

Thermal Unfolding of Membrane Proteins

Application Note NT-PR-002

Detergent Screen for solubilized membrane proteins – Case study on the SLAC-protein HiTehA from *Haemophilus influenzae*

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Abstract

The biophysical characterization of integral membrane protein stability is often challenging due to several factors: First, the expression and purification of membrane proteins is often impeded by low expression levels and protein stability. As a result, yields are usually low and do not allow for a thorough analysis or a screening approach to determine optimal conditions. Second, the use of detergents – which are necessary to solubilize membrane proteins – often introduces artifacts and other secondary effects, and most importantly precludes the use of reporter dyes to monitor protein unfolding. Label free methods – such as DSC or CD spectroscopy – on the other hand require large quantities of proteins, and are limited in throughput.

Here we use the 10 transmembrane-helix protein HiTehA, a protein of the slow anion channel family, to present label-free, native DSF as the method of choice to perform rapid and precise detergent screening projects for a solubilized membrane protein.

Introduction

Membrane proteins account for 20-30 % of the coding regions of all sequenced genomes and play crucial roles in many fundamental cell processes. For instance, ion channels, G-protein coupled receptors and carrier proteins are important in the regulation of a plethora of inter- and intramolecular processes. Defects in these proteins are often

linked to a number of severe diseases thereby rendering them promising targets for novel drugs [1, 2]. However, obtaining sufficient quantities of a purified integral membrane protein for downstream experiments, such as structural or functional analysis in high-throughput screening approaches, can be challenging due to low yields and often poor stability [3].

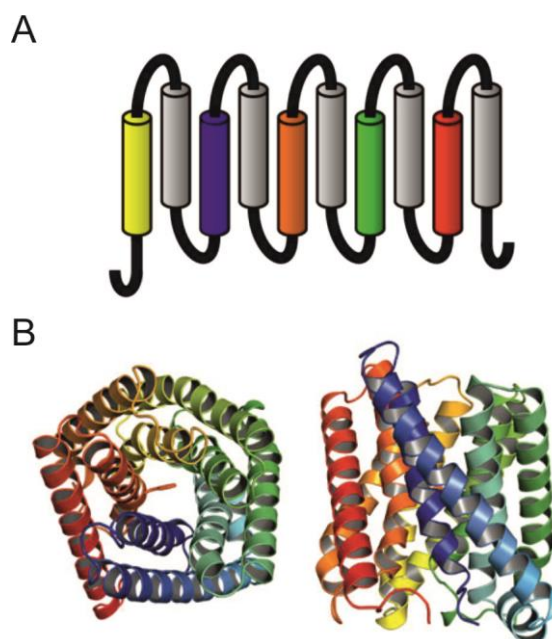


Figure 1: Structure of HiTehA.

A) Schematic representation of the domain organization of SLAC1-like transmembrane proteins. B) Crystal structure of HiTehA showing the quasi-five-fold symmetrical arrangement of transmembrane helices around the central pore.

One way to stabilize membrane proteins after purification and to make them amendable for subsequent biophysical analysis is solubilization with detergent additives [4].

Identifying the right detergent and buffer conditions however is a challenge by itself, since the low concentrations and yields of membrane proteins usually pose a bottleneck for buffer screening approaches by methods such as DSC or CD-spectroscopy. Moreover, commonly used fluorometric approaches with reporter dyes – such as DSF with SyproOrange – are not compatible with detergent-containing formulations.

Here we present a label-free, fluorometric high-throughput approach to monitor thermal stability of a transmembrane protein.

Using the Prometheus NT.48, we performed a detergent screen on the tellurite resistance protein A from *Haemophilus influenzae* (HiTehA). The integral membrane protein TehA together with TehB, a soluble cytoplasmic protein, confers resistance to tellurite in bacteria by an unknown mechanism [5]. Later studies showed that the integral membrane protein TehA possibly transports lipophilic quaternary ammonium compounds [6]. Sequence analyses predicted an architecture containing 10 transmembrane helices (Figure 1A) [6]. Crystal structure analysis of HiTehA confirmed this architecture, and revealed a novel transmembrane fold (Figure 1B): five helix-tandems are arranged in a quasi-five-fold symmetry, resulting in five inner and five outer helices traversing the cell membrane, surrounding the central pore [7].

