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Conformational behavior of alpha-2-macroglobulin: Aggregation and inhibition induced by TFE

Ahmed Abdur Rehman[a], Masihuz Zaman[b], Mohammad Khalid Zia[a], Haseeb Ahsan[c], Rizwan Hasan Khan[b], Fahim Halim Khan[a,*]

[a] Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh 202002, India
[b] Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, India
[c] Department of Biochemistry, Faculty of Dentistry, Jamia Millia Islamia, New Delhi, India

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ABSTRACT

Alpha-2-macroglobulin (α_2M), a pan-proteinase inhibitor, inhibits a variety of endogenous and exogenous proteinases and constitutes an important part of body's innate defense system. In the present study, we explored how trifluoroethanol (TFE) may modulate the structure, antiproteinase activity and aggregation of α_2M. TFE was sequentially added over a range of 0–20% (v/v) and the effects induced were studied by activity assay, intrinsic fluorescence, ANS fluorescence, circular dichroism, turbidity assay, Rayleigh scattering measurement and ThT fluorescence measurement. Decrease in activity and increase in fluorescence intensity of α_2M upon addition of TFE shows structural deviation from the native structure and suggests aggregation of protein upon solvent addition. Increase in turbidity and Rayleigh scattering of modified α_2M confirms the formation of aggregates. Insignificant ThT fluorescence intensity of TFE treated α_2M is indicative of amorphous or non-amyloid aggregation. Further, circular dichroism results indicate the changes in secondary structure of native α_2M as negative ellipticity decreased on addition of the polar solvent to the inhibitor. The turbidometric analysis, Rayleigh scattering, ThT fluorescence intensity of modified α_2M suggests that the protein might be driven towards non-amyloid or amorphous aggregation. Our studies provide important mechanistic insight how α_2M undergoes conformational and functional changes when exposed to TFE.

1. Introduction

Alpha-2-macroglobulin (α_2M) is an important component of innate immune system and has been evolutionarily conserved in the vertebrate species over half a billion years [1]. α_2M has many diversified and complex functions, but it is primarily known for its ability to inhibit virtually any proteinase by virtue of its unique mechanism of trapping of proteinase without the direct blockage of its active site. Due to the presence of multiple reactive sites, it is involved in a number of functions like binding, transportation and targeting of small molecules [2]. Apart from being a key player in antiproteinase barrier, α_2M binds and regulates the activity of a number of proteins such as, chymase, transferrin, prostate-specific antigen, defensin, etc [3–6]. It also acts as binding, carrier and targeting protein for a large number of biologically important cytokines and hormones [2]. It is also involved in metal ions binding [7] and is used as biomarkers in prognosis and diagnosis of a number of diseases such as liver fibrosis [8], myocardial infracted diabetic patients [9], HIV patients with cardiac manifestations [10], etc.

Trifluoroethanol (TFE), the simplest alcohol with a CF3 group (Supplementary Fig. S1), and its derivatives are most commonly used as anesthetics [11]. It is known for its toxicity to blood components, male reproductive system, brain, upper respiratory tract and eyes, in rats [12]. TFE has been found very effective in inducing conformational changes and protein denaturation. It decreases solvent polarity which weakens solvophobic interactions and simultaneously increases electrostatic interactions thereby stabilizing local secondary structures. At molecular level, TFE stabilize the secondary forms in helical, β-turn and β-hairpin structures of the peptides and proteins, while disrupting the tertiary structure of the native proteins [13]. The strengthening of intermolecular H-bonds in proteins is large enough to account for the increase in aggregate propensities in the presence of TFE. TFE induced fibrils

* Corresponding author.
E-mail addresses: fahimhkhan@rediffmail.com, fahimhalimkhan@hotmail.com, fahimhalimkhan@gmail.com (F.H. Khan).

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formed are β-sheet rich and resemble aggregates formed by protein α-synuclein involved in Parkinson's disease [14].

In this study, we explored the effect of TFE on one of the key antiproteinase of blood i.e. α_2M. We used battery of techniques like, intrinsic fluorescence, turbidity assay, Rayleigh scattering measurements, ANS and ThT binding, far-UV circular dichroism, TEM, etc. and demonstrated for the first time that α_2M was inactivated and aggregated in the presence of increasing concentration of this organic solvent – TFE. We further demonstrated TFE induced structural changes and induced amorphous aggregation of this key antiproteinase cum chaperon.

2. Materials and methods

2.1. Materials

Sodium phosphate monobasic and dibasic were purchased from SRL (Mumbai, India). Trifluoroethanol, 8-anilino 1-naphthalene sulphonic acid (ANS) and thioflavin-T (ThT) were obtained from Sigma-Aldrich, India. All other reagents used were of analytical grade.

