

Protein-Protein Interaction Analysis in Different Buffer Systems

Application Note NT-MO-012

Using MST to analyse the binding of the β -Lactamase TEM1 to BLIP

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Abstract

The analysis of protein-protein interactions plays an important role in understanding many biological processes. MicroScale Thermophoresis (MST) permits a robust and fast analysis of protein-protein interactions even in highly complex biological liquids. Here we have investigated the binding of TEM1 β -Lactamase to BLIP (β -Lactamase Inhibitory Protein) using MicroScale Thermophoresis. The experiments were performed as in 50% mammalian cell lysate, as well as in standard buffer. The TEM1-BLIP system is very well characterized by various biophysical methods, such as Surface Plasmon Resonance (SPR).

The role of residue substitutions at the interface of the proteins was easily studied using TEM1 and BLIP mutants. Dissociation constants (K_d) determined for TEM1 and BLIP show an excellent agreement with previously published data.

Introduction

Protein-protein interactions play an essential role in many biological processes. Specific non-covalent interactions stabilize the structure of macromolecular assemblies.

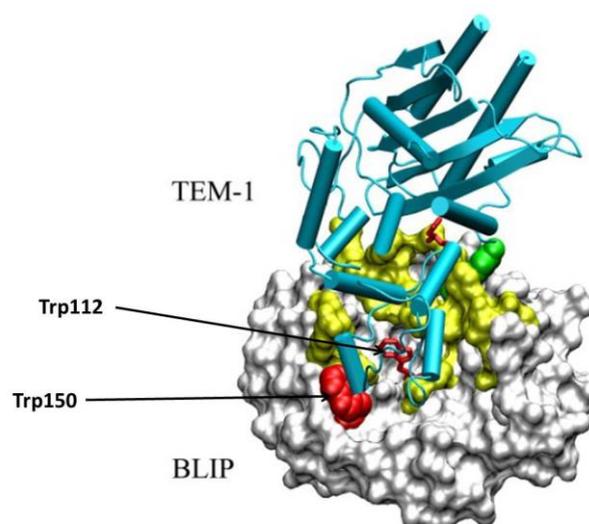


Fig. 1: Structural representation of the TEM1-BLIP complex. TEM1 is represented in cyan. BLIP is shown in white. TEM1 contact residues are in yellow. BLIP mutants with decreased affinity for TEM1 are represented in red, and the mutant W150A and W112A residues of BLIP are identified by a black arrow. The image is slightly modified from Wang *et al.*, 2007.

The binding of one protein to another involves many contact residues and a relatively large interface that is rather planar. Flexibility of contact residues, chemical and physical properties, as well as solvent molecules

trapped in the interface, can affect the binding. Interestingly only a small fraction of the contact residues contribute significantly to the free energy of binding, as studied by Alanine-scanning Mutagenesis (Wang *et al.*, 2007, Reichmann *et al.*, 2005).

A fast and reliable method to analyze binding affinities is important for understanding macromolecular interactions in biological systems. An example of such an analysis, using MicroScale Thermophoresis, is described in this application note. For more detailed information about MST Technology refer to Jerabek-Willemsen *et al.* 2011 and Wienken *et al.*, 2010.

β -Lactamase enzymes, such as TEM1, provide resistance to β -Lactam antibiotics, which are widely used as antimicrobial antibiotics. The enzyme can selectively cleave the active peptide bound in the β -Lactam ring.

BLIP (**β -Lactamase Inhibitory Protein**) is a 154- amino acid protein, which is expressed by the soil bacterium *Streptomyces clavuligerus*. This protein can inhibit the TEM1 - β -Lactamase (Albeck and Schreiber, 1999). The TEM1-BLIP complex is represented in Fig. 1.