Table 1: List of detergents used in this study

Detergent	Full name	CMC	final concentration (mM)
Z3-12	ZWITTERGENT 3-12 (ANZERGENT 3-12)	2.8	0.84
APO11	Dimethylundecylphosphine oxide	1.2	0.36
FC-U10-11	FOS-CHOLINE-UNSAT-11-10	6.2	1.55
7-DHPC	1,2-Diheptanoyl-sn-glycero-3-phosphocholine	1.4	0.42
LPC-14	14:0 LysoPC (1-tetradecanoyl-2-hydroxy-sn-glycero-3-phosphocholine)	0.06	0.3
T-20	Tween 20 (Anapoe-20)	0.059	0.59
FACADE-EM	Façade-EM	0.1	1
NG	n-Nonyl-β-D Glucopyranoside	6.5	1.625
DdαM	n-Dodecyl-α-D-maltopyranoside	0.15	0.75
DDTM	n-Dodecyl-β-D-thiomaltopyranoside	0.05	0.5
DDM	n-Dodecyl-β-D-maltopyranoside	0.17	0.85
DOMG	n-Dodecyl-N,N-dimethylglycine	1.5	0.45
CF-4	CYCLOFOS-4	14	2.8
FC-I11	FOS-CHOLINE-ISO-11	26.6	5.32
FC12	FOS-CHOLINE-12	1.5	0.45
C8E5	Pentaethylene glycol mono-octyl ether	7.1	1.775
C10E5	Pentaethylene glycol monodecyl ether	0.81	0.81
C6E3	Triethylene glycol monohexyl ether	23	4.6
C13E8	Polyoxyethylene(8)tridecyl ether (Anapoe C13E8)	0.1	1
C12E10	Polyoxyethylene(10)dodecyl ether (Anapoe C12E10)	0.2	1
CHAPS	CHAPS	8	2

