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Structural and enzymatic properties of Ageritin, a novel metal-dependent ribotoxin-like protein with antitumor activity

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ABSTRACT

Ageritin has been recently described as the first ribotoxin-like from Basidiomycota division (mushroom *Agrocybe aegerita*) with known antitumor activity (BBA 2017, 1861: 1113-1121). By investigating structural, catalytic and binding properties, we demonstrate that Ageritin is a unique ribotoxin-like protein. Indeed, typical of the ribotoxin family, it shows the specific ribonucleolytic activity against the ribosomal Sarcin-Ricin Loop in a rabbit reticulocytes assay. However, it displays several elements of novelty, as this activity is strongly metal-dependent and completely suppressed in the presence of EDTA, different from other representative members of the ribotoxin family. Consistently, we prove that Ageritin is able to bind magnesium ions with low micromolar affinity. We also show that Ageritin is significantly more stable than other ribotoxins in thermal and chemical denaturation experiments. These peculiar features make Ageritin the prototype of a new ribotoxin-like family present in basidiomycetes. Finally, given its high stability, this enzyme is a promising candidate as a new tool in immunoconjugates and nanoconstructs.

1. Introduction

Several interspecific interactions among organisms provide mechanisms for mutual survival [1]. These interactions can be indirect (e.g. shared resources, common enemies) or direct through various possibilities such as predation, herbivory, cannibalism or mutualism; all necessary to increase the fitness of the individual organism or an entire species [2]. In many cases such as host-parasite interactions these mechanisms have evolved at the molecular level [3]. Indeed, this bidirectional communication between parasites and their hosts includes an interweaving between parasite survival strategies and host defence mechanisms that involve the synthesis of secondary metabolites or proteins/enzymes [4]. The target of these metabolites or specific enzymes is often the ribosome, the complex macromolecular machinery responsible for protein synthesis and the main target also of antibiotics [5–7]. However, whereas secondary metabolites like antibiotics, bind the ribosome with high affinity and interfere with protein synthesis, in specific stages (initiation, elongation or termination) [8,9], enzymes directly damage ribosomes. In particular, enzymes such as colicin E3 [10], ribosome inactivating proteins (RIPs) [11] and ribotoxins [12] are able to damage the rRNA portion of ribosomes, thus interfering with the binding of elongation factors.

The cytotoxin colicin E3 specifically cleaves 16S rRNA of the 30S bacterial ribosomal subunit causing the inhibition of translation. Instead, RIPs and ribotoxins interact with a universally conserved sequence of 28S rRNA of the large 60S eukaryotic ribosomal subunit, known as Sarcin-Ricin Loop (SRL) [13], thus interfering with the association of elongation factors. RIPs are rRNA N-β-glycosidases that cleave a single N-glycosidic bond in the SRL region [14], while ribotoxins, belonging to RNases, cleave a specific phosphodiester bond in the same region [15].

Ribotoxins are a family of toxic extracellular fungal RNases [12] and the well-known prototype is α-Sarcin isolated from *Aspergillus* genus. They are basic proteins with a molecular mass of about 17 kDa with a high degree of identity, including two disulphide bridges conserved along the whole family [16]. More recently, several authors have reported the characterization of α-sarcin-like ribotoxins from various fungal species (e.g.: Hirsutellin A and Anisoplin, produced by the entomopathogenic fungi *Hirsutella thompsonii* and *Metarhizium anisopliae*, respectively [16–18] belonging to Ascomycota phylum). Hirsutellin A

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and Anisoplin have been exhaustively characterized as well as α-Sarcin. Interestingly, they show different structural and functional features, likely as a result of a different evolutionary pressure in Ascomycota phylum [19]. In particular, they show low sequence identity (about 25%) and are significantly smaller (130 aa) than the previously known ribotoxins, typically composed of 149/150 amino acids [20]. Besides their attributed biological function as defence proteins, ribotoxins have been also studied as part of immunotoxins for cancer therapy [20].

The recent discovery of a novel ribotoxin, Ageritin, the first identified in the basidiomycete mushroom *Agrocybe aegerita,* suggests that ribotoxins are more widely distributed among fungi than previously believed [21]. Despite its different amino acid composition [21], Ageritin is able to release the specific α-fragment from eukaryotic ribosomes in yeast lysates, thus confirming it is a ribotoxin. Moreover, it displays interesting additional functional properties as its cytotoxicity against central nervous system model cell lines (SK-N-BE(2)-C, U-251 and C6) [21], that make this enzyme an important candidate as an antitumor agent. In this work, we analyse similarities and differences in conformational properties, thermal and chemical stabilities, as well as the enzymatic activity of Ageritin compared to the prototype ribotoxins α-Sarcin and Hirsutellin A. Altogether, our data evidence that Ageritin is a completely new ribotoxin, with different structural and functional features.

2. Materials and methods

2.1. Purification of Ageritin

Native Ageritin was purified from *Agrocybe aegerita* (V. Brig.) Singer mushrooms as described previously using a general protocol for the preparation of basic proteins [21,22].

2.2. Analytical procedures

Homogeneity and relative molecular weight of protein were determined by SDS-PAGE with a Mini-Protean II mini-gel apparatus (Bio-Rad; Milan, Italy), using 6% (w/v) stacking polyacrylamide gel and 12 or 15% (w/v) separation gel with or without 2-mercaptoethanol [23].

