Protein-DNA Interaction Analysis

Application Note NT-MO-020

Dissection of complex interaction mechanisms by distinct thermophoresis signals – ssDNA binding to EcoSSB.

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Abstract

MircoScale Thermophoresis (MST) not only allows for a precise determination of binding constants, but can also be used to derive additional information about the molecular mechanism of the investigated interaction. For instance, MST can be used to discriminate between different binding modes, and can also be used to determine interaction stoichiometries. We illustrate the different information that can be derived from MST experiments on the example of the interaction of short Oligo(dT) nucleotides with single-strand binding protein from *E. coli* (EcoSSB). We show that MST can be used to easily and quickly determine the pM affinity, the binding mode and the stoichiometry of this interaction

Introduction

EcoSSB plays a key role during DNA replication by binding single-stranded DNA (ssDNA), thereby preventing re-annealing of the strands and allowing replication by DNA primases and polymerases (Alberts, 2003). Biophysical *in vitro* and crystallographic studies showed that one EcoSSB tetramer binds a single ssDNA oligonucleotide over a total length of 70 nucleotides (Figure 1) (Bujalowski & Lohman, 1989a; Bujalowski & Lohman, 1989b; Matsumoto et al, 2000). The affinity of the interaction of EcoSSB and oligo(dT)$_{70}$ ssDNA is known be extremely high at physiological salt concentrations, with K_ds in the low pM or even fM range (Bujalowski & Lohman, 1989b; Curth et al, 1994). However, the exact affinity could not be determined yet due to the limited sensitivity of conventional methods. Here we demonstrate that MST allows for the precise analysis of dissociation constants of high-affinity protein-DNA interactions that have been inaccessible until now. Moreover, the thermophoresis signals of the interaction of EcoSSB with Cy5-labeled Oligo-nucleotides contain additional information about binding modes and stoichiometries, allowing for a rapid and comprehensive analysis of complex molecular interaction mechanisms.

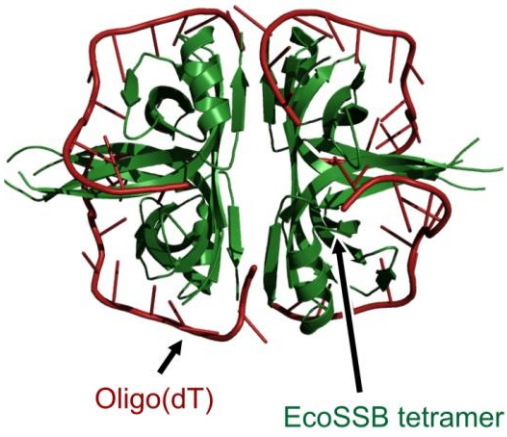

Figure 1: Crystal structure of the complex of Plasmodium falciparum SSB (pdb-code: 3ULP).

Results

By using Cy5-labeled oligo(dT)$_{70}$ at a concentration of only 20 pM and titrating EcoSSB from 5 nM down to 10 fM, a binding curve for the EcoSSB-oligo(dT)$_{70}$ interaction was resolved, yielding a K_d of 1 ± 0.1 pM (Figure 2).

MST is a powerful technique to precisely determine binding constants. In addition, this method can also be used to derive information about the molecular mechanism of the investigated interaction. For instance, MST can be used to discriminate between different binding modes, and can also be used to determine interaction stoichiometries.

