Stabilization of proteins for crystallization – how Thermofluor can help

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A major prerequisite for a successful structure determination project is that highly ordered, well-diffracting crystals have to be obtained of the molecule of interest. In this respect, the homogeneity (chemical and conformational), stability and solubility of a biological macromolecule are key factors that have a strong effect on the probability of obtaining crystals. It appears obvious, therefore, that optimization of these properties might improve the success rate of crystallization. One method to perform this analysis is the Thermofluor® approach. Thermofluor is a fluorescence-based thermal stability assay monitoring ligand effects on temperature-dependent protein unfolding [1].

The crystallization of macromolecules is a complex procedure which is strongly influenced by diverse environmental factors as pH, ionic strength, additives, precipitants, protein concentration and temperature. Moreover the crystallization behaviors are affected by the biochemical and biophysical properties of the protein sample itself, such as the chemical and conformational homogeneity, the stability and the solubility. Thermofluor presents a valuable method to monitor these properties as a function of the chemical composition of the solute. Consequently, these properties can be optimized, thereby enhancing the success rate of protein crystallization [2].

Thermofluor makes use of the observation that a hydrophobic fluorophore can be used to distinguish between the folded and unfolded state of the protein. More precisely, in an ideal case, at low temperature no fluorescence signal is observed because the protein is properly folded and no hydrophobic surface patches are present. However, with increasing temperature the protein starts to melt, thereby exposing hydrophobic areas, which can bind the fluoroprobe thus giving rise to a fluorescence signal. The resultant curve is used to determine the melting temperature T_m of the protein. Conditions and components which cause an increase in the melting temperature of the protein are therefore stabilizing the protein (Figure 1A). The basic assumption is now, that a more stable protein is more amenable to crystallization than a less stable one. Indeed, Ericsson *et al.* [2] observed a 2-fold increase in the number of crystallization hits when proteins were co-crystallized with stabilizing additives identified with Thermofluor.

A Thermofluor experiment is easy to perform and requires no prior knowledge of the protein properties. Every standard real-time PCR machine – available in many research labs – is suitable to perform this high-throughput experiment in a short time (1-2 hours). Only relatively little protein (500 µl of protein at 1-2 mg/ml for a 96-well experiment) and a dye (e.g. SYPRO® Orange, Invitrogen) are required. Proteins containing prosthetic groups (e.g. FAD) may not even require an external fluorophore if the intrinsic signal can be used during the Thermofluor experiment. For FAD-containing proteins this method has been termed ThermoFAD [3]. As a result, an 'optimal' buffer composition and/or potential physiological or non-physiological ligands causing an increased stability and homogeneity of the protein sample can be identified.

Subsequently, these new conditions can assist in optimizing the protein purification procedure yielding protein of higher quantity and quality.

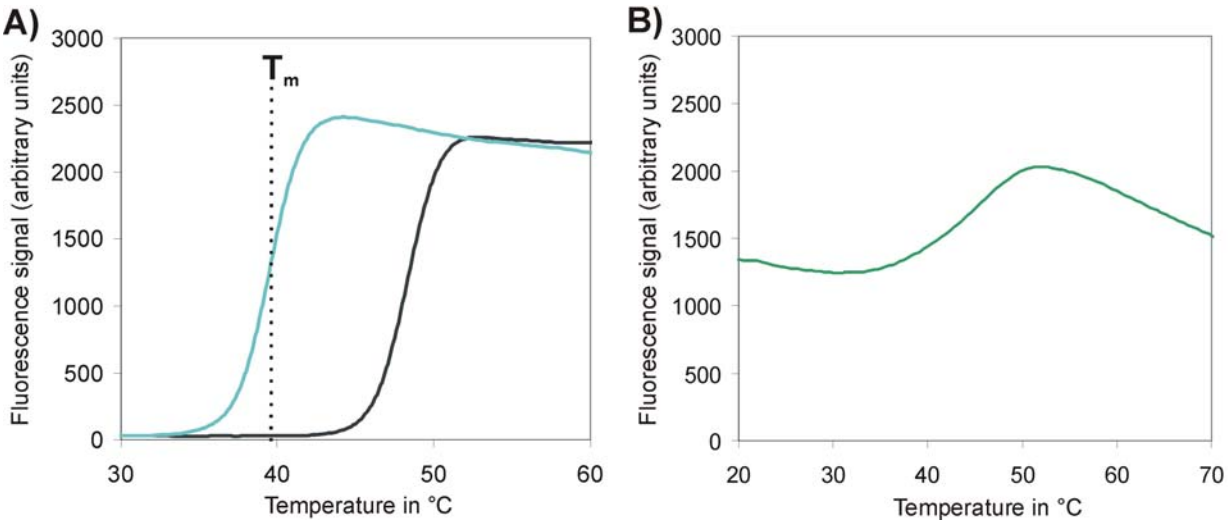

Figure 1: Thermofluor analysis of proteins from *Mycobacterium tuberculosis*. The melting temperature T_m is determined at the inflection point of the melting curve as indicated by the dotted line. **A)** The melting curve of the hypothetical secretory protein Rv0040c shows an ideal shape. The buffer 50 mM Bis-Tris pH 6.0, 100 mM NaCl (black curve; T_m=47°C) stabilizes the protein significantly compared to the buffer 50 mM Tris-HCl pH 8.5, 100 mM NaCl (blue curve; T_m=39.5°C). **B)** The melting curve of the protein DapE exhibits a high initial background fluorescence due to partially unfolded protein, however the melting process is still nicely visible. In both cases, the final protein concentration was 0.15 mg/ml and the concentration of the fluorophore SYPRO® Orange was 5x (based on the "5000x stock solution" as supplied by Invitrogen, the absolute concentration of the dye was not documented).

Needless to say, there is no experiment which guarantees 100% success and Thermofluor is no exception here. Not every protein sample is suitable for a Thermofluor experiment, simply because a hydrophobic core of sufficient size is required to produce a significant signal and some proteins, e.g. some DNA binding proteins, lack this feature. Additionally, the signal may be disturbed if the protein is already partially unfolded or hydrophobic patches are present (e.g. due to a lacking interaction partner) causing an increased background fluorescence (Figure 1B). However, Thermofluor experiments have successfully been applied to membrane proteins, although filtering out the signal out of the noise caused by the intrinsically present hydrophobic surface patches of membrane proteins is rather challenging [4].

References:

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