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Research article

Structural analysis of the interaction between Jaburetox, an intrinsically disordered protein, and membrane models

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ABSTRACT

Jack bean urease is entomotoxic to insects with cathepsin-like digestive enzymes, and its toxicity is mainly caused by a polypeptide called Jaburetox (Jbtx), released by cathepsin-dependent hydrolysis of the enzyme. Jbtx is intrinsically disordered in aqueous solution, as shown by CD and NMR. Jbtx is able to alter the permeability of membranes, hinting to a role of Jbtx-membrane interaction as the basis for its toxicity. The present study addresses the structural aspects of this interaction by investigating the behaviour of Jbtx when in contact with membrane models, using nuclear magnetic resonance and circular dichroism spectroscopies in the absence or presence of micelles, large unilamellar vesicles, and bicelles. Fluorescence microscopy was also used to detect protein-insect membrane interaction. Significant differences were observed depending on the type of membrane model used. The interaction with negatively charged SDS micelles increases the secondary and tertiary structure content of the polypeptide, while, in the case of large unilamellar vesicles and bicelles, conformational changes were observed at the terminal regions, with no significant acquisition of secondary structure motifs. These results were interpreted as suggesting that the Jbtx-lipids interaction anchors the polypeptide to the cellular membrane through the terminal portions of the polypeptide and that, following this interaction, Jbtx undergoes conformational changes to achieve a more ordered structure that could facilitate its interaction with membrane-bound proteins. Consistently with this hypothesis, the presence of these membrane models decreases the ability of Jbtx to bind cellular membranes of insect nerve cord. The collected evidence from these studies implies that the biological activity of Jbtx is due to protein-phospholipid interactions.

1. Introduction

Jaburetox (Jbtx) is an intrinsically disordered polypeptide (IDP) released from Jack bean (*Canavalia ensiformis*) urease (JBU) upon hydrolysis performed by cathepsin-like enzymes present in the digestive system of some insects [1–5]. Both JBU and Jbtx feature potential as insecticides, but the activity of the polypeptide is greater than that of urease and it is effective over a broader spectrum of insect orders, because it does not require prior hydrolysis to be active [6,7].

In 2009, Stanisçuaski and co-workers proposed a model in which Jbtx exerts its entomotoxic role by interacting with receptors at *Rhodnius prolixus* Malpighi tubule's membranes, thus altering the eicosanoid signaling pathway, increasing cGMP levels, and disturbing trans-membrane potential, with consequent diuresis inhibition in this insect model [8]. Recently, the central nervous system of *Triatoma infestans* was demonstrated to be a Jbtx target, with the polypeptide interfering in enzymatic pathways of neuronal tissue, both in vivo and in vitro [9]. Later, Fruttero et al. reported that Jbtx

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affects the defence mechanisms in *R. prolixus* by affecting both the cellular and the humoral immune responses [10]. In addition to insects, the activity of Jbtx against yeasts and filamentous fungi has also been reported, with its fungicidal mechanism of action involving alteration of ions transport, changes in morphogenesis and permeabilization of fungal cells [11].

The biological activities of Jbtx appear to correlate with its ability to interact with cellular membranes, specifically through a protein-lipid interaction that causes the alteration of membrane structures with the possible formation of pores [12–15]. In 2009, Barros and co-workers showed that incubation with Jbtx leads to disruption of large unilamellar vesicles (LUVs) and release of a pre-loaded fluorescent dye [12]. Additionally, Martinelli and co-workers [15] reported that both the N- and the C-terminal half portions of Jbtx were able to interact with lipid vesicles and disrupt them in a similar way as observed for the entire polypeptide.

Recently, the secondary and tertiary structures of Jbtx were established using circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopies [5]. The CD spectrum is typical of an intrinsically disordered protein, a result confirmed by NMR, which further revealed the presence of regions with transient secondary structure, such as a short α-helical motif in the N-terminal region and two turn-like structures, one located in the central region and another near the C-terminal of the polypeptide [5].

Intrinsically disordered proteins (IDPs) are described as molecules with a wide range of folding possibilities. Typically, IDPs are composed of *foldons*, portions of a protein that can be folded independently, and *unfoldons*, regions of the protein that dynamically move from order-to-disorder to be biologically functional [16,17]. This behaviour is classified as (i) *binding-induced folding*, when the disorder-to-order path is absolutely needed to achieve the biological function, (ii) *binding-induced transient folding,* when IDPs are critically involved in signalling interactions, participating in on-off type systems, (iii) *binding-induced folding divergence,* characteristic of proteins with the ability to bind to different partners, (iv) *binding-induced under-folding,* applied to a system in which the connection to the partner generates a partial fold in the IDP, and as (v) *binding and non-folding,* when the interaction is dependent on the flexibility of the protein [17,18].

Many IDPs are known to interact with cellular membranes [19], whether natural or artificial. To mimic cellular membranes, ionic surfactants have been largely and successfully used in the determination of protein structure, mainly for proteins that are unstructured, such as IDPs [20]. Sodium dodecyl sulphate (SDS), when in concentrations above its critical micelle concentration (CMC), can be used as a mimetic membrane due to its ability to influence protein secondary structure similarly to what is observed for natural membranes [20,21].

