International Journal of Biological Macromolecules 57 (2013) 63–68

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Myelin basic protein: Structural characterization of spherulites formation and preventive action of trehalose

Cinzia Di Salvo [a], Davide Barreca [a,*], Giuseppina Laganà [a], Marcella di Bella [b], Ester Tellone [a], Silvana Ficarra [a], Ersilia Bellocco [a]

[a] Dipartimento di Scienze Chimiche, Università di Messina, Viale F. Stagno d'Alcontres 31, 98166 Messina, Italy
[b] Dipartimento di Fisica e Scienze della Terra, Viale F. Stagno d'Alcontres 31, 98166 Messina, Italy

ARTICLE INFO

Article history:
Received 5 February 2013
Received in revised form 26 February 2013
Accepted 27 February 2013
Available online 13 March 2013

Keywords:
Myelin basic protein
Trehalose
FTIR-spectroscopy
Aggregation
Fluorescence
Spherulites

ABSTRACT

Myelin basic protein (MBP) is one of the main protein components of central nervous system that as shown in our work, under appropriate condition, forms spherulites. The structural and morphological features of these elements and the ability of trehalose to decrease or completely avoid their formation have been clarified with different but complementary techniques. The FTIR spectra provided compelling evidence for the presence of a small amount of elements organized in secondary structures such as helices and sheets. Polarized optical microscope observations show the presence of spherulites with an average size ranged from 41 to 61 μm, characterized by non-birifrangent core. The fluorescence data supply useful informations that are consistent with the presence of a molecular exclusion effect, following interaction of the disaccharide with protein that involves the environment around the single tryptophan residue. Moreover, study of protein conformational states by SDS-PAGE, let us to state that trehalose completely avoid autocatalytic cleavage properties of MBP up to 4 days of incubation at 37 °C and pH 7.4.

1. Introduction

It is known that the electrostatic interactions are important in protein stability. Nevertheless is not easy to understand in which manner these take place, because there are several different ways in which they contribute to the stabilization of protein native conformation. In addition, both attractive and repulsive electrostatic interactions are possible.

In general protein stability is also influenced by physical–chemical parameters such as pH and temperature. As regards the first factor, most proteins are significantly unfolded at low pH due to charge–charge repulsion emanating from multiple positively charged ammonium groups, while the presence of low anions concentration leads to substantial refolding, minimizing the repulsive interactions [1]. Moreover, a mechanism that in some case directly correlates with protein denaturation is the formation of aggregates. In fact, some experimental evidences seem to indicate that the leading process towards aggregation is due to conformational changes that starting from a native conformation lead to a partially unfolded state. The aggregation processes have been intensely studied in fields ranging from medicine, technology and food researches. Many systemic and neurodegenerative disorders, from light chain amyloidosis to Alzheimer's disease, have been found to be associated with the misfolding and aggregation of specific proteins [2,3]. Generally, if changes in environmental conditions lead to partial unfolding of the native state of a protein it will often aggregate, sometimes into well-defined fibrillar structures. Moreover as some proteins self-assemble *in vitro* into amyloid fibrils, it has been observed a further higher-order self-association of the protein into spherical structures, with diameter ranging from 10 to 150 μm [4,5]. These structures, called "spherulites", under polarizing light microscope, exhibit a "maltese-cross" extinction pattern and have been observed in brain sections of human patients with a particular strain of Creutzfeldt-Jacob disease and in a rat model of Alzheimer's disease [6], suggesting that they are formed by amyloid fibrils radially oriented into the spherulites. Each fibril contains multiple copies of a single polypeptide chain in an ordered array that is rich in β-sheet structures. According to this model, the β-strands that assemble to form sheet are oriented perpendicular to the fibril axis. Spherulites formation is also a phenomenon associated with the self-assemble of polymers during the phase of crystallization, in which the polymer chain folds back on itself repeatedly in a regular pattern forming lamellar crystals. Experimental evidences show as some protein are able to form spherulites such as lysozyme [7,8], carboxypeptidase under crystallization conditions [9] and bovine insulin [4].

The myelin basic protein (MBP) is one of the major components of the myelin sheath in the central nervous system of higher

* Corresponding author. Tel.: +39 090 6765187; fax: +39 090 6765186.
E-mail address: dbarreca@unime.it (D. Barreca).

0141-8130/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijbiomac.2013.02.022

vertebrates, and a member of a larger family of proteins with a multiplicity of forms and post-translational modifications (PTMs). The 18.5 kDa protein is the most abundant and the most studied isoform in adult myelin. It is a peripherally membrane associated monomeric protein of 169 amino acid residues, which constitutes ~30% of the total protein of the multilamellar myelin sheath that has been extensively studied for its implications in multiple sclerosis disease [10].

In this study we attempt to describe the conditions in which MBP shows aggregation phenomenons associated with a partially unfolded state of the protein; we also characterize the spherulites formed by optical microscopy and the reaction with CR. Changes in secondary structure have monitored by Fourier transform infrared spectroscopy (FTIR) showing that changes in pH medium, induce a little decrease in α-helix structures and an increase in turns, albeit with some degree of polymorphism and disorder. Moreover we evidence the direct effect of disaccharide trehalose on preventing both the phenomenon of aggregated structures formation and the autocatalytic degradation of MBP.

