Exploring Protein Stability by nanoDSF

Prometheus NT.48 – The Stability Expert

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Abstract

nanoDSF determines thermal and chemical protein stability with ultra-high resolution and with unmatched reproducibility. The detection of the intrinsic tryptophan and tyrosin fluorescence enables measurements at close-to-native conditions in any buffer, even in the presence of detergents. nanoDSF is applied in antibody engineering, membrane protein research, formulation and quality control.

Introduction

Protein unfolding and denaturation is one of the major causes for protein loss-of-function, and must therefore be carefully controlled in drug discovery and drug development. Many potential drug targets such as integral membrane proteins, cell surface receptors or kinases, must maintain their functionality during cost-intensive screening campaigns, and might react very differently to physical and thermal stress. Furthermore, optimal formulation conditions need to be identified for therapeutic antibodies which must maintain their functionality during storage and shipment.

The Prometheus NT.48 measures thermal unfolding profiles of proteins by detecting even minute changes in the emission properties of the amino acid tryptophan upon unfolding. By following the shift in the fluorescence emission wavelength, an unfolding curve can be generated (Figure 1). Importantly, the specific fluorescence ratios (F350/F330) of the folded and unfolded states can be directly correlated to the respective fraction of unfolded protein at any point during the unfolding process (Figure 1).

This also means that a single fluorescence scan of a protein sample contains information about the overall amount of unfolded protein, once the respective F350/F330 values of the folded and unfolded state are known. This information is valuable for protein quality control, either for evaluating long-term stabilities of biologicals or the effects of physical or thermal stress on protein stability.

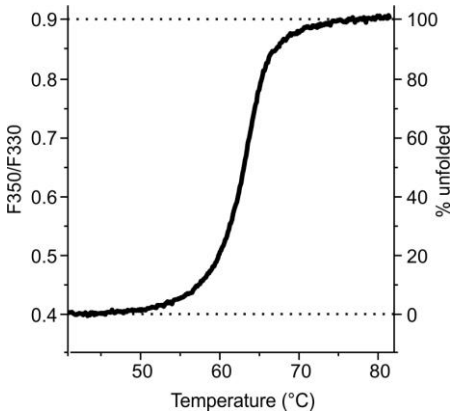

Figure 1 Example of a typical thermal unfolding curve by nanoDSF. The fluorescence ratio F350/F330 can be expressed as % unfolded if the fluorescence ratios of the folded and unfolded states are known.

The Prometheus NT.48 uses a capillary “dip-and-read” format, meaning that the capillaries can be directly loaded by dipping into a protein solution without additional pipetting steps. The measurement of the F350/F330 of up to 48 capillaries at a given temperature takes 7 seconds, and the Prometheus NT.48 instrument also does not require any calibration times, so that quality control measurements as we describe them here can be performed in less than a minute.

Results

Unfolding of an IgG-antibody as proof-of-concept

In order to verify the capability of the Prometheus NT.48 to exactly quantify the fraction of unfolded protein in a given formulation based on the F350/F330 ratio, we created an unfolding standard solution in which a monoclonal IgG antibody was subject to controlled thermal unfolding at 80 °C. Unfolded IgG was then mixed with folded IgG to different extends, resulting in formulations with defined amounts of unfolded antibody.

In thermal unfolding experiments, all formulations showed similar thermal unfolding profiles with unfolding transition temperatures around 74 °C.

However, the initial fluorescence ratio (F350/F330) at 25 °C increased with increasing concentrations of unfolded antibody (Figure 2A). A plot of the calculated and experimentally determined percentage of unfolded IgG versus the detected F350/F330 ratio displayed a clear linear correlation as well as a very good agreement between calculated and experimental data. Thus, the Prometheus NT.48 can pick up even small fractions of unfolded IgG (< 0.5 %) in formulations, and can thus be used as a quick and reliable method to follow the stability of antibodies, e.g. during long-term storage tests.

