Differential scanning fluorimetry (Thermofluor)

A complementary technique in protein crystallization

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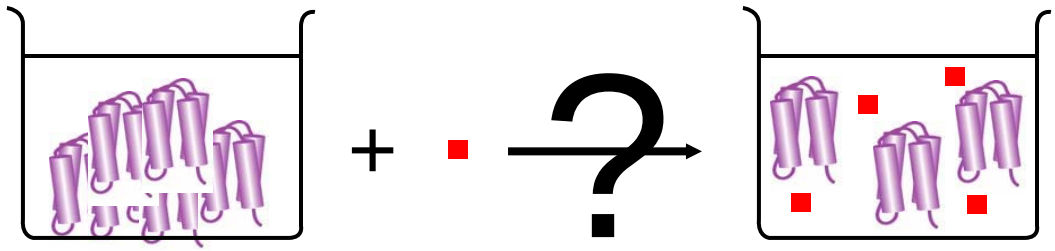
Common problems in protein science

Theory

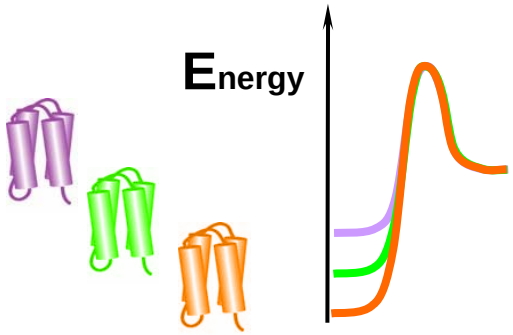
Applications

Protocol

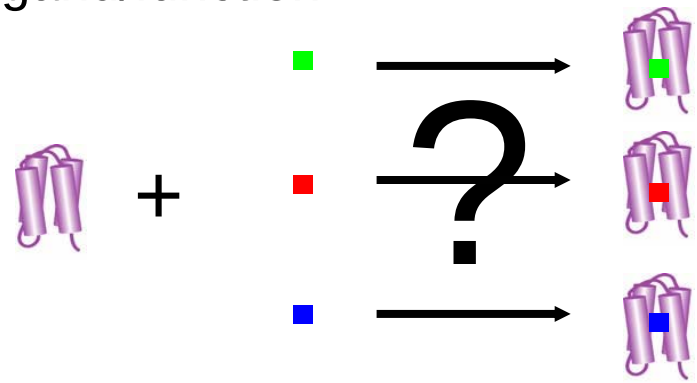
- Low solubility: what are the best storage conditions?



- A series of functional variants: which one is the more stable?



- Unknown ligand/function



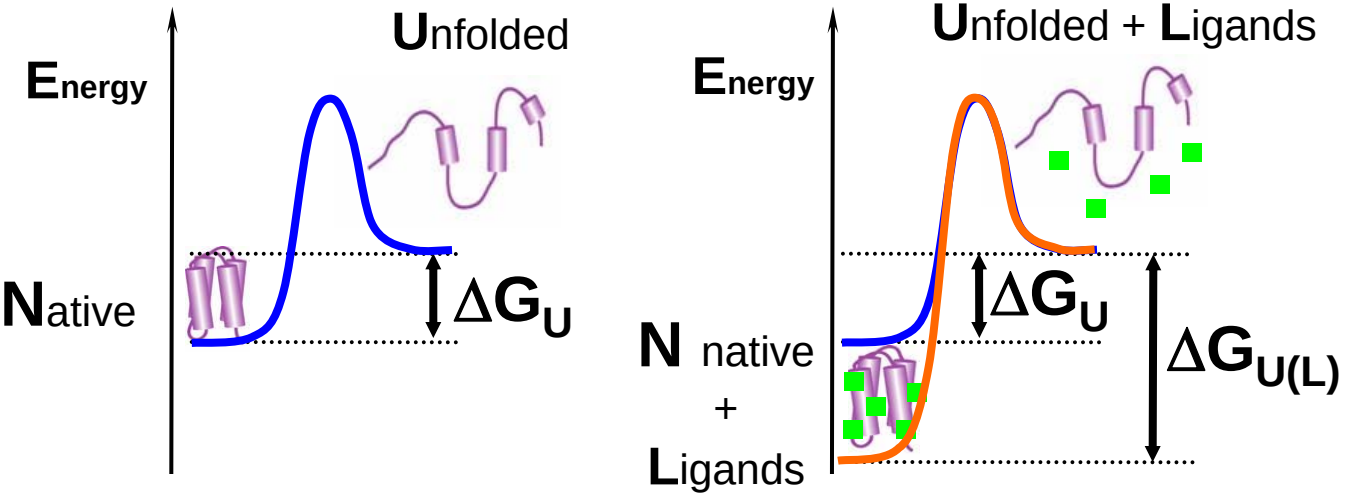
Measuring protein stability by analyzing the thermal unfolding

