MST untangles the intricacy of a multimeric protein complex in its native form

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Abstract

Proteins seldom act alone. They interact with a variety of other molecules and also with other proteins to form either homo-oligomers (self-association) or hetero-oligomers (heterologous association). Oligomerization provides diversity and specificity to many cellular pathways and play a central role in the regulation of gene expression, the activity of enzymes, ion channels, receptors, and cell-cell adhesion processes [1, 2]. The biophysical characterization of heterogenous oligomeric complexes is particularly difficult due to the complex nature and quality of the samples, and often the available material is not sufficient for most biophysical methods.

The ribosomal stalk is a heterogeneous pentameric complex directly involved in the regulation of protein translation. This process is often hijacked by toxins like ricin, which stalls translation in the cells by binding to subunits of the ribosomal stalk.

Here we show the versatility of MicroScale Thermophoresis (MST) to study the interaction between a subunit of the ricin toxin and the in-vitro reconstituted ribosomal stalk in its native form. We also compare the MST results to those obtained with other biophysical methods such and isothermal calorimetry (ITC), surface plasmon resonance (SPR) and bio-layer interferometry (BLI). MST allowed the study of this interaction in solution, without the need for immobilization using very little sample.

Introduction

Functional ribosomes are high molecular weight protein complexes responsible for protein synthesis. To fulfill their function as protein synthesis machinery, ribosomes

interact with battery of auxiliary proteins and RNA molecules. One of the essential functional elements of a eukaryotic ribosome is a P-stalk, a pentameric protein complex, composed of well conserved P1, P2 and uL10 proteins. Together with ribosomal uL10, uL11 and the sarcin-ricin loop (SRL), the P1-P2 heterodimers are part of the GTPase Associated Center (GAC) which is involved in recruitment of translational GTPases.

Function of the stalk proteins is related to interaction with auxiliary factors recruited to the GAC. Structurally a C-terminal part domain (CTD) of P1-P2 dimers is very important for the function of the complex [3]. CTD is very well conserved stretch of highly acidic and hydrophobic amino acids (EEEAKEESDDDMGFGLFD) and was shown to be involved in the interaction with translational GTPases and ribosome inactivating proteins (RIPs) [4].

Ricin is a type II RIP consisting of RTA and RTB subunits. RTA removes an adenine from a universally conserved SRL in the 28S rRNA and interacts with P-proteins of the stalk to depurinate SRL both in human and yeast cell [5, 6, 7]. P-proteins of the stalk represent the primary harboring site for ricin, with binding affinities in the low nanomolar range. Moreover, deletion of P-proteins reduces SRL depurination by ricin [8, 9, 10, 16].

In this application note we present a thorough characterization study of ricin's RTA chain interaction with human P1-P2 dimer. We demonstrate how the problem of characterizing the binding affinity between the multimeric assembly P1-P2 and toxin chain was solved with the help of MST. Additionally, we show the analysis with the alternative biophysical techniques and discuss benefits of MST platform in providing solution to similar biological questions.

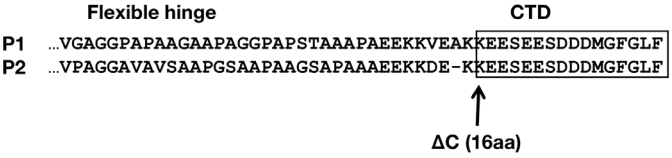
Results

To understand the binding mechanism between P1/P2 heterodimers and RTA binding affinities between RTA and several P1/P2 variants were determined by microscale thermophoresis. The individual proteins and their mutants lacking conserved 16-amino acid residues at the C-terminus, were purified in E.coli and assembled to form heterodimers. SDS-PAGE and native mass-spectrometry were used to confirm correct assembly of heterodimers with resolution of single Dalton in respect to molecular weight of heterodimers (Figure 1).