In this study we have analyzed the consequences of well characterized mutations of TEM1 and BLIP residues on the dissociation constant (K_d) in standard buffer, as well as in mammalian 293T cell lysate. For this analysis, we have used fluorescently-labeled TEM1 (NT-647 dye), in addition to a fluorescent-fusion protein - Ypet-BLIP. For a detailed description of the Ypet-BLIP system refer to Philipp *et al.*, 2011.

Results

In the first experiment, we investigated the binding of a NT-647-labeled TEM1 to WT BLIP or two other BLIP mutants, in which

alanine was substituted for tryptophan at positions 112 and 150 of BLIP.

The concentration of NT-647 labeled TEM1 was kept constant at 10 nM, while the concentration of BLIP was varied. After a short incubation, the samples were loaded into MST standard treated glass capillaries (K002, NanoTemper technologies GmbH, Germany) and MST analysis was performed. The calculated K_d for the interaction between NT-647-labeled WT TEM1 and WT BLIP was 3.5 nM +/- 0.6 nM. We observed, as expected, approximately a 100-fold weaker binding affinity of NT647-labeled TEM1-WT to BLIP-W112A. A mutation of tryptophan to alanine at position 150 resulted in a 600-fold weaker K_d , as compared to WT BLIP (Fig. 2). These results are in good agreement to previously published affinities determined by SPR (Table 1).

Interestingly the signal observed in Figure 2C for binding of NT647 labeled TEM1-WT to BLIP-W150A, showed an opposite thermophoretic behavior as compared to TEM1 binding to BLIP-WT (Fig. 2A) and BLIP-W112A (Fig. 2B).

Reichmann *et al.*, 2005 and Wang *et al.*, 2007 have described the anti-canonical nature of this mutation. Substitution of the tryptophan at position 150 of BLIP results in a strong conformational rearrangement, and significant change in the free energy of binding. Thermodynamic studies also indicate that this mutation leads to a significant loss of enthalpy. In addition, Wang *et al.* reported an increased number of water-bridged intermolecular hydrogen bonds in the complex of TEM-WT with BLIP-W150A. This contributes to a lower enthalpic driving force for binding and disrupts hydrophobic stacking interactions. Taken together, it is possible that the loss of enthalpy and change in the hydration shell is related to the different thermophoretic response.

