Characterization of Protein Interactions by ITC, SPR and BLI

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Outline

• Protein interactions: why bother?

• Calorimetry

• Optical methods: SPR and BLI

• Real-life example: hybrid approach
Protein interactions – why bother?
Protein interactions control the lives of cells

*Escherichia coli* drawn to molecular scale by David Goodsell
Protein interaction network

[Nature (2000)]
Protein interaction types

• **Homologous interactions:**
  • The same proteins
  • Oligomers
  • Coiled-coil
  • Amyloids

• **Heterologous interactions:**
  • Different proteins
  • Enzyme – inhibitors
  • Antibody – antigen
  • Protein complexes
Protein interactions: qualitative vs. quantitative

**Immunoprecipitation (IP)**

**Pulldown**

**Qualitative or semi-quantitative**

**ITC, SPR, BLI**

**Fluorescence anisotropy**

**Quantitative**
Protein interactions: binding affinity range

- **Low Affinity**
  - Weak interactions such as ubiquitin:ubiquitin receptor
  - $K_d < 10^{-4}$ M (mM)
  - $K_a < 10^4$ M$^{-1}$

- **Moderate Affinity**
  - Most protein interactions
  - $10^{-4} < K_a < 10^8$ M$^{-1}$
  - $10^{-8} < K_d < 10^{-4}$ M (mM – nM)

- **High Affinity**
  - Antigen:antibody
  - $K_a > 10^9$ M$^{-1}$
  - $K_d > 10^{-9}$ M (nM)
Dissociation constant: $K_d$

\[ P + L \stackrel{K_a}{\leftrightarrow} PL \]

\[ PL \stackrel{K_d}{\leftrightarrow} P + L \]

\[ K_a = \frac{[PL]}{[P][L]} = \frac{k_{on}}{k_{off}} \quad \text{M}^{-1} \]

\[ K_d = \frac{[P][L]}{[PL]} = \frac{k_{off}}{k_{on}} \quad \text{M} \]

\[ K_a = \frac{1}{K_d} \]
Isothermal Titration Calorimetry
Isothermal titration calorimetry (ITC): Measuring heat

- **Calor** (Latin, *heat*) + **metry** (Greek, *measure*)
- Direct measurement of heat *q* either released or absorbed in molecular binding during gradual titration
- Label-free measurement
- Microcalorimeters: as low as 100 μl
ITC theory: Thermodynamics

• Scenario: a ligand ($L$) binds to a protein ($P$) at temperature $T$

$$P + L \leftrightarrow PL$$

• Release of absorption of heat due to binding

$$q = \Delta H^0(T)n_{PL} = \Delta H^0(T)V[PL]$$

• $\Delta H^0(T)$ and $K_a$ (therefore $K_d$) can be determined by titration

$$q = \Delta H^0(T)V[P_T]\left(\frac{K_a[L]}{1 + K_a[L]}\right)$$
ITC theory: Thermodynamics

• Scenario: a ligand \( L \) binds to a protein \( P \) at temperature \( T \)

\[
P + L \xleftrightarrow[K_a]{\text{}^\uparrow} PL
\]

• Once you determine \( \Delta H^0(T) \) and \( K_a \) (therefore \( K_d \)), \( \Delta G^0 \) and \( \Delta S^0 \) can be calculated.

\[
\Delta G^0(T) = -RT \ln K_a
\]

\[
\Delta G^0(T) = \Delta H^0(T) - T \Delta S^0(T)
\]
Representative instruments
ITC: Instrument components

- Exothermic reaction
- The sample cell becomes warmer than the reference cell.
- Binding causes a downward peak in the signal.
- Heat released is calculated by integration under each peak.

[www.Malvern.com]
ITC: Data analysis

[Diagram showing ITC data analysis with peaks over time and a graph showing binding mechanism, affinity $\frac{1}{K_d}$, and stoichiometry $n$.]

