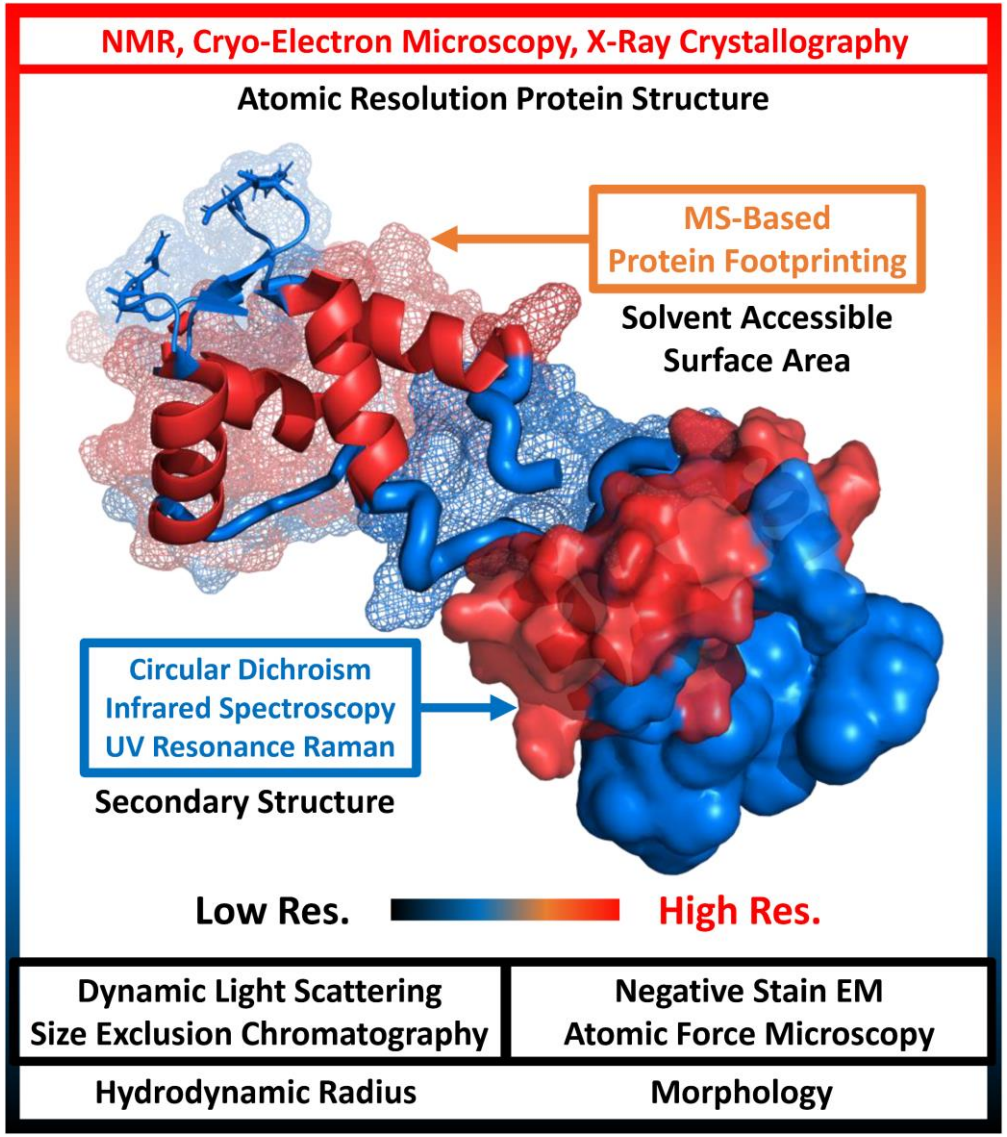
Binding Sites, Orders, Site-Specific Affinities, and Composite Allosteric Behavior Revealed by a Single Mass Spectrometry-Based Approach

Roger Liu

Global Product Development Bristol Myers Squibb™

04/14/2021

Mass Spectrometry-based Footprinting in Structural Proteomics

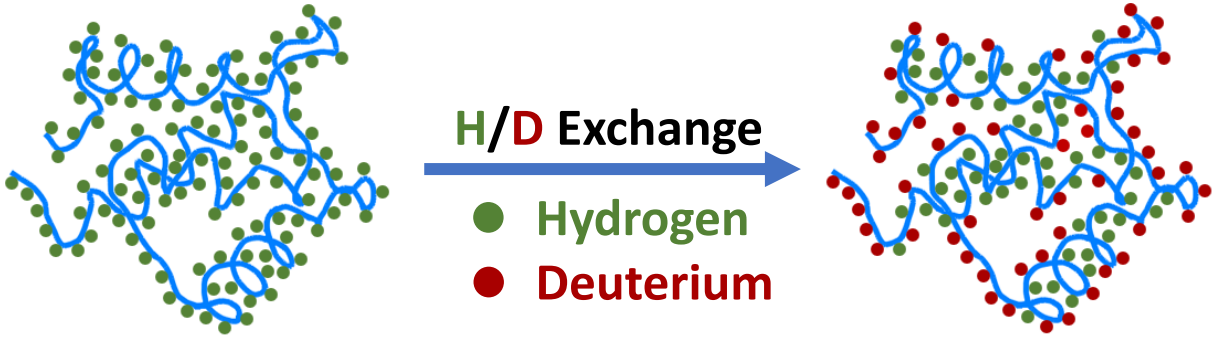


- **Protein Higher Order Structure (HOS)**
  - Secondary to quaternary structures
  - Enables various biological functions
- **Mass Spectrometry in HOS Analysis**
  - High throughput
  - Low sample amount requirement (ng - µg)
  - In-solution characterization
  - High sensitivity
  - Mid to high spatial resolution (peptide and amino acid residue level)

Liu, X. R.[†]; Zhang, M. M.[†]; Gross, M. L. *Chem. Rev.* **2020**, *120*, 4355-4454.

Reversible and Irreversible Approaches – Solvent Accessible Surface

- **Reversible Approaches**



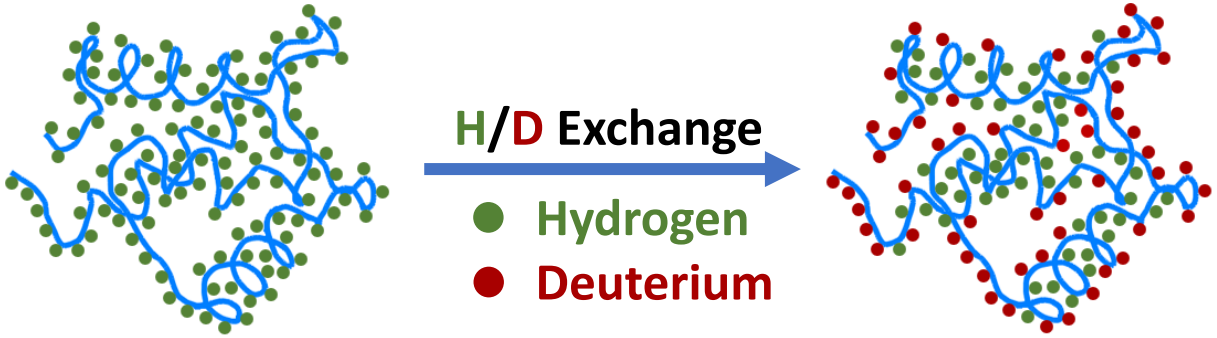
- Hydrogen Deuterium Exchange (HDX)

Solvent accessible backbone amide hydrogen reversibly exchange with deuterium in solvent

Liu, X. R.[†]; Zhang, M. M.[†]; Gross, M. L. *Chem. Rev.* **2020**, *120*, 4355-4454.

