state are more flexible and characterized by larger anharmonic nanosecond side-chains fluctuations, thus suggesting that in this dynamical range the relation ordered structure ↔ lower flexibility can be challenged.

Proteins are dynamic systems. Their flexibility and capability to perform anharmonic motions (e.g., thermal fluctuations among conformational substates) are essential to understand how proteins accomplish their function. A typical example is ligand binding to myoglobin: in the X-ray structure, no static path for the entry of ligands exists, so that only structural fluctuations can allow access to the active site.[4] This dynamical performance is due to fluctuations of side chains occurring on the nanosecond time scale. NS experiments on D_2O-hydrated protein powders allow observation of the nanosecond single particle dynamics of protein nonexchangeable H atoms.[5] Since H atoms are uniformly distributed in protein structure, NS gives information on the average protein dynamics, which is harmonic at low temperature, while two onsets of anharmonicity are revealed at 100−150 K and at ∼220 K: the first one is due to methyl group rotations, while the second, known as dynamical transition and whose physical origin is still a matter of discussion, is due to solvent-coupled large-scale fluctuations of protein backbone and amino acids side chains.[6−8]

Although the few NS studies on aggregation in the literature have suggested an alteration of dynamics due to the structural changes induced by aggregation,[9−11] there are no NS studies on the internal dynamics of a protein isolated in its amyloid state. Here we investigated by NS the nanosecond dynamics of aggregated states of ConA, a globular protein of the Lectin family that readily forms fibrils close to physiological

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conditions.[12] The aggregation process of ConA may evolve through two distinct pathways leading, respectively, to the formation of amyloids or amorphous aggregates. In particular, ConA is able to form long and thin fibrils via a non-nucleated mechanism involving the formation of intermolecular β-sheets with a fast rate and high repeatability of the aggregates morphology. Amorphous aggregates are formed as a consequence of ConA partial unfolding and are mainly stabilized by nonspecific hydrophobic interactions.[13,14] We selected ConA as a model system for its biological and structural peculiarities: (a) it induces apoptosis on tumoral cells with a mechanism related to its aggregation;[12,15] (b) it shows a large structural homology to the human serum amyloid protein, generally present in all the in vivo fibril deposits;[16] (c) its all-β secondary structure allows minimizing any dynamical effect related to changes in secondary structure, thus isolating the role of the supramolecular amyloid structure.

Elastic and quasi-elastic NS data were measured with the high-resolution IN16 neutron spectrometer at ILL (France). Aggregates were prepared according to a previously developed protocol[13] modified to obtain lyophilized powders with H→D isotopic substitution of the exchangeable H atoms. Samples were hydrated with D_2O at an hydration level (0.2 g D_2O/g ConA) low enough to get a scattering signal attributable to nonexchangeable H atoms with a negligible D_2O signal,[17] but high enough to allow the activation of anharmonic large-scale fluctuations. Details on sample preparations, experiments, and data analysis are given in the Supporting Information (SI).

Although the capability of ConA to form amyloid and amorphous aggregates in the selected conditions has been already demonstrated,[12−14] we think that it is of fundamental importance to check the aggregation state of the very same samples used for the present study. In Figure 1a we show diffuse reflectance Fourier transform infrared spectroscopy (FTIR) measurements on the hydrated powder samples. In Figure 1b,c we report FTIR absorption spectra and circular dichroism (CD) spectra on samples redissolved in D_2O after NS experiments. F-ConA FTIR spectra present an intense absorption peak between 1610 and 1620 cm^{-1}, typical of