[www.Malvern.com]
ITC: Limitations and competitive binding techniques

**Limits**

Can’t measure tight interactions $K_a$ by direct measurement:

$10^2$ M$^{-1}$ - $10^9$ M$^{-1}$

$K_d$ (dissociation constant) = $1/K_a$

**Work-around**

(1) Weak ligand binds to protein

(2) Strong ligand displaces weak ligand:protein complex

$$K_{app} = \frac{K_{strong}}{1 + K_{weak}[L_{weak}]}$$

Can measure tight interactions $K_a$ by competitive technique:

$10^9$ M$^{-1}$ - $10^{12}$ M$^{-1}$

Crystal structure of nicotinic acetylcholine receptor homolog AChBP in complex with an α-conotoxin PnIA variant

Patrick H N Celic1,5, Igor E Kashevorov2,5, Dmitry Y Mordvintsev2, Ronald C Hogg3, Pim van Nierop4, René van Elk4, Sarah E van Rossum-Fikkert1, Maxim N Zhmak2, Daniel Bertrand3, Victor Tsetlin2, Titia K Sixma1 & August B Smit4

Supplementary Table 1 Thermodynamic parameters

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ligand</th>
<th>$K_d$ (nM)</th>
<th>N (mol/mol)</th>
<th>H (kcal/mol)</th>
<th>T S (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ls-AChBP</td>
<td>PnIA[A10L,D14K]</td>
<td>27.5 ± 23.6</td>
<td>3.6 ± 0.1</td>
<td>-1.44 ± 0.06</td>
<td>-8.78 ± 0.16</td>
</tr>
<tr>
<td>Ls-AChBP</td>
<td>PnIA[A10L]</td>
<td>85.0 ± 32.8</td>
<td>5.7 ± 0.3</td>
<td>-1.13 ± 0.11</td>
<td>-8.46 ± 0.36</td>
</tr>
<tr>
<td>Ac-AChBP</td>
<td>PnIA[A10L,D14K]</td>
<td>32.6 ± 8.5</td>
<td>4.1 ± 0.3</td>
<td>-5.64 ± 0.19</td>
<td>-4.47 ± 0.19</td>
</tr>
<tr>
<td>Ac-AChBP</td>
<td>PnIA[A10L]</td>
<td>36.7 ± 16.6</td>
<td>5.3 ± 0.2</td>
<td>-3.91 ± 0.07</td>
<td>-6.19 ± 0.27</td>
</tr>
</tbody>
</table>

$K_d$ is dissociation constant in nM, N is number of binding sites in each pentamer, H is heat change in kcal/mol, S is entropy change in kcal/mol/deg. Data are the mean values from two independent experiments.
Mixed-lineage leukemia: A type of childhood leukemia in which a piece of chromosome 11 has been translocated (broken off and attached itself to another chromosome). Children with this type of leukemia have a particularly poor prognosis (outlook). They do not respond at all well to the standard therapies for ALL (acute lymphoblastic or lymphocytic leukemia) and often suffer from early relapse after chemotherapy.

On both the clinical and laboratory levels, chromosome 11 childhood leukemia appears therefore to be a distinctive disease and not a subset of ALL. Armstrong and coworkers (Nature, Jan 2002) named it "mixed-lineage leukemia."

[MedicineNet.com]
Protein: cofactor interaction

Dynamically driven protein allostery
Nataliya Popovych¹, Shangjin Sun¹, Richard H Ebright² & Charalampos G Kalodimos¹

CAP: catabolite activator protein (dimer)
cAMP: cyclic AMP
Protein:protein interaction – HIV Gag p6:Human Alix

Protein:protein interaction – Rabex-5:Polyubiquitin

Table 1. Binding affinities of Rabex-5(9-73) mutants to linear tetraubiquitin

<table>
<thead>
<tr>
<th>Rabex-5 (9-73)</th>
<th>Ubiquitin</th>
<th>$K_d$ (µM) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Ub</td>
<td>$18 \pm 11$</td>
</tr>
<tr>
<td></td>
<td>Linear-Ub4</td>
<td>$8 \pm 8$ $^b$</td>
</tr>
<tr>
<td>A58D</td>
<td>Ub</td>
<td>$41 \pm 20$</td>
</tr>
<tr>
<td></td>
<td>Linear-Ub4</td>
<td>$6 \pm 1$</td>
</tr>
<tr>
<td>Y25A/Y26A</td>
<td>Ub</td>
<td>$21 \pm 7$</td>
</tr>
<tr>
<td></td>
<td>Linear-Ub4</td>
<td>$10 \pm 2$</td>
</tr>
</tbody>
</table>

$^a$ Apparent dissociation constants based on a single-site model.