Reversible and Irreversible Approaches – Solvent Accessible Surface

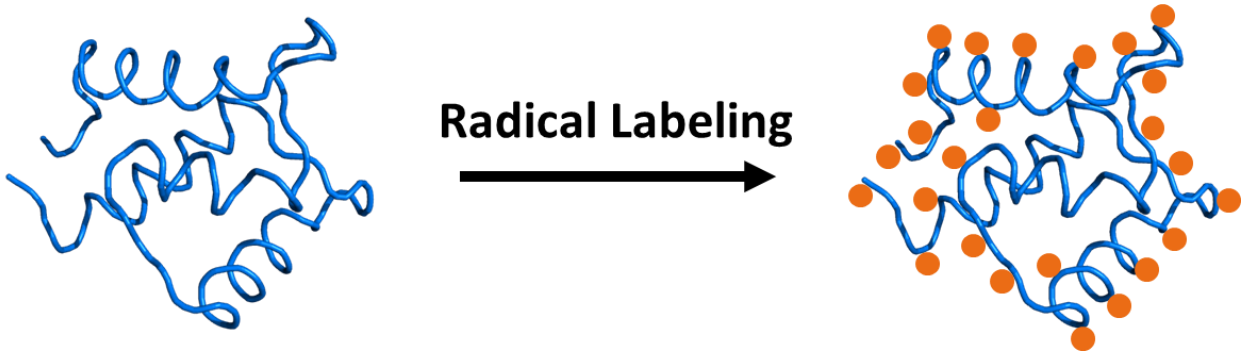
- **Reversible Approaches**



- Hydrogen Deuterium Exchange (HDX)

Solvent accessible backbone amide hydrogen reversibly exchange with deuterium in solvent

- **Irreversible Approaches**



- Radical labeling reagents

  e.g., $^{\bullet}OH$, $SO_4^{-\bullet}$, $^{\bullet}CF_3$, $CO_3^{-\bullet}$, $I^{\bullet}$

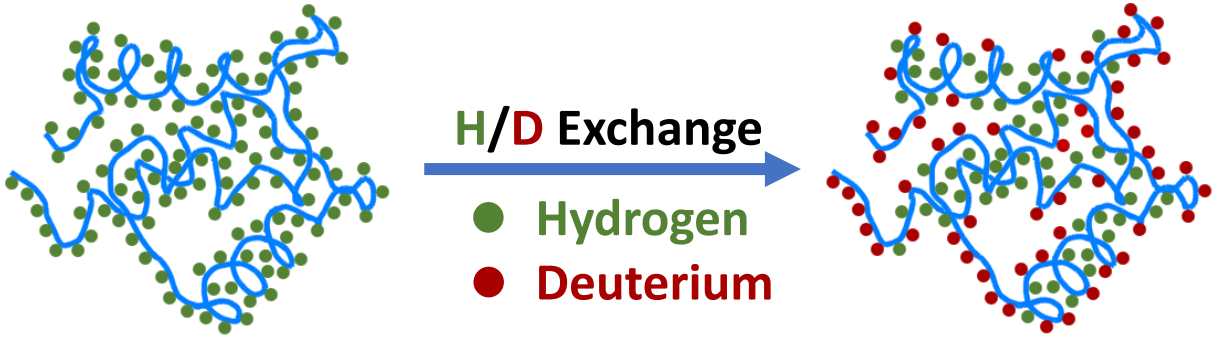
  RC:, photo cross-linking

Chemical labels covalently attach onto solvent accessible amino acid side chains

Liu, X. R.[†]; Zhang, M. M.[†]; Gross, M. L. *Chem. Rev.* **2020**, *120*, 4355-4454.

Reversible and Irreversible Approaches – Solvent Accessible Surface

- **Reversible Approaches**



  - Hydrogen Deuterium Exchange (HDX)

Solvent accessible backbone amide hydrogen reversibly exchange with deuterium in solvent

- **Irreversible Approaches**

  - Radical labeling reagents

    e.g., $^{\bullet}OH$, $SO_4^{-\bullet}$, $^{\bullet}CF_3$, $CO_3^{-\bullet}$, $I^{\bullet}$

    RC:, photo cross-linking
  - Targeted labeling reagents (covalent labeling)

    e.g., glycine ethyl ester: Asp & Glu

    Chemical cross-linking

Chemical labels covalently attach onto solvent accessible amino acid side chains

Liu, X. R.[†]; Zhang, M. M.[†]; Gross, M. L. *Chem. Rev.* **2020**, *120*, 4355-4454.

Measuring P-L Binding Affinity

$$K_a = \frac{k_{on}}{k_{off}} = \frac{[PL]}{[P][L]} \qquad K_d = \frac{k_{off}}{k_{on}} = \frac{[P][L]}{[PL]}$$

- **Concentration Measurement**

  Fluorescence Polarization, Circular Dichroism, NMR, FT-IR

- **Rate Constant Measurement**

  Surface Plasmon Resonance

- **Thermodynamic Measurement**

  Isothermal Titration Calorimetry (Heat flow during titration)

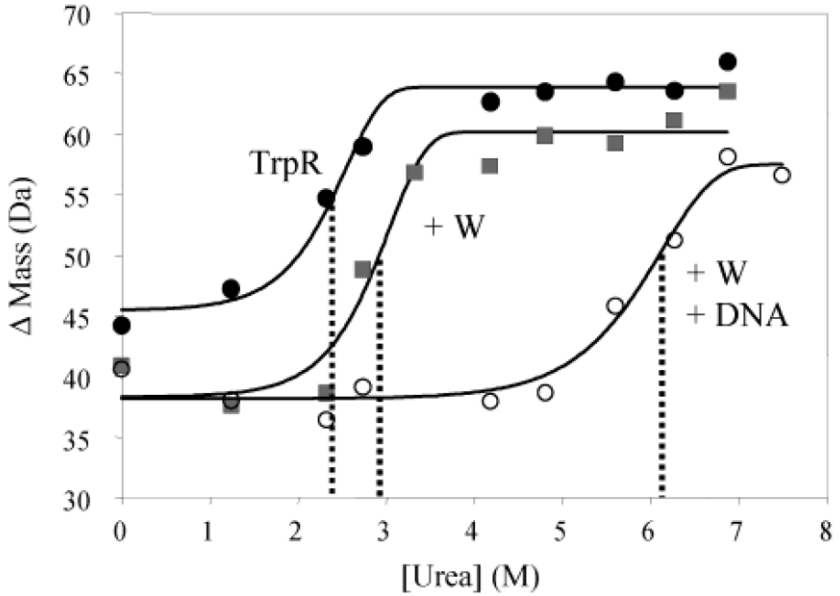
Specific sample preparation

Limited spatial resolution

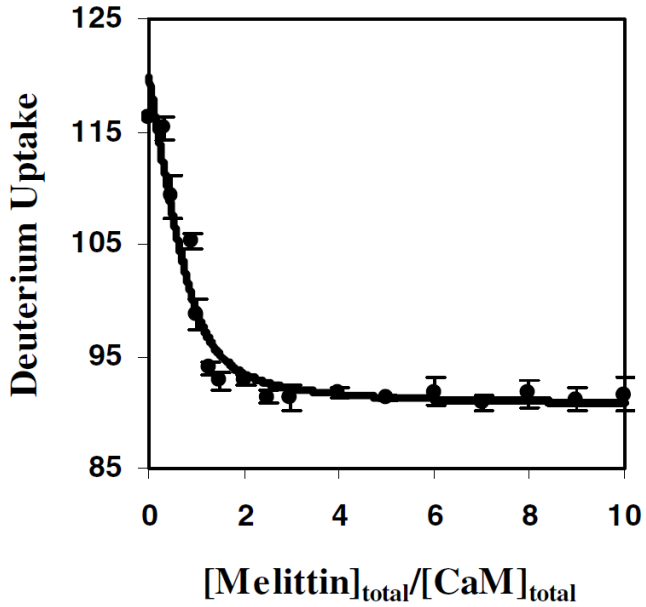
How could Mass Spectrometry contribute?

