Using protein thermal denaturation to characterize protein-ligand interactions

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Protein Chemistry Laboratory

- Provide protein chemistry support to National Institutes of Health scientists
  - Molecular interactions
    - Surface Plasmon Resonance Spectroscopy
    - Fluorescence polarization
    - Fluorescence lifetime measurements
  - Protein characterization/identification
    - Edman amino acid sequencing
    - HPLC purification of proteins and peptides
    - MALDI-TOF mass spectrometry
    - Thermal melting / aggregation onset of proteins
Differential scanning fluorimetry - extrinsic probes

- Sypro Orange quantum yield is environmentally sensitive

- Measure the change in Sypro Orange intensity as protein unfolds using qPCR instrument

Thermofluoro.org
Differential scanning fluorimetry - intrinsic probes

- Tryptophan is environmentally sensitive

Weichel et al., Bioprocess International 2008
Optim 1000 – differential scanning fluorimetry

- Two excitation lasers (266 and 473 nm)
- Measures the fluorescence emission of proteins and light scattering profile
- Reports $T_m$ and $T_{agg}$
- Requires 9 $\mu$L, 0.25 mg/mL
- Applications in PCL workflow
  - Characterize thermal stability/aggregation state prior to biophysical analysis
  - Change in thermal stability with binding to ligand to proteins
  - Buffer optimization for protein stability/function
Transcription factor characterization

- TF phosphorylated by receptor associated kinase, dimerizes, migrates to nucleus and initiates gene transcription.
- Phosphorylation status of TF is the read-out in high-content drug screen.
- Need to develop biophysical assays to evaluate compound binding.
- Protein buffer: 50mM Hepes pH 8.5, 100mM NaCl, 10% glycerol, 1mM DTT, 1mM EDTA, 0.1% NP-40
- Thermal melting to QC protein and monitor ligand binding
  - qPCR/Sypro Orange
  - Optim 1000
NP40 from TF buffer causes large intensity signal from Sypro Orange

Replicates of TF

Replicates of Carbonic Anhydrase
Thermal denaturation of TF protein using Optim 1000

Thermal transition

$T_m = 40^\circ C$
Aggregation of TF protein increases as the protein denatures.
Use thermal melting to optimize TF buffer

- His tagged-TF was purified using an NTA column using 20mM Hepes pH7.5, 300mM NaCl, 2mM mercaptoethanol, 300mM Imidazole. This material was then dialyzed into 6 different buffer:
  - Buffer 1: 20mM Hepes pH7.5, 300mM NaCl, 2mM mercaptoethanol, 300mM Imidazole (no dialysis)
  - Buffer 2: 20mM Hepes pH7.5, 300mM NaCl, 2mM mercaptoethanol, no Imidazole
  - Buffer 3: 50mM Hepes pH8.0, 100mM NaCl, 10% glycerol, 1mM EDTA, 1mM DTT, 300mM Imidazole,
  - Buffer 4: 20mM Hepes pH7.5, 300mM NaCl, 2mM mercaptoethanol, 300mM Imidazole, 50% glycerol
  - Buffer 5: 20mM Hepes pH7.5, 300mM NaCl, 2mM mercaptoethanol, 300mM Imidazole, 0.1% beta-octyl glucoside
  - Buffer 6: 20mM Hepes pH7.5, 300mM NaCl, 2mM mercaptoethanol, 500mM Arginine
  - Buffer 7: 20mM Hepes pH7.5, 300mM NaCl, 2mM mercaptoethanol, 300mM Imidazole, 10% sorbitol
Measure the thermal melting profile of the TF protein with phosphopeptide

• Dilute each TF protein prep into 10mM Hepes pH8.0, 50mM NaCl, 1mM EDTA, at 0.25mg/ml +/- 25uM peptide

• Measure the thermal transition and aggregation profile using the Optim 1000 and also using Sypro Orange/qPCR approach
Binding of the phospho-peptide stabilizes the TF protein (Buffer 4) – Optim data

Thermal transition 350:330

T_m 49°C
T_m + pep 53°C

Light scattering at 473nm

TF alone
TF + pep
Binding of the phospho-peptide stabilizes the TF protein (Buffer 4) – Sypro Orange data

Temperature (C)

