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Identification of a new alpha-2-macroglobulin: Multi-spectroscopic and isothermal titration calorimetry study

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ABSTRACT

A α_2M homologue was isolated from sheep (*Ovis aries*) blood plasma, using a simple two-step procedure, ammonium sulphate fractionation and gel filtration chromatography. Sheep α_2M was found to be a large tetrameric glycoprotein of 630 kDa with monomeric subunit of 133 kDa each. Each subunit of sheep α_2M was found to be made up of two fragments of 102 and 31 kDa respectively. The proteinase inhibitor from sheep was found to have Stokes radius of 79 Å, which makes it much more compact than its human homologue. It entraps only 1 mol of trypsin per mole of inhibitor, like its caprine counterpart. The use of isothermal titration calorimetry has become gold standard for exploring thermodynamics of binding interactions. In this study, binding interaction of trypsin with alpha-2-macroglobulin is studied using ITC. The thermodynamic signatures – enthalpy change (ΔH), entropy change (ΔS) and Gibb's free energy change (ΔG), along with number of binding sites (N) and affinity constant (K) are explored for α_2M-trypsin binding for the first time for any known α_2M molecule. The thermodynamics of proteinase-antiproteinase association suggests that trypsin–α_2M interaction is enthalpy driven event.

1. Introduction

Alpha macroglobulins are large glycoproteins which have been conserved throughout evolution in animals [77] and is present in the hemolymph of invertebrates, plasma of vertebrates, and the egg white of birds and reptiles [1–5]. Alpha macroglobulins can be monomeric (α_1-inhibitor 3 in rat and hamster, murinoglobulin in mice) [6,7], dimeric (human PZP, invertebrate alpha-2-macroglobulins) [8,9] or tetrameric (vertebrate alpha-2-macroglobulins) [4,10] structures. Alpha-2-macroglobulin (α_2M) is a prominent member of alpha macroglobulin family and is one of the major proteinase inhibitors of blood plasma of vertebrates [11]. The other members of alpha macroglobulin family such as alpha 1 macroglobulin [12], complement components C3, C4, and C5 [13], and pregnancy zone protein [5] are evolutionarily related to α_2M [14]. C_3, C_4 and α_2M are related by the presence of an internal β-cysteinyl-γ-glutamyl thioester [15,16].

α_2M is a unique proteinase inhibitor as it could inhibit virtually any proteinase, irrespective of its specificity, catalytic mechanism or source of origin [14,17]. Unlike other proteinase inhibitors, α_2M inhibit proteinases in such a way that they retain their peptidase or esterase activity toward low-molecular-weight substrates [18]. Human α_2M is composed of four identical subunits of 180 kDa each, while caprine α_2M is composed of four monomers of 136 kDa each [19] and mouse α_2M is composed of four monomers of 185 kDa each [20]. The α_2M tetramer is composed of a pair of monomeric subunits joined by disulphide bridges to form a dimer. Two such dimers are joined by non-covalent interactions to form a tetrameric molecule [21], that is why α_2M is often called "dimer of dimer". α_2M monomers could be single peptide as in human α_2M or could be cleaved subunits as reported for mouse and caprine α_2M [19,20].

In each subunit of α_2M, near the middle of the polypeptide chain, a unique sequence of amino acids is present which is highly susceptible to cleavage by almost all endopeptidases. This sequence is termed as the "bait" region. Cleavage of bait region triggers a conformational change in the structure of α_2M and consequent entrapment of the proteinase [17,22]. Conformational change in the structure of α_2M also results in the exposure and cleavage of an internal β-cystenyl-γ-glutamyl thiol ester bond which could bind covalently to the entrapped proteinase or other nucleophiles present [16].

The structural transformation following cleavage of the bait region by proteinase results in compact form of α_2M with an increased electrophoretic mobility, called the "fast" form [23]. This

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transformation of α_2M is termed as "slow-to-fast" transformation, because of its behaviour in polyacrylamide gel electrophoresis (PAGE) [24]. The cage formed by α_2M for entrapment of proteinases is a unique molecular structure [25]. The entrapped proteinase remains active and only sterically hindered from the access to the macromolecular substrates.

Apart from inhibition of proteinases, α_2M has many other diversified and complex functions, like binding, transportation and targeting of important molecules and ions. α_2M has a major role in transportation of zinc, binding and regulating the activity of a number of proteins and hormones in the plasma [14]. It also acts as a binding, carrier and targeting protein for many important cytokines, like, TNF, PDGF, IL6, etc [26–28]. α_2M is known for protecting the body from endotoxins, and regulate the apoptosis triggered during bacterial infections [29,30]. α_2M could be used as a marker protein for diagnosis and prognosis of diseases like liver fibrosis [31], myocardial infarcted diabetic patients [32], HIV patients with cardiac manifestations [33], pancreatitis [34], inflammatory bowel disease [35] and prostate cancer [36]. α_2M could also be used as a drug delivery system for vaccines [37,38]. In view of the diverse and immense importance, it would be interesting to investigate this unique antiproteinase in yet another unexplored source.

This is a first attempt to purify and characterise α_2M from sheep (*Ovis aries*) blood plasma. It is also first attempt on any known α_2M to study trypsin–α_2M interaction using ITC. ITC allows the determination of affinity constant (K), stoichiometry (N), free energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS) of binding and hence provides a plethora of information of previously ignored thermodynamic aspect of α_2M-proteinase interaction.