[1] Willams, M. A.; Daviter, T. *Protein-Ligand interactions, Methods and Applications*, 2nd ed.; Humana Press: New York, 2013.
[2] Weis, D. D. *Hydrogen Exchange Mass Spectrometry of Proteins, Fundamentals, Methods, and Applications*; Wiley: Chichester, 2016.

SUPREX and PLIMSTEX



Stability of Unpurified Proteins from Rates of H/D Exchange

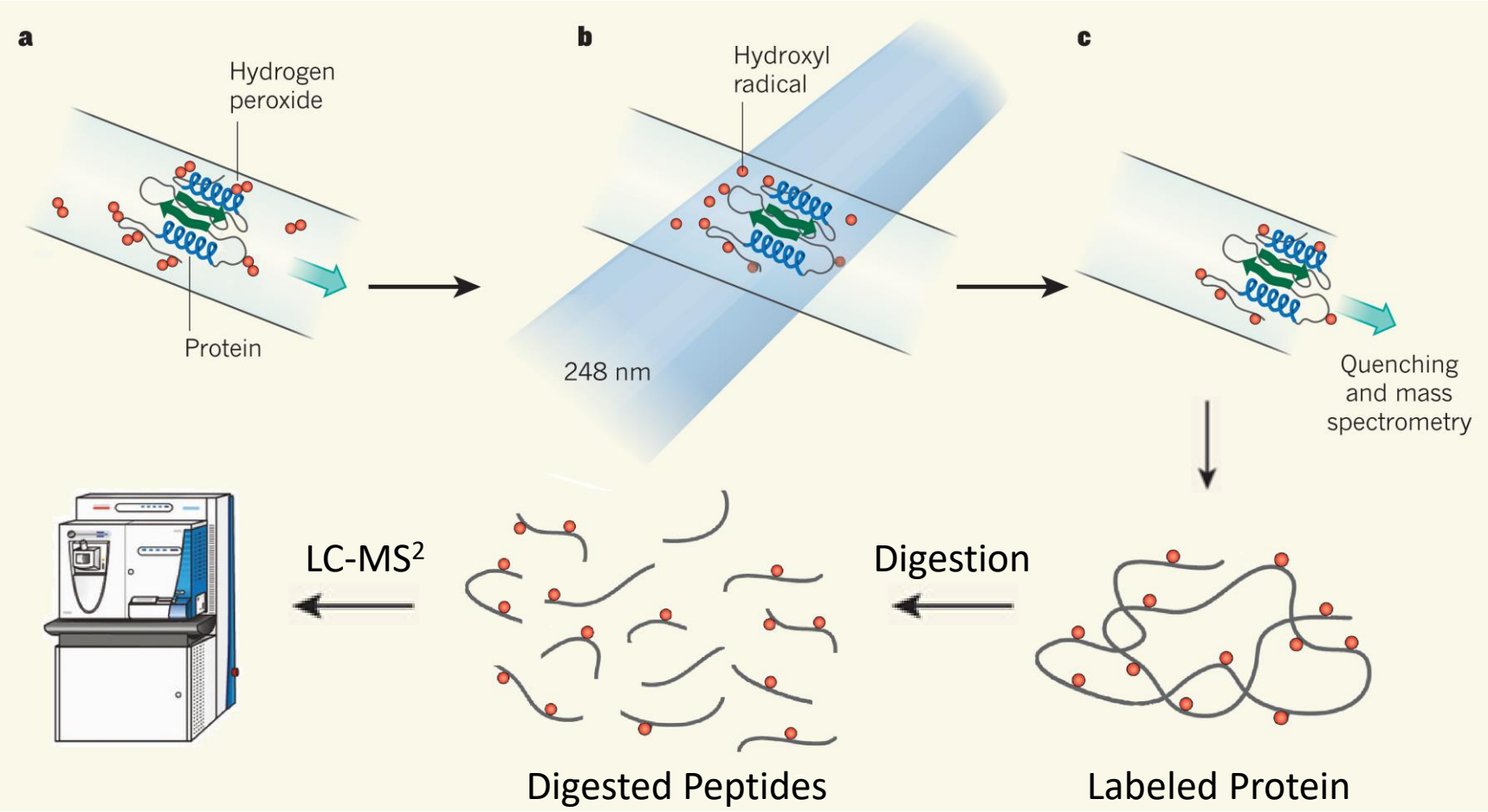


Protein-Ligand Interactions by Mass Spectrometry, Titration, and H/D Exchange

- Universal to various systems, low sample quantities, no special labeling, site-specific
- Backexchange, complicated picture by ligand off-rate and long H/D exchange time

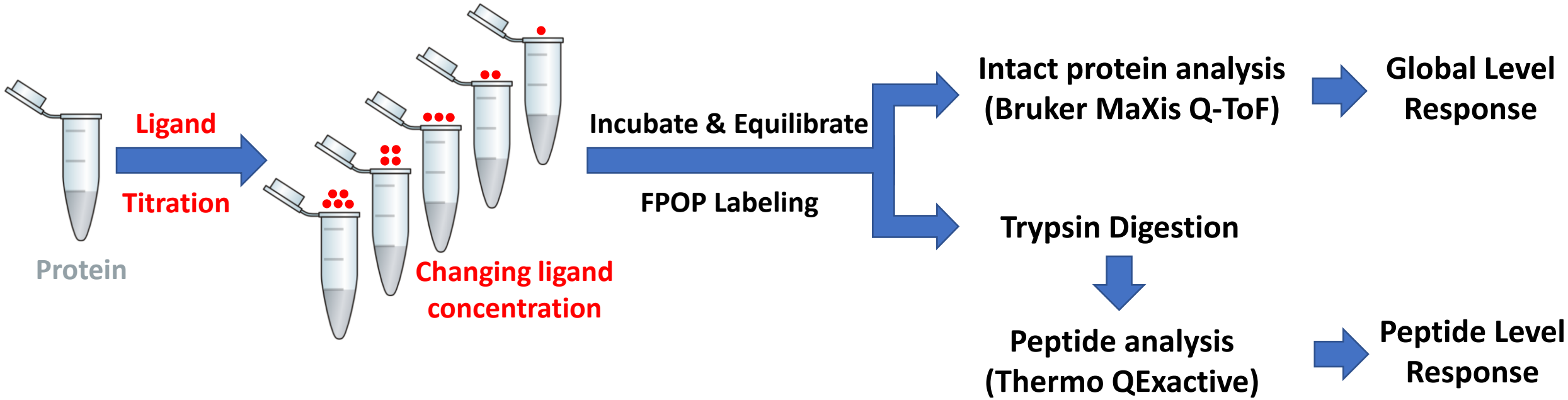
[1] Powell, K. D.; Ghaemmaghami, S.; Wang, M. Z.; Ma, L.; Oas, T. G.; Fitzgerald, M. C. *J. Am. Chem. Soc.* **2002**, *124*, 10256-10257.
[2] Zhu, M. M.; Rempel, D. L.; Du, Z.; Gross, M. L. *J. Am. Chem. Soc.* **2003**, *125*, 5252-5253.

