Biochimica et Biophysica Acta xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

The crowd you're in with: Effects of different types of crowding agents on protein aggregation

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ARTICLE INFO

Article history:
Received 21 September 2013
Received in revised form 1 November 2013
Accepted 11 November 2013
Available online xxxx

Keywords:
Amyloid
Crowding
Protein aggregation
Flexibility
Intrinsic disorder

ABSTRACT

The intracellular environment contains high concentrations of macromolecules occupying up to 30% of the total cellular volume. Presence of these macromolecules decreases the effective volume available for the proteins in the cell and thus increases the effective protein concentrations and stabilizes the compact protein conformations. Macromolecular crowding created by various macromolecules such as proteins, nucleic acids, and carbohydrates has been shown to have a significant effect on a variety of cellular processes including protein aggregation. Most studies of macromolecular crowding have used neutral, flexible polysaccharides that function primarily via excluded volume effect as model crowding agents. Here we have examined the effects of more rigid polysaccharides on protein structure and aggregation. Our results indicate that rigid and flexible polysaccharides influence protein aggregation via different mechanisms and suggest that, in addition to excluded volume effect, changes in solution viscosity and non-specific protein–polymer interactions influence the structure and dynamics of proteins in crowded environments.

1. Introduction

Protein aggregation is associated with a variety of human diseases including Alzheimer's, Parkinson's, and Huntington's diseases, prionopathies, and type II diabetes. Protein aggregation usually starts from the protein in a partially unfolded conformation similar to the pre-molten globule state [1]. In order to access this conformation, a folded protein needs to partially unfold, while an intrinsically disordered protein (IDP) needs to partially fold. In addition, the mechanism of aggregation is usually a rather complex process with many intermediate oligomeric states characterized by variable extent of secondary structure [2–5]. Some of these oligomers easily convert to fibrils while others have a high degree of kinetic stability.

Investigation of protein aggregation is complicated by the fact that the in vivo environment is crowded with macromolecules, such as proteins, nucleic acids, and carbohydrates, which occupy up to 30% of the available volume. Macromolecular crowding increases the effective protein concentration, decreases the protein diffusion rate, promotes partial folding of some intrinsically disordered proteins and facilitates their aggregation [6]. It has been previously shown that macromolecular crowding has a significant effect on protein–protein interactions including protein aggregation [7]. Crowding tends to stabilize compact protein conformations. These conformations can be either on the pathway to folding or on the pathway to aggregation depending on the protein and the assay conditions. Thus, the effect of crowding agents on protein aggregation depends on the nature of the protein.

Most studies of the effects of crowding on protein aggregation have used flexible, hydrophilic polymers (PEG, dextran and Ficoll). Due to their compact, largely spherical shape these polymers have relatively small surface to volume ratio. They are neutral and relatively hydrophilic minimizing their specific interactions with proteins. Thus these polymers are believed to act primarily via excluded volume effect by decreasing the effective volume available for the proteins in the cell and thus increasing the effective protein concentration. It has been shown that aggregation of many proteins and peptides is accelerated by the presence of dextrans and other neutral flexible crowding agents [8–12]. However, for some proteins with highly stable folded native states (e.g. lysozyme or superoxide dismutase) addition of crowding agents has been shown to inhibit aggregation [11].

A variety of other, more rigid biopolymers are also present in vivo including DNA, protein fibers and polysaccharide components of extracellular matrix. Solutions of rigid polymers have higher viscosity that may affect protein diffusion and slow down protein folding or aggregation. These polymers also have lower density making them more effective in creating excluded volume effect as intrinsic viscosity of a polymer is proportional to its volume [13]. Lower polymer density also increases the exposed surface of rigid polymers available for interactions with

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1570-9639/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bbapap.2013.11.004

proteins. Here we investigated the effects of both compact, flexible polysaccharides (dextrans) and more rigid cellulose derivatives (hydroxypropyl celluloses or HPCs) on the kinetics and mechanism of aggregation of several proteins with variable degrees of intrinsic disorder. We selected neutral, hydrophilic polysaccharides to minimize the specific protein–polymer interactions. In order to test the effects of these polymers on the protein structure and aggregation, we selected several small proteins with different structural and dynamic properties such as the degree of intrinsic disorder and oligomeric state. We tested aggregation of these proteins in the presence of polysaccharides in the conditions previously determined to be favorable for their conversion to amyloid fibrils. We examined the effects of the polysaccharides both on kinetics of aggregation and the structure of the aggregates.

2. Materials and methods

2.1. Materials

Recombinant α-synuclein was a gift from Dr. Munishkina (University of California Santa Cruz). Recombinant human insulin was from Akron Biotech (Boca Raton, FL). A commercially available mixture of core histones H2A, H2B, H3 and H4 from calf thymus (Calbiochem) was used without additional fractionation. Recombinant ^{13}C–^{15}N enriched α-synuclein was prepared as previously described [14]. All other proteins and chemicals were from Sigma, Fisher Scientific or VWR Scientific.