Ageritin was *S*-pyridylethylated with 4-vinylpyridine as previously reported [24]. Prior to MALDI-TOF mass spectrometry analysis both native and modified proteins were desalted by RP-HPLC using a C-4 column (0.46 × 15 cm; Alltech, Italy). Elution was obtained by a linear gradient of solvent B (solvent A: TFA 0.1%; solvent B: acetonitrile + TFA 0.1%) from 5.0 to 65%, over 60 min, at a flow rate of 1 mL/min, monitoring at 214 nm. The relative molecular masses (M*r*) of proteins were determined using a MALDI-TOF micro MX spectrometer (Waters, Manchester, UK) in linear mode as previously reported [25]. The protein concentration was determined through the BCA colorimetric assay (Pierce, Rockford, IL, USA) using BSA as protein standard.

2.3. Circular dichroism

To analyse the conformational state of Ageritin, far and near-UV CD spectra were registered at 20 °C in 20 mM sodium phosphate buffer at pH 7.5. All CD spectra were recorded with a Jasco J-810 spectropolarimeter equipped with a Peltier temperature control system (Model PTC-423-S), using a quartzs cuvettes. To avoid the formation of bubbles, all buffers were degassed and the cuvette was fully filled up. Molar ellipticity per mean residue, [θ] in deg. $cm^2 \cdot dmol^{-1}$, was calculated from the equation: $[\theta] = [\theta]obs \cdot mrw \cdot (10 \cdot l \cdot C)^{-1}$, where [θ]obs is the ellipticity measured in degrees, mrw is the mean residue molecular mass (108.2 Da), C is the protein concentration in $g \cdot L^{-1}$ and l is the optical path length of the cell in cm. Far-UV measurements (195–250 nm) were carried out at 20 °C using a 0.1 cm optical path length cell and a protein concentration of 0.2 $mg \cdot mL^{-1}$. Thermal denaturation was investigated by recording the CD signal at 208 nm and a heating rate of 1 °C/min. Near-UV experiments (250–320 nm) were carried out at 20 °C using a cuvette with 0.5 cm optical path and a protein concentration of 5.0 $mg \cdot mL^{-1}$.

The GuHCl-induced denaturation curves, at fixed constant temperature of 20 °C, were obtained by recording the CD spectra after an overnight incubation of independent protein samples with increasing concentrations of GuHCl, up to 7.0 M. The signal al 222 nm was also followed as a function of GuHCl concentration, to estimate the Cm.

2.4. Light scattering experiments

A MiniDAWN Treos spectrometer (Wyatt Instrument Technology Corp.) equipped with a laser operating at 658 nm was used connected on-line to a size-exclusion chromatography or in batch mode (*off-line*). Purified protein was analysed by size-exclusion chromatography connected to a triple-angle light scattering detector equipped with a QELS module (quasi-elastic light scattering) for mass value and Rh measurements. 500 μg of sample was loaded on a S75 10/30 column, equilibrated in 20 mM Tris·Cl (pH 7.5) and 200 mM NaCl. A constant flow rate of 0.5 mL/min was applied. Elution profiles were detected by a Shodex interferometric refractometer and a mini Dawn TREOS light scattering system. Data were analysed by using Astra 5.3.4.14 software (Wyatt Technology, Toulouse, France).

2.5. Enzymatic assays

All procedures were carried out using RNase-free materials and reagents. The specific ribonucleolytic activity of Ageritin was assayed on rabbit ribosomes in different buffer conditions and including α-Sarcin and Hirsutellin A as model ribotoxins. α-Sarcin and Hirsutellin A were obtained from their natural sources: *Aspergillus giganteus* MDH18894 and *Hirsutella thompsonii* var. thompsonii HTF72 respectively following previously described procedures [17,26].

Proteins (6, 60 and 600 nM) were assayed against ribosomes contained in a Promega rabbit cell-free reticulocyte lysate for 15 min at 25 °C [27]. Then RNA was phenol-extracted, precipitated with isopropanol and visualized by ethidium bromide staining after electrophoresis on denaturing 2% agarose gels as described [17,26,28]. The specific ribonucleolytic activity releases a 400-nt fragment (α-fragment) from the 28S rRNA. Band intensities were quantitated with QuantityOne Software (Bio-Rad Laboratories) and percentage of specific cleavage was expressed as: α/18S, considering 100% the α-fragment produced by 60 nM Hirsutellin A in buffer with EDTA [16]. Buffer and conditions assayed were 10 mM Tris·Cl pH 7.0, 10 mM KCl and 6 mM α-mercaptoethanol +/− 10 mM $MgCl_2$ and +/− 6 mM EDTA.

2.6. Determination of free sulfhydryl groups

Determination of SH groups was carried out according to Ellman [29] on the protein denatured in the presence of 6 M GuHCl.

2.7. ITC experiments

The interaction of Ageritin with magnesium ions was evaluated by isothermal titration calorimetry (ITC) using a MicroCal iITC200 calorimeter (GE Helthcare, Milan).

The experiment was performed at 25 °C by adding consecutive injections of 2.0 μL aliquots (at 150 s intervals) of magnesium chloride (0.6 mM) solutions to the calorimeter cell (0.280 mL) containing native Ageritin at a concentration of 0.03 mM. To assess the presence of metal ions binding to Ageritin, chelation titrations with EDTA was performed using an EDTA solution at a concentration 20 times that of the protein solution. Prior to further binding studies, Ageritin was dialyzed against 10 mM EDTA (pH 7.8) and then the chelating agent was removed by extensive dialysis against 20 mM Tris·Cl, pH 7.5. To evaluate the binding affinity of magnesium ions to the de-metallated Ageritin, ITC