Fast Photochemical Oxidation of Proteins (FPOP) & Mass Spectrometry



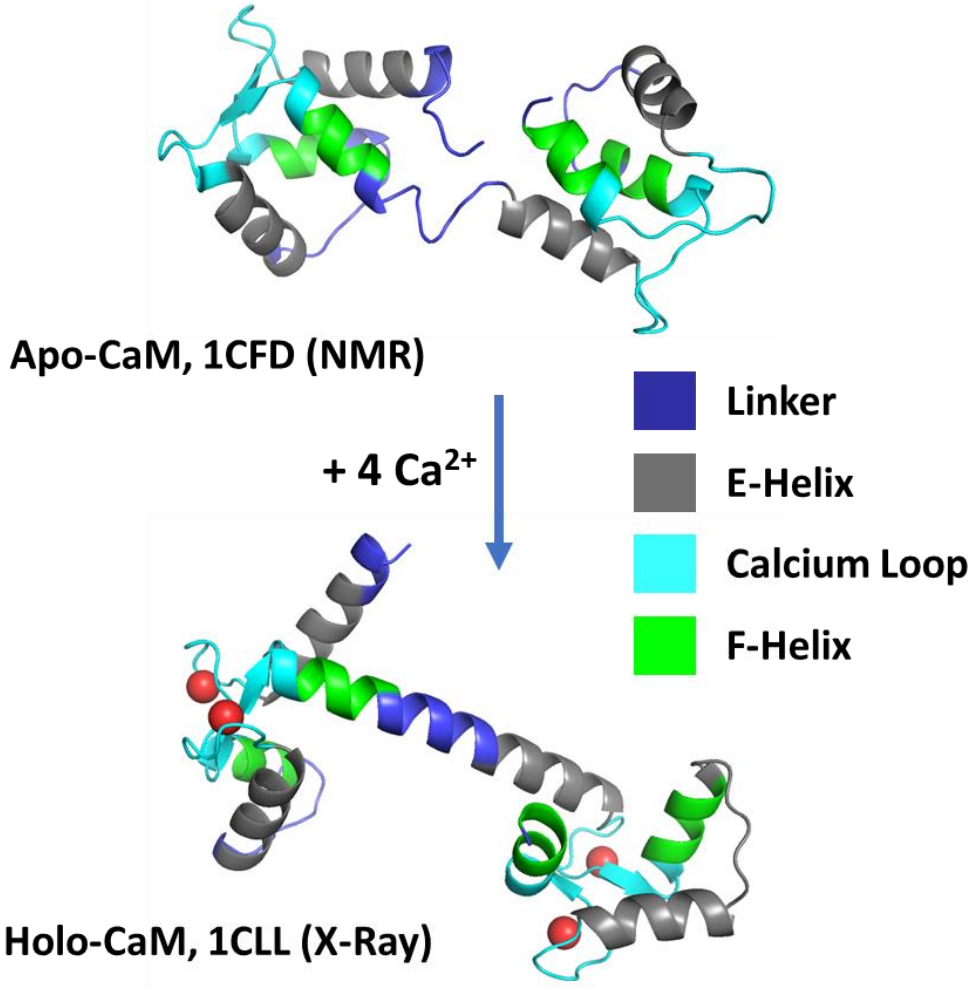
[1] Gruebele, M. *Nature* **2010**, *468*, 640-641.
[2] Hambly, D. M.; Gross, M. L. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 2057-2063.
[3] **Liu, X. R.**[†]; Zhang, M. M.[†]; Gross, M. L. *Chem. Rev.* **2020**, *120*, 4355-4454.
[4] **Liu, X. R.**; Rempel, D. L.; Gross, M. L. *Nat. Protoc.* **2020**, *15*, 3942-3970.

Protein-Ligand Interaction by Ligand Titration, Fast Photochemical Oxidation of Proteins and Mass Spectrometry: LITPOMS



[1] **Liu, X. R.**; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *J. Am. Soc. Mass Spectrom.* **2019**, *30*, 213-217.
[2] **Liu, X. R.**; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 5508-5512.

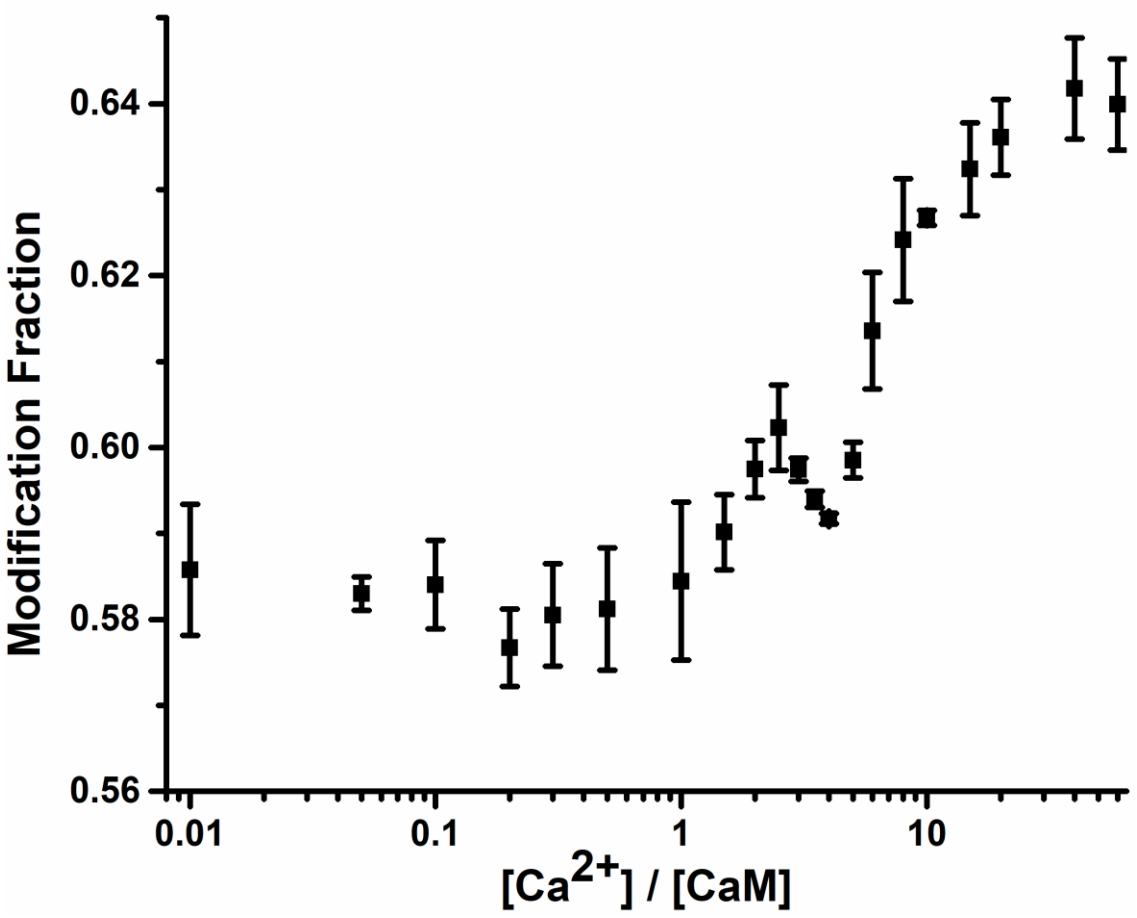
Calmodulin & Calcium in Tris – 1:4 System



- Found in all eukaryotic cells
- Major Ca^{2+} signaling pathway, regulating multiple intracellular process: cell growth, proliferation, apoptosis
- Four EF-hands with "helix-loop-helix" configuration
- Ca^{2+} binds to calcium loop through chelating with negatively charged residues
- Binding affinity of µM, sensitive to stimulations
- Hint of cooperativity / allosteric behavior during binding

[1] Koniwa, H.; Tjandra, N.; Grzesiek, S.; Ren, H.; Klee, C. B.; Bax, A. *Nat. Chem. Biol.* **1995**, *2*, 768-776.
[2] Chattopadhyaya, R., Meador, W.E., Means, A.R., Quiocho, F.A. *J. Mol. Biol.* **1992**, *228*, 1177-1192.
[3] Linse, S.; Helmersson, A.; Forsen, S. *J. Biol. Chem.* **1991**, *266*, 8050-8054.
[4] Sorensen, R.; Shea, M. A. *Biochemistry* **1998**, *37*, 4244-4253.