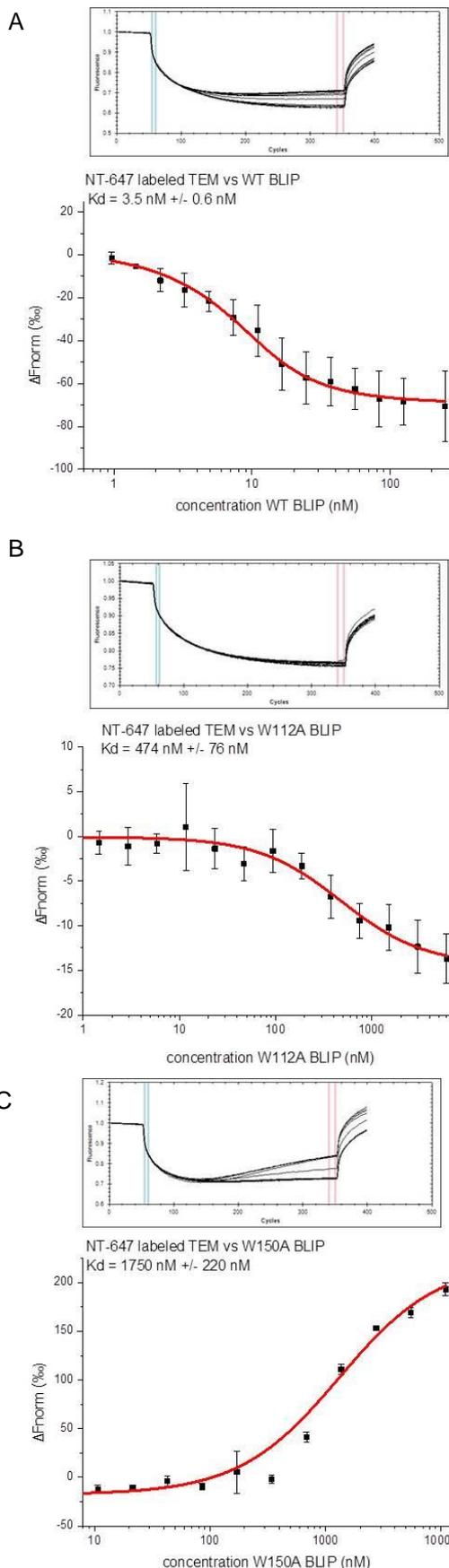


Fig. 2: Binding of NT-647-labeled TEM1 to BLIP protein. In the MST experiment, we kept the concentration of fluorescently-labeled TEM1-WT constant at 10 nM, while the concentration of BLIP was varied. After 10 min, an MST analysis was performed (n = 3). A) Binding of BLIP-WT to NT-647-labeled TEM1-WT. A K_d of 3.5 nM ± 0.6 nM was determined for this interaction. B) Binding of BLIP-W112A to NT-647-labeled TEM1-WT. A K_d of 474 nM ± 76 nM was determined for this interaction. C) Binding of BLIP1-W150A to NT-647-labeled TEM1-WT. A K_d of 1750 nM ± 220 nM was determined for this interaction. All MST time traces are shown above the binding curves.

In a second experiment we have used a fluorescence fusion protein in order to characterize the binding of Ypet-BLIP (Fig. 3) to TEM1-WT or TEM1-R243A. Fig. 4 illustrates the Ypet-BLIP TEM1 complex.

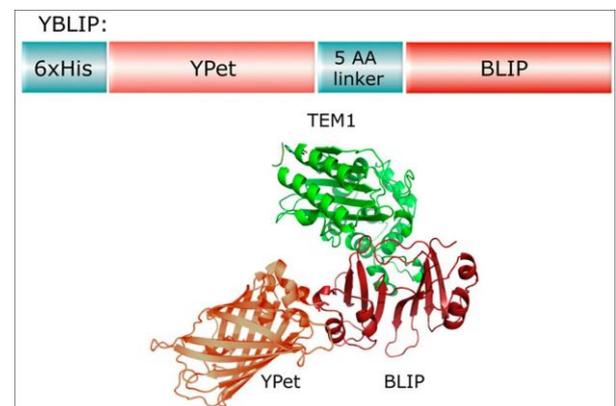
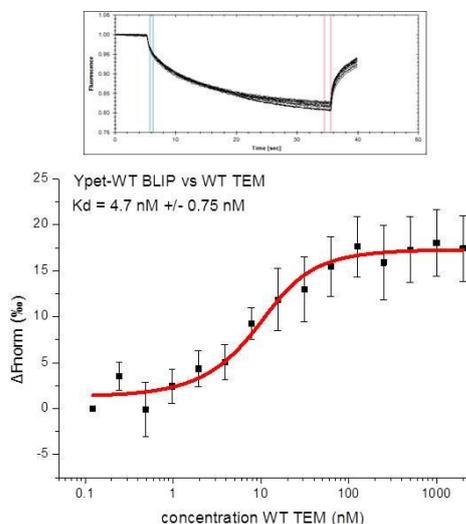


Fig. 3: The Ypet-BLIP construct. A 6-His tag and Ypet were fused to the N-terminus of BLIP with a five amino acid linker between them. A schematic of the Ypet-BLIP TEM complex is at the bottom.

The concentration of Ypet-BLIP-WT was kept constant at 15 nM, while the concentration of TEM1-WT or TEM1-R243A was varied. After a short incubation, the samples were loaded into MST standard treated glass capillaries (K002, NanoTemper technologies GmbH, Germany) and MST analysis was performed. A K_d of 4.7 nM ± 0.75 nM was determined for the binding of Ypet-WT BLIP to WT TEM (Fig. 4A), which correlates nicely with the binding affinity determined using NT-647-conjugated TEM1-WT. A 50-fold weaker affinity was determined for the binding of Ypet-BLIP-WT to TEM1-R243A (Fig. 4B). These results confirm published SPR-data,

which show that substitution of this residue results in a weaker affinity of BLIP to TEM1.