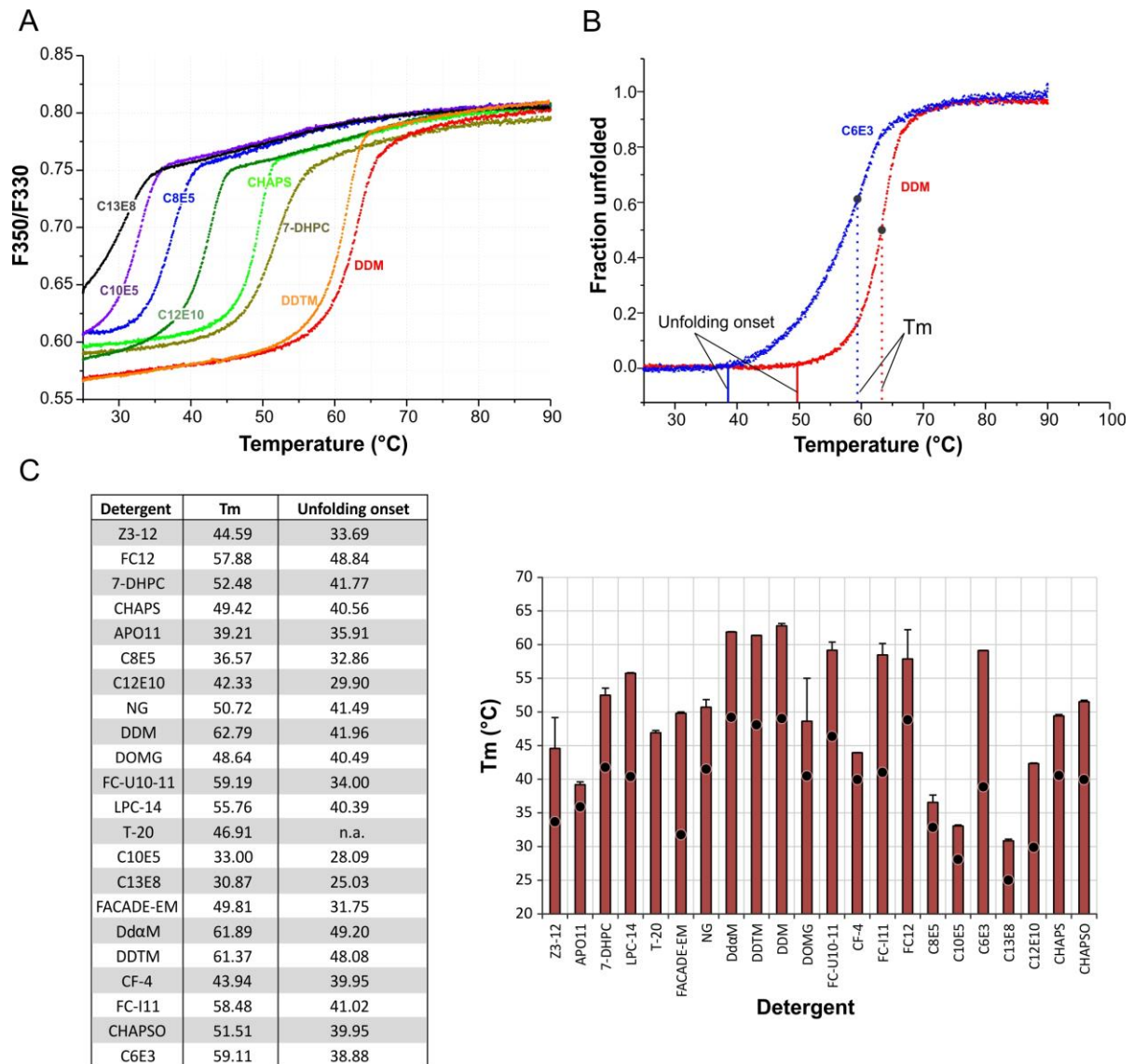


Figure 2: Detergent screen of HiTehA.

A) Representative thermal unfolding curves of HiTehA in 50 mM Tris HCl pH 8.0, 200 mM NaCl and the respective detergents. B) Example for different unfolding onset temperatures in presence of C6E3 and DDM. C) Summary of T_m values (red bars, right) and unfolding onset temperatures (black dots, right) in presence of different detergents.

The structure has gained great interest as TehA shares limited sequence homology with the plant anion channel proteins of the SLAC/SLAH family [7, 8]. The name-founding member of this plant anion channel family SLAC1 (slow anion channel 1) alters the turgor pressure in guard cells in accordance to release of the plant phytohormone abscisic acid, thereby closing or opening the stomata. The latter control the uptake of CO₂ required for efficient photosynthesis, however as open stomata also lead to water loss due to transpiration, a stringent control of opening and closing of these valves by altering the transport of the anion channel is essential for the plant's survival.

Results

Natively folded membrane proteins are most likely obtained by extracting them out of plasma membranes of the expression host by detergents. The detergent used for proper solubilization might interfere with stabilizing the protein in solution or is inconvenient for crystallization or other subsequent analysis processes thus requiring a detergent exchange during purification. As determining the best-suited detergent is an empirical process for each membrane protein a large number of different detergents have to be screened. However, limited supply due to low expression yields and low

purification efficiency necessitate a simple and fast screening method, which requires only minor amounts of protein. In this study we used the ion channel protein TehA from *Haemophilus influenzae* (HiTehA) as a test membrane protein to screen a number of different detergents to analyze their influence on thermal protein stability. For this, 10 μ l aliquots of purified HiTehA in purification buffer (50 mM Tris HCl pH 8.0, 200 mM NaCl, 0.02 % DDM) at a concentration of 1.2 mg/ml was first diluted 1:2 into buffers containing an excess of different detergents to promote detergent exchange at the protein (Table 1). Next, a detergent-removal-column-based detergent exchange was performed, in which HiTehA was eluted into 20 μ l of buffer with the respective detergent at the indicated final concentrations (Table 1). The final concentration of HiTehA for thermal unfolding experiments was 100 μ g/ml, corresponding to a molar concentration of \sim 2 μ M.

The HiTehA solutions were loaded into nanoDSF grade capillaries and subjected to thermal unfolding using the Prometheus NT.48. HiTehA stability was analyzed in presence of 22 different detergents (Table 1) in duplicate experiments within a single run. Plots of the tryptophan fluorescence ratio (F350 nm / F330 nm), which detects both, changes in tryptophan intensity and emission peak position, show a clear unfolding transition of HiTehA under all conditions tested. Since the analysis of the fluorescence ratio at 350 and 330 nm selectively monitors shifts in tryptophan fluorescence, this approach is basically unaffected by unspecific autofluorescence of the detergents.

A selection of thermal unfolding curves is shown in Figure 2A. Notably, detergent-dependent unfolding transition temperatures (T_m) differed by as much as 30°C. HiTehA was most stable in presence of the pyranoside-based detergents DDM ($T_m = 62.8 \pm 0.3$ °C), DDTM ($T_m = 61.37 \pm 0.05$ °C) and D α M ($T_m = 61.9 \pm 0.05$ °C), as well as in presence of the FOS-CHOLINEs FC-U10-11 ($T_m = 59.2 \pm 1.2$ °C), FC-I11 ($T_m = 58.5 \pm 1.0$ °C) and FC12 ($T_m = 58.5 \pm 1.6$ °C).