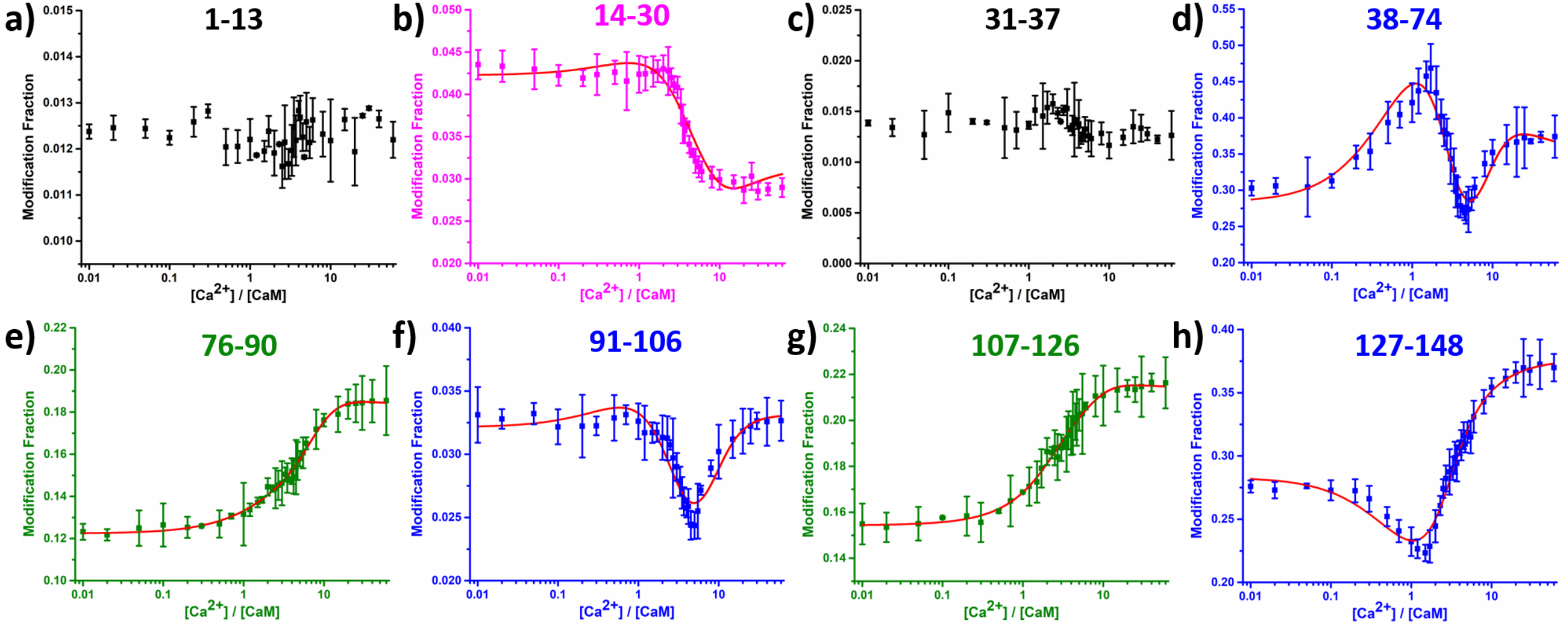
LITPOMS of Ca^{2+} & CaM at Global Level



- A more extended confirmation upon binding with calcium
- A decrease in modification fraction indicates a potential binding event
- A promising preliminary check before peptide & residue-level analysis

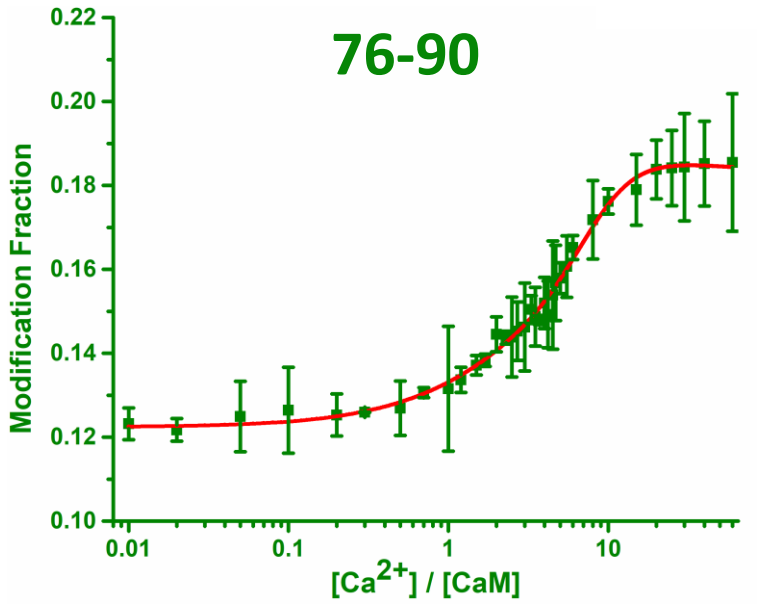
Liu, X. R.; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 5508-5512.

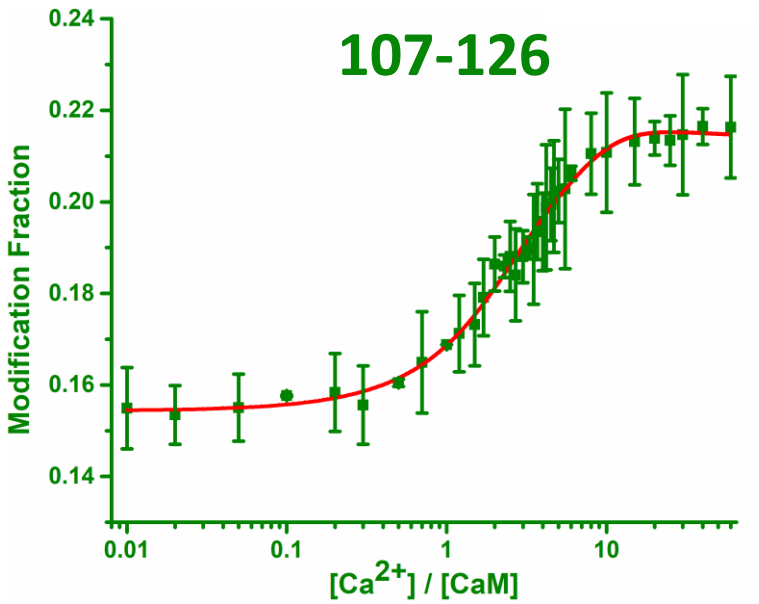
LITPOMS of Ca^{2+} & CaM at Peptide Level



Liu, X. R.; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 5508-5512.