For MST experiments, RTA was labeled using NHS NTT-647 fluorescent dye according to the manufacturer's protocol. Different P-protein complexes were titrated as ligands at concentrations starting from 50nM up to 100µM. Full-length P1-P2 heterodimer showed highest affinity toward RTA at K_d = 86nM. Deletion of conserved C-terminal fragment of P2 (P1-P2ΔC) resulted in six-fold weaker affinity (K_d =533 nM), when compared to native P1-P2 heterodimer. Interestingly, P2 formed homodimers showed a very weak affinity ~1-2 µM. In case of P1ΔC-P2ΔC we observed binding of this heterodimer with RTA but binding affinity could not be determined precisely, since saturation was not reached at 100 µM of ligand. RTA also did not show any interaction neither with P1ΔC-P2 heterodimer mutant nor P2ΔC-P2ΔC double homodimer mutant (Figure 2).

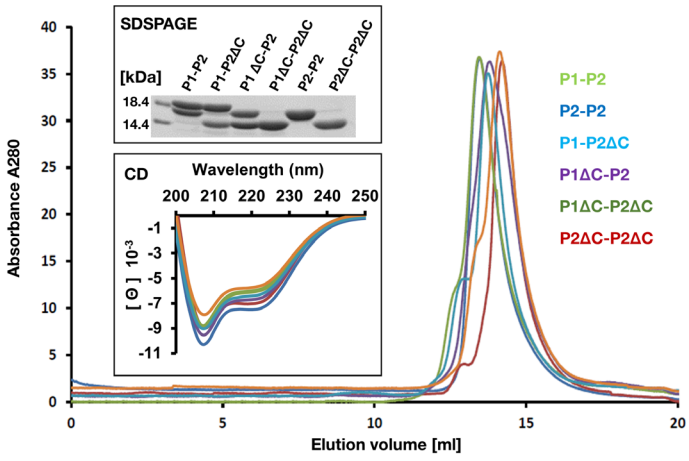
To validate the MST results, experiments using other biophysical methods including isothermal titration calorimetry (ITC), surface plasmon resonance (SPR) and bio-layer interferometry (BLI) were performed Using ITC, we were able to confirm that RTA directly interacts with P1-P2 heterodimer. However, we were not able to measure the K_d for the mutant proteins due to technical limitations of the method. BLI showed that mutations of individual dimer components as well as assembly of functional binding partner as homodimer results in weakening of affinity of the interaction based on BLI results. On the other hand, SPR allowed us to visually see the differences in binding potency of various assemblies of the functional dimer. However, the quantification of affinities of these binding events was hindered due lack of clear dissociation state (Table 1).

A



B

C



D

Protein complex	MMcal (Da)*	MMmeasured (Da)*
P1-met	12010.6	12009.98±06
P1ΔC-met	10357.8	10357.52±0.57
P2	12292.7	12292.66±0.10
P2ΔC-met	10357.8	10357.68±0.10
P1-met-P2	24302.6	24302.45±7.14
P1-met-P2ΔC-met	22368.4	22367.97±2.54
P1ΔC-met-P2	22499.5	22497.71±5.11
P1ΔC-met-P2ΔC-met	20564.6	20563.95±11.51
P2-P2	24585.4	24586.09±8.81
P2ΔC-met-P2ΔC-me	20715.7	20727.95±11.10

Figure 1: Characterization of human ribosomal stalk P1-P2 complexes. (A) Alignment of C-terminal amino acid sequences of human ribosomal P1, P2 protein. The arrow and box indicate the deletion site from the C-termini (ΔC). (B) Models of P1-P2 and P2-P2 complexes with deleted in the conserved C-terminal 16 amino acids sequences. (C) Size exclusion chromatography (SEC) of purified protein complexes. Purified complexes (10 µg of protein) were analyzed by SDS-PAGE (upper panel) and circular dichroism (CD) (lower panel). (D) Molecular masses of protein species detected by native mass spectrometry. *Calculated molecular masses (MMcal) compared with molecular masses determined by native-MS (MMmeasured).

