# Symposium: Top-Down Analysis of Antibodies and EU-US MS Conference

**June 20, 2014**



# "Protein Footprinting for Problem Solving in Biophysics"

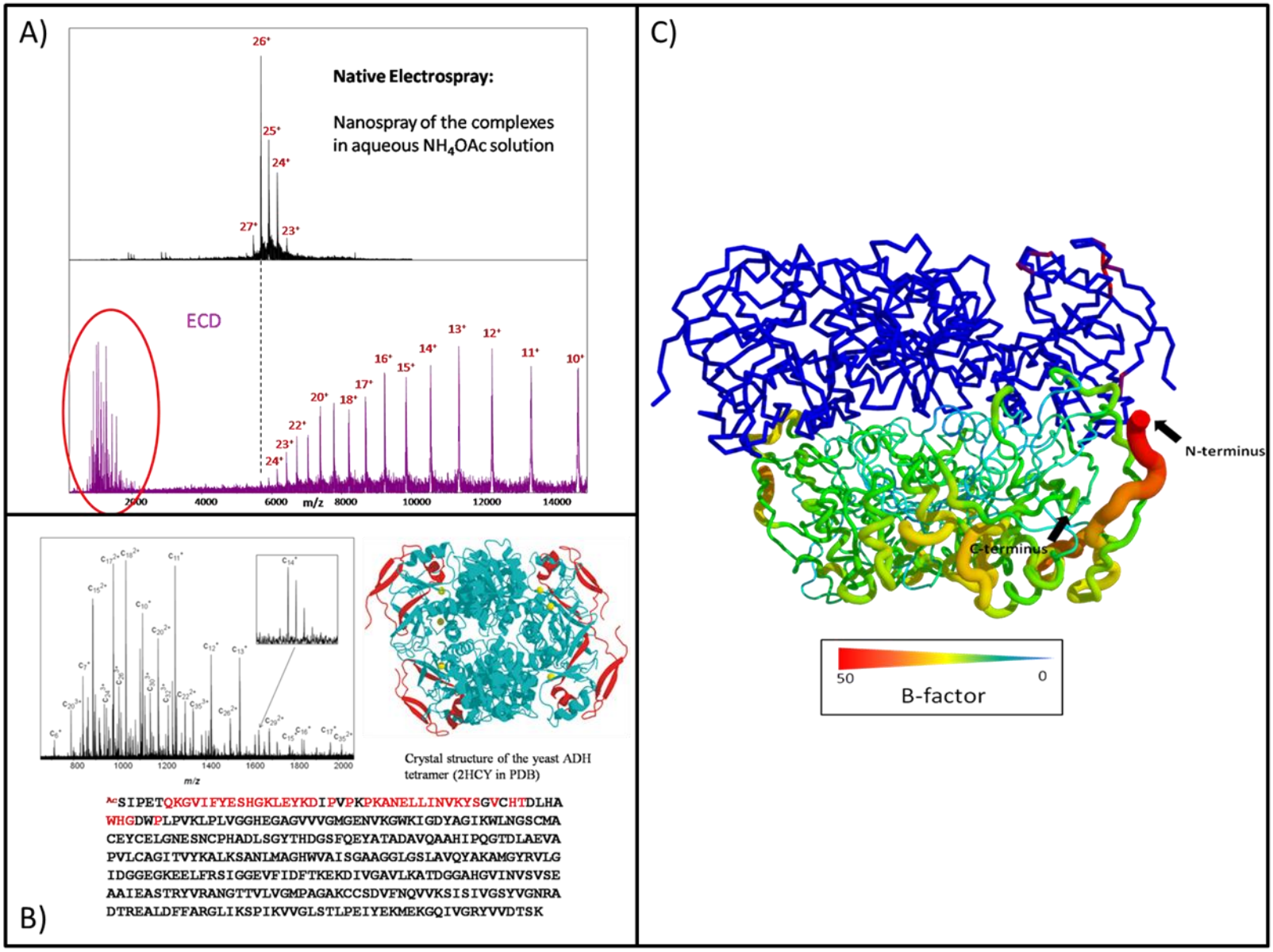
Hao Zhang, Weidong Cui, Yuetian Yan, Ying Zhang, Lisa Jones, Robert Blankenship, and Michael Gross

# Biophysics and Therapeutic Proteins

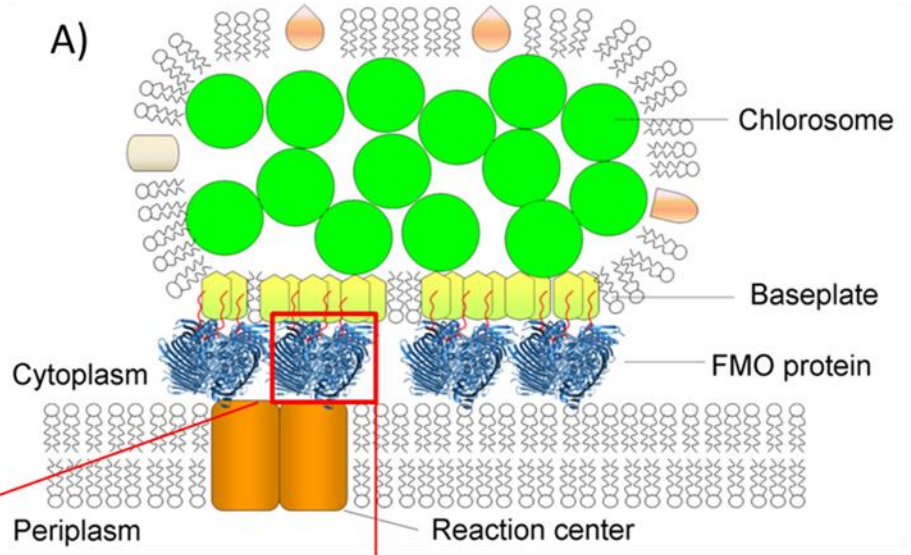
- Top Down of protein complexes, antibodies, by native spray, ECD, and other activation methods
- Ion mobility
- Protein Footprinting:  HDX (PLIMSTEX), FPOP, GEE labeling

