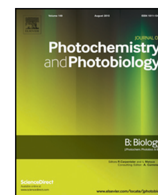
Journal of Photochemistry & Photobiology, B: Biology 169 (2017) 7–12

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

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



Aggregation of trypsin and trypsin inhibitor by Al cation

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ARTICLE INFO

Article history:
Received 17 December 2016
Received in revised form 16 February 2017
Accepted 21 February 2017
Available online 24 February 2017

Keywords:
Al cation
Trypsin
Trypsin inhibitor
Aggregation
AFM
Thermodynamic
Spectroscopy

ABSTRACT

Al cation may trigger protein structural changes such as aggregation and fibrillation, causing neurodegenerative diseases. We report the effect of Al cation on the solution structures of trypsin (try) and trypsin inhibitor (tryi), using thermodynamic analysis, UV–Visible, Fourier transform infrared (FTIR) spectroscopic methods and atomic force microscopy (AFM). Thermodynamic parameters showed Al-protein bindings occur via H-bonding and van der Waals contacts for trypsin and trypsin inhibitor. AFM showed that Al cations are able to force trypsin into larger or more robust aggregates than trypsin inhibitor, with trypsin 5 ± 1 SE ($n = 52$) proteins per aggregate and for trypsin inhibitor 8.3 ± 0.7 SE ($n = 118$). Thioflavin T test showed no major protein fibrillation in the presence of Al cation. Al complexation induced more alterations of trypsin inhibitor conformation than trypsin.
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1. Introduction

Extensive studies have been reported on the effect of aluminum on human health and disease [1]. Al accumulation in tissues and organs results in their dysfunction and toxicity, which can be correlated with the local concentration of the Al cation [2–4]. Al is known to induce formation of beta-sheet-rich fibrils that disrupt tissue structure and cause disease. Although not fully proven, Al accumulation in the brain is proposed to be associated with neurodegenerative diseases, including Alzheimer's dementia, Parkinson's disease [3]. It has been shown that metal ions including Al cation may trigger protein structural changes such as aggregation and fibrillation [5,6]. This study was designed to determine the effect of Al cations on the structural transformations of trypsin and trypsin inhibitor.

Trypsin a water soluble globular protein is a proteolytic enzyme that cleaves peptide bonds at the carboxylic groups of arginine and lysine [7]. Trypsin inhibitors are classified as small proteins or polypeptides that exhibit inhibitory activity against trypsin and can lead to certain diseases in animals and humans [8]. The inhibitory role of trypsin inhibitors comes from their bindings to trypsin and other proteins, causing major protein structural changes [9,10]. The hydrophobic and hydrophilic characteristics of trypsin and trypsin inhibitor are well known and their effects on enzyme-substrate interactions have been investigated [11–16]. Trypsin inhibitor with a large hydrophobic region showed different affinity than trypsin towards ligand interactions. Reports show hydrophobicity plays a major role in protein-protein and protein-polymer interactions [10–17].

We report the results of thermodynamic analysis, spectroscopic studies and AFM imaging for Al cation complexation with trypsin and trypsin inhibitor in aqueous solution at physiological conditions. Structural information regarding Al-protein binding and the effect of cation interaction on protein aggregation and fibrillation are presented here.

2. Experimental Section

2.1. Materials

Trypsin from bovine pancreas (MW = 23.8 kDa) and trypsin inhibitor type-1S (MW = 24 kDa) from glycine, max soyabean were purchased from Sigma Chemical Company (St-Louis, MO) and used as supplied. Hydrated $AlCl_3$ was from Aldrich Chemical Co and used as supplied. Other chemicals were of reagent grades.

2.2. Preparation of Stock Solutions

Solutions of trypsin (in H_2O) and trypsin inhibitor (in ethanol/H_2O 25/75%) 120 μM were prepared and diluted to various concentrations in 10 mM Tris-HCl (pH 7.4). Hydrated $AlCl_3$ was dissolved in 10 mM of Tris-HCl solution (pH 7.4) and diluted to various concentrations.

Abbreviations: Try trypsin, Tryi, trypsin inhibitor; FTIR, Fourier transform Infrared; AFM, atomic force microscopy.

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http://dx.doi.org/10.1016/j.jphotobiol.2017.02.018
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2.3. AFM Imaging

1:1 mixtures of aluminum chloride and trypsin and trypsin inhibitor at a concentration of 120 μM in 10 mM Tris-HCl, pH 7.4 were diluted 10 times in nanopure water. Freshly cleaved mica disks around 1 cm in diameter were incubated with 30 μl of sample solution for 1 min and washed thoroughly with several milliliters of nanopure water. The mica disks were then dried with compressed nitrogen and imaged in AC mode with an Agilent 5500 atomic force microscope (Keysight, USA). We used NTESP silicon cantilevers (Bruker, USA) with a typical resonant frequency around 300 kHz and a nominal tip radius of 10 nm. Images were acquired at a scan rate of 1 Hz and analyzed using the free software Gwyddion. For the measurement of particle sizes, the particles were marked using a height threshold of 0.5 nm and the pixel size was 1 nm for all the images used. In order to remove false-positive particles we only present results for particles with an equivalent radius above 3 nm.

To estimate the number of proteins per aggregate based on their dry volume, we used the molecular weight of trypsin and trypsin inhibitor to estimate their hydrated volume assuming a protein density of 1.37 g/cm^3. Assuming all the water leaves the protein upon drying which is an over simplification, the dry volume should be 37% of the wet volume, which gives us 10.7 nm^3 for the trypsins.