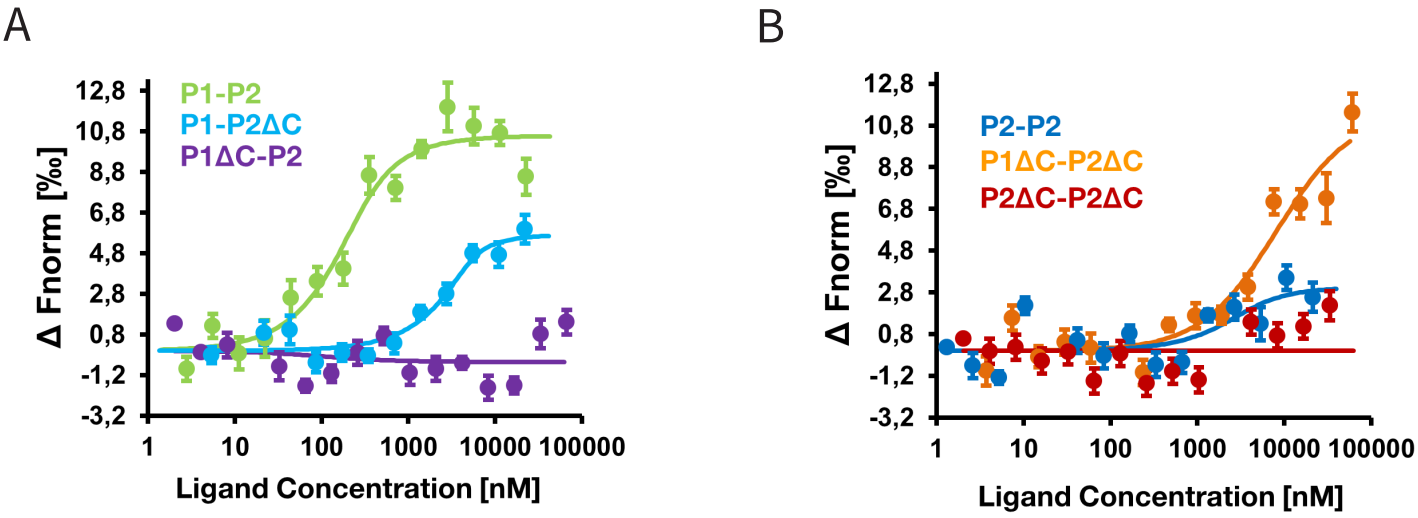


Figure 2: Interaction of RTA with P1-P2 protein complexes. (A) MST binding curves for the P1-P2, P1-P2ΔC and P1ΔC-P2 complexes with RTA. (B) MST binding curves for the P2-P2, P2ΔC-P2ΔC and P1ΔC-P2ΔC and RTA. To obtain ΔFnorm, the baseline Fnorm value is subtracted from all data points of the same curve. Thus, by definition, ΔFnorm is 0 in the unbound state. Error bars ± SE (n = 3).

Protein complex	MST	SPR	BLI	ITC
P1-P2	K_d = 86nM	Interaction visible, slow K_{off}, no fit possible	K_d = 21nM	Binding, low C value
P1-ΔP2	K_d = 533nM		K_d = 90nM	Binding, low C value
P2-P2	K_d = 1-2µM	Slow K_{off}, no fit possible	K_d = 2µM	No saturation, low C value
ΔP1-P2	No interaction	No interaction	No saturation	No saturation, low C value
ΔP1-ΔP2	No saturation	No interaction	No saturation	No saturation, low C value
ΔP2-ΔP2	No interaction	No interaction	No clear interaction	No saturation, low C value

Table 1: Summary of trimeric complex molecular interaction characterization with various biophysical techniques

Discussion

The ribosomal P-protein play a major role in functional ribosome serving as a docking site for variety of translational factors. This acceptor platform is also a target for variety of RIP protein, which utilize it to gain access to SRL and its depurination. Here, we show that the individual CTDs of P1 and P2 heterodimers directly interact with RTA. In addition, different mutants of P1 and P2 proteins were used to analyze the importance of CTD region. Interestingly, CTD of P1 protein is more critical than the CTD of P2 protein for efficient interaction of the stalk complex with RTA. These variations in binding capacity of P1-P2 dimer and CTD mutants could be explained by the unique behavior of the disordered nature of the CTD of P1 and P2 proteins, especially the hinge region [13, 14]. Previous studies showed that P2 forms a homodimer in solution, while in the absence of P2, P1 forms high-mass oligomeric aggregates. The formation of P1-P2 heterodimer is a favorable spontaneous process in which the less stable P2 homodimer is displaced by P1 to form a more stable P1-P2 heterodimer [15, 17].