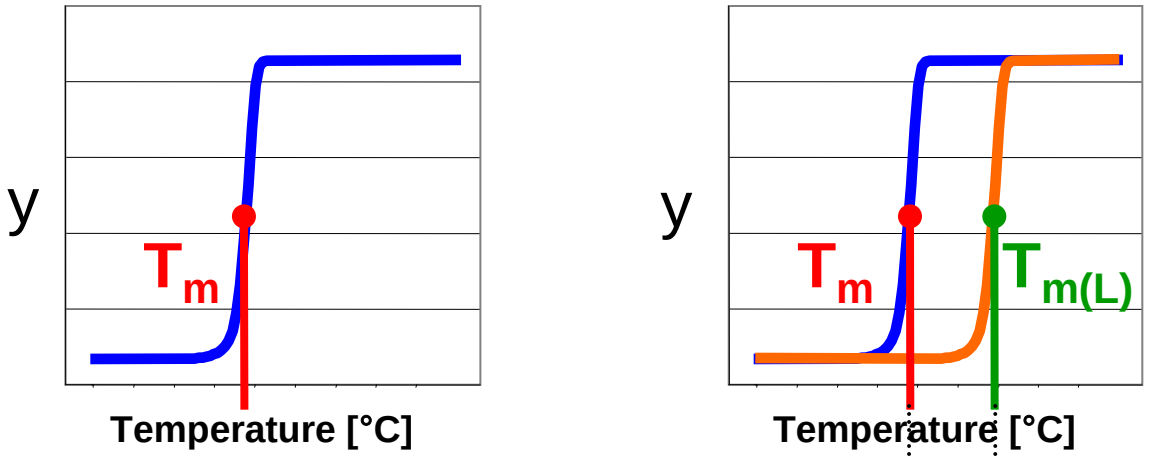
Theory

Applications

Protocol

protein folded/unfolded transition





DLS

DSC

CD

Abs/

Fluor

Y = fraction of native/unfolded state

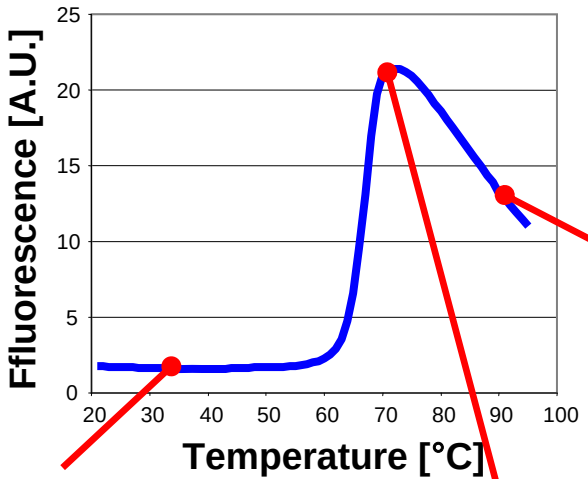
The ThermoFluor technique

Theory

Applications

Protocol

Fluorescence (a.u) vs T (ºC)