Conversely, several detergents such as C10E5 and C13E8 severely destabilized HiTehA, resulting in unfolding transition temperatures of 30.69 ± 0.24 °C and 33.17 ± 0.15 °C, respectively.

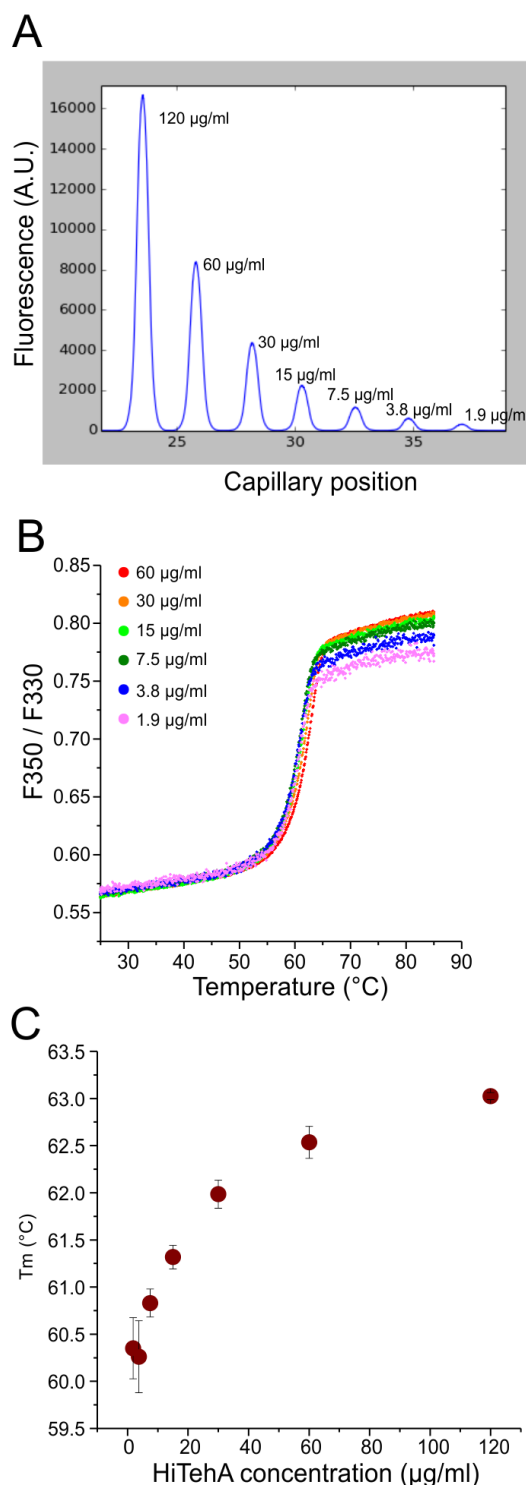


Figure 3: Concentration dependence of HiTehA thermal stability.

A) Initial discovery scan shows a broad range of fluorescence intensities prior to the thermal unfolding experiment. B) Thermal unfolding curves of HiTehA at different protein concentrations. C) Dependence of T_m of HiTehA on the total protein concentration.

Moreover, the unfolding curves revealed that some detergents greatly reduced the unfolding onset temperature. As an example, Figure 2B shows normalized unfolding curves of HiTehA in presence of DDM and C6E3, the T_m -values of which differ by only ~ 3.8 °C. However, the unfolding onset temperature was much reduced in presence of C6E3 compared to DDM, with a difference of 11 °C.

Since some membrane proteins can only be purified in very limited amounts, we tested whether it is possible to perform thermal unfolding experiments at very low protein concentrations. For this, a serial dilution of HiTehA in Tris buffer containing 0.02 % DDM was prepared and subjected to thermal unfolding. Notably, samples spanning a concentration range from 120 $\mu\text{g/ml}$ to 1.9 $\mu\text{g/ml}$ could be analyzed in a single run, showing that protein fluorescence intensities can differ by as much as ~ 70 fold and still allow for robust T_m determination in one experiment (Figure 3A and B). Interestingly, T_m -values decreased slightly with decreasing protein concentration, pointing towards a dilution-induced destabilization of the membrane protein (Figure 3C). Owing to the high sensitivity of the Prometheus NT.48, the entire detergent screen with 22 different detergents could have been performed with less than 0.5 μg of protein, demonstrating the efficiency of the Prometheus NT.48 for thermal unfolding experiments.