A



B

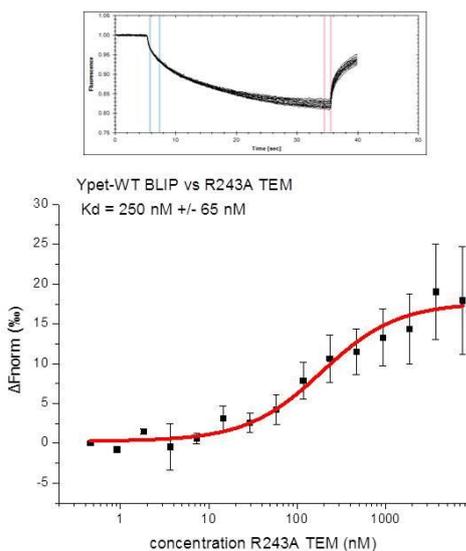


Fig. 4: binding of Ypet-BLIP to TEM1 protein. In the MST experiment we kept the concentration of Ypet-BLIP-WT constant at 15 nM, while the concentration of TEM1 was varied. After 10 min an MST analysis was performed (n = 3). A) Binding of Ypet-BLIP-WT to TEM1-WT. A K_d of 4.7 nM ± 0.75 nM was determined for this interaction. B) Binding of Ypet-BLIP-WT to TEM1-R243A. A K_d of 250 nM ± 65 nM was determined for this interaction.

Since biochemical processes take place in a highly crowded macromolecular environment *in vivo*, it is important to study interactions under conditions closer to those of the cells, such as in cell lysates. Additionally, using fluorescent fusion proteins to study binding in cell lysates is very

convenient, since the fluorescent fusion protein do not need further purification. Thus time and the amounts of biological materials needed can be saved.

Here, we have analyzed the binding of Ypet-BLIP-WT to TEM1-WT in mammalian cell lysates. First 12 dilutions of WT TEM1 were prepared in MST buffer. 15 nM BLIP was added to 293T cell lysate (20 million cells were lysed in 500 μl of RIPA-buffer). Then the cell lysate was centrifuged for 5 minutes at 15,000 rpm.

10 μl of Ypet-BLIP-containing lysate was added to each dilution of TEM-WT. After a short incubation, the samples were loaded into MST glass capillaries (K002, NanoTemper Technologies) and the analysis was performed. As expected, the K_d which was determined was in the low nM range as it was for the determination performed MST buffer.

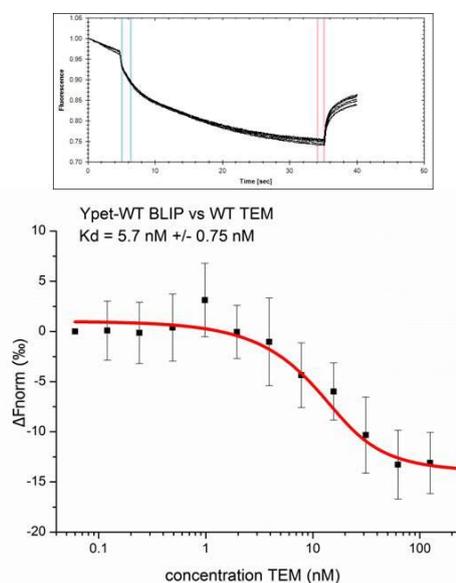


Fig. 5: binding of Ypet-BLIP to TEM1 protein analyzed in 50 % mammalian cell lysate. TEM1 was titrated in 1:1 dilutions in MST-buffer. Then Ypet-BLIP was added into 200 μl of 293T cell lysate to a final concentration of 20 nM. 10 μl of BLIP-containing cell lysate was mixed with 10 μl TEM1 dilutions. For measurements, samples were filled into standard treated capillaries (n = 3). A K_d of 5.7 nM ± 0.75 nM was determined for this interaction.