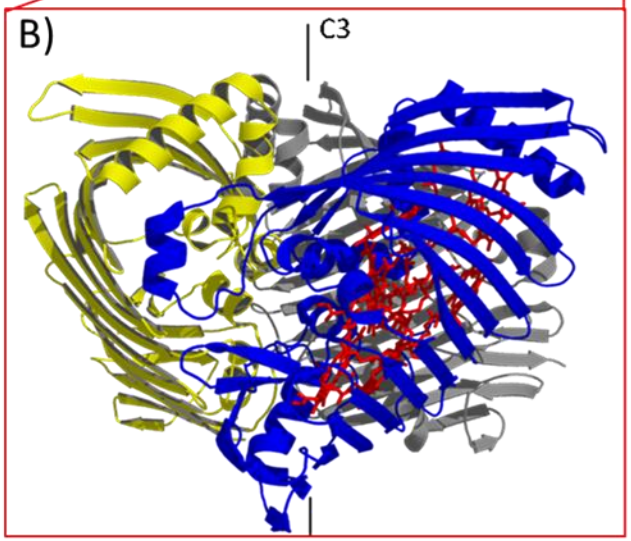
# ECD of Protein Assemblies and Antibodies

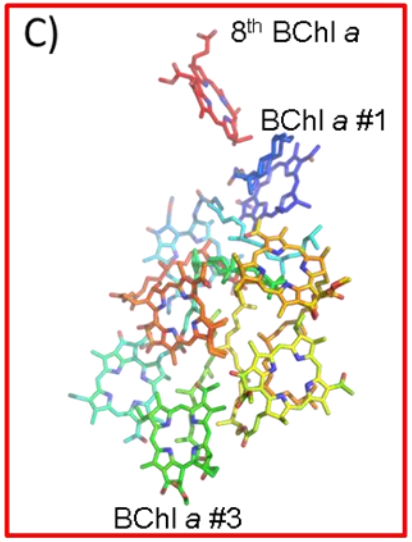
# Native MS of ADH tetramer

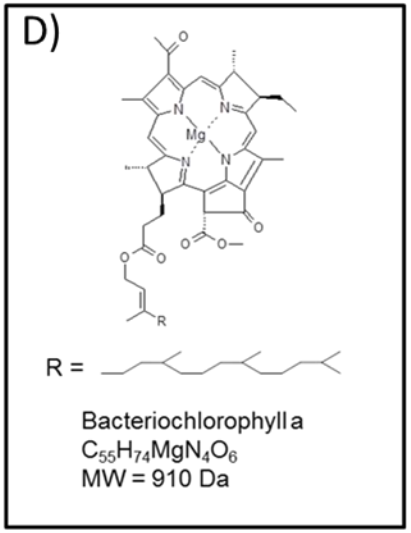


