Differential scanning fluorimetry (Thermofluor)

A complementary technique in protein crystallization

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Common problems in protein science

- Low solubility: what are the best storage conditions?

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

- Unknown ligand/function

Energy

\( E_{\text{energy}} \)
Measuring protein stability by analyzing the thermal unfolding

protein folded/unfolded transition

\[ \Delta G_U \]

\[ T_m \]  
Temperature [°C]

\[ \Delta T_m \]

\[ Y = \text{fraction of native/unfolded state} \]
The ThermoFluor technique

**Theory**

Fluorescence (a.u) vs T (°C)

- **Fluorescence [A.U.]**
- **Temperature [°C]**

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

**Applications**

- **fluorescent dye**
- **native state**
- **molten globule+dye**
- **unfolded +dye**

**Protocol**

- Enhanced quantum yield

Fluorescence excitation (---) and emission (-----)

- max ex.
- max em.
Theoretical treatment of the data

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

\[ y(x) = \frac{1}{1 + \exp \left( \frac{V_{50} - x}{a} \right)} \]

\[ F(T) = F_N \frac{F_U - F_N}{1 + \exp \left( \frac{Tm - T}{a} \right)} \]
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 °C; Matulis, *Biochemistry*, 2005)
- fast (∼45’ per run)
- allows the simultaneous screening of multiple conditions (up to 384)
Disadvantages

- requires compactly folded (globular) proteins

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

• **Buffer screening**

• **Test of the stability of different functional variants**

• **Kinetic studies**

• **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

- thermocycler with fluorescence excitation/emission filters
  - e.g. LightCycler 480 @ IMBV
- a suitable environment-sensitive dye
  - e.g. Sypro ORANGE (Sigma)

<table>
<thead>
<tr>
<th>Dye</th>
<th>Application</th>
<th>Stock Solution</th>
<th>Typical Concentration for Measurement (μm)</th>
<th>Extinction Coefficient (m⁻¹ cm⁻¹)</th>
<th>Excitation (nm)</th>
<th>Emission maximum (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANS</td>
<td>surface hydrophobicity unfolding/folding aggregation conformation</td>
<td>aqueous, ethanol</td>
<td>1–30 (33,35,52,99,107)</td>
<td>5,000 (350 nm, water) (19)</td>
<td>350–380</td>
<td>505°</td>
</tr>
<tr>
<td>Bis-ANS</td>
<td>surface hydrophobicity unfolding/folding aggregation conformation</td>
<td>aqueous, methanol, ethanol</td>
<td>1–20 (28,33,102)</td>
<td>4,950 (350 nm, water) (139)</td>
<td>385–400</td>
<td>515°</td>
</tr>
<tr>
<td>Nile Red</td>
<td>surface hydrophobicity unfolding/folding aggregation conformation</td>
<td>DMSO, ethanol, DMF</td>
<td>0.5–20 (22,108,123)</td>
<td>16,790 (385 nm, water) (140)</td>
<td>540–580</td>
<td>660°</td>
</tr>
<tr>
<td>Thioflavin T</td>
<td>fibrillation</td>
<td>aqueous</td>
<td>5–10 (32,105)</td>
<td>19,600 (552 nm, DMSO) (141)</td>
<td>450</td>
<td>480–490°</td>
</tr>
<tr>
<td>Congo Red</td>
<td>fibrillation</td>
<td>10 to 40% ethanol</td>
<td>10–300 (69,72,83)</td>
<td>36,000 (412 nm, water) (65)</td>
<td>550</td>
<td>480–505</td>
</tr>
<tr>
<td>DCVJ</td>
<td>microviscosity of protein environment rigidity</td>
<td>ethanol, DMSO</td>
<td>5 (23,24)</td>
<td>26,620 (416 nm, ethanol) (142)</td>
<td>639,000</td>
<td>480–505</td>
</tr>
</tbody>
</table>

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

*In water; blue shift in hydrophobic environment

*In the presence of amyloids
**Practical experiment – Pre opt experiment**

- ~80 μg of 2.5 mg/ml protein needed
- Set up an experimental grid
  
<table>
<thead>
<tr>
<th>Protein concentration [mg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>1:5000</td>
</tr>
<tr>
<td>1:1000</td>
</tr>
<tr>
<td>1:55</td>
</tr>
</tbody>
</table>

Dye dilution (SYPRO Orange)

96-well RT-PCR plate (25 μl/well)

- sealing with transparent foil (or oil)
- run the experiment (0.3°C/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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature</th>
<th>Fluorescence (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/ml</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.01 mg/ml</td>
<td>0.1</td>
<td>23.49</td>
</tr>
<tr>
<td>0.1 mg/ml</td>
<td>0.2</td>
<td>25.10</td>
</tr>
<tr>
<td>1 mg/ml</td>
<td>0.3</td>
<td>27.10</td>
</tr>
</tbody>
</table>

**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 analysis
   - “DSF Analysis” + GraphPad Prism
   - ftp://ftp.sgc.ox.ac.uk/pub/biophysics
   - “ThermoQ”
   - http://jshare.johnshopkins.edu/aherna19/thermoq/
Minimum Bibliography

• General interest:


• 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