Fluorescence Units

TF alone
TF + peptide

T_m 51°C
T_m + pep 56°C
Summary of peptide induced temperature shift on TF protein

Thermal shift - Optim data

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Melting temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 3</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>Buffer 3 + peptide</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>Buffer 4</td>
<td>50 ± 3</td>
</tr>
<tr>
<td>buffer 4 + peptide</td>
<td>53 ± 3</td>
</tr>
<tr>
<td>Buffer 6</td>
<td>52 ± 3</td>
</tr>
<tr>
<td>Buffer 6 + peptide</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>Buffer 7</td>
<td>54 ± 3</td>
</tr>
<tr>
<td>Buffer 7 + peptide</td>
<td>57 ± 3</td>
</tr>
</tbody>
</table>

Aggregation onset - Optim data

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Aggregation onset temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 3</td>
<td>40 ± 3</td>
</tr>
<tr>
<td>Buffer 3 + peptide</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>Buffer 4</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>buffer 4 + peptide</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>Buffer 6</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>Buffer 6 + peptide</td>
<td>50 ± 3</td>
</tr>
<tr>
<td>Buffer 7</td>
<td>50 ± 3</td>
</tr>
<tr>
<td>Buffer 7 + peptide</td>
<td>53 ± 3</td>
</tr>
</tbody>
</table>

Thermal shift - Sypro Orange data

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Melting temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 3</td>
<td>50 ± 3</td>
</tr>
<tr>
<td>Buffer 3 + peptide</td>
<td>53 ± 3</td>
</tr>
<tr>
<td>Buffer 4</td>
<td>52 ± 3</td>
</tr>
<tr>
<td>buffer 4 + peptide</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>Buffer 6</td>
<td>54 ± 3</td>
</tr>
<tr>
<td>Buffer 6 + peptide</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>Buffer 7</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>Buffer 7 + peptide</td>
<td>58 ± 3</td>
</tr>
</tbody>
</table>
Phospho-peptide shows a concentration dependent shift in TF melting temperature
Binding of the phospho-peptide to TF protein
Conclusions

• Optim 1000 provides information on thermal stability of TF protein.

• Able to identify buffers that stabilized the TF protein in the absence of NP40.

• Binding of the phospho-peptide increased the melting temperature of TF protein in a concentration dependent manner.

• The non-phosphorylated peptide does not change the melting temperature of the protein.

• Current work is directed to evaluating small molecule ligands for direct binding to TF protein.
Recombinase protein – oligonucleotide binding

- Recombinase protein
  - does not bind double stranded DNA
  - Binds weakly to single stranded oligo
  - Binds tightly to complementary oligonucleotides
- Forms large rings or filaments when bound to long oligonucleotides
Recombinase forms stable complexes in the presence of complementary oligos – SPR analysis

Time (s)

0 200 400 600 800 1000 1200

Response Units

-20

0

20

40

60

80

100

120

140

160

5 μM Rec

1.6 μM Rec

0.55 μM Rec

0.18 μM Rec

0.06 μM Rec

0 μM

Approx. response for 7 molecules of Recombinase bound

5 μM Rec + non complementary oligos
Oligonucleotide concentration dependence on recombinase stability

- Buffer: 20mM Hepes, pH7.5, 125mM Potassium Glutamate, 3mM EDTA, 1mM Spermidine

- Make 3 fold dilutions of the complementary oligos. Samples
  - 5µM Recombinase + 2µM 99mer
  - 5µM Recombinase + 2µM 99mer + 12µM comp oligos
  - 5µM Recombinase + 2µM 99mer + 4µM comp oligos
  - 5µM Recombinase + 2µM 99mer + 1.3µM comp oligos
  - 5µM Recombinase + 2µM 99mer + 0.44µM comp oligos
  - 5µM Recombinase + 2µM 99mer + 0.15µM comp oligos
Increasing oligo concentration increases the stability of recombinase.
Two complexes of Recombinase can be detected with variable oligo concentrations.

