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Aggregation of globular protein as a consequences of macromolecular crowding: A time and concentration dependent study

Gufran Ahmed Siddiqui, Aabgeena Naeem*

Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, 202002,India

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ABSTRACT

The living cells show profoundly crowded condition, called as macromolecular crowding. Crowding essentially impacts on protein structure and lead to its aggregation. Protein aggregates have been involved in a wide range of diseases including Parkinson, Alzheimer's, and Huntington's. Increased in normal physiological macromolecular crowding because of increasing age can be implicated as one of the leading cause of proteopathies. In the present study, we have demonstrated the effect of macromolecular crowding on native structure of hemoglobin using bovine serum albumin as a crowding agent. Conformational changes of Hb at different concentrations of BSA were monitored using intrinsic fluorescence and ATR-FTIR spectroscopy. These results showed the transition of native Hb to a non-native form. Thermodynamic parameters were analyzed by isothermal titration calorimetry. The measurements of turbidity, thioflavin T, congo red and intrinsic fluorescence revealed the formation of significant protein aggregates with time. The kinetics of protein aggregation using relative ThT and 8-anilino-1-naphthalenesulphonic acid spectra clearly showed acceleration of the process with time and in concentration dependent manner. The spectra at 80 g/l of BSA incubated for 64 h showed formation of maximum Hb aggregates. Transmission electron microscopy results clearly showed the formation of amyloid aggregates structures, thus supporting the spectroscopic data.

1. Introduction

Inside the living cells, total concentration of macromolecules is so high that a significant proportion of available volume is physically occupied and thus unavailable to other molecules. Strictly, crowding is termed as excluded volume effect that highlights the fact of being purely physical nonspecific effect arising from steric repulsions. Minton and Wilf in 1981 [1] brought the importance of crowding on macromolecules to the prominence in terms of theory and experiment and coined the term macromolecular crowding, one of the most important factor which influences the structure and function of proteins under physiological conditions [2,3]. In *Escherichia coli,* the total concentration of RNA and protein is ranges from 300 g/l to 400 g/l [4]. The intensity of crowdedness varies among different types of cells and their compartments. For example, human lens contains approximately 340 g/l protein [5]; the red blood cells contain about 350 g/l hemoglobin [3]; while the total protein content in the mitochondrial matrix may reach up to 500 g/l [6]. Not only inside the cell but also in extracellular matrix of tissues macromolecular crowding is observed, for example, protein content of blood plasma is ~80 g/l, enough to cause significant crowding effect [3]. Polysaccharides also contribute to crowding, especially in the extracellular matrix of tissues such as collagen. Thus the volume occupied by macromolecules is about 20–30% of available space, so when trying to mimic biological crowding conditions in vitro this range defines the relevant concentration of crowding agent to be used [3].

A lot of work has been done regarding in vitro mimicking the effect of macromolecular crowding on native structure of protein using concentrated solutions of a selected crowding agent such as polyethylene glycol [7], dextran, ficoll [8–11], hemoglobin (Hb), bovine serum albumin(BSA) [12–16]. Studies conducted so far showed that crowding can significantly impact on the structure of protein and can lead to its aggregation which disrupt the normal physiology of the living cell [8,17–19]. So far many diseases related to protein aggregation has been reported such as neurodegenerative diseases where the aggregation of specific proteins and amyloid deposition of many proteins have been found in the brain such as Alzheimer's disease, prion diseases, Parkinson disease, amyotrophic lateral sclerosis.

* Corresponding author at: Department of Biochemistry, Faculty of Life Sciences, AMU, Aligarh 202 0020, India.
E-mail addresses: aabgeenanaim@gmail.com, anaeem.bc@amu.ac.in (A. Naeem).

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Being a small globular protein, hemoglobin (Hb) can acts as an important model system for investigating the mechanism of amyloid formation and understanding the impact of these aggregates in vivo [20,21]. Many diseases like hemophilia, hemolytic anemia, sickle cell anemia occurs due to loss of Hb.

Hb is a well-known allosteric heme protein composed of two α and two β subunits having 141 and 146 amino acids respectively. It can transport molecular oxygen in RBCs. It can be an important model system to study helix/sheet transition as it possess 70% α helix and 30% β sheets. The transition of α helix to β sheet can be reliable strategy to discover new therapies to cure severe ailments [22].

The effect of macromolecular crowding on structure of hemoglobin has not been studied in vitro so far. Here in our studies we have demonstrated the macromolecular effect on hemoglobin using bovine serum albumin as a crowding agent. The concentration of BSA was varied from 0 g/l to 100 g/l. The samples were incubated at different time intervals up to 64 h to analyze the kinetics of the protein aggregate formation.

