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Lysozyme Fibrillation: Deep UV Raman Spectroscopic Characterization of Protein Structural Transformation

Abstract: *Deep ultraviolet resonance Raman spectroscopy was demonstrated to be a powerful tool for structural characterization of protein at all stages of fibril formation. The evolution of the protein secondary structure as well as the local environment of phenylalanine, a natural deep ultraviolet Raman marker, was documented for the fibrillation of lysozyme. Concentration-independent irreversible helix melting was quantitatively characterized as the first step of the fibrillation. The native lysozyme composed initially of 32% helix transforms monoexponentially to an unfolded intermediate with 6% helix with a characteristic time of 29 h. The local environment of phenylalanine residues changes concomitantly with the secondary structure transformation. The phenylalanine residues in lysozyme fibrils are accessible to solvent in contrast to those in the native protein.*

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INTRODUCTION

Amyloid depositions consisting of fibrils with a cross-β structure have been found in many neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and prion diseases, etc.[1–4] The amyloid fibrils are quite stable once they are formed, either in vivo or in vitro. It is still under debate whether the amyloid deposits are the cause or simply the result of the adverse cellular events.[3] Several mechanisms including inflammatory response, excitotoxicity, synaptic dysfunction, mitochondrial dysfunction, etc. have been proposed for the damaging role of amyloid deposits in the neurodegenerative diseases.[5] In vitro study of the biochemical mechanism of fibrillation could provide a new insight into the understanding of amyloid diseases and ultimately help in developing the therapeutic strategies for preventing or alleviating the onset of amyloid deposits.

Protein structural transformations on the molecular level during in vitro fibril formation are accompanied by substantial changes in macroscopic properties, such as formation of a gelatinous phase and the formation of insoluble particles.[6–10] These changes limit the application of conventional methods such as NMR, small-angle x-ray scattering, circular dichroism, Fourier transform infrared, intrinsic and 8-anilino-1-naphthalene sulfonate (ANS) fluorescence, etc. in the characterization of protein conformational transformations.[6–8] Raman spectroscopy has been proven to be an efficient technique for characterizing highly scattering and opaque samples. At deep ultraviolet excitation around and below 200 nm, Raman scattering is resonantly enhanced from the amide chromophore, a building block of a polypeptide backbone, providing quantitative information about the protein secondary structures.[11] A new deep UV Raman spectrometer, which requires only a 100-μL sample with protein concentration of at least 0.1 mg/mL, has been recently built.[12] No special sample preparation is required: the dynamic range has no limitations at the high concentration end,[12] although self-absorption might need to be taken into account for quantitative analysis of Raman spectra. We report here on the first application of deep ultraviolet resonance Raman (DUVRR) spectroscopy for studying the mechanism of amyloid fibril formation.

It has been postulated that fibril formation starts with a destabilized, partially unfolded intermediate that then self-associates to form a nucleus initiating the fibrillation.[1,3,4,6–10] The well-documented 3-D structure, the capability to form fibril in vitro,[6,8,9] and the linkage to the amyloid deposition[13] make lysozyme a good model for studying the fibrillation process. Lysozyme forms fibrils after a prolonged incubation in an acidic solution at high temperature.[8,9,14] A comprehensive study of the thermally induced formation of lysozyme fibrils has been recently reported.[14] Although many details of the lysozyme fibrillation process have been thoroughly discussed in the literature,[9,14–17] the changes of the protein structure in the first stage of fibrillation, including a partial denaturation, has not yet been described. We demonstrate here that deep UV resonance Raman spectroscopy can be used for quantitative structural characterization of protein at all stages of fibrillation.

MATERIALS AND METHODS

Hen egg white lysozyme (refer to as lysozyme) and sodium trifluoroacetate were purchased from Sigma–Aldrich, St. Louis, MO, USA and used as received. The method of Krebs, et al.[9] was used for fibril preparation. Briefly, pH 2.0 lysozyme solutions (14 mg/mL) were incubated at 65°C for various times. A gelatinous phase formed during the incubation was separated by centrifugation at 16,700*g* for 30 min. DUVRR measurements and data treatment have been described elsewhere.[12]