Conclusions

Our results show that monitoring of tryptophan fluorescence emission shifts during thermal unfolding using the Prometheus NT.48 is a perfect approach to determine the optimal conditions for membrane protein purification and storage. Since maximal stability of membrane proteins is a prerequisite for subsequent biophysical experiments and interaction studies, this approach enables the thorough and precise characterization of membrane proteins, which is particularly important in pharmacological screenings to identify novel drugs. Notably, micelle formation, autofluorescence or other secondary effects which are common for detergents, and which often interfere with biophysical characterization of membrane protein stability, do not impede measurements employing the Prometheus NT.48. Moreover, the high precision and sensitivity of the

instrument allows for detection of very low protein concentrations in the range of a few $\mu\text{g/ml}$ in as little as 10 μl of sample, thus protein consumption is very small compared to other methods. Last, since no equilibration times or washing steps are needed, more than 400 thermal unfolding experiments can be performed per day, thus guaranteeing fast and efficient screening of a variety of buffer conditions.

Material and Methods

Protein preparation

HiTehA was cloned into a modified pET28b vector using NcoI and NotI restriction site and protein was expressed with a C-terminal 3 x flag – 10 x His-tag in the *E. coli* strain C43 (DE3). Bacteria were grown in Terrific Broth Media (TB) in the presence of 50 mg l^{-1} kanamycin to an absorption at 600 nm of 0.6-0.8 at 37 °C. Then protein expression was induced with 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and continued for 4 hours at 37 °C. Cells were centrifuged and lysed by sonication in 50 mM Tris-HCl pH 8.0, 200 mM NaCl, protease inhibitor Set III (1:1000 final dilution). Bacterial membranes were obtained by ultracentrifugation and membrane proteins were extracted with 1 % (w/v) DDM (Carl Roth) over night under mild agitation at 8 °C. The supernatant of an ultracentrifugation step (150,000 g, 35 min, 4 °C) was loaded onto a Nickel-affinity-column (1 ml HisTrap Excel column, GE Healthcare). The column was washed with 20 CVs of 25 mM and 50 mM imidazole respectively. HiTehA was eluted with a gradient of 50 to 500 mM imidazole ranging over 20 CVs. Further protein purification was performed employing size exclusion chromatography using a Superdex 200 (XK 16/60, GE Healthcare) column and 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.02 % DDM as running buffer. HiTehA-containing fractions were pooled and concentrated up to 1.2 mg/ml using a VivaSpin6 ultrafiltration devices (100 kDa cut off). (Modified purification protocol after Chen et al., 2010)

In order to promote detergent exchange, DDM-solubilized HiTehA (in 50 mM Tris HCl pH 8, 200 mM NaCl and 0.02 % DDM) as obtained from the above-described preparation, was first diluted into a 10 x concentrated Tris-buffer indicated in Table 1. Selected detergents from the Memb-PASS™ Differential Filtration Detergent Screen

from Jena Bioscience (Jena, Germany) were used. After 30 minute incubation at RT, 20 μ l of the protein were applied to Pierce Detergent Removal Spin Column (0.5 ml), and prepared following the manufacturers protocol, except that the protein was directly eluted into 20 μ l of a 2 x concentrated Tris buffer (Table 1).

Thermal stability screen

HiTehA solutions were loaded into nanoDSF grade capillaries which were then loaded into the Prometheus NT.48. Each sample was measured in duplicate. Unfolding of HiTehA was detected during heating in a linear thermal ramp (1 $^{\circ}$ C/min, 25-90 $^{\circ}$ C) at low detector sensitivity and with an excitation power of 10 %, and unfolding transition points were determined from changes in the emission wavelengths of tryptophan fluorescence at 350 and 330 nm. Unfolding transition points were automatically identified by the Prometheus NT. Control software. Unfolding onset temperatures were determined after baseline correction and normalization of the data. The unfolding onset was defined as the point at which 1 % of protein was unfolded. To evaluate the concentration dependence of HiTehA unfolding, a 7 fold serial dilution of the protein in 50 mM Tris HCl pH 8, 200 mM NaCl and 0.02 % DDM was prepared, starting at a concentration of 120 μ g/ml.

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Notes

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