Module 2 overview

*lecture*
1. Introduction to the module
2. Rational protein design
3. Fluorescence and sensors
4. Protein expression

*SUPER BREAK*

*lab*
1. Start-up protein eng.
2. Site-directed mutagenesis
3. DNA amplification
4. Prepare expression system

5. Review & gene analysis
6. Purification and protein analysis
7. Binding & affinity measurements
8. High throughput engineering
9. Gene analysis & induction
10. Characterize expression
11. Assay protein behavior
12. Data analysis
Lecture 7: Binding and affinity measurements

I. Titration analysis
   A. Estimating $K_d$ & $EC_{50}$ from fluorescence data
   B. Multisite binding and cooperativity

II. Techniques for studying binding
Q: Our design task is to alter calcium sensitivity of pericam derivatives. We chose mutants to make, but **how will we determine the results** of our perturbations?

A: We will measure **titration curves**, by adding fixed amounts of calcium to samples of mutant pericam and measuring an indicator of binding (in this case fluorescence). From these data, we will extract estimates of binding parameters.

What is going on during a titration measurement?

We want to measure $K_d$ from fluorescence. $P_u$ and $P_b$ are fluorescent to different degrees, so that the total fluorescence is equal to the sum of contributions from the two species:

$$F = F_u [P_u] + F_b [P_b]$$

Fluorescence measurements can be converted to fractional saturation:

$$\frac{F_{\text{max}} - F}{F_{\text{max}} - F_{\text{min}}} = \frac{\Delta F}{\Delta F_{\text{max}}} = \frac{[P_b]}{[P]_{\text{tot}}}$$
ΔF/ΔF_{max} is equal to the fractional saturation of pericam calcium binding sites, often abbreviated Θ. Θ can be expressed as a function of calcium concentration as follows:

\[ \Theta = \frac{[P_b]}{[P]_{tot}} = \frac{[P_b]}{[P_b]K_d/\left[Ca^{2+}\right]^4 + [P_b]} \]

= \frac{\left[Ca^{2+}\right]^4}{K_d + \left[Ca^{2+}\right]^4}

= \frac{\left[Ca^{2+}\right]^4}{\left(EC_{50}\right)^4 + \left[Ca^{2+}\right]^4}

EC_{50} = (K_d)^{1/4} is the calcium concentration at which half-maximal binding and fluorescence change occurs.

Calcium sensing could realistically be performed for calcium concentrations near the EC_{50}, and our design goal is equivalent to shifting the EC_{50} of the pericam derivatives.
If we had fluorescence data over a range of calcium concentrations, and assuming these equations accurately describe calcium binding to pericam, we could determine the $\text{EC}_{50}$ and $K_d$ using a variety of methods:

1. Look for midpoint of the fluorescence change
2. Hill analysis
   • substitute $\Theta = \frac{\Delta F}{\Delta F_{\text{max}}}$
   • set $y = \log[\Theta/(1 - \Theta)]$
   • plot $y$ vs. $x = \log([\text{Ca}^{2+}])$ for transition region
   • slope tells number of cooperative binding sites, “Hill coefficient”
   • $x$ intercept is $\log(\text{EC}_{50})$

3. Curve fitting
   • consider the equation we derived:
     $$\frac{\Delta F}{\Delta F_{\text{max}}} = \frac{[\text{Ca}^{2+}]^4}{(\text{EC}_{50})^4 + [\text{Ca}^{2+}]^4}$$
   • express as $y = x^4/[c^4 + x^4]$
   • use Matlab or related to determine value of $c$ that best fits the data
   • could leave the exponent as a free parameter $n$, equal to Hill coeff.
Hill coefficient reflects **cooperativity**, a phenomenon of binding to multiple binding sites on an individual target: **positive cooperativity** means that binding to one site promotes binding to other sites; **negative cooperativity** means that binding to one site depresses binding to other sites.