2. Materials and methods

2.1. Purification of MBP

Bovine brains were obtained from 18 months old male animal. The entire brains were placed in approximately 1 l of physiological solution (0.9% NaCl). The meninges and cortex were removed, and blood was washed away with the same solution. The grey matter was carefully separated from white matter and this one was cut into small pieces and frozen until use.

All the sequent procedures were performed at 4 °C and without protease inhibitors. The protein was isolated essentially as described by Sedzik et al. [11], with some modifications. Briefly, 0.1 ml of 6.0 M hydrochloric acid was added to the 10% (w/v) white matter homogenate dispersed in 20% ethylene glycol (30 ml final volume). The acid-insoluble material was removed by centrifugation at 15,000 rpm for 1 h. An aliquot (6.0 ml) of the supernatant was withdrawn and brought to 65% saturation with ammonium sulphate and centrifuged for 10 min at 15000 rpm. The precipitate was dissolved with 2.5 ml of H_2O acidified solution and the extract was applied on a PD10 column equilibrated with the same solution. The protein (isoform of 18.5 kDa) was recuperate in 3.5 ml. Protein content was determined by the binding of Coomassie brilliant blue G-250 [12] using bovine serum albumin as standard. Protein purity was monitored by 10% SDS-PAGE electrophoresis according to Laemmli [13]. MBP purchased from Sigma Aldrich was utilized as reference.

2.2. Colorimetric determination of citrulline residues in MBP

The colorimetric determination of citrulline residues in purified protein was made according to Sugawara et al. [14].

2.3. Incubation conditions and optical microscopy

The H_2O acidified protein solution (0.8 mg ml^{-1}), in the presence and in the absence of 0.5 M trehalose was brought to pH 7.0 by addition of fixed amounts of NaOH 3.0 M. The solution was incubated in glass vials both at 37 °C and 55 °C for 2 h. After incubation, aliquots of MBP solutions were removed from the glass vials and put onto microscope slides. For each sample a corresponding blank has been prepared. The samples were air dried and studied by using an optical microscope without coverslips and magnifications of up to 40×. The sample was analyzed using both crossed and parallel-polarized illumination.

2.4. Differential spectra for Congo Red binding to MBP

Differential spectra were performed in 0.05 M borate buffer, pH 9.2 at 25 °C. The sample cuvette contained 6.4 μM MBP, while the concentration of the dye ranged from 5 to 40 μM. The dye concentration in the reference cuvette was the same as that used for the complex formation. Differential spectra were recorded on a Beckmann spectrophotometer in the wavelength region 400–800 nm and using a path length of 1.0 cm. Before recording the differential spectra the linearity of the absorbance *vs* concentration plot, was checked for dye concentration up to 80 μM. To test antiaggregative properties of trehalose, samples of MPB have been incubated at 37 °C and 55 °C in the absence or presence of 0.5 M of trehalose. After 30 min of incubation aliquots of the samples (100 μl) have been withdrawn and mixed with 20 μM Congo Red dye. Differential spectra were recorded on a Beckmann spectrophotometer in the wavelength region 400–600 nm using a path length of 1.0 cm and ΔAbs at 560 nm was taken into account for the analysis of aggregate structures.

2.5. Autodegradation of MBP

MBP solutions were added to 50 mM Tris–HCl pH 7.8 (ratio 1:2) and incubated in thermostated water bath at 37 °C in absence or in presence of 0.5 M trehalose. At various intervals of time (0–4 days) aliquots were removed, and frozen. Afterwards, these aliquots were separated on 10% SDS-PAGE electrophoresis according to Laemmli [13] and stained with Coomassie Blue-R. Stained gels, digitized by image scanner, were processed by ImageJ software (available at the website http://rsb.info.nih.gov/ij/) which included background subtraction, contrast enhancement, dye front baseline correction and signal to noise enhancement.

2.6. Fourier transform infrared spectroscopy (FTIR)

Prior to infrared experiments, the protein solutions were lyophilized and dissolved in acidified D_2O (pD 2.0) at 25 °C. Aliquots of these samples have been withdrawn and brought to pD 7.0 in deuterated phosphate buffer 10 mM. Successively the samples at pD 2.0 and pD 7.0 were incubated at 37 °C and 55 °C for 2 h before FTIR analysis. The FTIR spectra, between 1350 and 1750 cm^{-1}, were measured with a Bruker Vertex 80 spectrometer. The protein solution (6.8 mg ml^{-1}), placed between a pair of CaF_2 windows separated with a 25 μm Teflon spacer, was inserted in the fluid cell. For each measurement were collected 64 interferograms with a spectral resolution of 2.0 cm^{-1}. Each measure was performed under vacuum to eliminate minor spectral contributions due to residual water vapour.

2.7. Data analysis of IR spectra

Protein spectra were smoothed by Loess algorithm and the deconvolved spectra fitted with Gaussian band profiles. Initial values for the peak heights and widths were estimated from the deconvolved spectra. For the final fits, the positions, heights, and widths of all bands were varied simultaneously. The curve fitting procedure was calculated on Seasolve PeakFit v4.12 software.

2.8. Fluorescence measurements

Intrinsic fluorescence measurements of MBP (0.5 mg ml^{-1}) in 10 mM Tris–HCl buffer pH 7.4 were carried out in the absence or in the presence of 0.5 M trehalose and/or 6 M urea using a Jasco FP-750 spectrofluorimeter equipped with thermostat-controlled cell holder and the temperature was kept constant by using a circulating water bath. The excitation wavelength was set at 280 nm in