Thermal stability of membrane-bound proteins

Application Note NT-PR-012

nanoDSF Thermal Unfolding Analysis of a Membrane-bound Esterase in Various Detergents

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Abstract

nanoDSF, the miniaturized differential scanning fluorimetry technology, is a revolutionary method to determine the thermostability of proteins by following changes in their intrinsic fluorescence.
In this comparative study, the Prometheus NT.48 was used to determine the thermal stability of the membrane esterase PA2949 from *Pseudomonas aeruginosa* in presence of various detergents. The detergent type strongly affected enzyme thermal stability, which moreover correlated with enzyme activity. Thus, the Prometheus NT.48 can be used not only to rapidly screen for optimal purification conditions for enzymes, but also to evaluate enzyme activities based on their conformational stability in presence of detergents.

Introduction

Intrinsic protein fluorescence can mainly be attributed to the fluorescence of tryptophan (Trp) residues which is strongly sensitive to the polarity of the Trp micro-environment. Trp fluorescence is excited at 280 nm and emission occurs at 330 nm for Trp in non-polar environment and at 350 nm for Trp in polar environment [1]. Upon protein unfolding, Trp residues which are normally hidden in the protein hydrophobic core or in the detergent micelle get exposed to water resulting in a decrease of their fluorescence intensity and a shift of their emission maximum to longer wavelengths. Thus, by measuring the changes in Trp fluorescence intensity, the melting temperature (T_m) of proteins can be determined in a dye-free approach [2]. This approach is used by nanoDSF and has already been successfully applied for detergent-solubilized membrane proteins. This protein class is typically difficult to analyze with other techniques such as Thermofluor, since the hydrophobicity of the detergent micelles prevents the use of unfolding-reporting dyes such as Sypro-Orange. Also, differential scanning calorimetry is very sensitive towards different detergent types, limiting its applicability for experiments with membrane proteins. Since detergents are indispensable for successful purification and storage of membrane proteins, the screening for the optimal detergent is crucial, but is often still performed using a sub-optimal trial-and-error principle which is time consuming, unproductive and expensive.

Here, we analyzed the thermostability of a membrane-bound esterase (PA2949) from *Pseudomonas aeruginosa* PA01 [3]. PA2949 hydrolyses the racemic methyl ester of *β*-acetylthioisobutyrate to produce the (*D*)-*β*-acetylthioisobutyric acid (DAT). DAT is a valuable key intermediate for the synthesis of captopril (Figure 1), a drug used for treatment of hypertension [4].

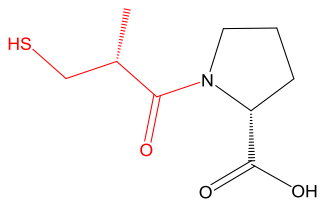

Figure 1: Chemical structure of captopril with (*D*)-*β*-thioisobutyrate group produced by PA2949 depicted in red.

Using the Prometheus NT.48 instrument, we could demonstrate that thermal stability of PA2949 strongly depends on the detergent type, and that enzyme activity directly correlated with the determined T_m-values.

Results

Using the Prometheus NT.48 instrument we have monitored the changes in fluorescence upon thermal unfolding of PA2949 in the presence of four different detergents: anionic sodium dodecyl sulfate (SDS), zwitterionic 3-(N,N-dimethylpalmitylammonio)propanesulfonate (SB3-16), and the neutral detergents *n*-octyl *β*-D-glucoside (OG) and *n*-dodecyl *β*-*D*-maltoside (DDM). All detergents were used at concentrations above the CMC (critical micelle concentration) which are necessary for stabilization of membrane proteins (Table 1). The ratio of fluorescence intensities at 350 nm and 330 nm as a function of temperature was used to determine the transition temperature, which can be interpreted as the melting temperature of PA2949 (Figure 2). Interestingly, SDS and SB3-16 caused immediate unfolding of PA2949 as demonstrated by the absence of an unfolding signal by nanoDSF. OG only slightly destabilized the enzyme, and DDM had no negative effect on PA2949 stability (Table 1).

