Protein Labeling

Purification-free Labeling in Whole Cell Lysate and Binding Characterization by MST

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Abstract

For researchers performing biophysical analysis of proteins, a common hurdle encountered is having sufficient amounts of materials with the appropriate purity to perform detailed analysis. Here we demonstrate the utility of a RED-tris-NTA dye from NanoTemper Technologies that can be used to specifically label His-tagged proteins for MicroScale Thermophoresis (MST) binding studies directly in cell lysates. The procedure requires minute amounts of sample, can be carried out without additional lab equipment and accurate K_d measurements are obtained within 45 minutes from cell lysis to measurement. As an example, we measured the affinity of a small molecule inhibitor to His-tagged p38α kinase expressed in mammalian cells. Our data demonstrate that MST assays are a rapid and cost-effective method for determination of affinities using unpurified proteins, and thus serves as a powerful tool in the early drug discovery, especially for proteins that are difficult to purify.

Introduction

Detailed biophysical characterization, whether for basic research or early drug discovery analysis, requires starting material of the highest quality and purity to generate meaningful results. Therefore, suitable purification protocols must be established to produce proteins at high enough concentration and quality. Also, biophysical measurements are typically carried out in artificial buffer systems, which can dramatically affect the outcome of binding studies when compared to the in vivo situation where natural ligands, substrates and ions are present. This study shows

how MicroScale Thermophoresis (MST) can be used to perform biophysical interaction studies with unpurified proteins, thereby saving valuable resources and time in a variety of research projects.

The interaction between p38α, a serine/threonine protein kinase belonging to the mitogen-activated protein kinase (MAPK) family (Dominguez et al. 2005), and a well-characterized small-molecule antagonist SB203580 (Davies et al., 2000) was used as a model system to test interaction studies by MST using purification-free labeling in cell lysate. p38α is known to play a major regulatory role in proinflammatory pathways and therefore is a key target for compound screening in the pharmaceutical industry. The interaction between purified p38α and SB203580 was successfully characterized previously using MST (Saxena et al., 2011). In this study, we show that the same interaction can be quantified in an entirely purification-free system.

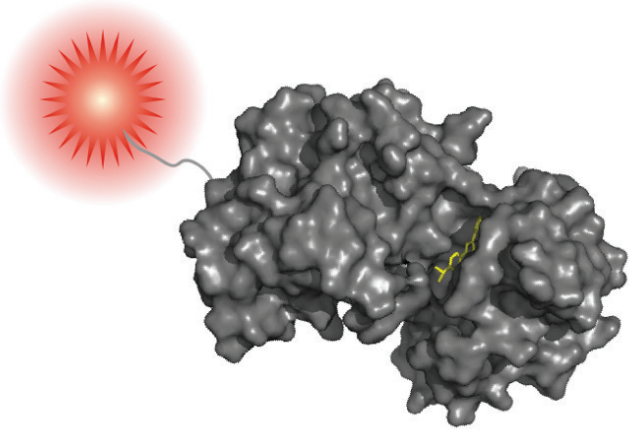

Figure 1. Schematic illustration of RED-tris-NTA labeled p38α bound to a small molecule inhibitor. (PDB 3ZS5)

His-tag labeling in cell lysate was achieved using the RED-tris-NTA dye, which is designed to specifically bind polyhistidine tags with high affinity (Tschammer et al., 2016). In addition to the His-tag, p38α was fused and co-expressed with an mNeonGreen protein, which allowed to validate the specificity of the labeling approach and to verify the precision of binding data by measuring MST of the fusion protein in response to the inhibitor.

Results

To ensure an optimal protein-to-dye ratio for subsequent His-tag labeling, MST experiments were conducted for estimating the concentration of p38α in the lysate. Concentration determination was achieved by titrating the lysate containing p38α-mNeonGreen-6xHis against the RED-tris-NTA dye. By using the previously published K_d value for the affinity of RED-tris-NTA dye towards a His-tagged purified p38α (2.3 nM) (Tschammer et al., 2016), the p38α concentration in the cell lysate was estimated to be between 50 and 100 nM.