2.4. UV–Visible Spectroscopy

The UV–Vis spectra were recorded on a Perkin-Elmer Lambda spectrophotometer with a slit of 2 nm and scan speed of 400 nm min^{-1}. Quartz cuvettes of 1 cm were used. The absorbance measurements were performed at pH 7.4 by keeping the concentration of protein constant (60 μM), while increasing Al cation concentrations (1 μM to 60 μM). The binding constants of Al-protein adducts were obtained according to the published method [18,19].

2.5. FTIR Spectroscopic Measurements

Infrared spectra were recorded on a FTIR spectrometer (Impact 420 model), equipped with deuterated triglycine sulphate (DTGS) detector and KBr beam splitter, using AgBr windows. Solution of Al cation was added dropwise to the protein solution with constant stirring to ensure the formation of homogeneous solution and to reach the target Al concentrations of 15, 30 and 60 μM with a final protein concentration of 60 μM. Spectra were collected after 2 h incubation of trypsin and trypsin inhibitor with Al solution at room temperature, using hydrated films. Interferograms were accumulated over the spectral range 4000–600 cm^{-1} with a nominal resolution of 2 cm^{-1} and 100 scans. The difference spectra [(protein solution + Al solution) − (protein solution)] were generated using water combination mode around 2300 cm^{-1}, as standard [20]. When producing difference spectra, this band was adjusted to the baseline level, in order to normalize difference spectra.

Analysis of the secondary structures of trypsin and trypsin inhibitor and their Al complexes were carried out on the basis of the procedure previously reported [21,22]. The curve-fitting analysis was performed using the GRAMS/AI Version 7.01 software of the Galactic Industries Corporation.

2.6. Fluorescence Spectroscopy

Fluorimetric experiments were carried out on a Varian Cary Eclipse. Solution containing thioflavin T 25 μM in Tris-HCl (pH = 7.4) was prepared at room temperature and maintained at 24 °C. Solutions of trypsin and trypsin inhibitor containing 50 μM in 10 mM Tris-HCl (pH = 7.4) were also prepared at 24 °C. The fluorescence spectra were recorded at excitation 450 nm and emission from 480 to 520 nm.

3. Results and Discussion

3.1. AFM Analysis and Protein Aggregation by Al Cation

We observed the presence of aggregates on the mica surface for Al-protein complexes (Fig. 1). A protein concentration of 1.2 μM was used for incubation to limit unwanted crowding of the aggregates on the surface. As a result we were able to measure the average volume of the protein aggregates and estimate the number of protein per aggregates assuming that all the water is removed upon drying. For trypsin we obtained 5 ± 1 SE ($n = 52$) proteins per aggregate and for trypsin inhibitor 8.3 ± 0.7 SE ($n = 118$) (Fig. 1A–C). These numbers clearly indicate that aluminum ions are able to force trypsin and trypsin inhibitor into large aggregates. The difference between trypsin and trypsin inhibitor is the presence of elongated aggregates in the former but not in the latter (Fig. 1A–C).

3.2. Stability of Al-Protein Complexes by UV–Visible Spectroscopy

Al-protein binding results in changes in the absorption spectra of the protein, and the observed changes can be used to calculate the Al-protein binding constants [18,19]. The UV spectra of Al-protein complexes are presented in Fig. 2. Al-protein complexation occurred with a decrease in the intensity of trypsin and trypsin inhibitor absorption band at 280 nm (Fig. 2).

The Al-protein binding constants were calculated as described earlier in materials and methods [18], using plots of $1/(A_0 - A)$ vs (1/Al concentrations) (Fig. 2). The double reciprocal plot is linear and gives the overall binding constant for each complex with $K_{\text{Al-trypsin}} = 5.24 (\pm 0.5) \times 10^3 M^{-1}$ and $K_{\text{Al-trypsin inhibitor}} = 7.48 (\pm 0.9) \times 10^4 M^{-1}$ (Fig. 2 and Table 1). The calculated binding constants show stronger affinity for Al trypsin inhibitor than trypsin and this correlate well with protein hydrophobicity. The increase of protein surface hydrophobicity in trypsin inhibitor, results in an increase of the binding affinity for Al-protein complexes (Fig. 2 and Table 1). However, evidence regarding hydrophobic, hydrophilic or H-bonding contacts come from the thermodynamic analysis of Al-protein complexes discussed below.

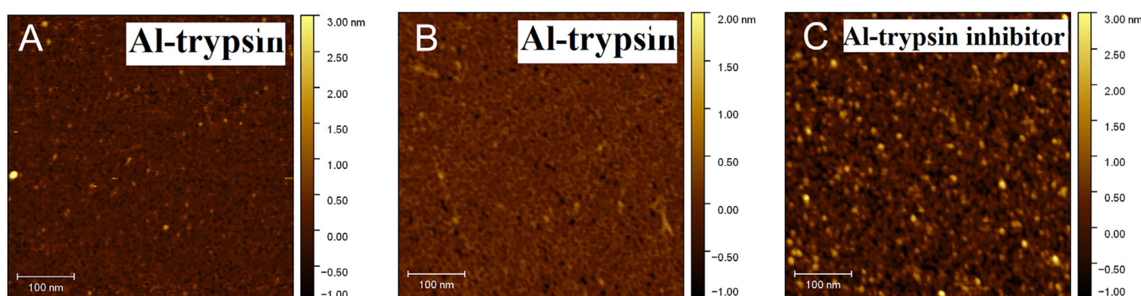

Fig. 1. AFM height images of the trypsin and trypsin inhibitor aggregates induced by aluminum cations, A and B) trypsin, C) trypsin inhibitor.

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