$^b$ Values calculated from experiments in duplicates.

Surface plasmon resonance
Surface plasmon resonance (SPR): Assay objectives

**ITC**
- Equilibrium analysis
  - $K_D$
  - Determination of binding strength
- Kinetic rate analysis
  - $k_a$, $k_d$
  - Modelling binding reactions to determine the dynamic behaviour of a system

**SPR**
- Yes/No data
- Specificity studies
- Ranking
  - Early selection of binders

**BLI**
- [BiaCore]
Surface plasmon resonance (SPR): Theory

- To measure the refractive index near to a sensor surface
- Polarised light is directed through a prism to the under surface of the gold film where surface plasmons are generated at a critical angle of the incident light.
- This absorption of light is seen as a decrease in intensity of the reflected light. Resonance or response units (RU) are used to describe the increase in the signal, where 1 RU is equal to a critical angle shift of $10^{-4}$ deg or $10^{-12}$ g mm$^{-2}$.
- When a steady-state is achieved (all binding sites occupied), the maximum RU is determined ($n$: No. of binding sites in Ligand)

$$RU_{max} = nRU_L \left(\frac{MW_A}{MW_L}\right)$$

[Patching, Biochim. Biophys. Acta (2014)]
Surface plasmon resonance (SPR): Sensorgram

[Diagram showing the process of association, dissociation, and regeneration with concentration and time on the x-axis and resonance signal on the y-axis.]

[BiaCore]
Surface plasmon resonance (SPR): Components

Detection System

Sensor Chip

Microfluidics

[BiaCore]
Surface plasmon resonance (SPR): Sensor chips

Sensor Chip CM5
- CM dextran

Sensor Chip CM4
- Low COOH density

Sensor Chip C1
- Flat COOH

Sensor Chip L1
- CM dextran + Lipophilic Tail

Sensor Chip HPA
- Thiolalkane

Sensor Chip NTA
- NTA

Sensor Chip CM3
- Short dextran

Sensor Chip SA
- Short dextran + SA

Sensor Chip SA
- Short dextran + SA

Sensor Chip AU
- Gold

Hydrophilic vs. Hydrophobic
High charge density vs. No charge
Extended matrix vs. No matrix
Kinetic analysis: Why important?

$$K_d = \frac{k_{off} (s^{-1})}{k_{on} (M^{-1}s^{-1})}$$

[BiaCore]
Kinetic analysis: Same affinity, different kinetics

Compare sensorgrams for three different interactions

- Same 1 nM affinity ($K_d$)
- Different kinetics

\[ K_d = \frac{k_d}{k_a} \]
Things to consider: Analyte concentration

- Run analyses over a wide range of analyte concentrations, ideally 100-fold or more: The range should span 10x below the $K_d$ to 10x above the $K_d$.
- Accurate analyte concentration is critical!
- Include a zero-concentration sample in the analyses.

![Graphs showing different analyte concentration levels](BiaCore)

- **Too high** concentration
- **Too low** concentration
- Optimized
Things to consider: Mass transfer

- If the diffusion rate is slower than the association rate, mass transfer effects can be observed.
- Low $RU_L$ reduces analyte consumption in “no-flow zone”.
- Apparent rate constants are smaller when mass transport limited binding occurs (inaccurate kinetic data).
- Work-arounds: higher flow rates, lowest ligand density.

At different flow rates

Mass transfer limitation  No limitation
Things to consider: Conformational changes

- Conformational changes during interaction may cause kinetic parameters to change
- Inject analyte at a fixed concentration
- Vary contact times
- Overlay the sensorgrams

Do relative dissociation rates change?
If so, a conformational change is occurring.