Conclusions

This study demonstrates that MicroScale Thermophoresis (MST) is a very powerful tool for the analysis of protein-protein interactions in solution. The binding affinities which were determined are in excellent agreement with those previously measured on the ProteOn XPR36 system (Table 1).

Interaction	K_d	
	MST (nM)	Proteon (nM)
WT TEM vs WT BLIP	3.5 +/- 0.6	3.2 +/- 0.6
WT TEM vs W112A BLIP	474 +/- 76	360 +/- 60
WT TEM vs W150A BLIP	1750 +/- 220	3800 +/- 600
Ypet BLIP vs WT TEM	4.7 +/- 0.75	3.5 +/- 0.5

Table 1: Comparison of binding affinities of TEM1 to BLIP as analyzed by MicroScale Thermophoresis or SPR.

An important advantage of MST-Technology is the buffer independency. The technology is suited to analyze binding interactions in very complex biological samples, such as cell lysates and blood serum.

Since interactions take place in biological fluids, it is important to measure interactions under similar experimental conditions. The use of fluorescence-fusion proteins and MST-technology, as was demonstrated in this application note (Fig. 5), opens new possibilities to analyze binding affinities using *in vivo*-like conditions. This can be useful in order to improve our understanding of macromolecular interactions in crowded macromolecular environments.

Materials and Methods

Assay conditions

Binding of NT-647-labeled TEM1 to BLIP

Protein Labeling: 10 μ M TEM1 was labeled using NanoTemper's Protein Labeling Kit RED-NHS (L001, NanoTemper technologies, Germany).

Sample Preparation: The concentration of labeled TEM1 protein was kept constant at a concentration of 10 nM. The unlabeled binding partners were titrated in 1:1 dilutions. The highest concentration of BLIP was about 20-fold above the expected K_d , which was previously determined by SPR. Samples were diluted in MST optimized buffer (50 mM Tris-HCl buffer, pH 7.6 containing 150 mM NaCl, 10 mM MgCl₂ and 0.05 % Tween-20). For measurements, samples were filled into standard treated capillaries (Cat#K002).

Binding of Ypet-BLIP to TEM1

The concentration of Ypet-BLIP protein was kept constant at 15 nM. The unlabeled binding partners were titrated in 1:1 dilutions. The highest concentration of TEM1 was about 20-fold above the expected K_d , which was previously determined by SPR. Samples were diluted in MST optimized buffer (50 mM Tris-HCl buffer, pH 7.6 containing 150 mM NaCl, 10 mM MgCl₂ and 0.05 % Tween-20). For measurements, samples were filled into standard treated capillaries (Cat#K002, NanoTemper Technologies, Germany).

Binding of Ypet-BLIP to TEM1 in cell lysate

Preparation of cell lysate:

2 x 10⁷ 293T cells were lysed using 500 μ l of RIPA-buffer. The cell lysate was centrifuged at 15,000 rpm for 5 minutes in order to remove large aggregates and cell debris.

Sample Preparation:

TEM1 was titrated in 1:1 dilutions (in MST-buffer). Then Ypet-BLIP was added into 200 μ l of 293T cell lysate to a final

concentration of 20 nM. 10 μ l of BLIP-containing cell lysate was mixed with 10 μ l TEM1 dilutions. For measurements, samples were filled into standard treated capillaries (Cat#K002).

Instrumentation

The measurements were conducted on a NanoTemper Monolith NT.115 instrument.

The Analysis was performed at 50 % LED power and 80 % MST power, Fluo. Before time was 5 seconds, MST on time was 30 seconds and “Fluo. After” time was 5 seconds.

References

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Notes

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