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Unusual spiral structures formed by glycated β-casein in the presence of thioflavin T: amyloid transformation?

Ivan A. Zanyatkin, Yulia Yu. Stroylova, Aleksandra K. Melnikova, Ali Akbar Moosavi-Movahedi, Ali Akbar Saboury, Thomas Haertle and Vladimir I. Muronetz

Experimental details

Native β-CN was purified according to the two-step purification procedure with additional reversed-phase chromatography.[S1] In order to prepare aggregates, protein solution (78 μM β-CN) in 50 mM MES buffer, pH 6.7, containing 100 mM NaCl, was incubated at 94°C during 1 hour.

β-CN glycation protocol

0.2 mM β-CN was incubated in 50 mM Tris-HCl buffer, pH 8.0, containing 0.01% sodium azide in the presence of 200 mM glucose at 37°C during 24, 48 or 72 hours with gentle shaking. After the incubation during specified time intervals, the protein solution was purified from D-glucose and low molecular weight compounds using reverse phase chromatography on a Sep-Pak 20 CC column containing 30 RPC gel (Amersham Biosciences, Piscataway, NJ). The column was equilibrated with water containing 0.01 % trifluoroacetic acid. Protein was eluted with 80 % acetonitrile, containing 0.005 % trifluoroacetic acid, v/v). Then samples were dialyzed against 50 mM MES buffer, pH 6.7, concentrated using SpeedVac and frozen. In case of methylglyoxal β-CN solution (5 mg/ml) was incubated in 50 mM borate buffer, pH 8.0 in the presence of 200 mM methylglyoxal at 37°C during 1-24 hours with gentle shaking. Further purification from methylglyoxal and low molecular weight compounds was performed by dialysis against 20 mM MES buffer, pH 6.7. In both cases 35 mM sodium borohydride reduction was used if necessary.

ThT fluorescence protocol

A freshly prepared water solution of ThT was added to 4 μM β-CN sample in 20 mM Tris-HCl buffer, pH 7.5 at a molar ratio of 100:1. ThT was incubated avoiding light at room temperature for 20 min with protein samples before the measurements. Spectra of ThT fluorescence at 490-600 nm were acquired on Hitachi F 4500 spectrofluorimeter at 20ºC using an excitation wavelength of 445 nm.

Amadori product determination

To determine Amadori products content 100μl of the tested solution, containing 0.02-0.08 mM β-CN, was added to 900 μl of a solution containing 0.25 mM nitro blue tetrazolium in 100 mM sodium carbonate, pH 10.8, and after 10 min of incubation at 50 °C, absorption of the sample at 530 nm was measured.

Fluorescence microscopy

Freshly prepared water solution of 2.5 mM ThT was added to β-CN solution (0.06 мМ) in 20 mM Tris-HCl buffer, pH 7.5 directly mixing at slides. Samples were incubated avoiding light at room temperature for 20 min in wet conditions before the measurements. Samples were observed with an inverted fluorescence microscope (Leica DMRB) using band-pass excitation filter 450–490 nm, reflection short pass beam splitter RKP 510 and long pass filter >490 nm. Digital pictures were acquired using a Nikon DS-1QM camera and NIS software.

Transmission Electron Microscopy

Samples of β-CN (0.2 мМ) were glycated in with 200 mM glucose at 37°C during 48 hours. Freshly prepared water solution of 2.5 mM ThT was added to glycated β-CN solution (0.06 мМ) in 20 mM Tris-HCl buffer, pH 7.5 The prepared samples were adsorbed on the Formvar film applied to the copper mesh (200 mesh) and stained with uranyl acetate for the negative contrast of

the structures. After air drying and carbon spraying, the samples were investigated using an Jeol JEM-1400 transmission electron microscope (Jeol, Japan) at an accelerating voltage of 100 kV.

Turbidimetry measurement

Usual turbidimetric detection of optical density at 320 nm was hindered by the presence of ThT in the samples, so wavelength was changed to 600 nm. Absorbance measurement of β-CN solutions (0.02 mM) in 50 mM Tris-HCl buffer, pH 7.5, was performed using Hitachi U-2900 spectrophotometer.

