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The thermal unfolding of the ribosome-inactivating protein saporin-S6 characterized by infrared spectroscopy

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ABSTRACT

Saporin-S6 is a plant toxin belonging to the type 1 ribosome-inactivating protein (RIP) family. Since it was extracted and isolated from *Saponaria officinalis* for the first time almost thirty years ago, the protein has been widely studied mainly for its potential applications in anti-tumour and anti-viral infection therapy. Like other RIPs, saporin-S6 is particularly effective in the form of immunotoxin conjugated with monoclonal antibodies and its chemico-physical characteristics made the protein a perfect candidate for the synthesis, development and use of saporin-S6-based chimeric toxins. The high stability of the protein against different denaturing agents has been broadly demonstrated, however, its complete thermal unfolding characterization has not already been performed. In this work we analyse in detail structure, thermostability and unfolding features by means of infrared spectroscopy coupled with two-dimensional correlation spectroscopy. Our data showed that saporin-S6 in solution at neutral pH exhibits a secondary structure analogue to that of the crystal and confirmed its good stability at moderately high temperatures, with a temperature of melting of 58 °C. Our results also demonstrated that the thermal unfolding process is non-cooperative and occurs in two steps, and revealed the sequence of the events that take place during the denaturation, showing a higher stability of the N-terminal domain of the protein.

1. Introduction

Ribosome-inactivating proteins (RIPs) are a class of toxins that act on ribosomes in a highly specific way, inhibiting protein synthesis in eukaryotic cells by depurinating a specific region of the 28S rRNA in the 60S subunit. These proteins are mainly produced by plants and to a lesser extent, by fungi, algae and bacteria [1–3]. RIPs are classified into three groups based on their physical properties: Type 1 RIPs, formed by single-chain proteins with a molecular weight in the range of 26–31 kDa; type 2 RIPs (56–69 kDa), consisting of an A-chain with catalytic activity connected by a disulphide linkage to a slightly bigger B-chain with lectin activity; and type 3 RIPs, synthesized as inactive precursors, and after proteolytic processing events, consist of two associated polypeptides of about 16.5 and 8.5 kDa [2,4–8]. RIPs possess several antiviral and anti-proliferative properties [9] and have been employed in popular medicine. For example trichosanthin, a RIP purified from a traditional Chinese herb medicine, has been used as abortifacient, immunosuppressive, anti-tumour and inhibitor against HIV-1 replication [10]. Thus, numerous RIPs have been and still are under investigation for their potential application in therapy of cancer or viral infection, mainly conjugated with monoclonal antibodies for the construction of immunotoxins, and particularly useful are the type I RIPs as they lack the non-specific cytotoxicity shown by the type 2 RIPs.

Among all RIPs, saporin-S6 is one of the most studied because of its strong activity both in cell-free systems and in cell lines and for their demonstrated numerous potential applications [11].

Saporin-S6 belongs to a multigene family of the type 1 RIP group which includes nine different isoforms. Saporin-S6 was isolated from *Saponaria officinalis*, a plant also known as soapwort, and it is the most relevant of the seed isoforms, representing approximatively the 7% of the total seed protein content [12,13]. This protein displays several interesting properties including a potent in vitro anti-tumour activity against cell lines of CD80/CD86 tumours [14], Hodgkin lymphoma [15], breast cancer [16], human bladder tumour [17] and HSP 65 tumours [18]. In addition saporin-S6 was found to be active as antiviral agent against HIV [19], as antimicrobial against *Trypanosoma brucei* and *Leishmania infantum* [20] and for the pain management [21].

Saporin-S6 shares the typical three-dimensional structure of type 1 RIPs and the A-chain of type 2 RIPs, showing a two-domain conformation with a predominantly β-sheet N-terminal domain and an α-helix-rich *C*-terminal domain forming an α + β structure [22]. The

Abbreviations: FTIR, Fourier transform infrared spectroscopy; 2D-COS, two-dimensional correlation spectroscopy; RIP, ribosome-inactivating protein.

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highly conserved active site region is located at the cleft between these subdomains [6]. Saporin-S6 molecular weight is 28.7 kDa and it is composed by 253 amino acids. The overall sequence homology of the protein with other RIPs is quite low, in particular the C-terminal region presents the highest variability [8,23].

The three dimensional structure of saporin-S6 has been already resolved at high resolution and presents a high homology with the characteristic RIP structural motif [22]. The protein shows the N-terminal domain composed by six strands (β1 to β6) arranged in four central antiparallel strands and two parallel strands at the edges. Two α-helices and one 3_{10}-helix are part of the crossover connection between the parallel strands. Otherwise, the C-terminal consists in eight α-helices including two 3_{10}-helices arranged in the canonical geometry and two antiparallel β-strands (β7 and β8) connected by a loop that is shorter than many other RIPs and may contribute to an increased accessibility to the substrate [22,24]. The chemico-physical properties of the protein have been widely studied; saporin-S6 shows an extraordinary resistance to denaturing agents, extreme pH, proteolytic enzymes and those chemical modifications necessary to create the immunotoxin [25,26]. Furthermore, the protein is stable at moderately high temperatures also in the presence of guanidine [25,27]. However, a complete characterization of the thermal unfolding of saporin-S6 has not already been performed. In this study, Fourier transform infrared (FTIR) spectroscopy was employed to investigate in detail the thermal stability and the temperature-induced changes of saporin-S6 at neutral pH. This powerful, sensitive and highly reproducible technique allows identifying structural moieties of proteins on the basis of their infrared absorption and combined with two-dimensional correlation spectroscopy (2D-COS) can provide detailed information on protein thermal unfolding as it is very sensitive to any structural and conformational changes.