# FMO antenna protein complexes

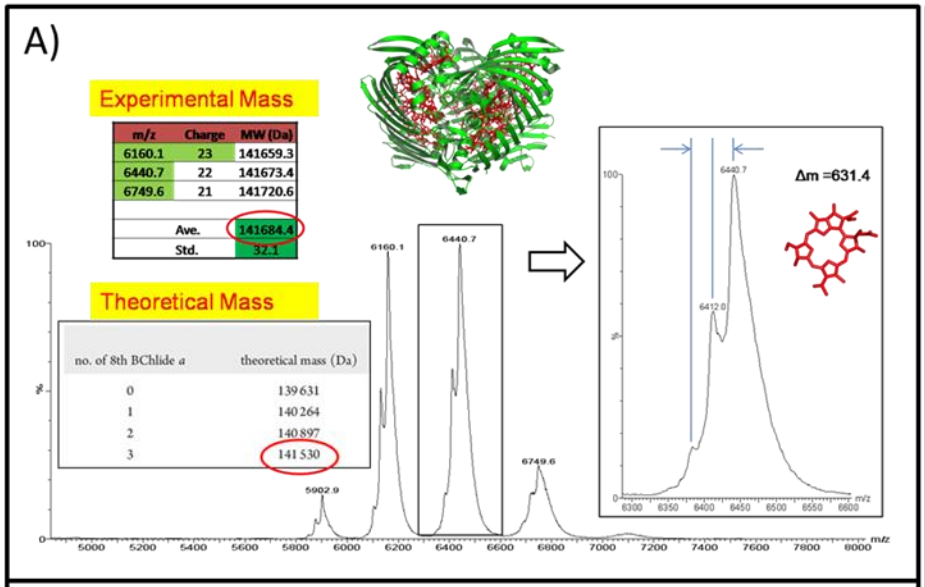


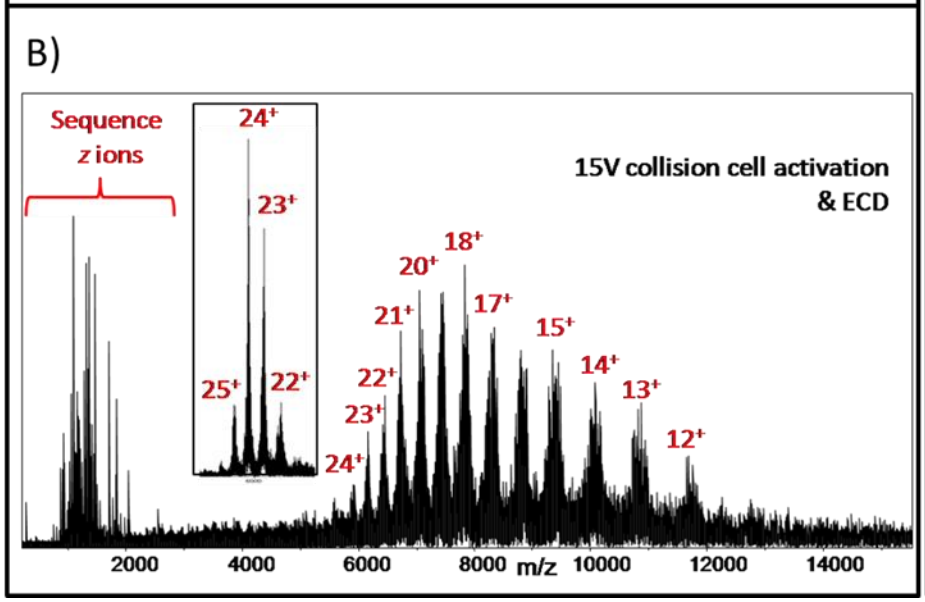



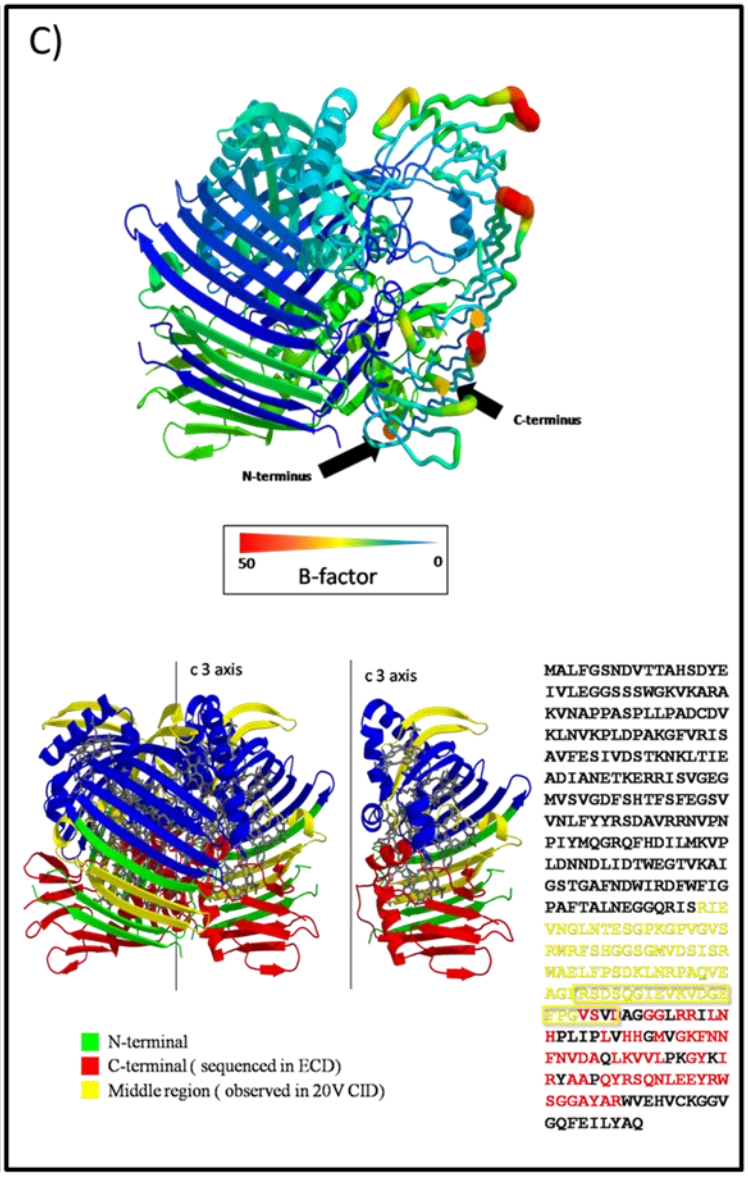



# Native MS and Top-down of FMO

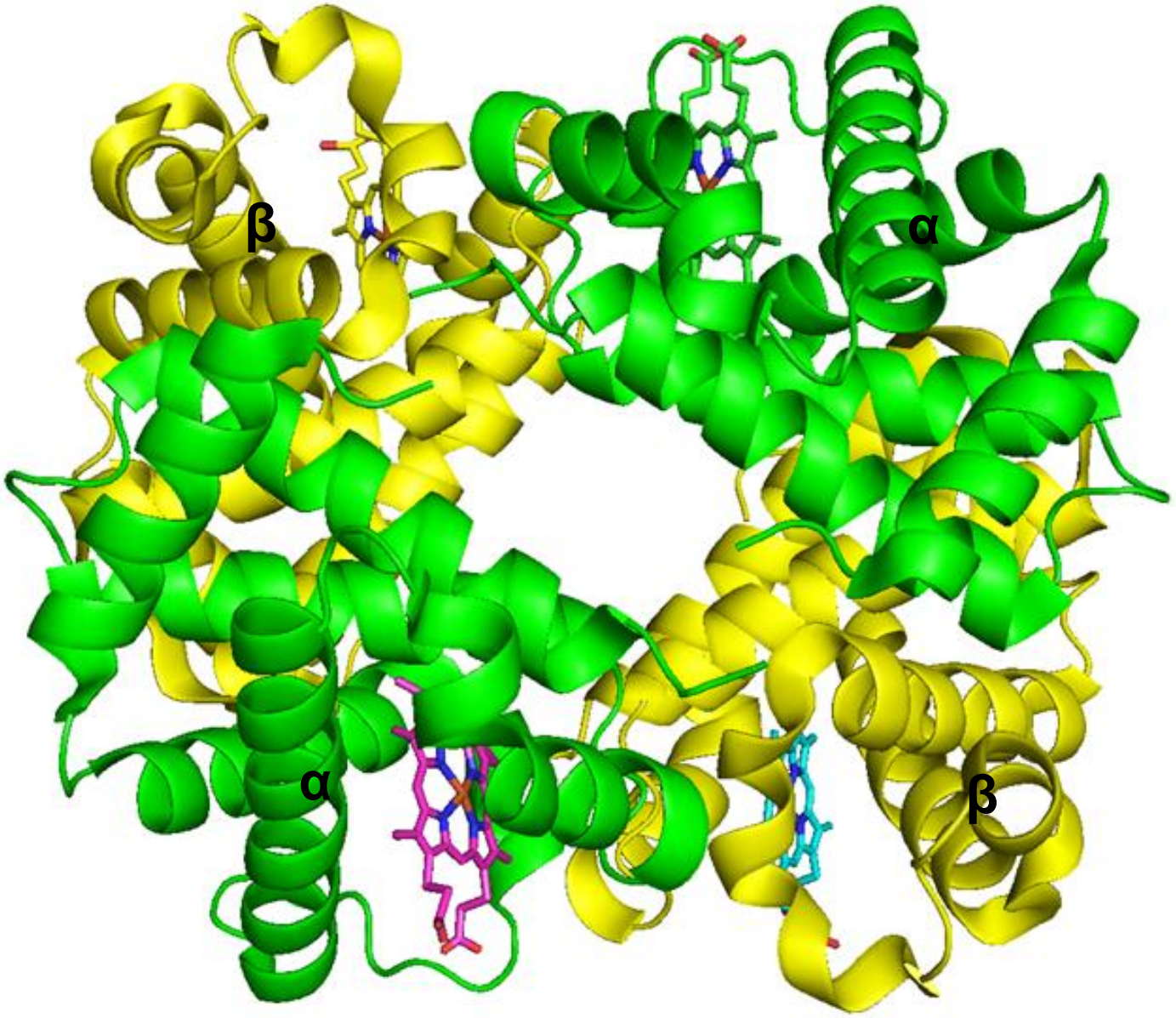