We also show that MST was the only method that allowed us to characterize in solution the interactions between the trimeric complex (P1-P2 and RTA) and P1-P2 mutants with RTA that exhibit lower affinities. In addition, MST required less material which was essential as purification of these recombinant proteins and in-vitro reconstitution of dimeric P1-P2 complexes is extremely challenging and time consuming.

Materials and Methods

Protein Expression and Purification

Expression, purification of recombinant human P1, P2 proteins, truncated forms and preparation of the complexes were performed according to the procedure established previously [11, 12] (9,10). The P1-P2 heterocomplex was prepared following the denaturation/renaturation procedure established for the yeast P protein complex [15] (13). RTA was expressed in Escherichia coli BL21(DE3) RIL cells and N-terminal 10xHis-tagged recombinant RTA was purified using Ni-NTA agarose from QIAGEN (Valencia, CA, USA).

MicroScale Thermophoresis

Purified RTA was labeled with amine reactive protein labeling kit RED-NHS Monolith (NanoTemper Technologies MO-L001) using red fluorescent dye NT-647-NHS. 60µM of the dye was mixed in ratio 3:1 with 20µM of RTA in total volume of 200µL. Free dye was removed by buffer-exchange column chromatography into MST reaction buffer: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl2, 0.05 % Tween-20 (assay buffer). The concentration of labeled RTA was adjusted to approximately 5–20 nM in 200µL of assay buffer that was supplemented with 1mg/mL of bovine serum albumin. 16 twofold dilutions starting from 50 or 100 µM of reconstituted P1-P2 dimers were prepared in a final volume of 10 µL of assay buffer and mixed with 10µL of previously adjusted labelled RTA. Thermophoresis was measured using a Monolith NT.115pico instrument (NanoTemper) at an ambient temperature of 25 °C. The

initial fluorescent measurement and MST-ON time was set to 5 and 30 sec respectively. The LED power and MST power were set to 90 % and 20 % respectively. The data were analyzed using NanoTemper's NT analysis software (version 1.5.41). Titration of the non-fluorescent ligand resulted in a gradual change in MST signal, which was plotted as ΔFnorm to yield a binding curve, which was fitted to derive the binding constants.

Circular Dichroism Spectroscopy and Mass Spectrometry

CD spectra were recorded with application of a Chirascan Plus Spectrometer (Applied Photophysics, UK.). The purified stalk dimers and their truncated variants were dialyzed against 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl2. Spectra were recorded in the range 200–250 nm, at 25 °C temperature, with a 1 nm resolution. The scan rate was 60 nm/min. Protein samples for CD were scanned three times and averaged. The averaged baseline spectra were then subtracted from the averaged sample spectra and converted to molar ellipticity. The recorded spectra were analyzed with application of the Grams/AI software from Thermo Scientific (USA).

All complexes were analyzed using SYNAPT G2-Si High Definition Mass Spectrometer (Waters, Manchester, U.K.). For the analysis, all protein solutions were buffer exchanged into 200 mM ammonium acetate (pH 7.5) using Micro Bio-Spin chromatography columns (Bio-Rad). Aliquots (~2 µL) were introduced into the mass spectrometer via nanoflow capillaries using the following conditions: capillary voltage 1.2 kV, sampling cone 120 V, source offset 20 V. The source temperature was set up for 25 °C. The collision voltage was adjusted for the optimal signal level. Maximum entropy (MaxEnt, Waters) deconvolution was applied to electrospray data to recalculate the gas phase existing masses.

Acknowledgments

The data presented in this application note were published in the article Human ribosomal P1-P2 heterodimer represents an optimal docking site for ricin A chain with aprominent role for P1 C-terminus by by Grela, P. et al.[19].

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