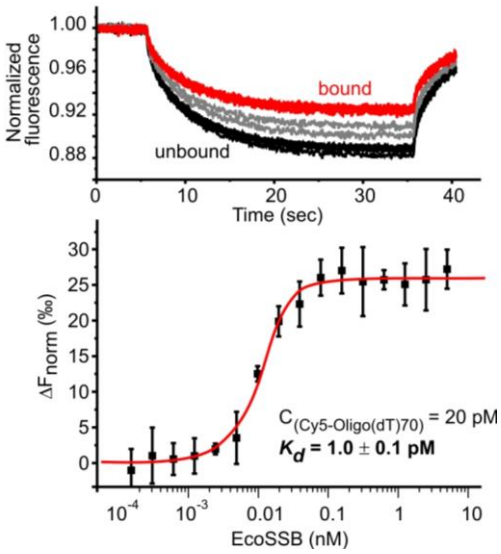

Figure 2: Determination of the pM dissociation constant of the EcoSSB-Oligo(dT)$_{70}$-interaction. Association of single strand binding protein from *E. coli* (EcoSSB) with a Cy5-labeled oligo-nucleotide (dT)$_{70}$ was monitored by titrating EcoSSB from 5 nM to ~100 fM against 20 pM Cy5-labeled DNA. Changes in thermophoresis were plotted, yielding a *Kd* of 1 ± 0.1 pM. Error bars = s.d.; n=3.

The thermophoresis signal that is detected by the NT.115 and NT.LabelFree devices contains multiple pieces of information: Upon activation of the IR-laser (which establishes the local temperature gradient) the temperature jump (T-jump) of the fluorophore is detected. It describes the rapid change in the photo-physical properties of the fluorophore upon heating, and occurs within less than a second. The T-jump predominantly depends on the local surroundings of the fluorophore and can thus be affected e.g. by binding of ligands in close vicinity of the fluorophore. In contrast, thermophoresis depends globally on the properties of the entire molecule/complex, and is a comparably slow process that occurs on a second to minute time scale (Figure 3A). In many cases, either the thermophoresis or T-Jump signal can be used to derive a dissociation constant. In addition, both signals contain different information about the molecular interaction mechanism, which is owed to their different physical origin, and can thus be used independently to address different aspects of molecular interactions. Here we demonstrate that T-jump and thermophoresis signals provide different mechanistic information about binding stoichiometries as well as about binding modes for the interaction of ssDNA with EcoSSB. As shown in Figure 2, EcoSSB binds to oligo(dT)$_{70}$ with very high affinity. Extensive biophysical characterization of this interaction has shown earlier that EcoSSB and oligo(dT)$_{70}$ bind with a 1:1 stoichiometry. A shorter oligonucleotide, oligo(dT)$_{35}$, was shown to bind to EcoSSB with almost equally high affinity, but in a 2:1 stoichiometry (Bujalowski & Lohman, 1989b).
Both DNA oligos were employed to investigate the interaction and to determine the exact binding affinity by MST. Intriguingly, the MST time traces of the titration of Cy5-labeled oligo(dT)$_{70}$ and oligo(dT)$_{35}$ with EcoSSB as well as the corresponding thermophoresis signals showed striking differences (Figure 3A and B): While the titration curve of oligo(dT)$_{70}$ displays a typical, sigmoidal shape, the titration curve of oligo(dT)$_{35}$ displays an atypical peak in the thermophoresis signal close to the apparent point of saturation. Interestingly however, this peak disappears when plotting the T-jump signal (Figure 3C), suggesting that the local changes that affect fluorescence upon binding of either oligo(dT)$_{70}$ or oligo(dT)$_{35}$ are identical, but that different molecular species with different thermophoretic properties are formed by binding of EcoSSB to the different oligo-nucleotides.