Dynamic Light Scattering

DLS experiments were carried out by using a Zetasizer Nano-ZS apparatus (Malvern Instruments, Malvern, U.K.) equipped with 173° optics for detection of scattered light intensity. Size distribution by number was used for interpretation of results. β-CN (0.2 mM) was glycated with 200 mM glucose at 37°C during 48 hours. DLS measurements were carried out at 25 °C using 0.02 mM β-CN in the same buffer. Each reported data point is an average of 7 runs, 15 s each. Data sets obtained were analyzed using the Malvern DTS software. Distribution of particles by number, volume and intensity were reported. Presence of soluble particles with the hydrodynamic diameter less than 2 μm was monitored.

Immunochemical Analysis

β-CN samples were applied onto slides with preliminarily fixed nitrocellulose membrane and air-dried during 10 min, then slides were washed five times with 25 мM Tris-HCl buffer, pH 7.5, containing 0.1% Tween-20 and 5 more times with 25 мM Tris-HCl buffer, pH 7.5. Next, slides were blocked with 1% BSA in 25 мM Tris-HCl buffer, pH 7.5, containing 0.1% Tween-20 for 40 minutes and washed with the same buffer. The slides were incubated with primary polyclonal rabbit antibodies against bovine β-CN (50 μg/ml) for 1 hour. After washing, secondary monoclonal goat anti-rabbit antibodies with Alexa Fluor 555, Thermo Fisher Scientific) were applied for 1

hour. Representative images were taken with a microscope Carl Zeiss Axiovert 200M combined with a camera Hamamatsu Orca 2GF2.

Advanced glycation end-products detection

Advanced glycation end-products (AGE) were detected using its characteristic fluorescence.[S2] Fluorescence measurement of 0.02 mM β-CN solutions in 50 mM Tris-HCl buffer, pH 7.5, was performed in 96-well plates and fluorescence was excited at 335 nm and registered at 410 nm (PerkinElmer 2030 Multilabel Reader Victor X5).

Determination of free amino groups

β-CN sample (at 4 μM final concentration) was incubated in 50 mM sodium borate buffer, pH 9.3, containing 0.2% N,N - dimethylmercaptoethylammoniumchlorid (DMMAC) as reducing agent, and 5 mM o-phtalaldehyde during 10 minutes [S3]. Absorbance was measured at 360 nm using spectrophotometer Hitachi U2900.

Supplementary results

The results of the turbidity measurements of β-CNs solutions after ThT addition are presented in Figure S1. The measurements were performed at a wavelength of 600 nm due to the high absorption of ThT at 320 nm, usually used in such experiments. As follows from the data, the turbidity of unmodified β-CN does not change upon heating and addition of ThT (Figure S1, curve 1). The turbidity of glycated β-CN increases with heating, reaching a maximum value of 0.1 after 30 min of incubation (Figure S1, curve 2). Addition of ThT to glycated β-CN leads to almost instant (within 5-30 seconds) development of the solution turbidity. The effect of ThT on the aggregation of glycated β-CN at room temperature and while heated does not differ significantly (Figure S1).

Using the DLS method, it was shown that unmodified β-CN preparations mainly contain particles with a diameter of about 25 nm, characteristic of micelles formed by this protein at room

temperature, as well as particles with a diameter of about 400 nm. Modification of β-CN with glucose leads to a slight decrease in micelle size to 22 nm, as well as to the appearance of particles with a diameter of 8 nm, corresponding to the monomeric form of the protein. Heating unmodified casein does not significantly affect the particle size distribution, while particles with a diameter of > 5 μm appear in glycated β-CN. The addition of ThT to unmodified β-CN causes a slight change in the hydrodynamic radius of the micelles to 20 nm, but does not lead to the appearance of particles with a diameter of more than 1 micron both with and without heating, which corresponds to the data obtained by the turbidimetry measurements. The effect of ThT on glycated β-CN was difficult to analyze by DLS, since the addition of ThT led to an instant turbidity development and precipitation of large particles.