3. Methods

3.1. Purification of α_2M

α_2M was isolated from sheep plasma by the method of Rehman et al. [15]. Fresh sheep blood was routinely collected at slaughter (within a few minutes after slaughtering) into bottles containing 1/10 vol of acid citrate dextrose containing 1 mM PMSF/STI (50 mg/l of blood). Sheep α_2M was purified in high yield by a simple two-step procedure. Sheep plasma was fractionated with ammonium sulphate and the fraction precipitating between 20% and 40% saturation was extensively dialyzed against 50 mM sodium phosphate buffer, pH 7.4, containing 50 mM KCl. This was subsequently chromatographed on Sephacryl-S300 HR column (100 × 1.5 cm) in the same buffer. The fractions containing inhibitory activity against trypsin were pooled and concentrated. The preparation thus obtained was electrophoresed both in the presence and absence of SDS. All purification steps were performed at 4 °C.

3.2. Preparation of samples for biophysical studies

Purified α_2M (1 mM) in sodium phosphate buffer (pH 7.4), was incubated separately with varying concentrations of trifluoroethanol (2–20% v/v) for 2 h at 37 °C, before any spectroscopic measurements were performed. All the experiments were performed in triplicates before analysis for the final result.

3.3. Assay of α_2M antiproteinase activity

The ability of α_2M to protect trypsin from inhibition by soybean trypsin inhibitor (STI) was used to quantitate the activity of inhibitor as described by Gollas-Galván et al. [16]. The activity of α_2M was measured at the end of its incubation period with TFE. The activity of untreated α_2M was taken as reference.

3.4. Intrinsic fluorescence measurements

The fluorescence spectra were recorded with a Shimadzu RF-5301 spectrofluorophotometer (Tokyo, Japan) in a quartz cell of path length 1 cm. The excitation wavelength was 280 nm and the emission was recorded in the range of 300–400 nm [17]. The final concentration of α_2M was 10 μM.

3.5. Turbidimetric aggregation analysis

Turbidity assay was performed as an indicator to suggest the formation of protein aggregates [18]. Absorbance of the native and TFE modified protein samples was recorded at 350 nm on a UV–vis spectrophotometer (UV-VIS 1700 Shimadzu, Japan) in a cuvette of 1 cm path length. The concentration of the protein was 10 μM.

3.6. Rayleigh scattering measurements

Rayleigh scattering measurements were carried out on Shimadzu RF-5301 spectrofluorophotometer (Tokyo, Japan), in a quartz cell of path length 1 cm. The protein samples were excited at 350 nm and the emission spectra were recorded from 300 to 400 nm. Fluorescence intensities at 350 nm were plotted. Both excitation and emission slits were fixed at 3 nm. The final concentration of protein in the samples was 10 μM.

3.7. Thioflavin T (ThT) fluorescence assay

ThT fluorescence was measured to monitor the formation of amyloid fibrils of the treated α_2M. The samples were excited at 440 nm and the emissions were recorded from 450 to 600 nm, and the path length was 1 cm. ThT was prepared in 50 mM sodium phosphate buffer, pH 7.4. The concentration of α_2M in the sample was 10 μM while the concentration of ThT was 40 μM [19].

3.8. ANS (8-anilino 1-naphthalene sulphonate) fluorescence measurement

Binding of ANS to α_2M was measured by fluorescence emission spectra with excitation at 380 nm and emission was recorded from 400 to 600 nm. ANS concentration was 100 molar excess of protein concentration, and concentration of α_2M was 10 μM [20].

3.9. Circular dichroism measurements

CD measurements were carried out in a JASCO spectropolarimeter (J-815) calibrated with ammonium D-10-camphorsulphonic acid. Spectra were collected between 190 and 250 nm in a cell of path length 1 mm. Spectra were collected with a scan speed of 100 nm/min and a response time of 2 s. Each spectrum was an average of 3 scans. Concentration of α_2M used for the scans was 10 μM. All spectra were smoothed by the Savitzky-Golay method with 11 convolution width.

3.10. Transmission electron microscopy (TEM)

The micro-architecture of α_2M aggregates was observed using transmission electron microscopy (TEM). Protein sample was placed on a carbon coated copper grid and left to absorb for 1 min. Afterwards the grid was washed with distilled water and air dried. This grid was further negatively stained with (2%, w/v) aqueous solution of uranyl acetate for 45 s. Excess stain was removed and the samples were then left to dry. The samples were then analyzed using JOEL JEM-2100 (Japan) transmission electron microscope operating at 200 kV. The protein concentration was 20 μM.

4. Results

4.1. Antiproteinase activity

The effect of organic solvent TFE on antiproteinase activity of α_2M was evaluated as is shown in Fig. 1. The native antiproteinase, in the absence of organic solvent, showed maximum activity and was taken as reference. On increasing the concentration of TFE, the