Subsequently, p38α was directly labeled in cell lysate following the RED-tris-NTA labeling protocol. After serial dilution of SB203580 in PBST and addition of unpurified, RED-tris-NTA-labelled p38α, the affinity of the interaction was measured by following binding-induced changes in the MST signal. As shown in Figure 2, a clear binding-induced change in MST was detected, yielding a K_d of 247 nM, which is comparable to previously published results (Cuenda et al, 1995). To test for specificity of the binding signal, negative control experiments were performed using whole cell

lysate expressing mNeonGreen-6xHis fusion protein but lacking p38α. First, the His-tag was labeled with RED-tris-NTA in cell lysate and the ligand SB203580 was titrated. As shown in Figure 2 (grey), binding could not be detected, demonstrating that the changes in the MST signal of RED-tris-NTA-labelled p38α are a result of its specific interaction with SB203580.

In addition, orthogonal MST experiments were carried out in which the mNeonGreen fluorescence of the protein was detected using the Monolith NT.115 system's blue detection channel. As shown in Figure 3, clear binding was detected and the affinity of SB203508 for p38α-mNeonGreen-6xHis was determined to be 82 nM, which strongly correlates with the previous results. Interestingly, comparison of the results with the first experiment, where the RED-tris-NTA labeled protein was tested (Figure 2), show that the labeling with RED-tris-NTA resulted in a larger binding amplitude and a highly-improved signal-to-noise ratio.