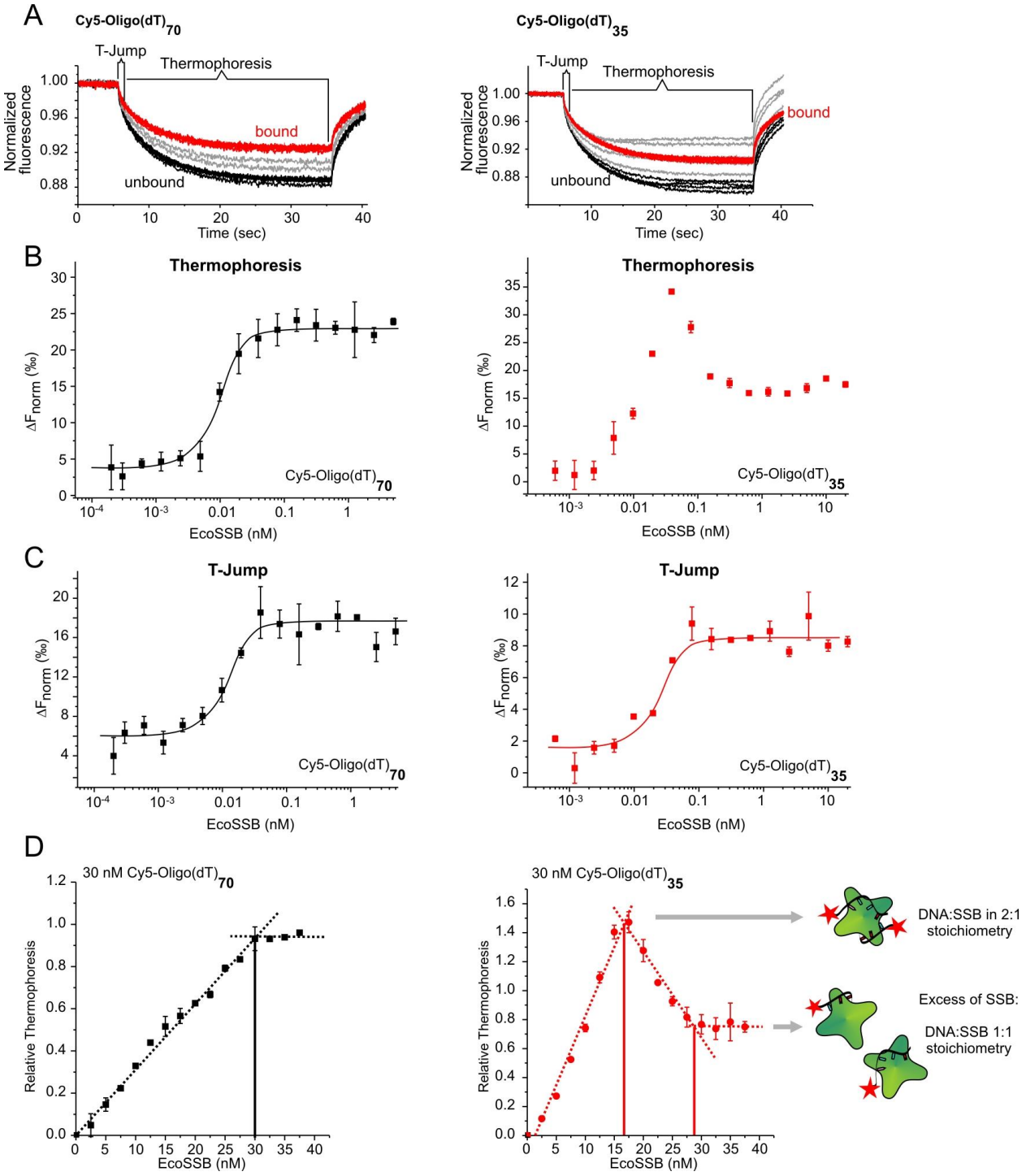

Figure 3: MST of the interaction of EcoSSB with Cy5-oligo(dT)$_{70}$ and Cy5-oligo(dT)$_{35}$ ssDNA allows for determination of stoichiometries and binding modes. (A) Comparison of MST traces of titrations of EcoSSB against 20 pM Cy5-oligo(dT)$_{70}$ (left) and 100 pM Cy5-oligo(dT)$_{35}$ (right). Traces corresponding to bound and unbound states are colored black and red, respectively, traces corresponding to partially bound intermediates are shown in grey. Regions of the traces corresponding to T-Jump and thermophoresis signals are highlighted. (B) Thermophoresis signal from experiments equivalent to those shown in (A). Error bars = s.d.; n=3. (C) T-Jump signal from experiments equivalent to those shown in (A). Error bars = s.d.; n=3. (D) Thermophoresis signals of the titration of EcoSSB from 40-0 nM against 30 nM Cy5-oligo(dT)$_{70}$ (left) and Cy5-oligo(dT)$_{35}$ (right). Dotted lines represent linear extrapolations. Solid lines highlight "kinks" in those saturation curves, which correspond to different occupancies of EcoSSB with Cy5-oligo(dT) constructs. Error bars = s.d.; n=2.