Confirm with other techniques.
Data analysis: Curve fitting in kinetic analysis

\[ k_{\text{on}}, \ k_{\text{off}}, \ \text{and} \ RU_{\text{max}} \ \text{are calculated by global curve fitting} \]

\[
A + B \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} AB
\]

[BiaCore]
Data analysis: Steady-state affinity determination

- Kinetic determinations give an independent value
  
  \[ K_a = \frac{k_{on}}{k_{off}} \quad K_d = \frac{k_{off}}{k_{on}} \]

- Steady-state response levels give one value for affinity constants

- Steady-state can be used for fast interactions where kinetics are not available

Kinetics and affinity

Affinity only
Data analysis: Steady-state affinity determination

- Response at equilibrium can be plotted against the concentration to determine the affinity
  - Response should be at or close to equilibrium at all concentrations for a reliable measurement
Qualitative and quantitative interaction analysis: Rabex-5 and ubiquitin

- Rabex-5: guanine exchange factor (GEF) for Rab5 in intracellular trafficking
- Two ubiquitin binding domains: A20_ZF, MIU

[Image of molecular structures and interaction analysis graphs]

Biolayer interferometry
Biolayer interferometry (BLI): Theory

Optical thickness change at the sensor tip due to binding causes wavelength shift $\Delta \lambda$

[Ligand: Analyte]

[ForteBio; Citartan et al. Analyst (2013)]
BLI: Experimental platforms
BLI: Practical considerations

- pH Scouting is done for optimal ligand immobilization on a sensor.
- Molecular weight of the analyte matters.
- Choice of data analysis method (kinetic or steady state) depends on the nature of protein interactions.
BLI example system: Rabex-5 and polyubiquitin

Experimental design

(Ligands)

**Analytes**
- GST-Rabex-5<sub>9-73</sub>
- GST
- Rabex-5<sub>9-73</sub>

**Ligands**
- Linear Ub<sub>4</sub>
- K63-linked Ub<sub>4</sub>
- K48-linked Ub<sub>4</sub>
Experimental design

(Ligands)

Analytes

GST-Rabex-59-73

GST

Rabex-59-73

Ligands

Linear Ub_4

K63-linked Ub_4

K48-linked Ub_4
Ligand immobilization: pH Scouting

3. **Ligand preparation** - Dilute the ligand to be immobilized in 100 mM MES at the appropriate pH. Follow the guidelines below for selecting the appropriate pH of MES buffer.

   c. If the pI of the protein is unknown it may be necessary to test the immobilization of the ligand at all three pHs provided (pH 4.0, 5.0, 6.0).

<table>
<thead>
<tr>
<th>Target pI</th>
<th>Volume of 100 mM MES pH 4.0 (mL)</th>
<th>Volume of 100 mM MES pH 5.0 (mL)</th>
<th>Volume of 100 mM MES pH 6.0 (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>9.45</td>
<td>0.55</td>
<td>----</td>
</tr>
<tr>
<td>4.2</td>
<td>9.2</td>
<td>0.8</td>
<td>----</td>
</tr>
<tr>
<td>4.3</td>
<td>8.7</td>
<td>1.3</td>
<td>----</td>
</tr>
<tr>
<td>4.4</td>
<td>8.1</td>
<td>1.9</td>
<td>----</td>
</tr>
<tr>
<td>4.5</td>
<td>7.3</td>
<td>2.7</td>
<td>----</td>
</tr>
<tr>
<td>4.6</td>
<td>5.4</td>
<td>4.6</td>
<td>----</td>
</tr>
<tr>
<td>4.7</td>
<td>4.9</td>
<td>5.1</td>
<td>----</td>
</tr>
<tr>
<td>4.8</td>
<td>4.0</td>
<td>6.0</td>
<td>----</td>
</tr>
<tr>
<td>4.9</td>
<td>3.3</td>
<td>6.5</td>
<td>----</td>
</tr>
<tr>
<td>5.1</td>
<td>----</td>
<td>9.4</td>
<td>0.6</td>
</tr>
<tr>
<td>5.2</td>
<td>----</td>
<td>8.9</td>
<td>1.1</td>
</tr>
<tr>
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<td>2.6</td>
<td>7.4</td>
</tr>
<tr>
<td>5.9</td>
<td>----</td>
<td>1.2</td>
<td>8.8</td>
</tr>
</tbody>
</table>
Ligand immobilization: pH Scouting