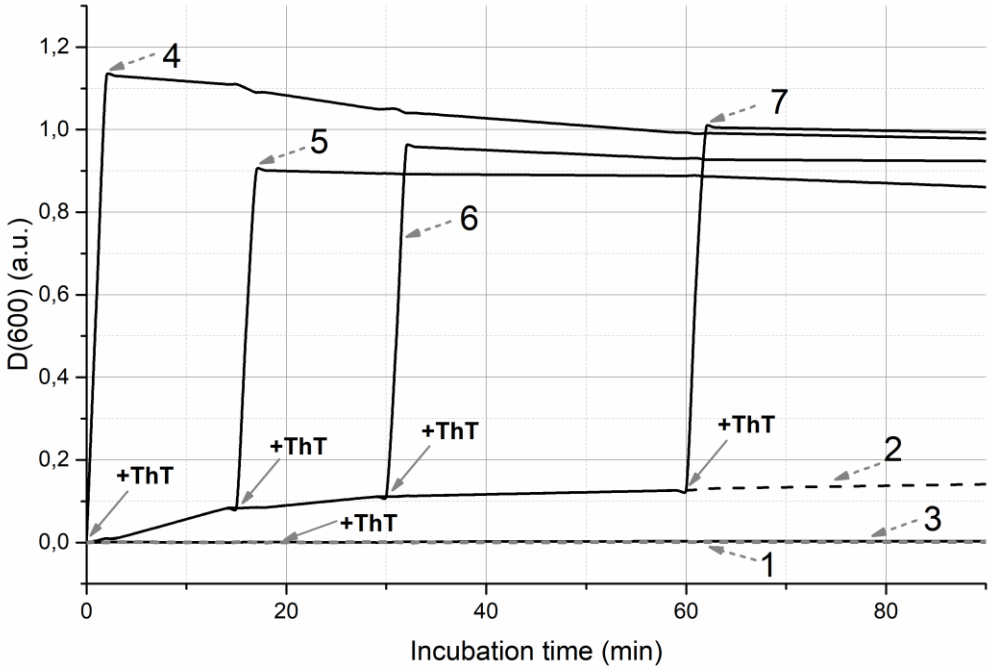

Figure S1. Influence of ThT on turbidity of unmodified and glycated β-CN upon heating. Turbidity measurements were performed at 600 nm with heating at 94^0C of unmodified (curve 1, dashed line) and glycated with glucose (curve 2, dashed line) β-CN (0.078 mM protein in 50 mM MES buffer, pH 6.7, containing 100 mM NaCl) for 90 min. Arrows indicate time points of ThT addition to a sample, added in a 100-fold molar excess relative to protein.
Curves 3 – 7 (solid lines) – after ThT injection.

To prove the presence of β-CN in spiral structures and aggregates, immunochemical staining with specific antibodies to this protein was performed (Figure S2). Due to the presence of intensive washing step during immunostaining, the experiment was performed on a nitrocellulose membrane to preserve ThT in the sample.

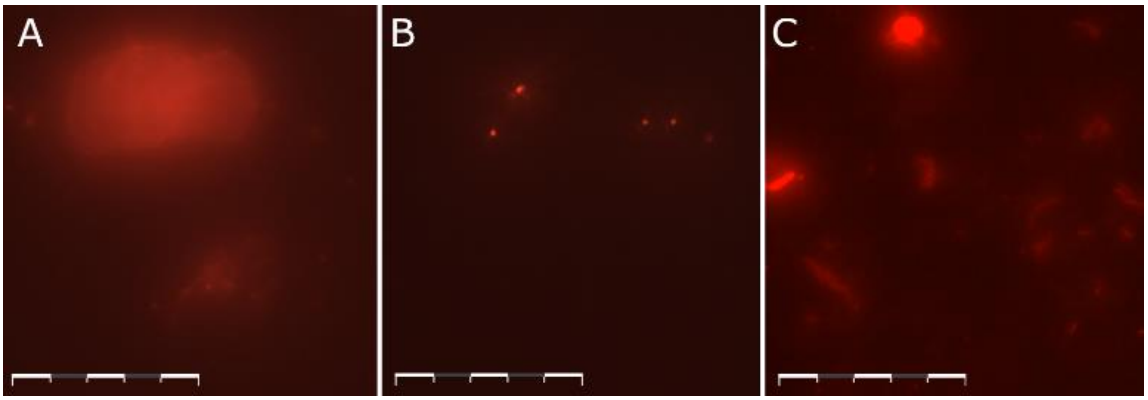

Figure S2. Immunostaining of spiral structures of glycated β-CN.
A – amorphous aggregates of glycated β-CN before heating, B – spiral structures of glycated β-CN before heating, C - spiral structures and amorphous aggregates of glycated β-CN after incubation in the presence of ThT at 37°C during 60 minutes. B-CN (5 mg/ml) was glycated in 50 mM Tris-HCl buffer, pH 8.0, containing 0.01% sodium azide with 200 mM glucose at 37°C during 48 hours. Scale bar: 100 μm.

As can be seen in Figure S2, we confirmed immunochemically the presence of protein, namely β-casein in amorphous and, which is more important, in specific helix-like aggregates.

References

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