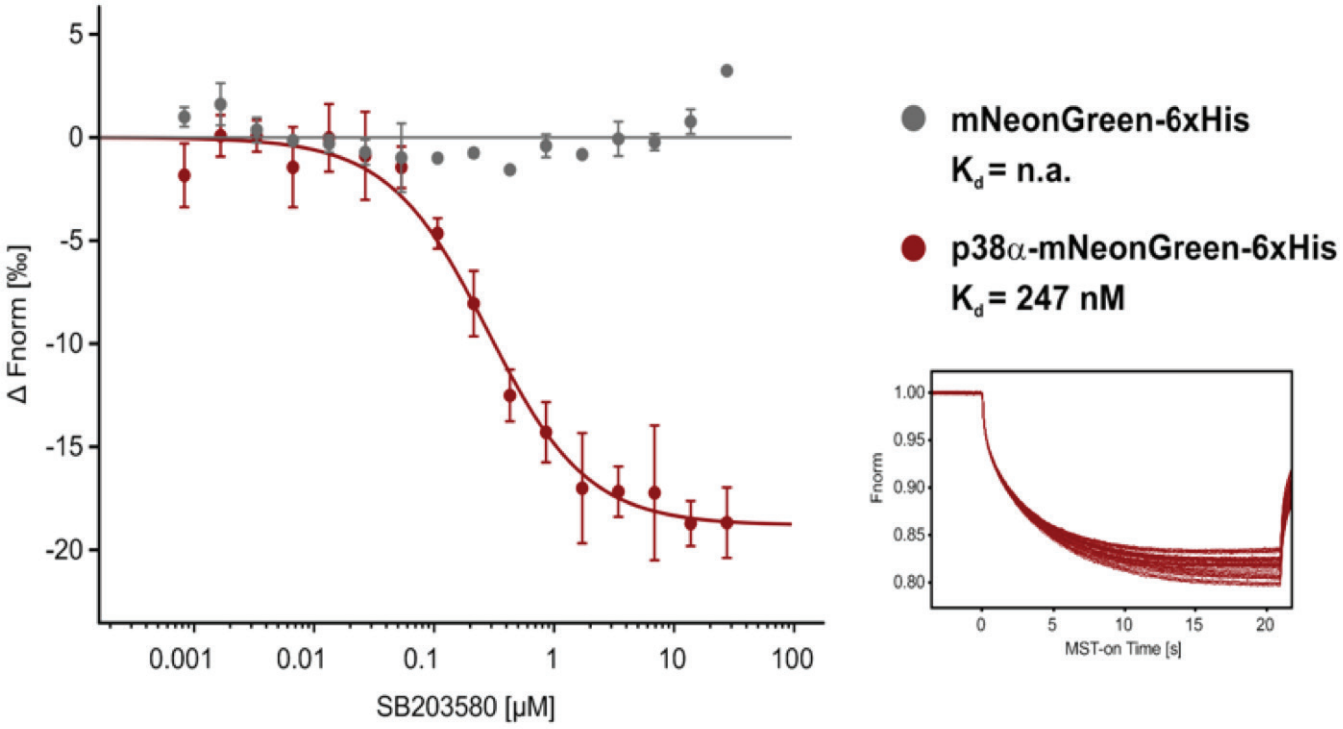

Figure 2. MST traces and dose-response curve for the binding interaction between RED-tris-NTA labeled p38α and SB203580 in whole cell lysate. The concentration of RED-tris-NTA labeled p38α-mNeonGreen-6xHis (red) or mNeonGreen-6xHis (grey) in lysate is constant, while the concentration of SB203580 varies between 26.5 μM and 1.62 nM. A K_d of 247 nM was determined for p38α-mNeonGreen-6xHis. The negative control employing mNeonGreen-6xHis did not yield a binding curve. Concentrations on the x-axis are plotted in μM. RED-tris-NTA fluorescence was used for detection. The corresponding MST time traces are shown on the right. n = 3

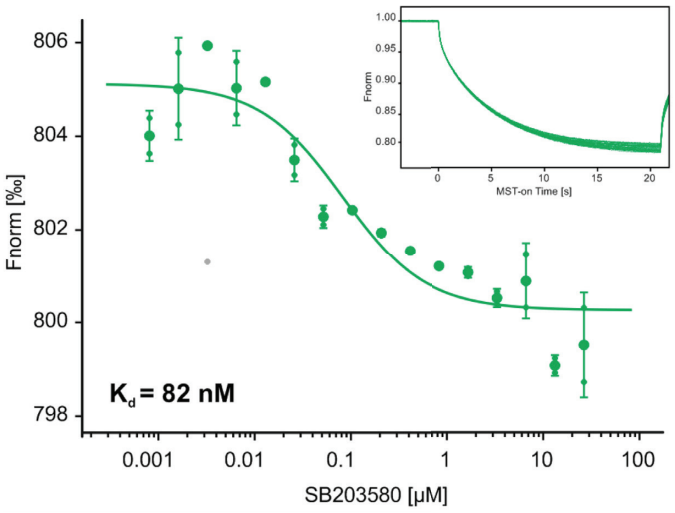

Figure 3. MST traces and dose-response curve for the binding interaction between p38α-mNeonGreen-6xHis and SB203580. The concentration of p38α-mNeonGreen-6xHis in lysate is constant, while the concentration of the non-labeled SB203580 varies between 26.5 µM – 1.62 nM. A K_d of 82 nM was determined for this interaction. Concentrations on the x-axis are plotted in µM. mNeonGreen fluorescence was used for detection. The corresponding MST time traces are shown as an inset. n = 3

Conclusion

In this report, we demonstrate the applicability of RED-tris-NTA labeling of proteins in cell lysates to provide rapid and quantitative characterization of biomolecular interactions using MST. This approach can be useful in providing initial binding characterizations during the early stages of drug discovery, particularly for targets which are difficult to purify, thus saving time and money. This application is not limited to protein-small molecule interactions as shown here and can also be applied to other systems, including protein-protein, protein-sugar or protein-nucleic acid interactions.

Methods

Plasmids

The p38α coding sequence originates from reverse transcription from mRNA from A549 cells and was fused on a mammalian expression vector behind a CMV promotor to the mNeongreen-6xHis coding sequence separated by a linker ESGSGS.

Production of p38 in HeLa cells

3x106 HeLa cells were transfected with p38α-mNeonGreen-6xHis, and mNeonGreen-6xHis respectively, in individual T-75 flasks. Cells were harvested after 24 hours, when each pellet contained approximately $10x10^6$ cells. Cells were lysed in 1 ml PBS-T buffer (137 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4; 0.05 % Tween-20) supplemented with protease inhibitors and disrupted with a Dounce homogenizer. Cell debris was removed by centrifugation at 20000 × g for 30 minutes. The lysate was diluted 1:10 in PBS-T containing protease inhibitors.