2. Materials and methods

2.1. Materials

Trypsin, soya bean trypsin inhibitor (STI), N-benzoyl-DL-arginine-p-nitroanilide (BAPNA), phenylmethylsulphonylfluoride (PMSF), bovine serum albumin (BSA) and Sephacryl S300HR were obtained from Sigma–Aldrich, India. The chemicals for electrophoresis and for calibration were obtained from Merck. All the other reagents used were of analytical grade commercially available.

2.2. Methods

(a) ***Purification of*** α_2***M***: α_2M was isolated from sheep blood by the method of Khan et al. [19]. Fresh sheep blood was routinely collected at slaughter (within a few minutes after slaughtering) into bottles containing 1/10 volume 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 dialysed against 50 mM sodium phosphate buffer pH 7.4 containing 50 mM KCl. This was subsequently chromatographed on Sephacryl-S-300 HR column (100 cm × 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.

(b) ***Polyacrylamide gel electrophoresis***: SDS-PAGE and native SDS-PAGE using 5% gels were run essentially as outlined by the method of Laemmli [39] using Tris-glycine buffer pH 8.3. SDS-PAGE was run both in the absence and presence of 5% β-mercaptoethanol.

(c) ***Assay of*** α_2***M***: The ability of α_2M to protect trypsin from inhibition by STI was used to quantitate the inhibitor as described by Gollas-Galván et al. [40]. Sheep α_2M was also examined for its ability to interfere with the caseinolytic activity of various proteinases at appropriate pH [41].

(d) ***Methylamine treatment***: To determine the effect of methylamine, purified α_2M in 50 mM sodium phosphate buffer pH 7.4, was incubated with increasing concentration (25 mM to 200 mM) of methylamine for 30 min at 37 °C. The protein was exhaustively dialysed against 50 mM sodium phosphate buffer pH 7.4 and then assayed for residual α_2M activity [42].

(e) ***Stokes radius***: The Stokes radius of sheep α_2M was computed from its elution volume on a calibrated Sephacryl S300 column. The column was calibrated by determining the elution volume of several globular proteins with known Stokes radii. The standard proteins used with their Stokes radii in parenthesis were thyroglobulin (86 Å), apoferritin (59 Å), bovine serum albumin (35.5 Å), ovalbumin (27.3 Å) and soybean trypsin inhibitor (22.6 Å). The data was analysed according to the theoretical treatment of Laurent and Killander [43]. The linear plot between Stokes radii and $[-\log K_{av}]^{1/2}$ was used for calculating the Stokes radius of sheep α_2M.

(f) ***Saturation of*** α_2***M with trypsin***: Sheep α_2M (0.2 nmoles) was incubated in the presence of increasing amount of trypsin (0.01–0.161 nmol) in a total reaction volume of 1900 μl in 50 mM sodium phosphate buffer pH 7.4 at 37 °C. After 15 min, 100 μl of soyabean trypsin inhibitor (0.45 nmol) was added and the incubation was continued for an additional 15 min at 37 °C. 2.0 ml of BAPNA (2.28 μmol) was added and change in absorbance at 410 nm was observed after 30 min. The control contained 0.161 nmol of trypsin, 0.45 nmol of soyabean trypsin inhibitor, 2.28 μmol of BAPNA at the condition described above.

(g) ***Effects of temperature on*** α_2***M activity***: To determine the effect of temperature, purified α_2M was incubated for 30 min at 10 °C intervals from 10 °C to 90 °C, separately and directly put into an ice water bath prior to the activity assay as described above. The activity of the sample incubated at 4 °C was used as a control (100% of relative inhibitory activity).

(h) ***Effects of pH on*** α_2***M activity***: To determine the effect of pH, purified α_2M was added to buffers with the pH adjusted from 1.0 to 11.0, and incubated for 30 min at 37 °C, and then assayed as previously. The antiproteolytic activity at pH 7.4 was used as control (100% of relative inhibitory activity).

(i) ***Determination of carbohydrates***: Total neutral carbohydrate were determined by the phenol/H_2SO_4 method of Dubois et al. [44], using glucose as standard.

(j) ***Determination of –SH groups***: The trypsinisation and methylamine induced appearance of free thiol groups in the α_2M was followed by titration with DTNB as described by Ellman [45]. To the appropriate amount of α_2M was added 0.1 M DTNB in 0.05 M Tris–EDTA buffer pH 8.0 and absorbance measured after 15 min. The appearance of thionitrobenzoate ion was monitored at A_{412} nm in UV-VIS 1700 Shimadzu, Japan, recording spectrophotometer with an extinction coefficient of 13,600 M^{-1} cm^{-1}.

(k) ***Determination of molecular mass***: The molecular mass of native sheep α_2M was computed from its elution volume on a Sephacryl S300 HR column. The column was calibrated by determining the elution volume of several globular proteins whose molecular mass were known. The standard proteins were thyroglobulin (668 kDa), apoferritin (440 kDa), β-galactosidase (116 kDa), hemoglobin (64 kDa) and ovalbumin (45 kDa). The data was analysed according to the theoretical treatment of Andrews [46]. The linear plot between V_e/V_o and $\log M$ was used in calculating the molecular mass of sheep α_2M. The molecular mass of subunits of α_2M was