Class I Behavior – Loss of Protection

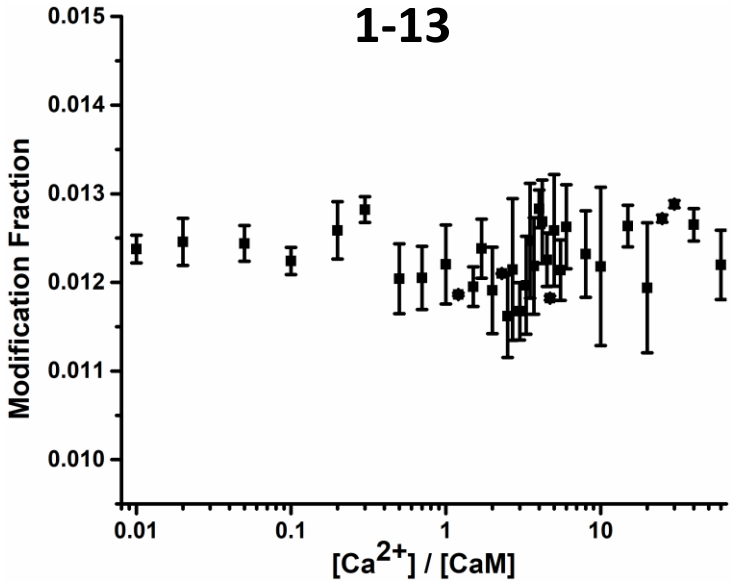


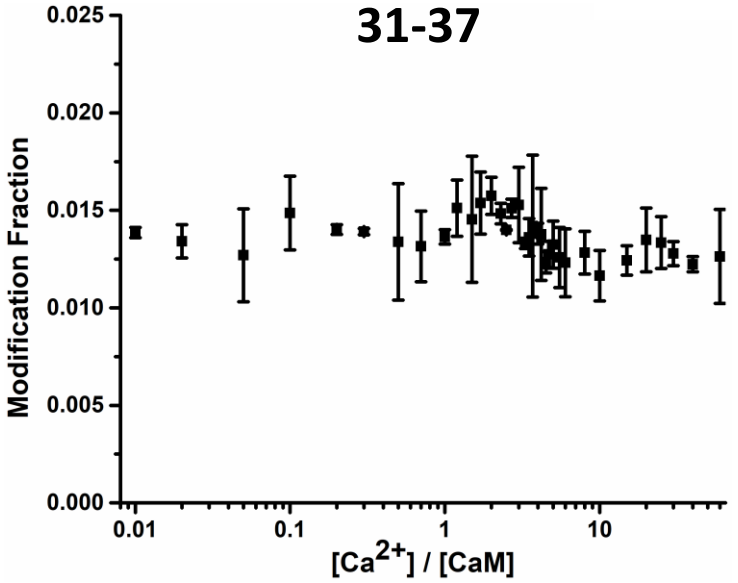


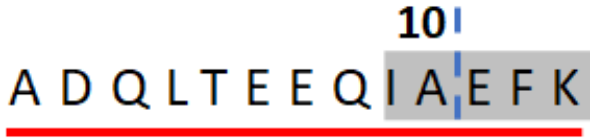
- Loss of protection upon binding with calcium, reporting the overall structural transition
- Linker region between EF-hands / more exposed helices

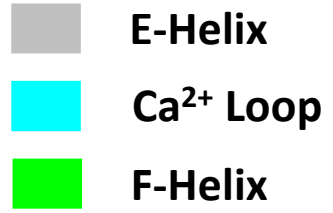
Liu, X. R.; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 5508-5512.

Class II Behavior – Constant Protection





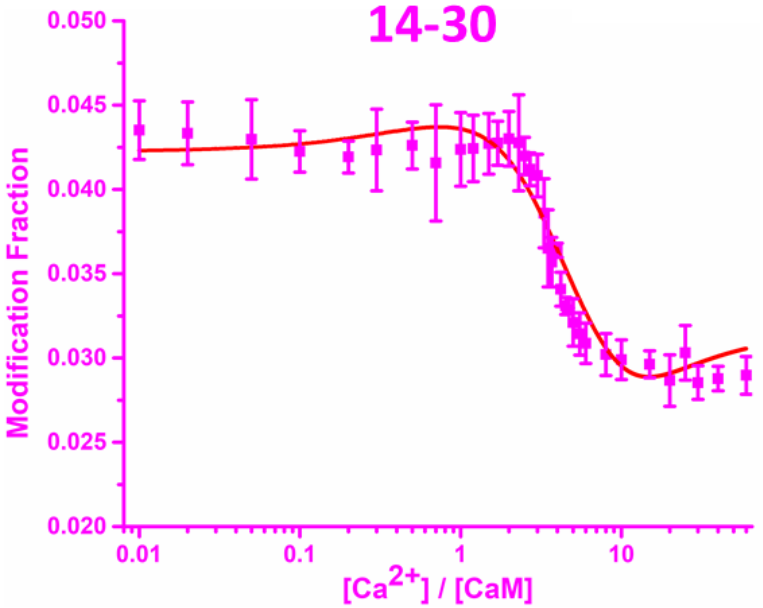




- Constant protection upon binding with calcium
- Protein N-terminus / F-helix of EF-1

Liu, X. R.; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 5508-5512.

Class III Behavior – Classical Binding

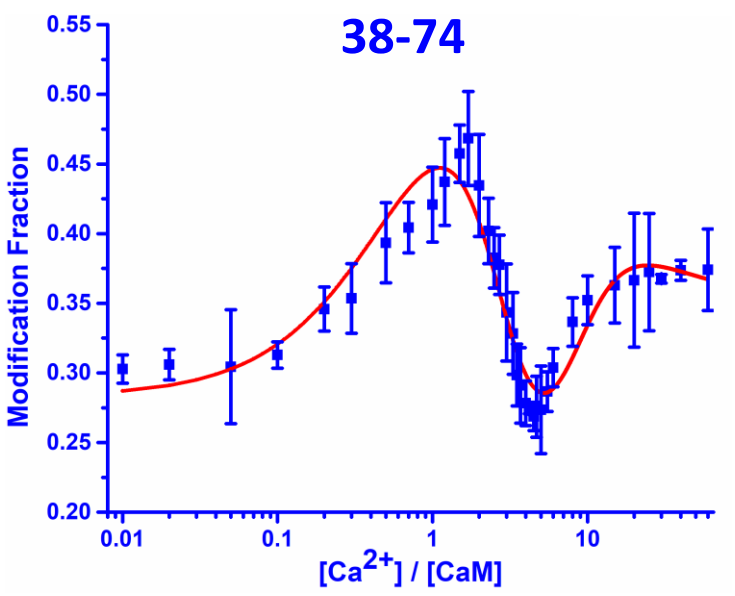


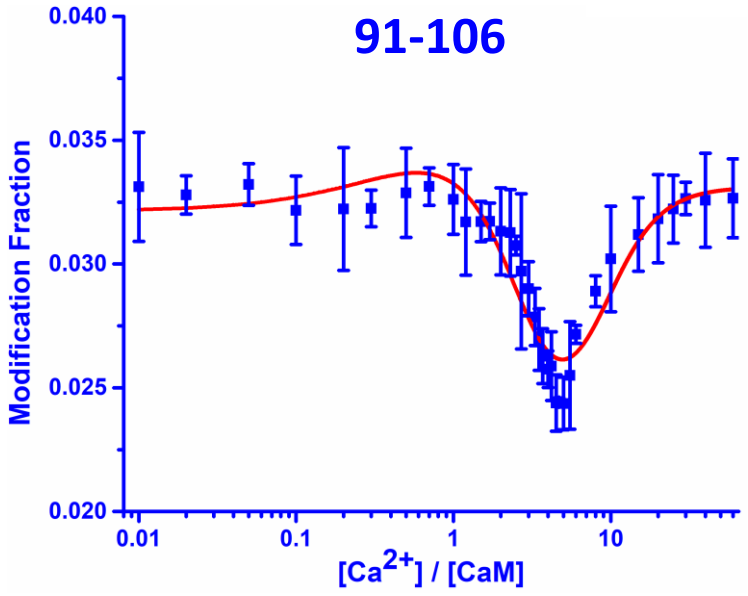
- E-helix & calcium loop of EF-1

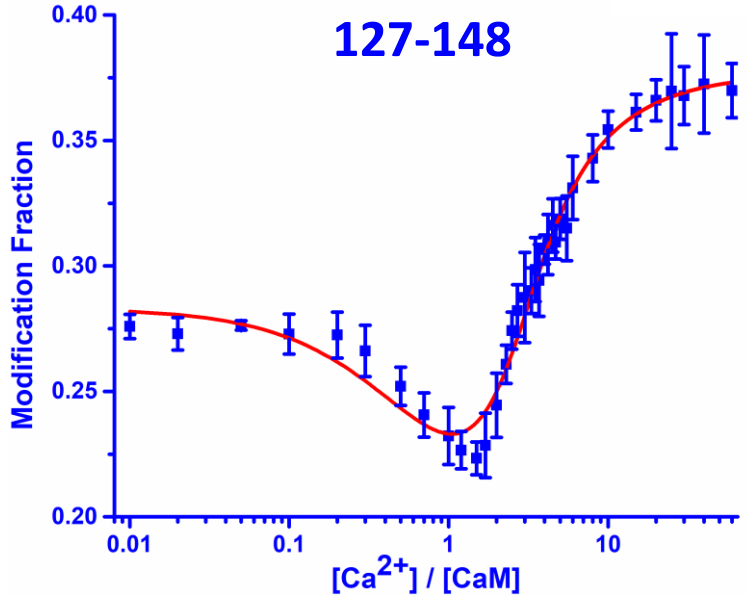
- More protected upon binding with calcium, a classical binding behavior

Liu, X. R.; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 5508-5512.