<table>
<thead>
<tr>
<th>Complementary oligo concentration (µM)</th>
<th>$T_m$ 1 (°C)</th>
<th>$T_m$ 2 (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>55.9±0.1</td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>54.6±0.3</td>
<td>70.7±0.6</td>
</tr>
<tr>
<td>0.44</td>
<td>55.0±0.4</td>
<td>71.4±0.1</td>
</tr>
<tr>
<td>1.3</td>
<td></td>
<td>71.6±0.1</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>72.1±0.1</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>71.9 ± 0.2</td>
</tr>
</tbody>
</table>
Results

• We observe a significant increase in the thermal stability of the recombinase protein with the addition of complementary oligonucleotides

• We can detect two $T_m$’s for recombinase with variable concentrations of complementary oligos, suggesting the presence of two conformations, annealed and unannealed.
Length dependence of oligonucleotide binding on recombinase stability

- Buffer: 20mM Hepes, pH 7.5, 125mM Potassium Glutamate, 3mM EDTA, 1mM Spermidine
- Incubate 5μM recombinase protein with 2μM single stranded or 2μM complementary oligos.
  - Vary oligo length between 15-23 bases
  - Vary oligo length between 21-27 bases
No length dependence on recombinase melting profile with single stranded oligos
Length dependence on melting profile of recombinase with complementary oligos
## Length dependence in recombinase melting temperature

<table>
<thead>
<tr>
<th>Length complementary oligos</th>
<th>$T_m$ 1°C</th>
<th>$T_m$ 2°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>No oligo</td>
<td>51.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>15mer</td>
<td>53.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>16mer</td>
<td>52.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>17mer</td>
<td>54.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>18mer</td>
<td>53.9 ± 0.3</td>
<td>60.1 ± 0.3</td>
</tr>
<tr>
<td>19mer</td>
<td>52.2 ± 0.3</td>
<td>66.4 ± 0.7</td>
</tr>
<tr>
<td>20mer</td>
<td>52.1 ± 0.8</td>
<td>69.0 ± 0.3</td>
</tr>
<tr>
<td>21mer</td>
<td>52.6 ± 0.1</td>
<td>67.1 ± 0.3</td>
</tr>
<tr>
<td>22mer</td>
<td>52.3 ± 0.5</td>
<td>66.3 ± 0.3</td>
</tr>
<tr>
<td>23mer</td>
<td>51.7 ± 0.3</td>
<td>71.5 ± 0.3</td>
</tr>
</tbody>
</table>

* Significant differences
Recombinase shows enhanced stability with 23mers
Length dependence in recombinase melting temperature

<table>
<thead>
<tr>
<th>Length</th>
<th>$T_m$ 1°C</th>
<th>$T_m$ 2°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>No oligo</td>
<td>52.4 ± 0.3, 52.5 ± 0.2</td>
<td>66.0 ± 0.1, 65.8 ± 0.3</td>
</tr>
<tr>
<td>21mer</td>
<td>53.6 ± 0.2, 53.5 ± 0.2</td>
<td>66.0 ± 0.1, 66.4 ± 0.2</td>
</tr>
<tr>
<td>22mer</td>
<td>53.6 ± 0.2, 53.8 ± 0.2</td>
<td>66.6 ± 0.1, 66.4 ± 0.2</td>
</tr>
<tr>
<td>23mer</td>
<td>53.0 ± 0.2, 52.0 ± 0.2</td>
<td>71.3 ± 0.2, 71.0 ± 0.2</td>
</tr>
<tr>
<td>24mer</td>
<td>53.0 ± 0.3, 53.0 ± 0.2</td>
<td>70.0 ± 0.2, 69.0 ± 0.2</td>
</tr>
<tr>
<td>26mer</td>
<td>53.4 ± 0.5, 52.5 ± 0.6</td>
<td>71.4± 0.2, 71.0± 0.3</td>
</tr>
<tr>
<td>27mer</td>
<td>52.4 ± 0.6</td>
<td>68.5± 0.4, 68.4± 0.3</td>
</tr>
</tbody>
</table>

Each condition run in duplicate
Results

- Binding of recombinase to single stranded oligos does not show a concentration dependent shift in $T_m$.
- Length dependence of recombinase to complementary oligos show 2 shifts in $T_m$.
- With 17mer to 18mer recombinase is stabilized 6 °C and with 22mer to 23mer is stabilized 5 °C.
- There also appears to be an impact of the oligo sequence on the stability of the complex.
- Biological significance.....
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