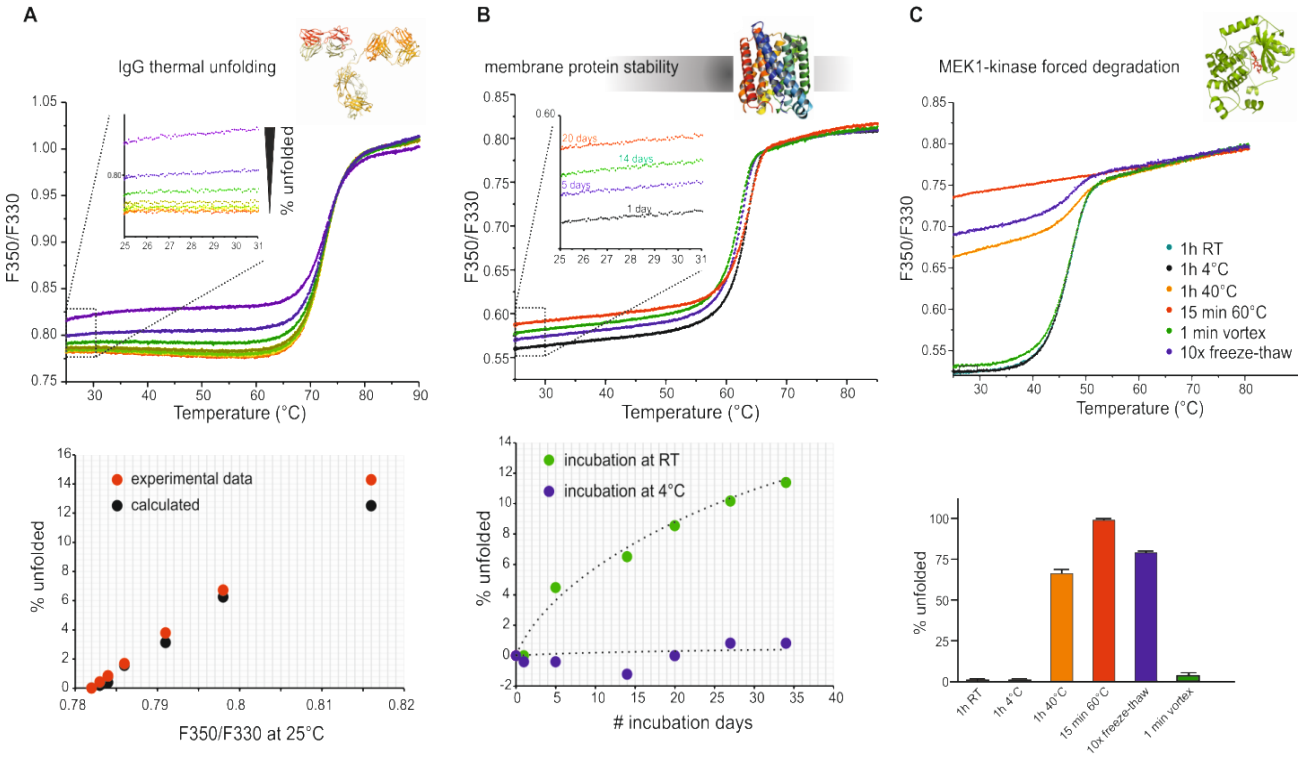

Figure 2 A) Establishing a protein unfolding standard. Unfolded IgG at different concentrations was mixed with folded IgG and subject to thermal unfolding. The percentage of unfolded IgG in the solution was quantified based on the F350/F330 ratio measured at 25 °C.
B) Long-term storage test on HiTeha. Aliquouts of the integral membrane protein HiTeha were stored at 4 °C and at RT, respectively, and thermal unfolding curves were measured over a time period of 34 days. %unfolded protein was calculated based on the F350/F330 ratio.
C) Forced-degradation stress-test on MEK1
MEK1 protein was subject to the indicated stresses, and the fraction of unfolded protein was calculated based on the F350/F330 ratio at 25 °C. Error bars are s.d. from three measurements.