2. Materials and methods

2.1. Materials

Bovine hemoglobin, bovine serum albumin, fluorescent dyes, viz., ANS, CR, ThT and NaN_3 were purchased from Sigma (St. Louis). Sodium phosphate dibasic and monobasic salts were purchased from SRL (Mumbai, India). All other reagents were of analytical grade.

2.2. Stock preparation and protein concentration determination

Hemoglobin was dissolved in 20 mM sodium phosphate buffer pH 7.2 to make a stock solution of 6 mg/ml and dialyzed against the same buffer. The concentration of protein was determined by using molar extinction coefficient (at 405 nm) of 179 mM^{-1} per heme (Hb) on Shimadzu UV-1700 spectrophotometer. The path length of the cuvette was 1 cm.

2.3. Effect of BSA on hb

Hb samples, using a stock solution of 6 mg/ml in varying concentration, i.e. 0–100 g/l of BSA were prepared in 20 mM phosphate buffer, pH 7.2 then incubated for 64 h at 37.0 °C, the incubated samples were diluted to make the final concentration of Hb to 3 μM due to the instrument limitations. Three replicates for all the samples were analyzed for results. All the experiments were performed in 20 mM phosphate buffer, pH 7.2.

2.4. Turbidity assay measurements

Turbidity assay was performed on Shimadzu UV-1700 spectrophotometer in a cuvette of 1 cm path length. The turbidity of native Hb and also with different concentrations of BSA were determined by monitoring the change in the absorbance at 350 nm. Before measurement the samples were incubated at 37.0 °C for respective time.

2.5. Intrinsic fluorescence measurements

The fluorescence spectra were recorded on a Shimadzu RF-5301 spectrofluorophotometer (Tokyo, Japan) using 1 cm path length quartz cell. The excitation wavelength was 280 nm and the emission range was set from 300 to 400 nm [23]. The final concentration of Hb was 3 μM.

2.6. Soret absorption spectroscopy

Soret absorption spectra of the heme group was taken with the help of Shimadzu UV-1700 Spectrophotometer with 1 cm path length of cell. The final concentration of Hb was 3 μM. the spectra was taken in the range of 400–700 nm.

2.7. 8-Anilino-1-naphthalenesulphonic acid (ANS) fluorescence measurements

Fluorescence spectra were performed using a Shimadzu (RF-5301 PC) fluorescence spectrophotometer. Post-incubation, the protein samples were supplemented with ANS with the ratio of 1:20 (protein:ANS), and further incubated for 30 min in the dark chamber. The final concentration of Hb was 3 μM The excitation wavelength was 380 nm and the emission range was 400–600 nm [24].

2.8. Attenuated total reflection fourier transform infrared spectroscopy (ATR-FTIR) measurements

ATR-FTIR spectra were recorded with Interspec 2020 FTIR spectrometer in deuterated water. Protein concentration was 30 μM. The scanning wave number was set from 1000 to 4000 cm^{-1} [25].

2.9. Thioflavin t (ThT) assays

After incubation the samples were supplemented with ThT solution with a ratio of 1:5 and incubated in the dark chamber for 30 min. The protein concentration was 3 μM. The excitation wavelength was set at 440 nm and the emission spectra was recorded with in the range of 450–600 nm [26].

2.10. Congo red (CR) assays

The aggregates formation was analyzed with congo red dye by measuring the absorbance in the range of 400–700 nm. The spectra was recorded using Shimadzu UV-1700 spectrophotometer. 0.2 mg/ml protein samples were mixed with a solution containing 20 μM congo red dye dissolved in 20 mM sodium phosphate buffer [27].

2.11. Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry performed at 27.0 °C to analyze the thermodynamic parameters. The Hb was titrated with BSA using a VP-ITC titration micro calorimeter (MicroCal Inc., Northampton, MA) to examine the energetics of the binding. Prior to the titration experiment, the protein samples and buffer were degassed in a thermovac. The sample cell of the calorimeter was loaded with 1.43 ml Hb solution (20 μM) in sodium phosphate buffer (20 mM, pH 7.2) and reference cell with 20 mM sodium phosphate buffer pH 7.2. Titration was carried out using 295 μl syringe filled with BSA solution, with stirring at 242 rpm. The concentration of BSA was 300 μM, injections were started after baseline stability had been achieved. Titrations were performed by adding an aliquot of 5 μl for the first injection and aliquots of 10 μl for the following injections with 3-min interval between injections. MicroCal ORIGIN was used to determine the site binding model [28,29].

2.12. Transmission electron microscopy (TEM)

The ultrastructure of protein aggregates was observed using transmission electron microscopy. Samples of native hemoglobin (Hb) and Hb supplemented with 80 g/l of bovine serum albumin (BSA) were incubated for 64 h and was placed on a carbon coated