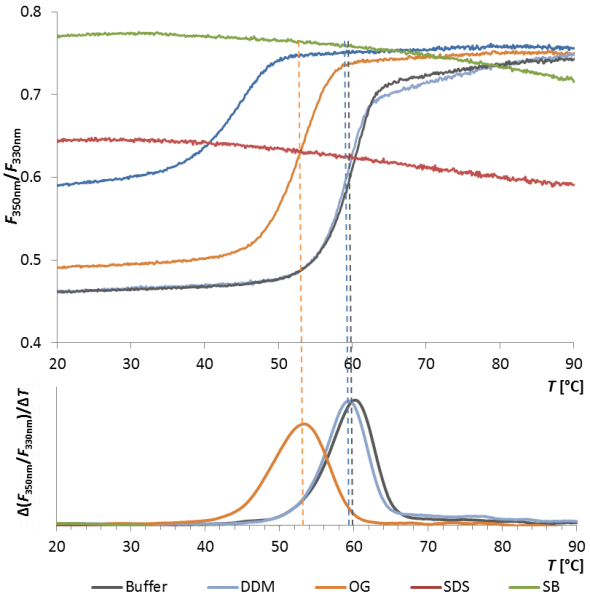

Figure 2: Thermal stability of PA2949 measured with the fluorescence-based nanoDSF method. The fluorescence ratio (F_{350nm}/F_{330nm}) and the derivative of the fluorescence ratio ($\Delta(F_{350nm}/F_{330nm})/\Delta T$) as a function of temperature are shown in the upper and lower diagram, respectively. Colored dashed lines indicate the melting temperatures (T_m) in the presence of different detergents.

We further determined thermal unfolding of PA2949 using a classical biochemical method, which relies on measurement of the residual esterase activity after exposure of PA2949 to different temperatures. These results revealed a complete loss of PA2949 activity in SDS and SB3-16, OG inactivated PA2949 only slightly and DDM did not cause any inactivation (Figure 3, Table 1).

The comparison of thermal stability of PA2949 in detergents studied by fluorescence and enzymatic methods revealed the same correlation of melting temperatures, T_m (DDM) > T_m (OG) > T_m (SDS) = T_m (SB3-16). However, the absolute melting temperatures of PA2949 in buffer measured enzymatically (T_m (buffer) = 52.1 °C) was approximately 8 °C lower than the one measured with the fluorescence method (T_m (buffer) = 59.9 °C).

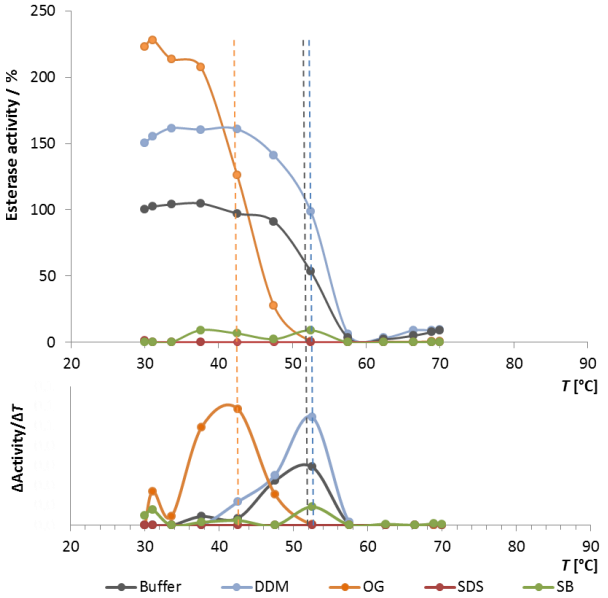

Figure 3: Thermal stability of PA2949 measured by determination of enzymatic activity. The relative esterase activity and the derivative of esterase activity (ΔActivity/ΔT) as function of temperature are shown in the upper and lower diagram, respectively. 100 % activity corresponds to the activity of PA2949 in buffer at 30 °C. Colored dashed lines indicate the melting temperatures (T_m) in the presence of different detergents.