Assessing long-term stability of integral membrane proteins

Next, we asked whether this approach can even be used for long-term storage tests of detergent-solubilized membrane proteins. For this, we used the slow-anion channel protein HiTeHa and incubated the protein at 4 °C and at RT, respectively (Maschberger et al, 2015). Then, we determined F350/F330 ratios and measured thermal unfolding curves over a period of 34 days (Figure 2B). Again, a clear storage temperature- and incubation time-dependent shift in the F350/F330 ratio could be observed, while the Tm-values of the thermal unfolding curve did not change significantly. The results show that HiTeha is rather stable, but that it tends to degrade slowly when stored at room temperature. Thus, the F350/F330 ratio can be used to measure the amount of unfolded, detergent-solubilized membrane protein.

Forced denaturation of the drug target MEK1

We next used a forced degradation approach to test protein stability. Such an approach can be valuable to identify critical handling steps during protein purification, storage or setup of biochemical assays. Information about the stability of a protein under different conditions is particularly important for biophysical screening campaigns, in which multiple biochemical assays are applied. We chose the popular drug target MEK1 kinase, which already has been used in a large number of different drug discovery screening campaigns, including a MST-based fragment screen (see Application Note NT-MO-021 (Breitsprecher et al, 2014) and (Amaning et al, 2013; Oyarzabal et al, 2010)). MEK1 solutions were subjected to a number of thermal and physical stresses, namely incubation at different temperatures, heavy vortexing, and repeated freeze-thaw cycles. Again, the fluorescence ration F350/F330 served as an indicator for the fraction of unfolded protein (Figure 2C).

Incubation of MEK1 for 1 h on ice or at RT resulted in no significant difference in the F350/F330 ratio, showing that the protein can be handled at RT over this time period without risking protein denaturation. This finding is in agreement with the previously performed MST-based fragment screening campaign, in which the MEK1-nucleotide interaction was shown to be stable for > 12 h at RT (Breitsprecher et al, 2014). In contrast, incubation at an elevated temperature of 40 °C for 1 h, which is above the unfolding onset temperature, already resulted in a fraction of unfolded protein of ~ 65 %. Incubation of the protein solution for 15 minutes at 60 °C led to a complete unfolding of the protein, as expected, indicated by the absence of a unfolding transition in the thermal unfolding curve. Interestingly, the protein was rather resistant towards mechanical stress: vortexing for 1 minute at maximum rpm resulted in only a small fraction of unfolded protein (< 5 %). In contrast, repeated freeze-thaw cycles strongly denatured the protein, leading to > 75 % unfolded protein (Figure 2C).

Thus, this set of experiments quickly revealed that MEK1 is stable at ambient temperature and rather insensitive against mechanical stress such as vortexing, whereas freeze-thaw cycles and elevated temperatures promote the denaturation of the protein and should be avoided.

This type of assay is also well suited to easily test and optimize conditions for protein immobilization on biosensor chips.

Antibody formulation screen

To identify optimal storage conditions for the therapeutic monoclonal antibody mAb1, the protein was subjected to a thermal unfolding formulation screen. 45 different buffer conditions were tested to address the thermal unfolding transition temperatures of the antibody in dependence of pH, salt and buffer substances. The buffer screen comprised the buffer substances Na-Citrate (pH 3.5-6), Na-Acetate (pH 3.2-6), $NaPO_4$ (pH 6-8.5), and Tris (pH 7-8.5), each at 25 mM final concentration, each in the absence and presence of 130 mM NaCl.

Thermal unfolding experiments were carried out on the Prometheus NT.48 at a heating rate of 1 °C/min. The antibody showed multiple unfolding transitions in the plot of the fluorescence ratio (F330/F350) versus temperature (Figure 3A). The distinct unfolding events can be attributed to the different thermal stabilities of FAB- and FC-domains of the antibody.

A plot of the unfolding transition temperature versus pH revealed that the thermal stability strongly depended on the pH of the buffer (Figure 3B): While the antibody displayed constant thermal

stabilities at a pH range from 6 to 8.5, with Tm1-values around 70 °C, a strong decrease in unfolding transition temperatures at pH values < 6 was observed, pointing towards a significant destabilization under these conditions. Conversely, the respective buffer substance and salt concentration had little to no effect on the thermal stability of the antibody.