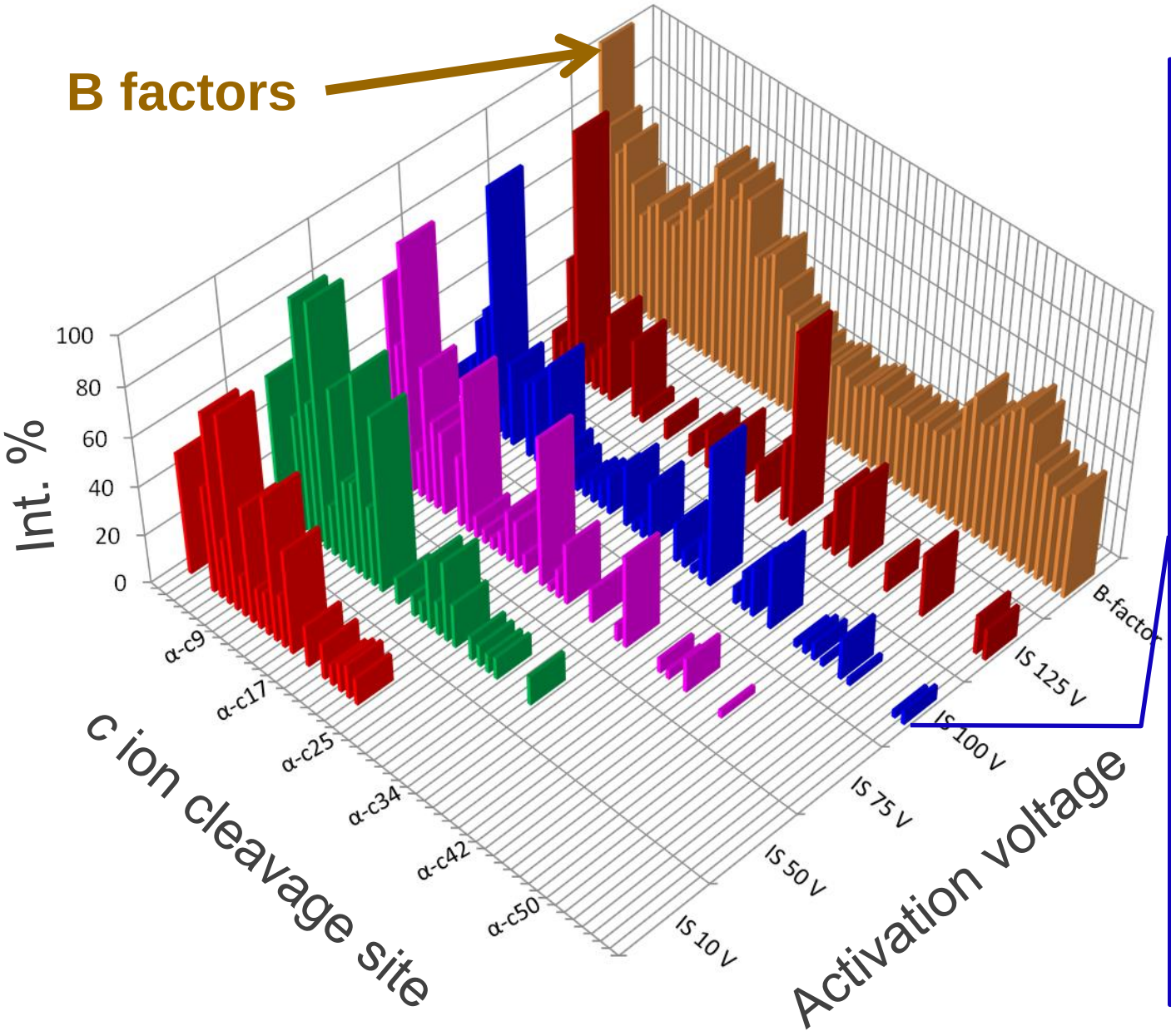



# ECD of Heterogeneous Protein Complexes

Structure of Human Hemoglobin Tetramer



# ECD cleavage sites on chain α

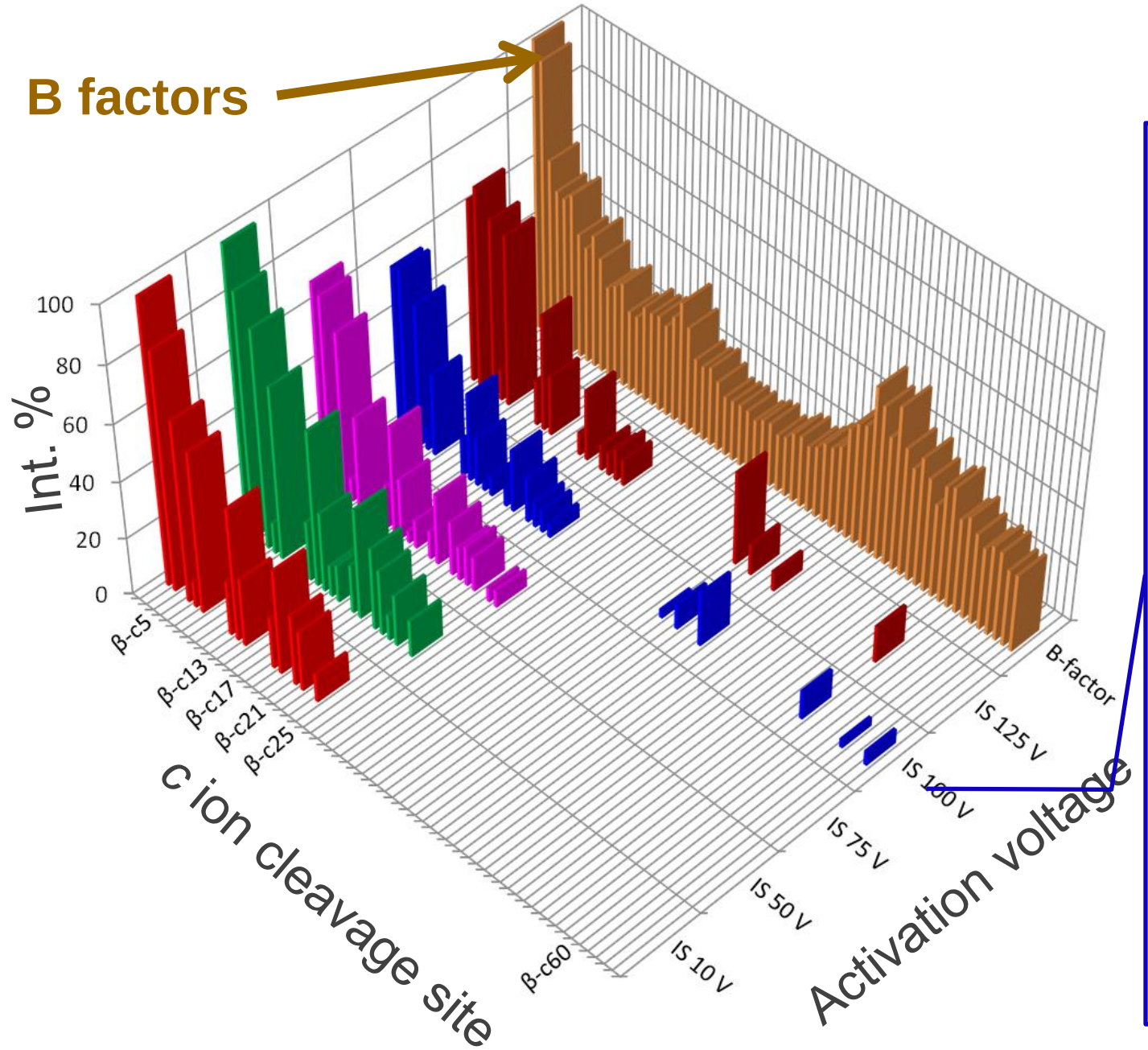



IS 100V&ECD

Chain α :

VLSPADKTNVK
AAWGKVGAHAG
EYGAEALERMF
LSFPTTKTYFP
HFDLSHGSAQV
KGHGKKVADAL
TNAVAHVDDMP
NALSALSDLHA
HKLRVDPVNFK
LLSHCLLVTLA
AHLPAEFTPAV
HASLDKFLASV
STVLTSKYR