Estimation of the concentration of His-tagged protein in cell lysate by MST

A sixteen-point 1:1 serial dilution of cell lysate expressing p38α was prepared using non-transfected HeLa cell lysates in 10 µl of final volume. 10 µl of 50 nM RED-tris-NTA dye were added to each reaction tube, resulting in a final dye concentration of 25 nM. The reaction mixture was incubated for 30 minutes at room temperature and loaded into premium coated capillaries.

Since the affinity of the RED-tris-NTA dye towards the 6xHis-tag is in the low nM to pM range and therefore well below the concentration of RED-tris-NTA, the binding curve will display a “kink” at the point where equimolar concentrations of dye and His-tagged protein are present (Figure 4). Since there is a single binding site for the RED-tris-NTA dye, one can assume that dye and protein concentrations are equivalent at the “kink”, so that the concentration of the protein at the start of the dilution can be calculated. In this experiment, saturation of the binding curve was not reached, suggesting that the concentration of the protein in the lysate was less than 2-fold above the concentration of the dye (less or equal to 50 nM, data not shown).

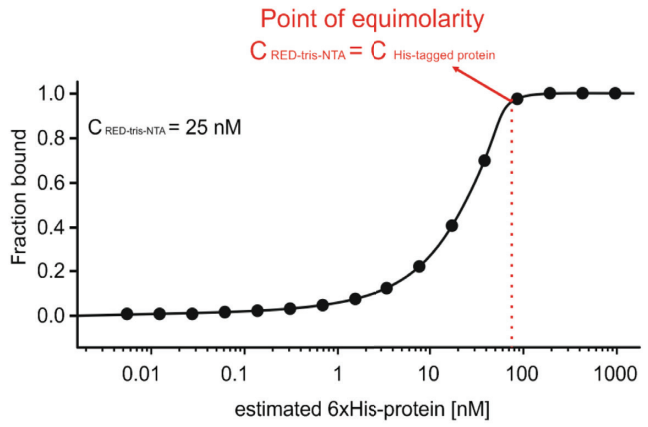

Figure 4. Simulation of a binding curve for an interaction between RED-tris-NTA dye and a 6xHis-tagged protein. The concentration of the target (RED-tris-NTA) is above the K_d. Therefore, the binding curve shows a saturation “kink” at the concentration where the dye is fully saturated by 6xHis-tagged protein.

Labeling of p38α-mNeonGreen-6xHis

The RED-tris-NTA dye was diluted in PBS-T to 100 nM. Diluted lysate and dye were mixed in 1:1 volume ratio and incubated for 30 minutes at room temperature.

MST experimental protocol

SB203580 stock solution (2.65 mM, 100 % DMSO) was diluted 1:50 in PBS-T buffer. The dilution series was prepared by mixing 10 µl of 53 µM SB203580 with an equal volume of PBS-T buffer containing 2 % DMSO in 1:1 steps, resulting in a precise 16-step dilution series of the ligand. 10 µl of lysate containing the fluorescent target protein was added to each reaction tube, decreasing the final DMSO concentration to 1 % and the highest ligand concentration to 26.5 µM, whereas the concentration of supernatant was constant in each tube. All interactions were measured in Monolith NT.115 MST Premium Coated Capillaries at room temperature with an MST-on time of 20 seconds and an MST-off time of 5 seconds. The interaction of p38α-mNeonGreen and SB203580 was measured at 100% LED and High MST power. The interaction of RED-tris-NTA-labeled p38α-mNeonGreen-6xHis and SB203580 was measured at 20 % LED and High MST power. The data were collected by the MO.Control Software and analyzed using the MO.Affinity Analysis Software.

Instrumentation

Measurements were performed using a NanoTemper® Technologies Monolith® NT.115 system.

References

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