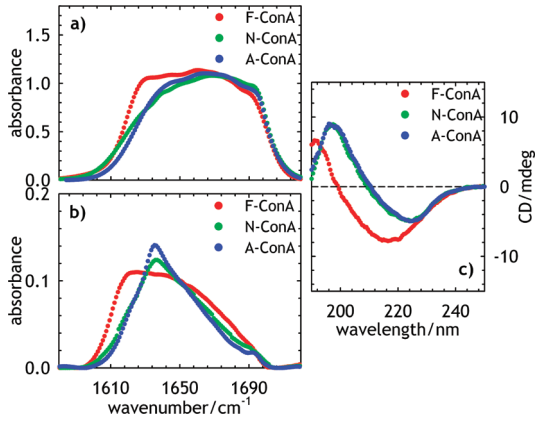

Figure 1. (a) Spectra on the amide I′ band region measured by diffuse reflectance FTIR spectroscopy on the hydrated powder samples used for neutron experiments. (b) Spectra measured by standard FTIR spectroscopy on samples redissolved in D_2O after neutron experiments. The differences in the overall band profiles obtained with the two different techniques are due to the line shape distorsions tipically occurring in the reflectance spectra on powders. (c) Far-UV CD spectra on samples redissolved in D_2O after neutron experiments.

aggregate β-sheets with very strong intermolecular hydrogen bonds and considered a fingerprint of amyloid structures,[12] while CD measurements reveal the usual signal of cross-β structures in amyloid fibrils.[18] Note that neither the FTIR nor the CD spectra show any sign of the presence of disordered random coil structures in the F-ConA sample. As expected, N-ConA and A-ConA have similar spectral features, confirming that amorphous aggregation is not accompanied by changes at the secondary structure level.[13]

In Figure 2 we report the characterization of the aggregate samples by means of two different imaging techniques, which

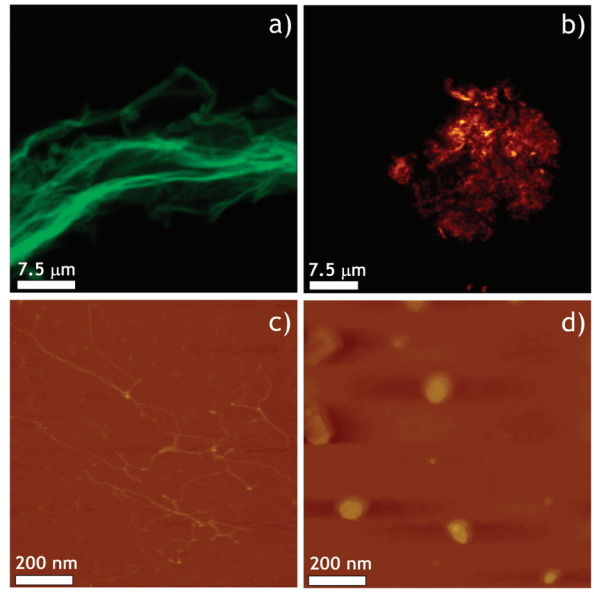

Figure 2. (a) Two-photon fluorescence microscopy images (λ_{exc} = 885 nm) of F-ConA stained with ThT; (b) confocal fluorescence microscopy images (λ_{exc} = 496 nm) for A-ConA stained with Sypro Orange; (c) AFM images (height scale = 10 nm) of F-ConA; (d) same as panel c, for A-ConA (height scale = 15 nm). Note that all the experiments were performed on the very same samples used for NS.

allow exploring the samples both at micrometric and nanometric resolution. In Figure 2a we show a two-photon fluorescence microscopy image of F-ConA samples stained with Thioflavin T (ThT): the F-ConA aggregates have a long fibrillar morphology constituted by intertwined thin filaments that bind ThT, a well recognized dye for amyloid studies, as its fluorescence is considered a test for the presence of amyloid structures.[19] In Figure 2b, A-ConA morphology is shown by means of confocal fluorescence microscopy with Sypro Orange staining. The use of this dye, which binds hydrophobic regions of protein molecules, was necessary because no detectable fluorescence signal was observed in A-ConA stained with ThT. We also note that the F-ConA sample stained with both probes shows colocalization of the two dyes (data not shown) indicating the absence of appreciable amounts of amorphous aggregates. In Figure 2c,d we report atomic force microscopy measurements relative to F-ConA and A-ConA samples, respectively. These data, in line with the results already shown by Vetri et al.,[13] indicate the presence of simple thin fibrils in F-ConA and flat and roundish aggregates in A-ConA. The presented data consistently indicate that F-ConA is indeed in the fibrillar state and does not contain substantial amounts of

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disordered structures, while A-ConA is in the amorphous aggregate state.