For comparison with pericam, consider (noncooperative) calcium binding to BAPTA, a commonly used calcium-specific chelator:

\[
\text{Ca}^{2+} + \text{B}_u \xrightarrow{K_d} \text{K}_d = \frac{([B_u][\text{Ca}^{2+}])/[B_b]}{[B]_{tot} = [B_u] + [B_b]}
\]

In this case, the equation that describes a titration curve (fraction of bound sites vs. calcium concentration) is:

\[
\Theta = \frac{[B_b]}{[B]_{tot}} = \frac{[B_b]}{[B_b]K_d/[\text{Ca}^{2+}]+[B_b]} = \frac{[\text{Ca}^{2+}]}{K_d+[\text{Ca}^{2+}]} \quad \text{Hill coeff. } = 1
\]
Simulated binding curves for BAPTA and pericam compare as follows:

saturation vs. $\log([Ca^{2+}])$

- pericam: sharper transition
- BAPTA: broader transition

saturation vs. $[Ca^{2+}]$

- pericam: sigmoidal transition
- BAPTA: smooth transition

Hill plot

- pericam: greater slope (Hill coefficient)
- BAPTA: lesser slope
The titration behavior we’ve been discussing for pericam is idealized, because we have been assuming **complete cooperativity** among the calcium binding sites (all four Ca$^{2+}$ ions bind at once). In fact, binding to individual sites *can occur independently*, but each site’s apparent affinity depends on whether the other sites are occupied.
No titration curve provides enough detail (features) to accurately fit all the independent equilibrium constants in the “full picture.” One possibility is to settle for a compromise that fits data from CaM-based sensors:

\[
\frac{\Delta F}{\Delta F_{\text{max}}} = f_1 \times \frac{[\text{Ca}^{2+}]^{n_1}}{K_{d1} + [\text{Ca}^{2+}]^{n_1}} + f_2 \times \frac{[\text{Ca}^{2+}]^{n_2}}{K_{d2} + [\text{Ca}^{2+}]^{n_2}}
\]

\(n_1\) and \(n_2\) are the Hill coefficients associated with the transitions from \(P_u\) to \(P_i\) and from \(P_i\) to \(P_b\), respectively. \(f_1\) and \(f_2\) are the fractional fluorescence changes associated with these two steps.

Why is modeling pericam with \textbf{two calcium-dependent} transitions particularly appropriate?
One- and two-step transition behavior of CaMeleons (Miyawaki et al.):

- your mutations in pericam may affect the apparent $K_d$s (or EC$_{50}$s) and Hill coefficients for two transition steps
- the "resolution" of the two steps may be affected—note that the parent pericam appears to have a single transition, in part due to the E104Q mutation (cf. curve with black circles above)
- in some cases, your mutations may affect the relative fluorescence changes of the two transition steps

Note on calcium concentrations

How is a range of known calcium concentrations be produced?
• note that EC₅₀s are typically around 1 μM; the most useful calcium concentrations will be near the EC₅₀
• you may need > 1 μM protein to make robust measurements
• need to make sure that we know the concentration of unbound calcium, as opposed to total calcium concentration

\[
\frac{\Delta F}{\Delta F_{\text{max}}} = f_1 \times \frac{[Ca^{2+}]^{n_1}}{K_{d1} + [Ca^{2+}]^{n_1}} + f_2 \times \frac{[Ca^{2+}]^{n_2}}{K_{d2} + [Ca^{2+}]^{n_2}}
\]

We will solve this problem by using a calcium buffer
• analogous to a pH buffer, with pCa²⁺ determined by affinity of the buffer (in our case EGTA) and absolute amount of Ca²⁺ present

\[
[Ca^{2+}] = \frac{[Ca^{2+} - \text{EGTA}]}{[\text{EGTA}] \cdot K_a}
\]

• with 10 mM Ca²⁺-EGTA/EGTA mixtures, the amount of pericam present is unlikely to have much effect
Binding may be quantified using methods other than fluorescence

Other techniques for titration curve measurement:

- absorbance spectroscopy, e.g. $O_2$ binding to Hb,
- circular dichroism, e.g. $Ca^{2+}$ binding to TrC fragments


• surface plasmon resonance, *e.g.* antibody binding to a target

De Santis *et al.* (2003)
*Br. J. Cancer* 88: 996-1003


• enzymatic activity, *e.g.* CaM-dependent enzyme activation

Shen *et al.* (2002)
*EMBO J.* 21: 6721-32

• calorimetry, e.g. peptide-protein interaction

\[ K_D = 192 \pm 26 \, \mu M \]

Schütz et al. (2006) *EMBO J.* 25: 4245-52

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• analytical ultracentrifugation, e.g. ligand-protein binding