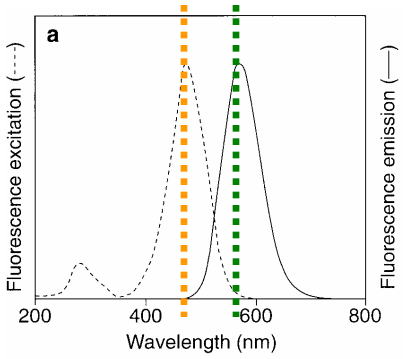
fluorescent dye

native state

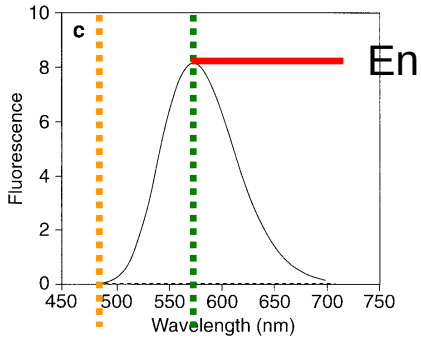
molten globule+dye

unfolded +dye

max ex. max em.



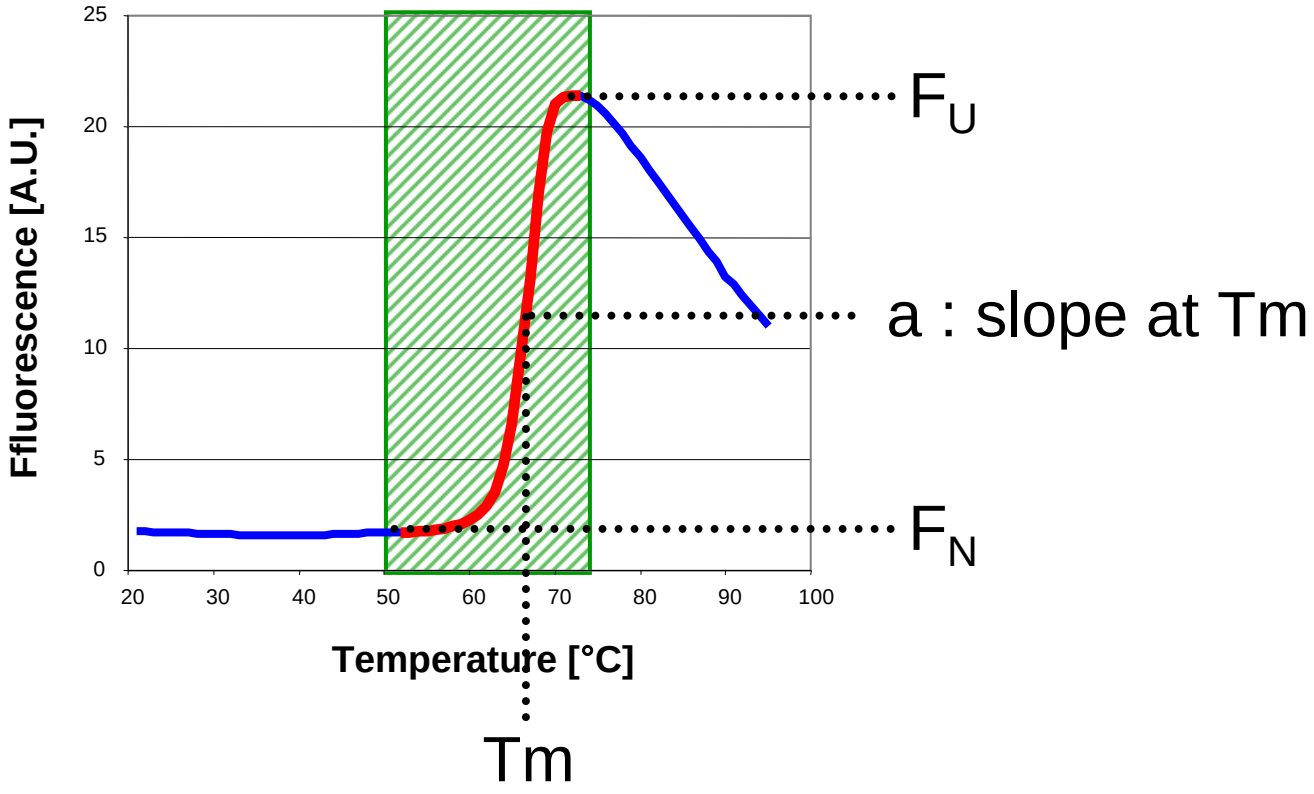
max ex. max em.