In Figure 3a we report the total mean square displacements (msd's) of non exchangeable H atoms as a function of temperature, obtained from the analysis of elastic NS data. The typical temperature dependence of msd in protein powders is observed, and the dynamics of the aggregates is nearly identical to that of the native one up to 220 K. Above the dynamical transition temperature, msd's in F-ConA are larger than those in N-ConA and A-ConA. The coincidence of msd temperature dependence below 220 K and, in particular, of the anharmonic onset temperatures reveals that aggregation state does not perturb harmonic/local motions and does not modify the energy barriers of thermally activated motions (for a discussion of the transition temperatures dependence on the time window accessed by the spectrometer used, see refs 20 and 21). This is an interesting observation, since it shows that aggregation, although affecting protein function in living systems, does not influence the energy barriers for the activation of nanosecond anharmonic dynamics, related to functionally relevant motions; this strengthens recent evidence of the lack of direct correlation between dynamical transition and protein function.[22,23]

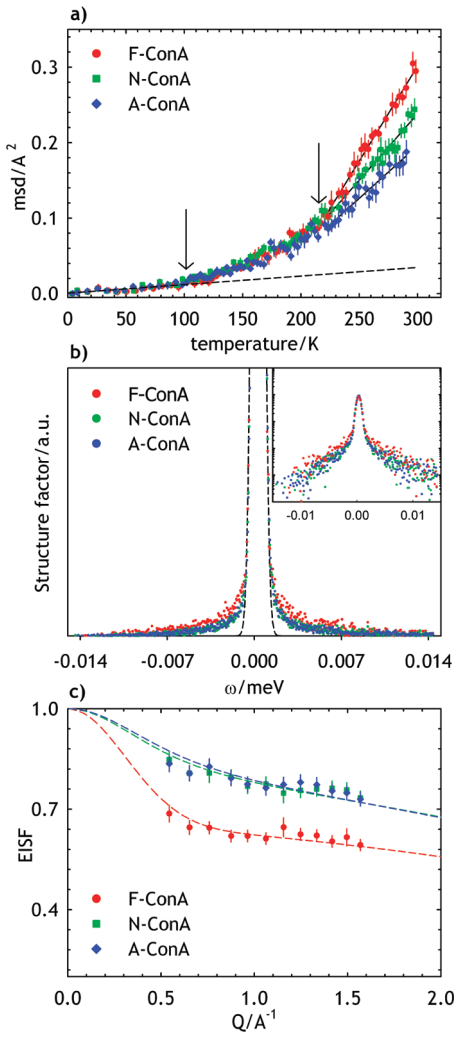

Figure 3. (a) Total msd as a function of temperature obtained by analyzing the elastic data using the Gaussian approximation in the Q range satisfying the condition $Q^2 < u^2 > \leq 2$ (see the SI); dashed line is a linear fit in the harmonic temperature region, solid lines are linear fits to calculate the force constant $k = 0.00138/(\partial \mathrm{msd}/\partial T)$; the arrows mark the temperatures at which anharmonic onsets occur. (b) Normalized quasi-elastic spectra at 300 K, binned over the observed Q-range; dashed line is the instrumental resolution, obtained by collecting spectra of the samples at 5 K; in the inset, the same spectra in logarithmic scale. (c) EISF vs Q obtained from the analysis of quasi-elastic spectra; solid lines were obtained using the Volino–Dianoux model (see text and SI).

Importantly, solvent-coupled motions, responsible for the steep increase of msd's above 220 K, are enhanced in the amyloid state and reduced in the amorphous one. The slopes of msd versus T above the dynamical transition give the environmental force constant k (see Table 1 and Figure 3a)

Table 1. Environmental Force Constants k and Parameters Obtained from the Analysis of Quasi-Elastic Data (See Text)

	F-ConA	N-ConA	A-ConA
k (N/m)	0.55	0.78	1.0
f	0.64	0.76	0.75
r_0 (Å)	5.0	3.5	3.0
σ (Å)	1.0	2.0	2.0

introduced to quantify the molecular softness of a protein structure[24] and highlight the fact that F-ConA, in the time/space regime investigated, is softer than N-ConA and A-ConA.