Class IV Behavior – Composite Behavior







E-Helix

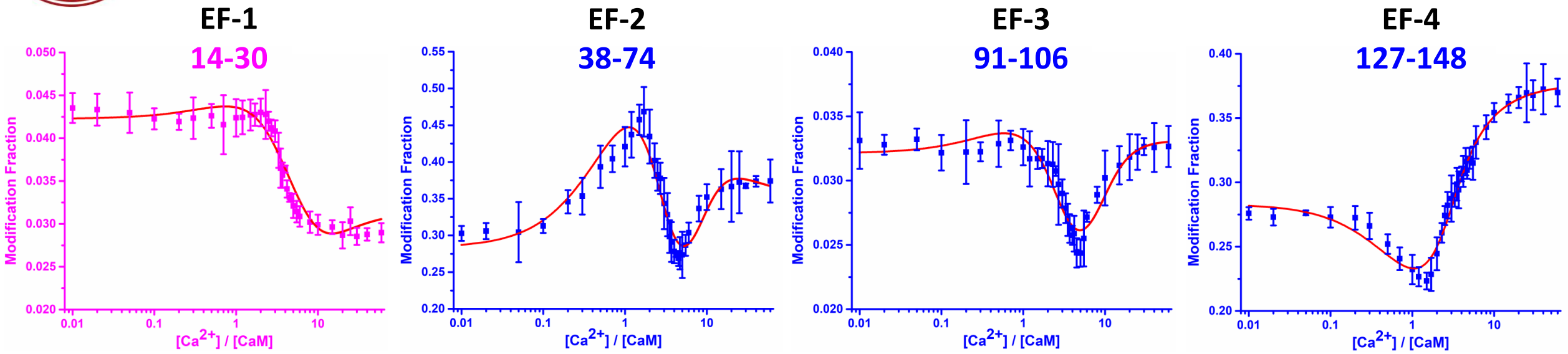
Ca^{2+} Loop

F-Helix

- Covers EF-2 (38-74), EF-3 (91-106) and EF-4 (127-148)
- Binding & Allostery (Conf. change by a remote binding event)

Liu, X. R.; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 5508-5512.

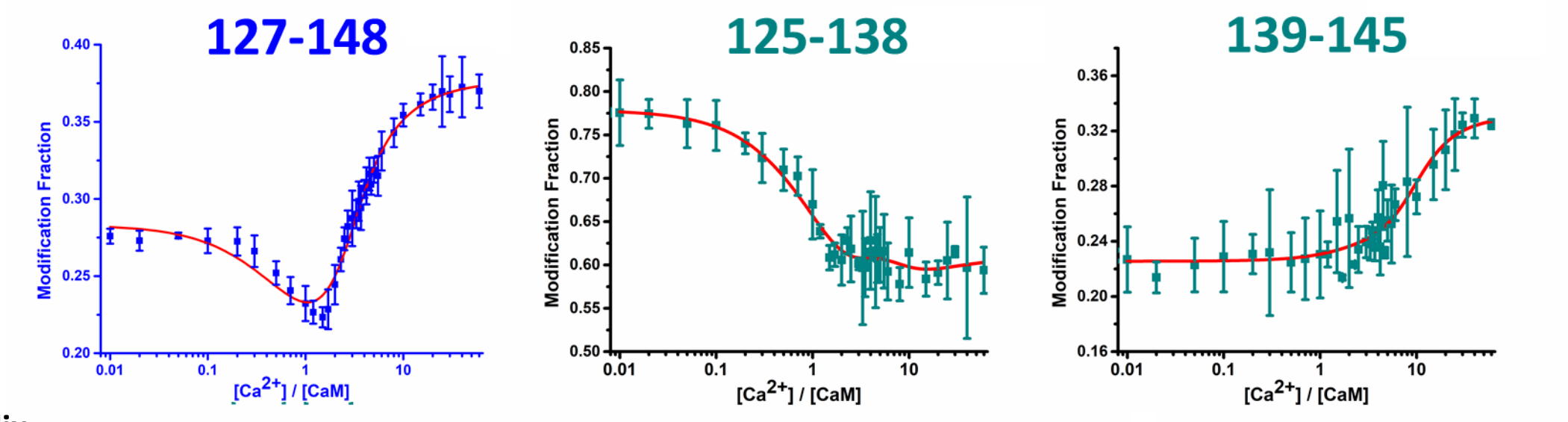
Allosteric Behavior / Binding Dynamics



- EF-4 (127-148) binds first, followed by a conformational change over helix F (more exposed)
- EF-2 (38-74) becomes more exposed owing to binding at EF-3 (91-106) and EF-4 (127-148), preparing EF-2 for Ca^{2+} binding
- EF-2, 3 and 4 become more exposed at high Ca^{2+} owing to binding at EF-1 (14-30)
- Calmodulin is saturated at $[Ca^{2+}]$ / [CaM] = 15

Liu, X. R.; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 5508-5512.

Dissect Composite LITPOMS by Chymotrypsin



E-Helix

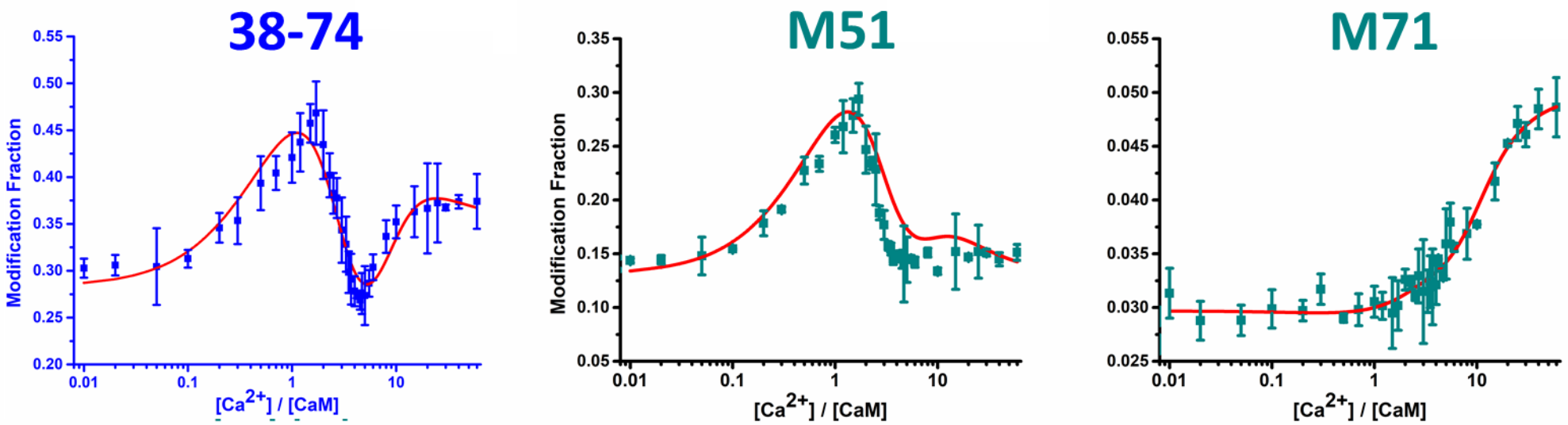
Ca^{2+} Loop

F-Helix

- Tryptic Peptide 127-148 covers EF-4 of calmodulin
- Chymotryptic peptides 125-138 and 139-145 show simple behavior of binding and opening

[1] **Liu, X. R.**; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 12560-12567.
[2] **Liu, X. R.**; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 5508-5512.