These results show that the Prometheus NT.48 delivers highest quality thermal unfolding data for antibody buffer screening campaigns.

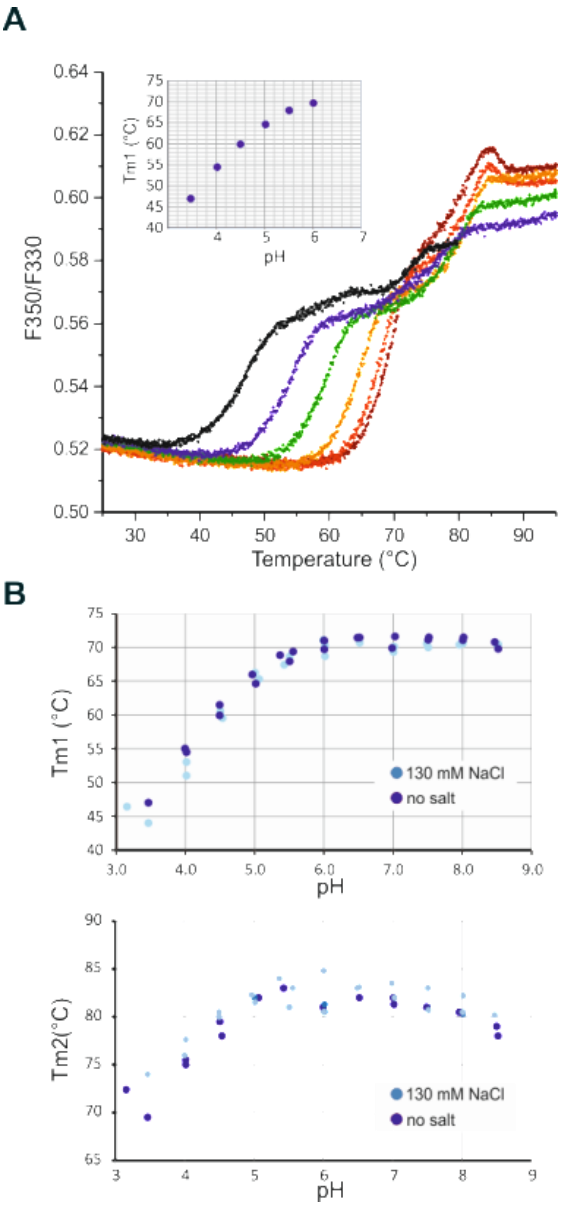

Figure 3 Thermal unfolding curves and unfolding transition midpoints. (A) Thermal unfolding curves in presence of 25 mM Na-Citrate at different pH values. Insets show the pH-dependence of the first unfolding transition midpoint (Tm1). (B) Dependence of Tm1 and Tm2 on the pH of the buffer for all tested conditions.

Conclusion

The presented data demonstrate that the Prometheus NT.48 can be employed to quickly detect and quantify unfolded proteins for quality control purposes with unmatched speed, at the same time offering unique ease of use. NanoTemper's *on-the-fly* technology allows to measure 48 samples in parallel providing more than 10 data points per minute. Formulation development as well as antibody engineering projects benefit from ultra-high resolution which is not compromised by aggregation. Thus, nanoDSF enables the identification of unfolding transitions for single domains which can then be selectively targeted. In addition, the large dynamic range of the Prometheus NT.48 allows for analyzing thermal unfolding in solutions containing antibody concentrations of more than 200 mg/ml down to few µg/ml. Thus, it can be utilized for both, stability screening during early phases of antibody development where only small amounts of protein are available, as well as for final formulation screenings campaigns in highly concentrated and even very viscous samples.

By using high-precision capillaries and the dual-UV technology, a so far unmatched reproducibility of data is achieved, which is essential for long term stability tests. Furthermore, the temperature diverges less than 0.1 °C throughout the capillary tray leading to stability results deviating by less than 0.05 °C.

Thus, the Prometheus NT.48 with its nanoDSF technology sets novel standards for fluorescence-based analysis of protein stability.

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