Enhanced quantum yield

Data for the SYPRO orange dye (Steinberg, 1996, *Analytical Biochemistry*)

Theoretical treatment of the data



- non-linear regression using a sigmoidal curve (e.g. Boltzmann eq.)

$$y(x) = \frac{1}{1 + \exp\left(\frac{V_{50} - x}{a}\right)} \longrightarrow F(T) = F_N \frac{F_U - F_N}{1 + \exp\left(\frac{Tm - T}{a}\right)}$$

Advantages

- very small quantities of protein (~ 300 μg, 96-well plate)
- low protein concentration needed: 0.01÷1 mg/ml
- reproducible results (s.d. 0.2 ºC; Matulis, *Biochemistry*, 2005)
- fast (~45’ per run)
- allows the simultaneous screening of multiple conditions (up to 384)

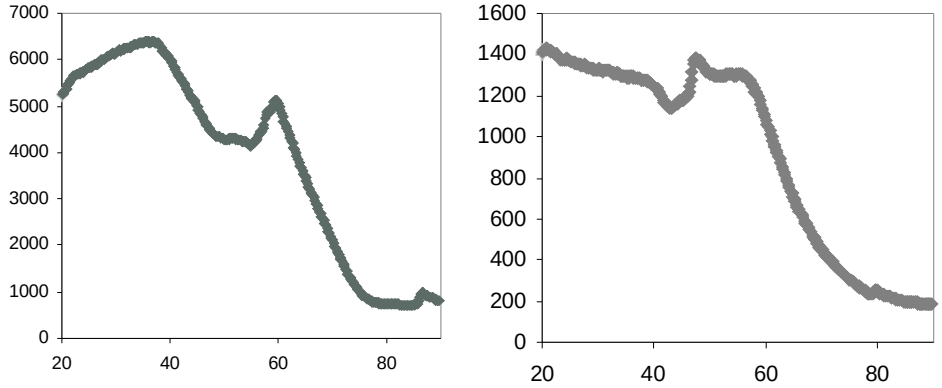
Disadvantages

Theory

Applications

Protocol

- requires compactly folded (globular) proteins



multiple transitions non-specific

Berglund H. (SGC Stockholm), *Topics in protein crystallization workshop*, 2011, Uppsala

- thermodynamics-based intepretation of the data
  - correlates well with ITC (Lo et al., *Anal Bioch.*, 2004)
  - correlates well with DSC and CD (Ericsson et al., *Anal. Bioch.*, 2006
  - needs further confirmation with other techniques

Applications

Theory

Applications

Protocol

- **Buffer screening**
  - Phillips et al., "*The Combined Use of the Thermofluor Assay and ThermoQ Analytical Software for the Determination of Protein Stability and Buffer Optimization as an Aid in Protein Crystallization*", Current Protocols in Molecular Biology, 2011
- **Test of the stability of different functional variants**
  - Lavinder et al., "*High-Throughput Thermal Scanning: A General, Rapid Dye-Binding Thermal Shift Screen for Protein Engineering*", JACS, 2009
- **Kinetic studies**
  - Matulis et al., "*Thermodynamic stability of carbonic anhydrase: measurements of binding ad stoichiometry using ThermoFluor*", Biochemistry, 2005
- **Ligand screening/ Functional studies**
  - Carver et al., "*Decrypting the Biochemical Function of an Essential Gene from Streptococcus pneumoniae Using ThermoFluor Technology*", JBC, 2005

Practical experiment - materials

Theory

Applications

Protocol

- thermocycler with fluorescence excitation/emission filters

e.g. LightCycler 480 @ IMBV

- a suitable environment-sensitive dye

e.g. Sypro ORANGE (Sigma)

Table II. Overview of Extrinsic Fluorescent Dyes Applied for Protein Characterization