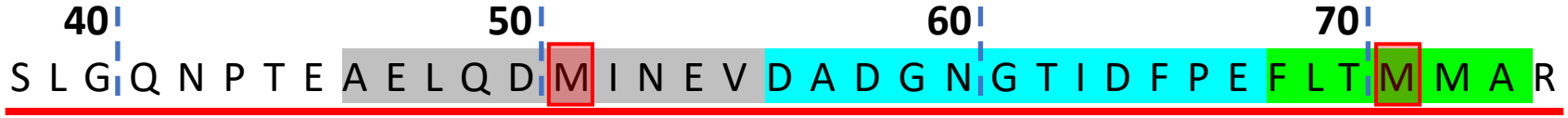
Dissect Composite LITPOMS by Residue-Level Analysis



E-Helix

Ca^{2+} Loop

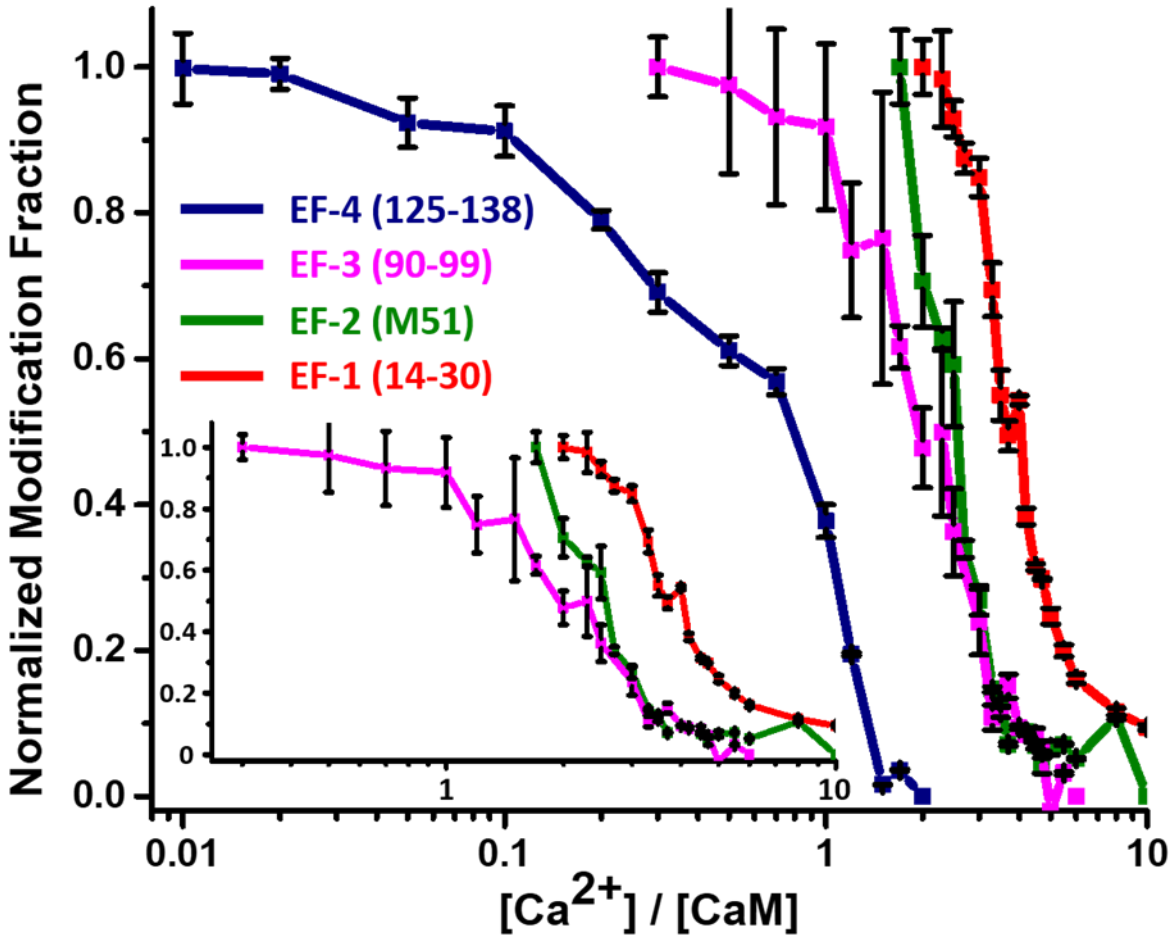
F-Helix



- Tryptic Peptide 38-74 covers EF-2 of calmodulin
- Residue M51 show deprotection initially, followed by binding induced protection and stay protected

[1] **Liu, X. R.**; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 12560-12567.
[2] **Liu, X. R.**; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 5508-5512.

Ca^{2+} Binding Order by LITPOMS



EF-4 > EF-3 > EF-2 > EF-1

[1] **Liu, X. R.**; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 12560-12567.
[2] **Liu, X. R.**; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 5508-5512.

Site-Specific Binding Affinity

- Four distinct binding affinities through fitting

Binding Order	EF-hand	LITPOMS K_i (M^{-1})	Literature K_i (M^{-1})
1	EF-4 (127-148)	1.4×10^6	8.0×10^4
2	EF-3 (91-106)	6.2×10^6	4.0×10^6
	C-Term Lobe	8.6×10^{12}	3.2×10^{11}
3	EF-2 (38-74)	4.1×10^4	2.5×10^4
4	EF-1 (14-30)	2.9×10^6	4.0×10^5
	N-Term Lobe	1.2×10^{11}	1.0×10^{10}

- Agree with literature values within 20-fold

[1] **Liu, X. R.**; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 5508-5512.
[2] Weis, D. D. *Hydrogen Exchange Mass Spectrometry of Proteins, Fundamentals, Methods, and Applications*; Wiley: Chichester, 2016.
[3] Linse, S.; Helmersson, A.; Forsen, S. *J. Biol. Chem.* **1991**, *266*, 8050-8054.

Conclusion

- LITPOMS for the first time reveals the **calcium binding order** and **allostery** of calmodulin upon binding with calcium
- March through the protein region-by-region, characterize binding at peptide & residue level
- Composite LITPOMS behavior can be dissected by a different protease or by analyzing residue-level LITPOMS curves
- Successfully demonstrate the capability of dealing with
    - Systems with various binding stoichiometries
    - Systems with binding affinities of μM to nM (both loose and tight binders)
    - Systems that bind with small peptides
    - Systems that bind with metal ions
- Readily applicable to other systems with complex binding schemes, e.g., signaling proteins

[1] **Liu, X. R.**; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *J. Am. Soc. Mass Spectrom.* **2019**, *30*, 213-217.
[2] **Liu, X. R.**; Zhang, M. M.; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 5508-5512.
[3] **Liu, X. R.**; Rempel, D. L.; Gross, M. L. *Anal. Chem.* **2019**, *91*, 12560-12567.

Acknowledgement

5P41GM103422
1S10OD016298
R24GM136766
R01GM131008

National Institute of General Medical Sciences

PROTEIN METRICS

Stay Safe!