# ECD cleavage sites on chain β



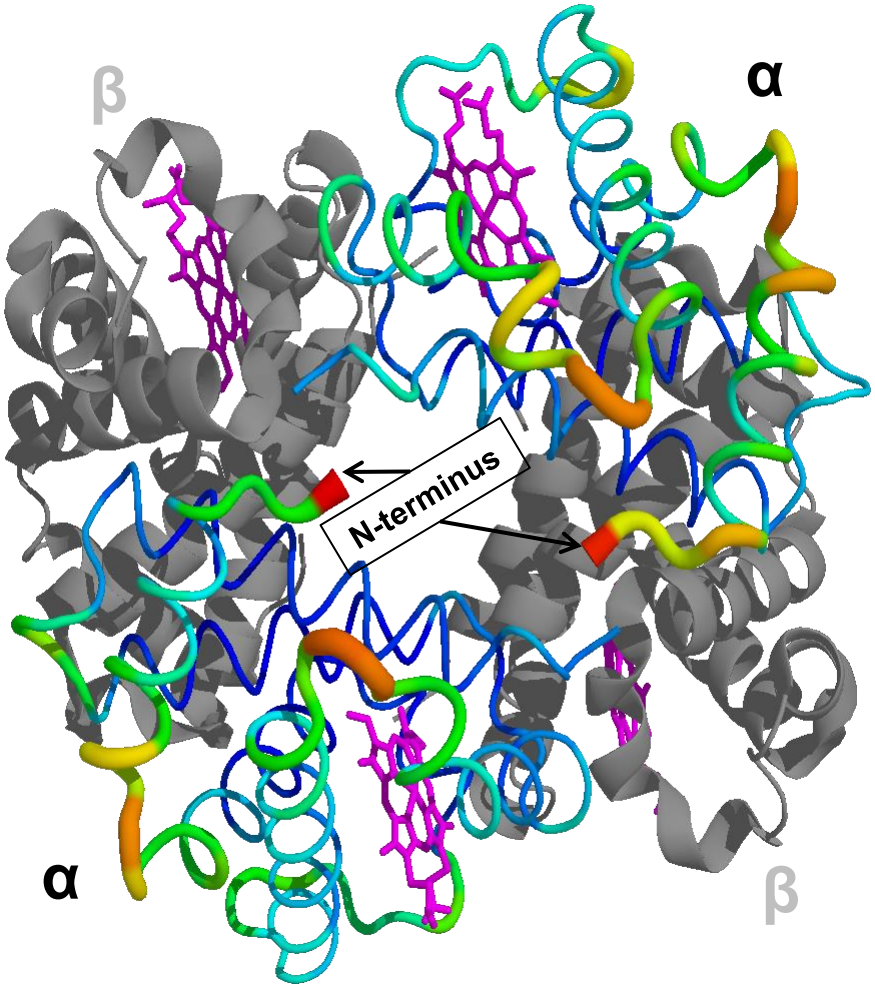

IS100V&ECD

Chain β :

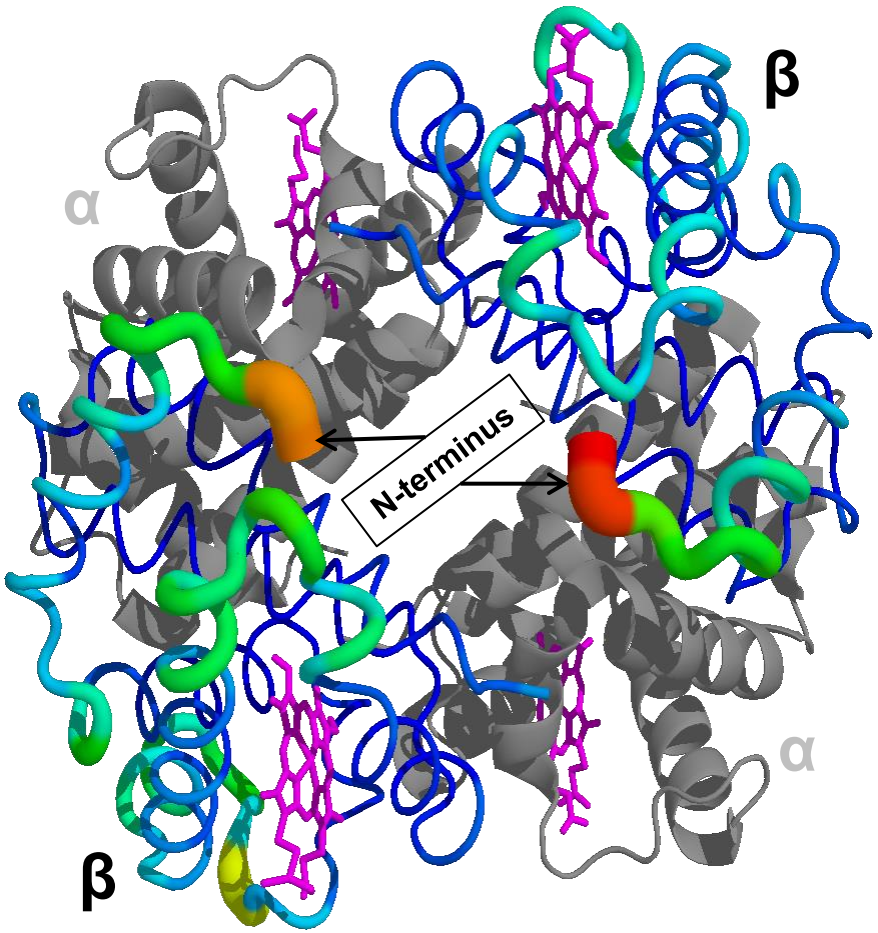
VHLTPEEKSA
VTALWGKVNV
DEVGGEALGR
LLVVYPWTQR
FFESFGDLST
PDAVMGNPKV
KAHGKKVLGA
FSDGLAHLDN
LKGTFATLSE
LHCDKLHVDP
ENFRLLGNVL
VCVLAHHFGK
EFTPPVQAAY
QKVVAGVANA
LAHKYH