To analyze the differences in thermophoresis in more detail, a saturation experiment was performed, in which a narrower range of EcoSSB (0 to 40 nM) was titrated against higher concentrations of Cy5- labeled oligonucleotides (30 nM). Under these conditions, added EcoSSB is completely bound to the oligonucleotide until saturation is reached, resulting in a linear increase of the MST signal. At EcoSSB concentrations at and above the saturation point, the thermophoresis signal does not change and remains constant despite further addition of EcoSSB. For the interaction of Cy5-oligo(dT)$_{70}$ with EcoSSB, a saturation curve was obtained with a clear saturation "kink" at 30 nM EcoSSB, validating the previously reported 1:1 stochiometry of this interaction (Figure 3D). Interestingly, the same experiment with Cy5-oligo(dT)$_{35}$ yielded different thermophoresis signals. Here, the signal first increased with increasing EcoSSB concentrations and reached a peak at around 15 nM, but then decreased again to reach a second "kink" at around 30 nM EcoSSB. The positions of the different "kinks" indicate two different binding stoichiometries. In this example, this can be correlated to two distinct species of oligonucleotide-EcoSSB complexes: at 15 nM EcoSSB, each EcoSSB molecule is saturated with two Cy5- oligo(dT)$_{35}$ molecules, resulting in a 2:1 stoichiometry (Bujalowski & Lohman, 1989a; Bujalowski & Lohman, 1989b) (Figure 3D). Addition of EcoSSB at concentrations >15 nM results in a decrease of occupancy, until only one Cy5-oligo(dT)$_{35}$ molecule is bound per EcoSSB, which is reflected by the second kink around 30 nM (Figure 3D). This effect is particularly pronounced since ssDNA fragments bind to EcoSSB with negative cooperativity (but high affinity), resulting in a homogenous mixture of oligo(dT)$_{35}$-EcoSSB 1:1 complexes. Importantly, the different species can only be identified by analyzing the thermophoresis signal, while the T-jump signal does not allow to discriminate the different complex species (compare Figure 3B and 3C). The T-Jump signal solely depends on the local surrounding of the fluorophore, which should be independent of the occupancy of EcoSSB, while thermophoresis detects the different migration of the formed complexes in the temperature gradient. This movement is dictated by its size and hydration shell and thus harbors information on the overall structure of the newly formed complex.

Conclusion

The presented example shows that MST signals contain binding information that exceed simple equilibrium constants, and that these information can be used to infer binding stoichiometries and binding modes for a given biomolecular interaction.

Material and Methods

Instrument and settings: All experiments were performed on a Monolith® NT.115^{Pico}. K_d determination was performed with 40 % MST power and 100 % LED power.

EcoSSB was a kind gift from Dr. Ute Curth and was purified as described previously (Curth et al, 1994). 5´-Cy5-labeled oligo(dT)$_{70}$ was obtained from Axolabs (Kulmbach, Germany). Cy5-labeled oligo(dT)$_{35}$ was obtained from Metabion (Martinsried, Germany). Measurements were carried out in SSB-buffer (20 mM HEPES pH 7.4, 300 mM NaCl, 0.05 % tween-20) and standard capillaries. Photobleaching was suppressed using the NanoTemper Anti-Photobleaching kit. All pre-dilutions and dilution series were pipetted in low-binding tubes to prevent sample loss on tube walls. Error bars reflect the standard deviation from three independent experiments.

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