[Diagram showing the process of ligand immobilization]

- NHS
- EDC
- Linear Ub

0.25 μM Linear Ub₄

[Graph showing the response over time for different pH values]

- pH 4.5
- pH 4.7
- pH 4.9
- pH 5.1
- pH 5.3
- pH 5.5
- pH 5.7
- pH 5.9

[Graph showing the response vs pH]

- pH 4.3
- pH 4.6
- pH 4.9
- pH 5.2
- pH 5.5
- pH 5.8
Analyte selection: Size matters

Rabex-5_{9-73}  
GST-Rabex-5_{9-73}  
N-C

0.25 μM K63-linked Ub$_4$

0.25  \textbf{No saturation}  
1.05  \textbf{Saturation}
Full sensorogram: Everything optimized

Baseline  Activation  Loading  Quenching  Baseline  Association  Dissociation

0.25 μM Linear Ub₄

GST-Rabex-5₉₋₇₃

10 μM
5 μM
1 μM
0.5 μM
0.1 μM

GST
10 μM
Full sensorogram: Everything optimized

---

**Baseline**

**Activation**

**Loading**

**Quenching**

**Baseline**

**Association**

**Dissociation**

---

0.25 μM Linear Ub₄

GST-Rabex-5₉₋₇₃

- 10 μM
- 5 μM
- 1 μM
- 0.5 μM

GST

10 μM
Data analysis: Kinetic vs. steady-state

Kinetic

Steady-state
Qualitative and quantitative interaction analysis: Rabex-5 MIU domain and polyubiquitin


### Table: Binding affinities of Rabex-59-73 to linkage-specific tetraubiquitin chains

<table>
<thead>
<tr>
<th>Rabex-59-73 mutant</th>
<th>$K_d$ (μM)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linear Ub$_4$</td>
</tr>
<tr>
<td>WT</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Y25A/Y26A</td>
<td>0.28 ± 0.14</td>
</tr>
<tr>
<td>A58D</td>
<td>2.6 ± 1.2</td>
</tr>
</tbody>
</table>

$^a$ Each linkage-specific Ub$_4$ chain was immobilized on a sensor and GST-Rabex-59-73 mutants were added.  
$^b$ $K_d$ values were calculated based on triplicate data and limited to two significant figures.

Qualitative and quantitative interaction analysis: Rabex-5 MIU domain and polyubiquitin

[Yoshikatsu Aikawa, Sangho Lee et al. (2012) J. Biol. Chem.]
Real-life example: hybrid approach
Regions between UBZ and LRM of Rad18 Are Involved in Polyubiquitin Recognition


[Trung Thanh Thach, Namsoo Lee, Donghyuk Shin, Seungsu Han, Gyuhee Kim, Hongtae Kim, and Sangho Lee (2015) Biochemistry]
SAXS-based model for Rad18(201-240):Linear Ub$_2$

[Trung Thanh Thach, Namsoo Lee, Donghyuk Shin, Seungsu Han, Gyuhee Kim, Hongtae Kim, and Sangho Lee (2015) *Biochemistry*]
Validation of Rad18(201-240):Linear Ub$_2$ Interaction

[Trung Thanh Thach, Namsoo Lee, Donghyuk Shin, Seungsu Han, Gyuhee Kim, Hongtae Kim, and Sangho Lee (2015) Biochemistry]
## Summary

<table>
<thead>
<tr>
<th>Affinity range ($K_d$)</th>
<th>ITC</th>
<th>SPR, BLI</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM to sub-mM (pM with competition)</td>
<td>nM to low mM</td>
<td></td>
</tr>
</tbody>
</table>

### Pros
- Thermodynamic parameters ($\Delta G$, $\Delta H$, $\Delta S$)
- No immobilization
- Kinetic parameters ($k_{on}$, $k_{off}$)
- “Dirty” samples possible
- “Less” sample required
- High throughput

### Cons
- “More” sample required
- Lows to medium throughput
- Mass transfer limitation
- Immobilization artifacts