2. Materials and methods

2.1. Materials

Deuterium oxide (99.9% ^2H_2O), ^2HCl, NaO^2H, Na_2HPO_4, and NaH_2PO_4 were purchased from Sigma-Aldrich. All other reagents and solvents were commercial samples of the highest purity.

The isoform 6 of saporin (saporin-S6) was purified from homogenates of the seeds of *S. officinalis* according to previously reported procedures [12].

2.2. Preparation of samples for infrared measurements

An amount of 1.5 mg of lyophilized saporin-S6 was dissolved in 200 μl of 50 mM sodium phosphate buffer, prepared in ^2H_2O, p^{2}H 7.4, and concentrated into an approximate volume of 30 μl by centrifugation in an Amicon Ultra-0.5 Centrifugal Filter with Ultracel-10 membrane (Millipore) at 10,000 *g* and at 4 °C. Then, a further 200 μl of the same buffer was added, the sample was centrifuged again, and the solution was re-concentrated. This procedure was repeated 5 times in order to completely hydrate the protein with the chosen buffer. Before the last concentration the protein was incubated overnight at 4 °C in order to maximize the $^1H/^2H$ exchange and then the sample volume was brought to 30 μl used for the FTIR experiment. The final protein concentration and p^{2}H of the sample was 50 mg/ml and 7.4, respectively. The p^{2}H value was measured with a standard pH electrode, and the value was corrected according to $p^2H = pH + 0.4$ [28].

2.3. Infrared spectra

The concentrated protein sample was placed in a thermostated GS20500 cell equipped with CaF_2 windows and 25 μm Teflon spacers used with a water heating jacket cell holder GS20710 (Graseby—Specac Ltd, Orpington, Kent, UK). FTIR spectra were recorded by means of a Perkin-Elmer 1760-x Fourier transform infrared spectrometer using a deuterated triglycine sulfate (DTGS) detector and a normal Beer–Norton apodization function. A total of 32 scans were performed for each spectrum with a nominal resolution of 2 cm^{-1}. A sample shuttle accessory was used to obtain the average background and sample spectra. At least 24 h before and during data acquisition, the sample chamber of the spectrometer was continuously purged with dry air at a dew point of −40 °C. Sample and buffer were scanned between 20 and 85 °C at 5 °C intervals with a 6 min delay between each scan for the stabilization of the temperature inside the cell using an external bath circulator. The temperature of the cell was controlled by a thermocouple placed directly onto the CaF_2 windows. Spectra were collected and processed using the Spectrum software from Perkin-Elmer. Subtraction of ^2H_2O buffer from the sample spectrum was performed interactively removing the ^2H_2O bending absorption close to 1220 cm^{-1} and maintaining a flat baseline between 2000 cm^{-1} and 1300 cm^{-1} [29,30]. Second derivative spectra were calculated over a 9 data-point range (9 cm^{-1}) and the parameters of the deconvoluted spectra were set with a γ value of 2.5 and a smoothing length of 60.

The quantitative analysis of the protein secondary structure was performed by curve fitting of the amide I′ band [31,32] using the peak fitting module of the Origin software (OriginLab Corporation, Northampton, MA 01060, USA). The band shape was set to a Gaussian curve, and the fitting was obtained by iteration in two steps, the first iteration was performed fixing the peak positions as obtained by second derivative and deconvolution, while in the second step the bands were free [33].

2.4. "Phase Diagram" method of FTIR

The phase diagram method is a sensitive approach for the study of protein unfolding/refolding [34]. This method can be applied to detect thermodynamic intermediates, like molten globule state or protein monomerization, and to determine if a denaturation process is cooperative or not. This approach is based on the elaboration of a diagram of $I(\nu_1)$ versus $I(\nu_2)$, where $I(\nu_1)$ and $I(\nu_2)$ are the FTIR absorbance spectral intensity values measured on wavenumber ν_1 and ν_2 under different experimental conditions, in our case at different temperatures, for a protein undergoing structural transformations. Increasing the temperature, the transitions during the denaturation process from the initial to the final state can be detected. A linear phase diagram represents a cooperative denaturation process (all-or-none) with the existence of only two thermodynamic states, the folded and the unfolded. Otherwise, in the presence of a multi-state transition from the initial to the final state that corresponds to a non-cooperative process, a non-linear phase diagram is generated and contains two or more linear portions which reflect individual all-or-none transitions.

2.5. Two-dimensional correlation spectroscopy

Two-dimensional correlation spectroscopy (2D-COS) analysis of saporin-S6 deconvoluted spectra, with heat as the perturbation, was performed as described by Noda [35,36] using the 2DShige software (Shigeaki Morita, Kwansei-Gakuin University, 2004–2006). 2D-COS generates synchronous and asynchronous spectra from a set of dynamic spectra calculated from perturbation-induced dynamic fluctuations of spectroscopic signals. A synchronous spectrum corresponds to the real part of the cross correlation and it represents simultaneous or coincidental changes of spectral intensities at two wavenumber (ν_1 and ν_2 on x- and y-axes respectively), while an asynchronous spectrum corresponds to the imaginary part of the cross correlation and it represents sequential or unsynchronized spectral intensity changes at ν_1 and ν_2. A synchronous 2D map shows auto-peaks and cross-peaks. Auto-peaks are always positive and located in the diagonal; they represent the main changes in spectral intensity. Cross-peaks can be positive or negative and are located at off-diagonal positions; a positive cross-peak indicates that both spectral intensities at corresponding