Dye	Application	Stock Solution	Typical Concentration for Measurement (μm)	Extinction Coefficient (m^{-1} cm^{-1})	Excitation (nm)	Emission maximum (nm)
ANS	surface hydrophobicity unfolding/folding aggregation conformation	aqueous, ethanol	1–30 (33,35,52,99,107)	5,000 (350 nm, water) (19) 4,950 (350 nm, water) (139)	350–380	505[a]
Bis-ANS	surface hydrophobicity unfolding/folding aggregation conformation	aqueous, methanol, ethanol	1–20 (28,33,102)	16,790 (385 nm, water) (140)	385–400	515[a]
Nile Red	surface hydrophobicity unfolding/folding aggregation conformation	DMSO, ethanol, DMF	0.5–20 (22,106,123)	19,600 (552 nm, DMSO) (141)	540–580	660[a]
Thioflavin T	fibrillation	aqueous	5–40 (32,105)	36,000 (412 nm, water) (68) 26,620 (416 nm, ethanol) (142)	450	480–490[b]
Congo Red	fibrillation	10 to 40% ethanol	10–300 (69,72,83)	45,000 (498 nm, water) 59300 (505 nm, ethanol) (143)	550	
DCVJ	microviscosity of protein environment rigidity	ethanol, DMSO	5 (23,24)	659,000 (453 nm, ethanol) (23)	450	480–505

[a] In water; blue shift in hydrophobic environment
[b] In the presence of amyloids

Hawe et al., *Pharm. Research*, 2007

Practical experiment – Pre opt experiment

- ~80 μg of 2.5 mg/ml protein needed
- Set up an experimental grid

Protein concentration [mg/ml]

Dye dilution (SYPRO Orange)	0	0.01	0.1	1
1:5000				
1:1000				
1:55				

96-well RT-PCR plate

(25 μl/well)

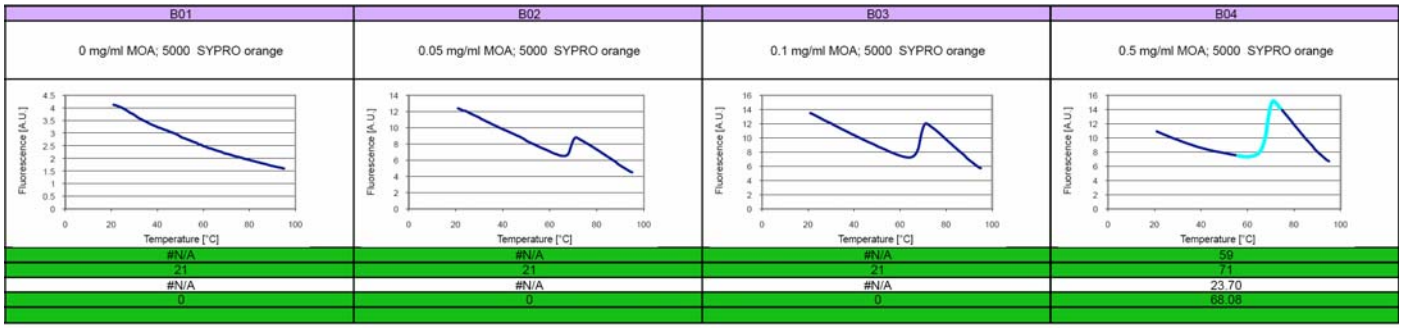
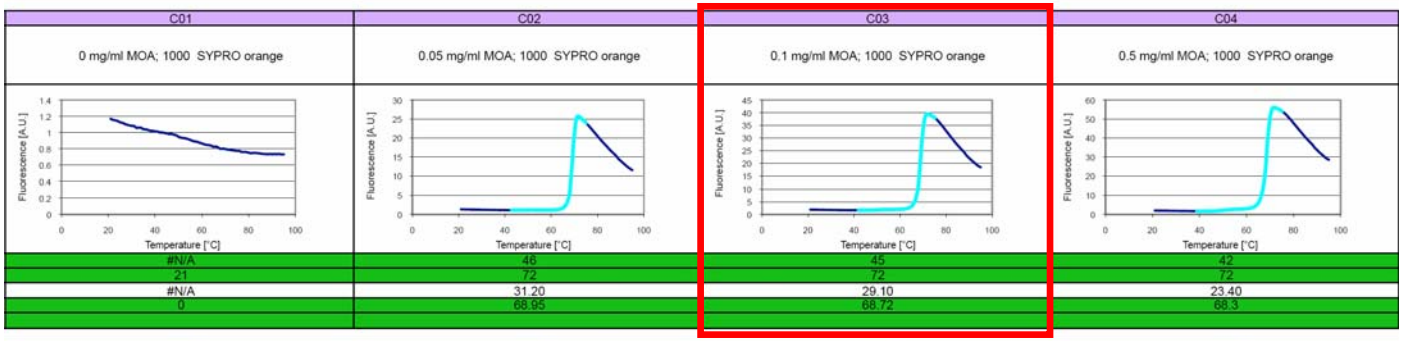
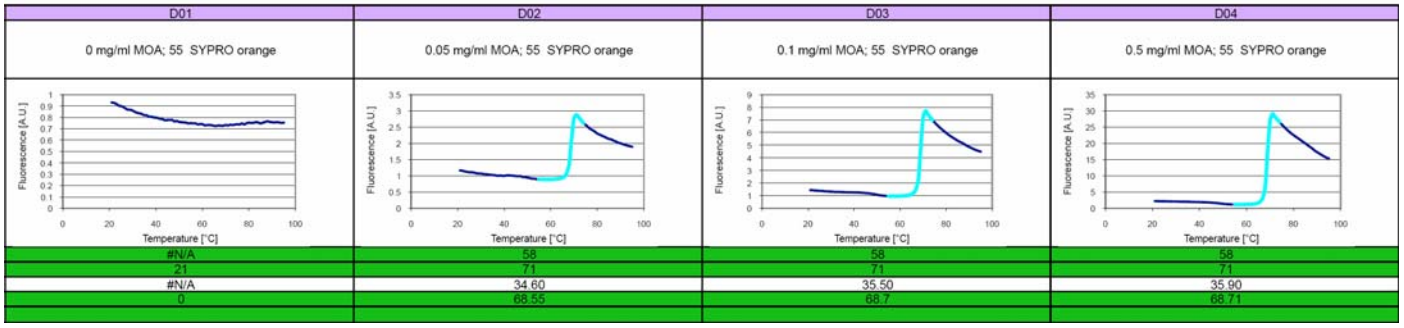
- sealing with transparent foil (or oil)
- run the experiment (0.3ºC/min, 3s hold time, ex 483 nm -em 568 nm)
- Data analysis and choice of the optimal conditions
  - sharpest transition
  - highest quantum yield vs minimum protein concentration