RESULTS AND DISCUSSION

The DUVRR spectra of briefly heated lysozyme (above 65°C) showed an increase in the intensity of the C_α–H bending band (1390 cm^{-1}), which indicated the melting of the α-helix into random coil.[12] These changes are completely reversible: the initial Raman spectrum of native lysozyme completely restores at room temperature after a brief heating (less than 30 min) to high temperatures.[12] Prolonged incubation of lysozyme at 65°C and pH 2.0 resulted in irreversible changes. During the first 2 days, an irreversible α-helix melting occurred, which is evident from the room temperature DUVRR spectra obtained after the incubation (Figure 1A,B). In the range of 0.01 to 10 mM, the rate constant of this process was independent of lysozyme concentration, which could indicate an intramolecular transformation. The latter result was consistent with the earlier observation that the lag time of the lysozyme fibril formation was practically concentration independent.[14] This also indicated that the lysozyme irreversible unfolding documented here was an important stage of the fibrillation process. Furthermore, the irreversible character of the partial unfolding at the initial stage of fibrillation might play a key role in the amyloid diseases since it could allow further fibrillation at a relatively low concentration of fibrillogenic protein in vivo; although the conditions causing an irreversi-

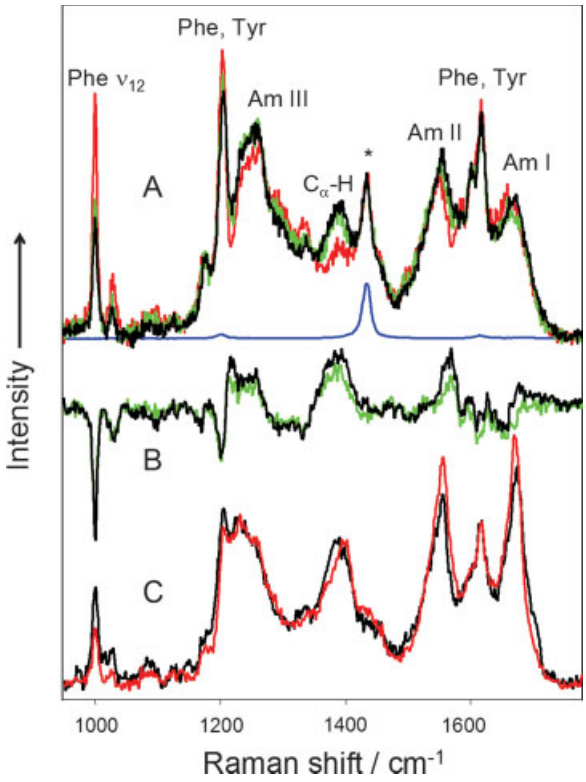

FIGURE 1 (A) 197-nm excited Raman spectra of native lysozyme (~14 mg/ml, pH 2.0, red) and the solution phase of lysozyme incubated at 65°C for 2 (green) and 8 days (black).[8] The incubated samples were centrifuged at 16,700*g* for 30 min to remove a gelatinous phase. The spectra were normalized to the protein concentration in solutions. 30-mM trifluoroacetate was used as the internal standard (blue curve). Both lysozyme DUVRR spectra with and without trifluoroacetate were measured to determine quantitatively the contribution of the protein and the internal standard. (*) Trifluoroacetate band. Amide I mode (Am I) consists of carbonyl C=O stretching, with a small contribution from C–N stretching and N–H bending. Amide II and Amide III bands involve significant C–N stretching, N–H bending, and C–C stretching. The C_α–H bending vibrational mode involves C_α–H symmetric bending and C–C_α stretching.[18] (B) Difference spectra obtained for incubated lysozyme (2 days, green; 8 days, black) and native lysozyme. (C) DUVRR spectra of the lyophilized fibril powder dispersed in water (red) and acetonitrile (black).

ble partial unfolding in vivo should be quite different from the low pH and high temperature utilized for the in vitro studies.

A gelatinous phase, which appeared after incubation for more than 2 days, was separated from the solution by centrifugation and both parts were analyzed separately. The DUVRR spectrum of the 8-day incubated solution (supernatant after centrifugation) showed a small contribution from β-sheet, which could not be eliminated by centrifugation. It turned out that all DUVRR solution spectra obtained at various incubation times fit well with a linear combination of three spectra corresponding to the native protein, a 6% α-helix–containing conformer, and the lysozyme gel. The last spectrum might not exactly represent the soluble β-sheet–rich conformer, but its contribution was relatively small and did not impair the overall fitting. Figure 2A shows the contribution of the three components as a function of the incubation time. The concentration of the native protein declined and that of the partially unfolded conformer grew monoexponentially with a characteristic time of 28 ± 2 h. In addition, the contribution of β-sheet increased monotonically. The DUVRR spectra of gels were dominated by the contribution from β-sheet and showed no change with the incubation time that was consistent with the formation of fibrils[1] (Figure 1C). The amount of protein deposited into the gel increased with the incubation time as shown in Figure 2C. Further