In Figure 3b we report the normalized quasi-elastic spectra at 300 K, binned over the observed momentum transfer (Q) range. F-ConA shows a larger quasi-elastic broadening evidenced by the more intense Lorentzian tail over the Gaussian elastic peak, while A-ConA and N-ConA spectra are similar. This suggests that proteins in the fibril state are characterized by increased diffusive-like motions, responsible for quasi-elastic signal. Figure 3c shows the elastic incoherent structure factor (EISF) versus Q in the range 0.5–1.5 Å^{-1}. In line with the msd results, F-ConA shows an increased decay with respect to the other samples; different from, and apparently at odds with the msd behavior, N-ConA and A-ConA have a similar trend. Since a fraction of quasi-elastic contribution included within the instrumental resolution function is considered when calculating the msd but not the EISF,[20,21] this latter result may be traced to the finite energy resolution of the used spectrometer (for a deeper discussion, see SI). The analysis of EISF data gives information on diffusive motions of side chains: solid lines in Figure 3c were obtained using the Volino–Dianoux model[25] for diffusive motions confined in a spherical volume of radius r by neighboring side chains, modified as to take into account a distribution of radii.[26] According to this model,

$$\mathrm{EISF}(Q) = e^{-Q^2 \mathrm{msd}_{vib}}\left[f + (1-f)\int_0^\infty G(r, r_0, \sigma)\left(\frac{3j_1(Qr)}{Qr}\right)^2 dr\right]$$

where msd_{vib} is the vibrational term to the msd, extrapolated from the low temperature trend, f is the fraction of H atoms not diffusing in the explored time scale, $G(r,r_0,\sigma)$ is a Gaussian distribution of width σ centered at r_0, j_1 is the first-order spherical Bessel function of the first kind. The parameters obtained from this analysis (see Table 1) reveal that, compared

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with native and amorphous samples, fibrils are characterized by a larger number of mobile H atoms (note that this result is model independent), by larger confinement radii and by a lower heterogeneity, suggested by the smaller σ value and indicative of a more ordered structure.

In this Letter we have presented the first NS analysis of amyloid fibrils. Our data evidence enhanced nanosecond atomic fluctuations in ConA amyloid fibrils, at temperatures above the dynamical transition, where protein motions are coupled to hydration water dynamics. This is a particularly intriguing and surprising result, in view of the fact that data obtained with FTIR and CD spectroscopy on the very same samples used for NS experiments do not show any indication that relevant parts of the proteins are either looped out or are disordered rather than forming the characteristic fibrillar amyloid structure. Our tentative explanation of this counter-intuitive result is based on the coupling between amino acid side chains and hydration water dynamics. We suggest that the structural constraints leading to amyloid formation bring proteins to expose side-chains to the fibril surface, thus lowering the steric hindrance from other amino acids and allowing the "plasticizing" interaction with hydration water;[27] conversely, amorphous aggregation, via nonspecific interactions, hides protein surfaces from hydration water, thus reducing water coupled motions.

From a more general point of view, our data indicate that the usually accepted paradigm ordered structure ↔ lower flexibility can be violated when focusing on the local fast side-chains protein dynamics, as recently suggested by A. Sokolov and colleagues for transfer RNA (t-RNA).[28] In fact ConA, when involved in the fibril structure, shows reduced resilience and increased diffusive side chains motions. Accordingly, recent NMR and simulative studies showed that amyloid fibrils of the prion-forming part of the HET-s protein contain large rigid and well ordered parts but also highly flexible parts.[29]

In view of the structural variability (polymorphism) of amyloids, where F-ConA is representative of simple and thin fibrils, these surprising results constitute the first step toward a complete picture of the aggregation−dynamics relationship, fundamental for a deep understanding of fibril functional properties. In order to generalize the dynamical behavior observed here, further NS studies are in progress on different amyloid structures: glucagon fibrils with twisted and straight morphologies[30] and various fibril architectures obtained by freezing kinetic intermediates during the insulin aggregation pathway.[31]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details on sample preparation; experimental procedures for infrared spectroscopy, circular dicrhoism, fluorescence microscopy, atomic force microscopy, and neutron scattering measurements; elastic and quasi-elastic neutron scattering data reduction and analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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