# B-factor view of chain α and β

## How about B-factor?

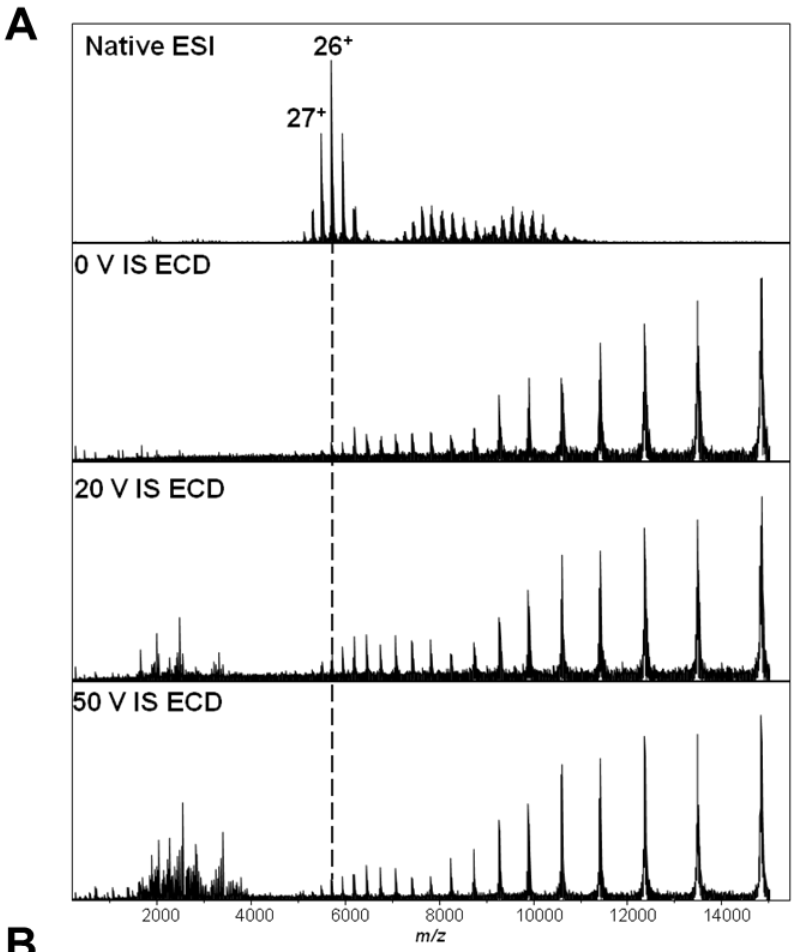


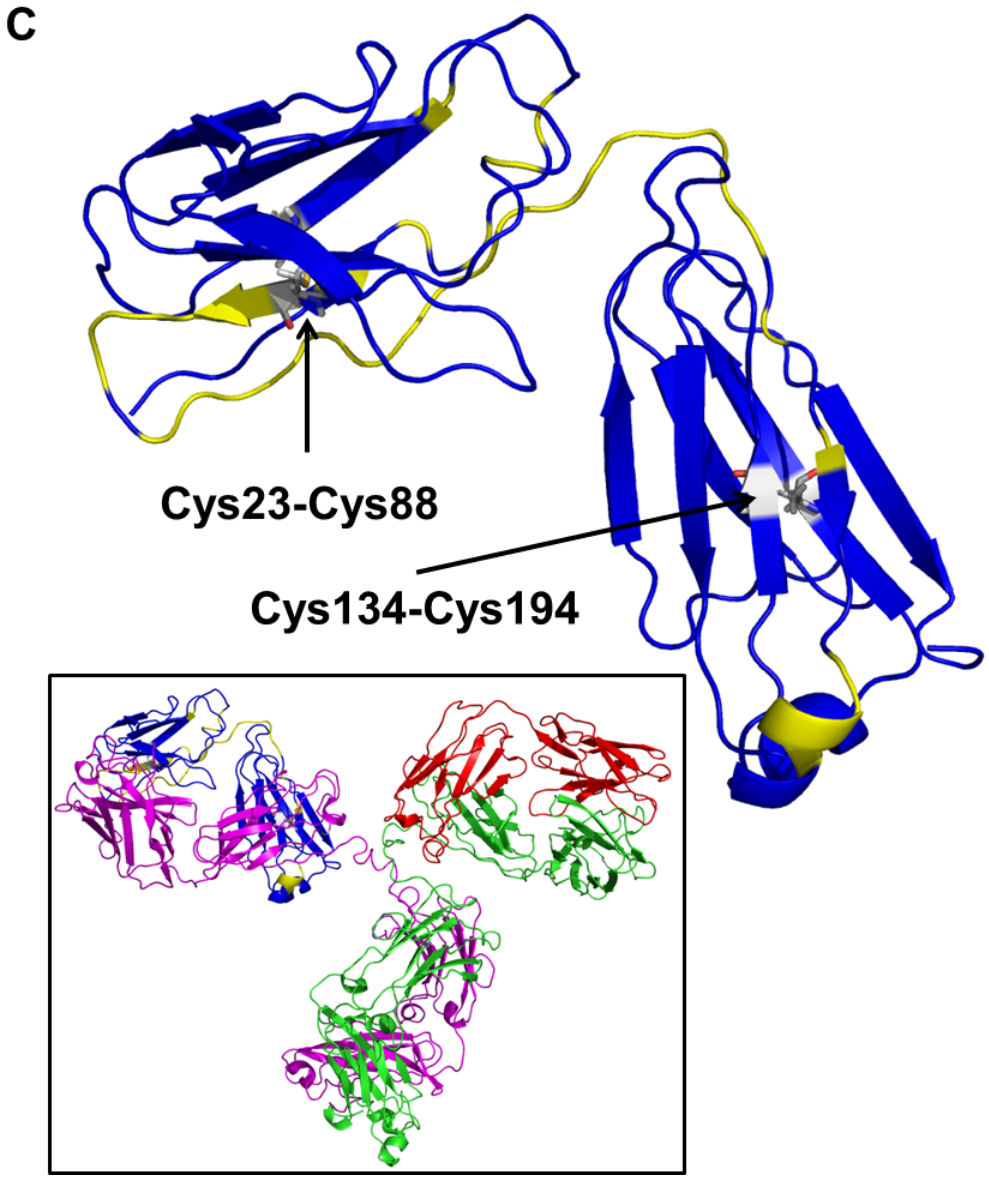

Chain α shown in B-factor scale



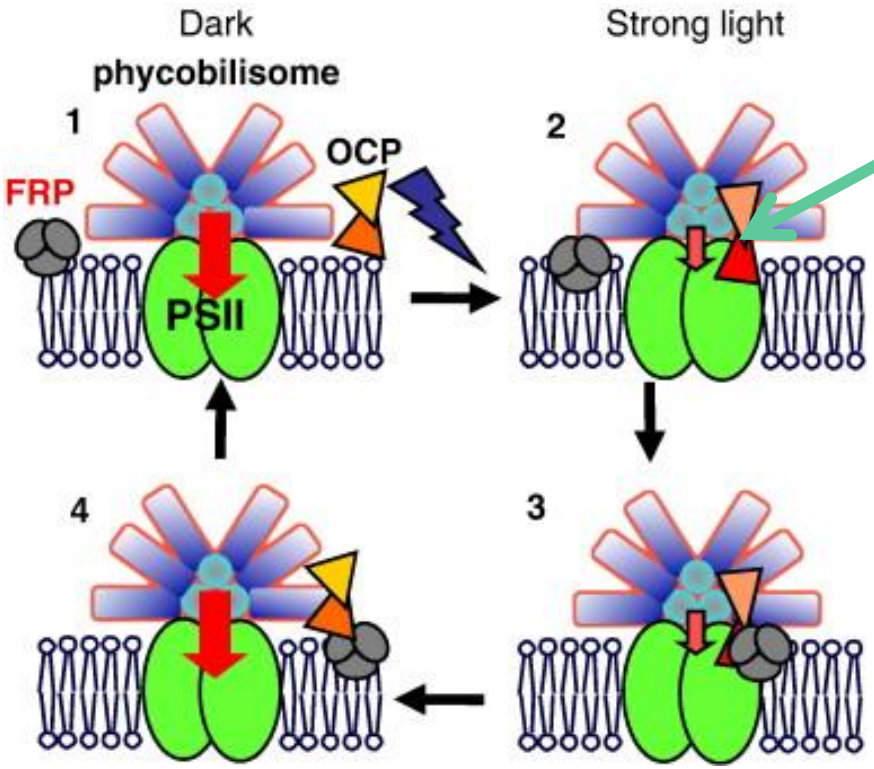

Chain β shown in B-factor scale