2.2. Methods

2.2.1. Protein aggregation assays

Conditions for protein aggregation were optimized for each protein. Aggregation of insulin was studied in two sets of conditions: low pH, which stabilizes the monomeric form of the protein, and neutral pH, which stabilizes the insulin hexamer. At low pH aggregation of insulin (0.5 mg/ml) was conducted in glycine buffer (20 mM, pH 2.5) at 37 °C. The protein was dissolved directly in this buffer and incubated for 5 min prior to the start of the reaction. At neutral pH insulin (1.2 mg/ml) was aggregated in 20 mM sodium phosphate (pH 7.5) at 37 °C. Aggregation of histones (0.75 mg/ml) was conducted in the glycine buffer (20 mM glycine, pH 2.5) in the presence of 0.7 M NaCl at 37 °C. Histones were initially dissolved in 5 mM HCl at 4 mg/ml, incubated in this solution for 5 min and diluted into the final reaction buffer. Aggregation of α-synuclein (0.4 mg/ml) was conducted in 20 mM acetate buffer, pH 3.5 in the presence of 0.1 M NaCl at 37 °C. Protein was initially dissolved in 5 mM NaOH at 4 mg/ml, incubated in this solution for 1 min and diluted into the final reaction buffer. α-Lactalbumin (0.2 mg/ml) was aggregated in 40 mM phosphate buffer (pH 7.0) in the presence of 0.1 M NaCl, 1 mM EGTA and 2 mM DTT at 30 °C. Lysozyme (0.25–0.75 mg/ml) was aggregated in 25 mM potassium phosphate buffer (pH 2.0) in the presence of 225 mM NaCl at 39 °C using a modification of the method of Hill et al. [15].

Protein aggregation in the automated format was carried out in a reaction volume of 0.1 ml in black, flat-bottomed 96-well plates in the presence of 10 μM ThT. Two Teflon or polyethylene balls (2.38 mm diameter, Precision Ball, Reno, PA) were placed into each well of a 96-well plate. The reaction mixture containing protein and ThT (350 μl) was split into three wells (100 μl into each well), the plates were covered by Mylar septum sheets (Thermo), and incubated with continuous orbital shaking at 280 rpm in an Infinite M200 Pro microplate reader (Tecan). The kinetics was monitored by top reading of fluorescence intensity every 3–8 min using 444 nm excitation and 485 nm emission filters. Data from replicate wells were averaged before plotting fluorescence vs time. The data were fit to a sigmoidal equation using Origin (OriginLab, Northampton, MA). The equation [16,17] was:

$$F = A + B/(1 + \exp(k \times (t - t_m))) \quad (1)$$

where A is the initial level of ThT fluorescence, B is the difference between the final level of ThT fluorescence and its initial level, k is the rate constant of amyloid accumulation (h^{-1}) and t_m is the midpoint of transition. The lag time (t_l) of amyloid formation was calculated as $t_l = t_m - 2/k$. The parameters derived from this equation are: yield of amyloid (B), lag time (t_l), and elongation rate (k) of amyloid. Initiation rate was defined as the inverse of lag time. Although Eq. (1) gave good fits for the ThT kinetic profiles, the expression is strictly an empirical means of deriving kinetic parameters from the data and does not necessarily reflect the underlying complex kinetic scheme.

2.2.2. Electron microscopy

10 μl aliquots of protein solutions (0.1–0.3 mg/ml) were adsorbed onto 200 mesh formvar/carbon-coated nickel grids for 5 min. The grids were washed with water (10 μl), stained with 2% uranyl acetate for 2 min and washed with water again. The samples were analyzed with a JEM 1400 transmission electron microscope (JEOL) operated at 80 kV.

2.2.3. NMR

All the spectra were acquired at a 21.1 T Bruker AVANCE spectrometer operating at 898.71 MHz for ^{1}H. Samples were measured at 285.5 K, unless otherwise specified, by using a cryogenically cooled triple-resonance probe head. The stock α-synuclein solution was 0.6 mM ^{13}C, ^{15}N labeled α-synuclein in 20 mM potassium phosphate buffer, pH 6.5, 200 mM NaCl, 0.5 mM EDTA. Generally the samples were prepared by taking 150 μl of the stock solution and adding either buffer or the appropriate amount of crowder and buffer up to a final volume of 200 μl in a 3 mm NMR tube. Final concentrations of polymers were 5% for dextran 100 and 2.5% for HPC 100. The peak volumes were calculated using a routine implemented in the program CARA [18].

2.2.4. FTIR

FTIR spectra were measured with a Bruker Tensor 37 FTIR instrument (Bruker Optics, Billerica, MA) equipped with a DTGS detector. Aqueous protein solutions (25 μl, 1.2 mg/ml) were loaded into the BioATRcell II. 512 scans at 2 cm^{-1} resolution were collected for each sample, corrected for water vapor, and background spectra were subtracted.

2.2.5. CD

Far-UV CD (195–260 nm) spectra of proteins were measured using a JASCO J-815 spectropolarimeter at room temperature. A solution of protein (110 μl, 1 mg/ml) was placed into a 0.2 mm pathlength cell (0.1 mm pathlength for histones), and the CD spectra were acquired with 20 nm/min scan speed at 0.1 nm step size and 1.0 nm bandwidth under constant purging with nitrogen. Four spectra were accumulated and averaged for each sample. The same buffer was used for CD and FTIR measurements and for protein aggregation experiments.

2.2.6. Viscosity measurements

Viscosity of polymer solutions was determined with an Ostwald viscometer (Cannon Instruments) at 25 °C. In a typical experiment, a solution of polymer in water (2 ml) was loaded into the viscometer and the time taken for it to pass between the marks was measured with a stopwatch. Each measurement was performed at least in triplicate.

2.2.7. Determination of global stability

Aggregated samples (1–3 μl) were suspended in HEPES buffer (20 μl total volume, 50 mM, pH 7.5) containing different concentrations of GdnSCN. The solution was incubated for 1 h at 24 °C and then diluted to 300 μl with 6 M GdnSCN and HEPES buffer (50 mM, pH 7.5) adjusting the final concentration of GdnSCN to 0.2 M. Fluorescence spectra were recorded in the presence of 10 μM ThT. Excitation wavelength was 442 nm and emission spectrum was recorded in 470–500 nm range. Excitation slit was at 2.5 nm and emission slit at 5 nm.

Please cite this article as: L. Breydo, et al., The crowd you're in with: Effects of different types of crowding agents on protein aggregation, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbapap.2013.11.004