Practical experiment – Pre-experiment

Theory

Applications

Protocol

	0 mg/ml	0.01 mg/ml	0.1 mg/ml	1 mg/ml
1:5000				
1:1000				
1:55				

0.1 mg/ml, SYPRO Orange 1:1000

Excel-based processing using Frank Niesen's (SGC Osxford) analysis tool

Practical experiment - protocol

1. 96-well plate (25 μl/well)

- 10 μl, 2.5X base
- 7.5 μl, 3.33X dye
- 2.5 μl, 10X target
- 5 μl, 5X protein

2. set optimal conditions from pre-opt experiment, thermocycler run

3. Data analsys

- "*DSF Analysis*" + GraphPad Prism

ftp://ftp.sgc.ox.ac.uk/pub/biophysics

- "*ThermoQ*"

http://jshare.johnshopkins.edu/aherna19/thermoq/

Minimum Bibliography

Theory

Applications

Protocol

- General interest:
  - Pantoliano et al., "*High-Density Miniaturized Thermal Shift Assays as a General Strategy for Drug Discovery*", Journal of Biomolecular screening, 2011
  - Ericsson et al., "*Thermofluor-based high-throughput stability optimization of proteins for structural studies*", Analytical Biochemistry, 2006
  - Niesen et al., "*The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability*", Nature protocols, 2007
- Thermodynamic analysis of the data:
  - Matulis et al., "*Thermodynamic stability of carbonic anhydrase: measurements of binding ad stoichiometry using ThermoFluor*", Biochemistry, 2005
  - John et al., "*van't Hoff enthalpies without baselines*", Protein Science, 2000