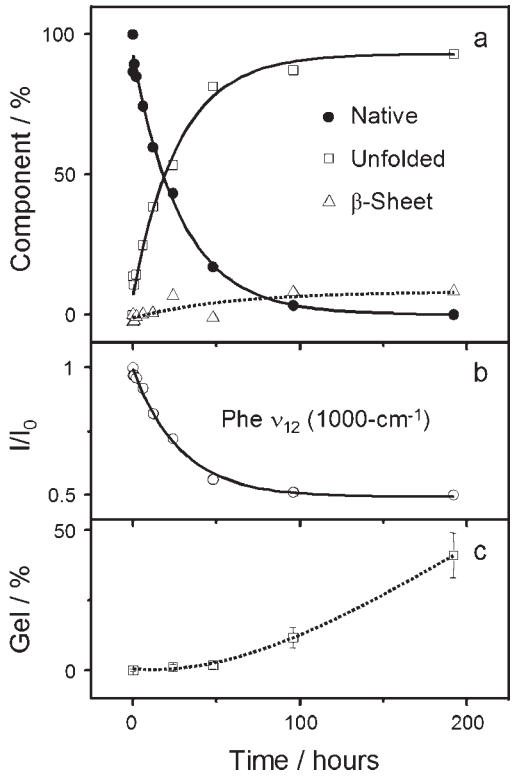

FIGURE 2 (A) A three-component composition of the solution part of incubated samples (see text). (B) A relative intensity of the 1000-cm^{-1} phenylalanine band in the Raman spectra of lysozyme solutions incubated for various times. I_0 corresponds to the band intensity in the spectrum of a nonincubated solution. I value was normalized to the protein concentration in solutions. (C) The percentage of lysozyme deposited in a gelatinous form. The solid curves represent monoexponential fits.

experiments are required to clarify the exact conformation of the soluble β-sheet–rich conformer and to verify whether it plays a role as the fibrillation nucleus.

Unlike tryptophan and tyrosine, phenylalanine has a low fluorescence quantum yield that limits its application as a natural fluorescence probe.[19] The Raman scattering of phenylalanine is resonantly enhanced on excitation with wavelengths below 200 nm.[20] The 1000-cm^{-1} (ν_{12}) phenylalanine Raman band was evident in all 197-nm spectra of lysozyme (Figure 1). A dramatic decrease of its Raman cross-section with temperature[12] and incubation time was assigned to the increased exposure of phenylalanine residues to water resulting from lysozyme unfolding. The assignment was based on the observations that the Raman cross-section of phenylalanine amino acid residue (i) is independent of temperature[12] and (ii) increases dramatically on the addition of acetonitrile into an aqueous solution (data not shown). A decreased 1000-cm^{-1} band was also evident in the spectrum of the partially unfolded intermediate (Figure 1A) and the difference spectrum (Figure 1B). The intensity of this Raman band decreased monoexponentially with incubation time with a characteristic time of 29 $\pm$ 2 h (Figure 2B). Consequently, the local environment of phenylalanine residues changes as a result of lysozyme unfolding concomitantly with the secondary structure changes.

A further decrease in the relative intensity of the 1000-cm^{-1} Raman band was found in the lysozyme fibril spectrum, indicating even stronger exposure of phenylalanine to water (Figure 1C, red). The gelatinous part of an incubated sample was lyophilized and dispersed again in water and acetonitrile. The former sample showed exactly the same spectrum as before the lyophilization. The DUVRR spectrum of the sample suspended in acetonitrile (Figure 1C, black) showed almost no change in the amide bands, indicating that the secondary structure of fibrils was preserved, yet the intensity of the 1000-cm^{-1} band was increased, indicating the exposure of phenylalanine to the acetonitrile environment. A similar experiment involving lyophilization of the protein and its dispersion in acetonitrile was performed with native lysozyme. No increase was found for the intensity of the 1000-cm^{-1} band, indicating that phenylalanine residues are buried in a hydrophobic core and are not accessible to solvent in the native protein (data not shown).

CONCLUSIONS

Deep UV Raman spectroscopy was utilized for the first time for structural characterization of protein at all stages of fibril formation. The evolution of the protein secondary structure as well as the local environment of phenylalanine, a natural deep ultraviolet Raman marker, was documented for the fibrillation of lysozyme in vitro. Concentration-independent irreversible helix melting was quantitatively characterized as the first step of the fibrillation.

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