Table 1: Comparison of melting temperatures of PA2949 measured with nanoDSF and enzymatic activity assay

Condition	CMC*	T_m (fluorescence) [°C]	T_m (enzymatic) [°C]
Buffer	-	59.9 ± 1.0	52.1 ± 0.7
DDM, 0.2 mM	0.15 mM	59.3 ± 0.1	52.8 ± 0.5
OG, 34.2 mM	25 mM	53.1 ± 0.4	43.4 ± 0.5
SDS, 1.5 mM	8 mM	unfolded	unfolded
SB3-16, 10 mM	3 mM	unfolded	unfolded

*CMC: the critical micelle concentration is defined as the concentration of detergent above which micelles form.

Conclusion

Our results show that the Prometheus NT.48 by NanoTemper Technologies is suitable for monitoring fluorescence emission changes of membrane proteins in the presence of detergent micelles.
The nanoDSF method allowed to identify DDM as the best suited detergent for PA2949 stabilization. This result was confirmed with an enzymatic activity assay. Moreover, nanoDSF showed that SDS and SB3-16 completely denature PA2949 in agreement with a complete loss of esterase activity of PA2949 measured enzymatically. Apparently, the nanoDSF method can be used for the fast and reliable identification of detergents suitable for the solubilization and storage of membrane proteins.
These results demonstrate the potential of the Prometheus NT.48 for determination of detergent-protein compatibility, which is particularly interesting for proteins which cannot be assayed enzymatically.

Material and Methods

Protein Preparation

PA2949 was expressed in *P. aeruginosa* PA01 carrying pBBRplbFH6 expression plasmid [3] cultivated in LB media supplemented with 100 µg/ml tetracycline at 37 °C. The cells were harvested when the culture reached an optical density of 1 by centrifugation for 10 min at 3000*g* and 4°C. Cells suspended in 100 mM Tris-HCl (pH 8.0) were disrupted by French press and total cell membranes were isolated by ultracentrifugation for 1 h at 200,000 *g* and 4 °C.
The total membrane fraction containing PA2949 was solubilized with Tris-HCl (100 mM, pH 8.0) containing 1 % (v/v) Triton X-100 by gentle agitation overnight at 4 °C. PA2949 was purified from the solubilized membranes by immobilized metal affinity chromatography using Ni-NTA material [3]. Elution buffer was exchanged to Tris-HCl (100 mM, pH 8) containing 0.22 mM DDM by PD-10 desalting column (GE Healthcare, Solingen, Germany) according to the manufacturers´ protocol. This PA2949 sample was furthermore purified by anion exchange chromatography using UNOsphere Q material (BioRad, Hercules, CA, USA).

Thermal unfolding experiments

The samples for thermal unfolding experiments were prepared by combining the same volume of the pure PA2949 sample (6 µM) and Tris-HCl buffer (100 mM, pH 8) containing the respective detergent. The final concentrations of each detergent given in the table 1 were above the CMC. PA2949 was incubated with detergent for 30 min at room temperature. The same PA2949 samples were used for determination of fluorescence and enzymatic activity.
Fluorescence based thermal unfolding experiments were performed using the Prometheus NT.48. The capillaries containing 10 µl PA2949 samples were inserted into the machine, the temperature was increased by the rate of 1 °C/min from 20 °C to 90 °C and the fluorescence at emission wavelengths of 330 nm and 350 nm was measured.
Enzyme activity based thermal unfolding experiments were performed by measuring the residual esterase activity of purified PA2949 samples incubated at temperatures from 30 °C to 70 °C. Enzyme assay was performed in a 96-well microplate by combining 10 µl of the enzyme sample with 150 µL of *p*-nitrophenyl butyrate substrate [5].

References

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