Due to the remarkable resemblance of lipid bilayers to natural membranes, artificial lipid vesicles have been used as mimetic cellular membranes to provide structural and quantitative information regarding protein-membrane interactions [21,22]. In the past twenty years, bicelles have also emerged as a new class of membrane model for solid-state NMR that provides structural information of hydrophobic molecules in their native state [23,24]. Bicelles are composed of a mixture of phospholipids and detergent. The most used combination is the lipid dimyristoyl-phosphatidylcholine (DMPC) with the detergent dihexanoyl-phosphatidylcholine (DHPC), which can be manipulated in order to control the size and its ability to orientate itself in a magnetic field by controlling the lipid/detergent concentration rate (q-ratio) [23,25]. Small bicelles have been used to evaluate protein structure as well as protein-membrane interaction [20,26].

In this work, we investigated the interaction of the intrinsically disordered Jbtx polypeptide with detergents and lipids (micelles, large unilamellar vesicles and bicelles) at the molecular level, by using CD and NMR spectroscopy, as well as fluorescence microscopy.

2. Materials and methods

2.1. Cell culture and protein purification

The construct containing the Jbtx with a C-terminal His-tag sequence was introduced into a pET-23a vector as described in Postal et al. [11], and the corresponding protein sequence is composed of 100 amino acids (MGPVNEANCK-AAMEIVCRREFGHKEEEDASEGVTTGDPDCPFTKAIPREEYANKYG-PTIGDKIRLGDTDLIAEIEKDFALYGDESVFGGGKVISHHHHHH), as reported in Lopes et al. [5]. This vector was used to transform *Escherichia coli* BL21(DE3) (Novagen, Madison, WI, USA) by heat shock. The expression was performed using a previously described protocol [5]. ^{15}N labelled protein expression was performed by addition of $(^{15}NH_4)_2SO_4$ in M9 medium (Sigma-Aldrich, St. Louis, MO, USA). The purification followed the previously reported protocol [5] with some modifications. In detail, the cellular pellet was harvested by centrifugation (11,000*g* for 10 min at 4 °C) and resuspended in 30 mL of buffer A (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 20 mM Imidazole). Cells were disrupted by three passages of the crude extract through a French pressure cell system (SLM, Aminco, Haverhill, MA, USA) at 20,000 psi. Following the removal of cell debris by centrifugation at 27,200*g* for 40 min at 4 °C, the supernatant was applied onto a Ni(II)-loaded 5 mL His-Trap HP column (GE Healthcare, Little Chalfont, UK) previously equilibrated in buffer A. The affinity resin was washed with 20 column volumes (CV) of buffer A. After this step, the imidazole concentration was increased up to 70 mM in order to remove weakly bound contaminants. The affinity column was then connected in tandem to a Superdex 75 16/60 column (GE Healthcare, Little Chalfont, UK) pre-equilibrated in 50 mM sodium phosphate buffer (NaPB) at pH 7.5, containing 1 mM EDTA and 1 mM TCEP. The two connected columns were eluted, first using 25 mL of buffer A containing 500 mM imidazole, and subsequently using 50 mM sodium phosphate buffer (NaPB) at pH 7.5, containing 1 mM EDTA and 1 mM TCEP (Fig. S1A). The fractions containing Jbtx were collected, concentrated using 3 kDa Amicon Ultra Centrifugal Filters (Merk Milllipore, Darmstadt, Germany) and loaded onto a Superdex 75 10/300 column (GE Healthcare, Little Chalfont, UK), equilibrated with the same buffer at pH 6.5 (Fig. S1B); the final fractions were concentrated as above and stored at −80 °C.

Fractions of all purification steps were analyzed by SDS-PAGE using NuPAGE Novex 12% Bis-Tris gels (Life technologies, Carlsbad, CA, USA) and staining with ProBlue Safe Stain (Giotto Biotech, Sesto Fiorentino, Italy). Protein quantification was performed by the Bradford assay [27] and by absorbance at 280 nm considering a molar extinction coefficient of 4595 M^{-1} cm^{-1} [28]. The final yield was ca. 30 mg of pure protein per liter of culture.

2.2. Preparation of model membranes

2.2.1. SDS micelles

SDS micelles were prepared starting from 300 mM SDS (Sigma Aldrich, St. Louis, MO, USA) stock solutions. The critical micelle concentration (CMC) of SDS in 50 mM NaPB pH 6.5, 1 mM EDTA, 1 mM TCEP and in MilliQ water (control) were estimated by measuring the conductivity at controlled temperature, as previously described [29].

2.2.2. Large Unilamellar Vesicles (LUVs)

Large unilamellar vesicles (LUVs) were prepared by the extrusion method [30,31], using lipids (*10* mg/mL in *chloroform*) purchased from Avanti Polar Lipids Inc. (Alabaster, AL,