# ECD fragments antibody. . .



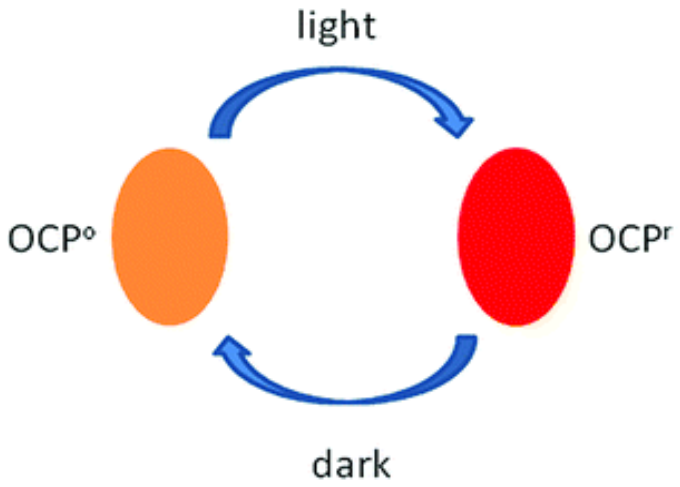



# Orange carotenoid protein (OCP)




**Photosynthesis system in Cyanobacteria**

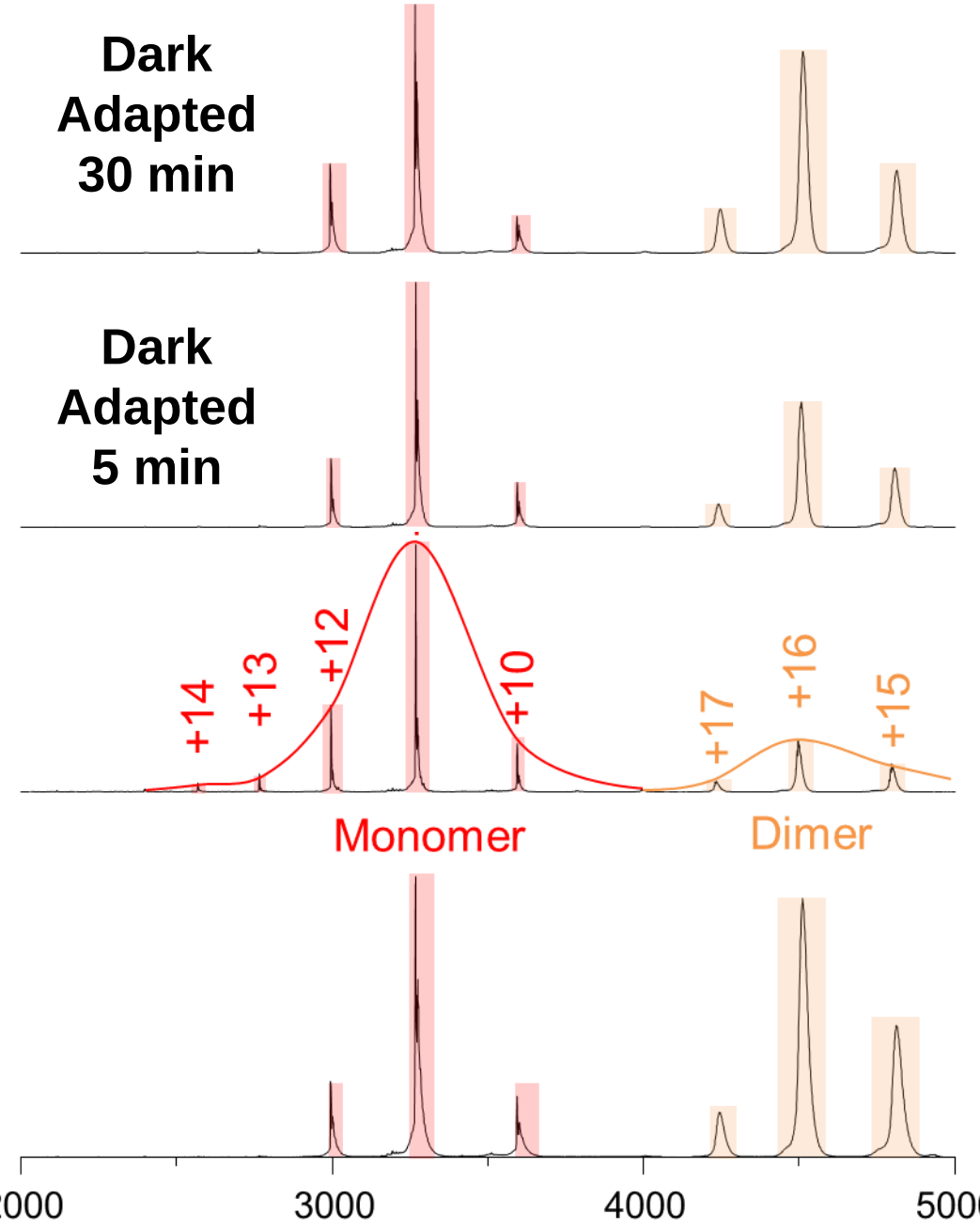
## Putative Dimer of OCP



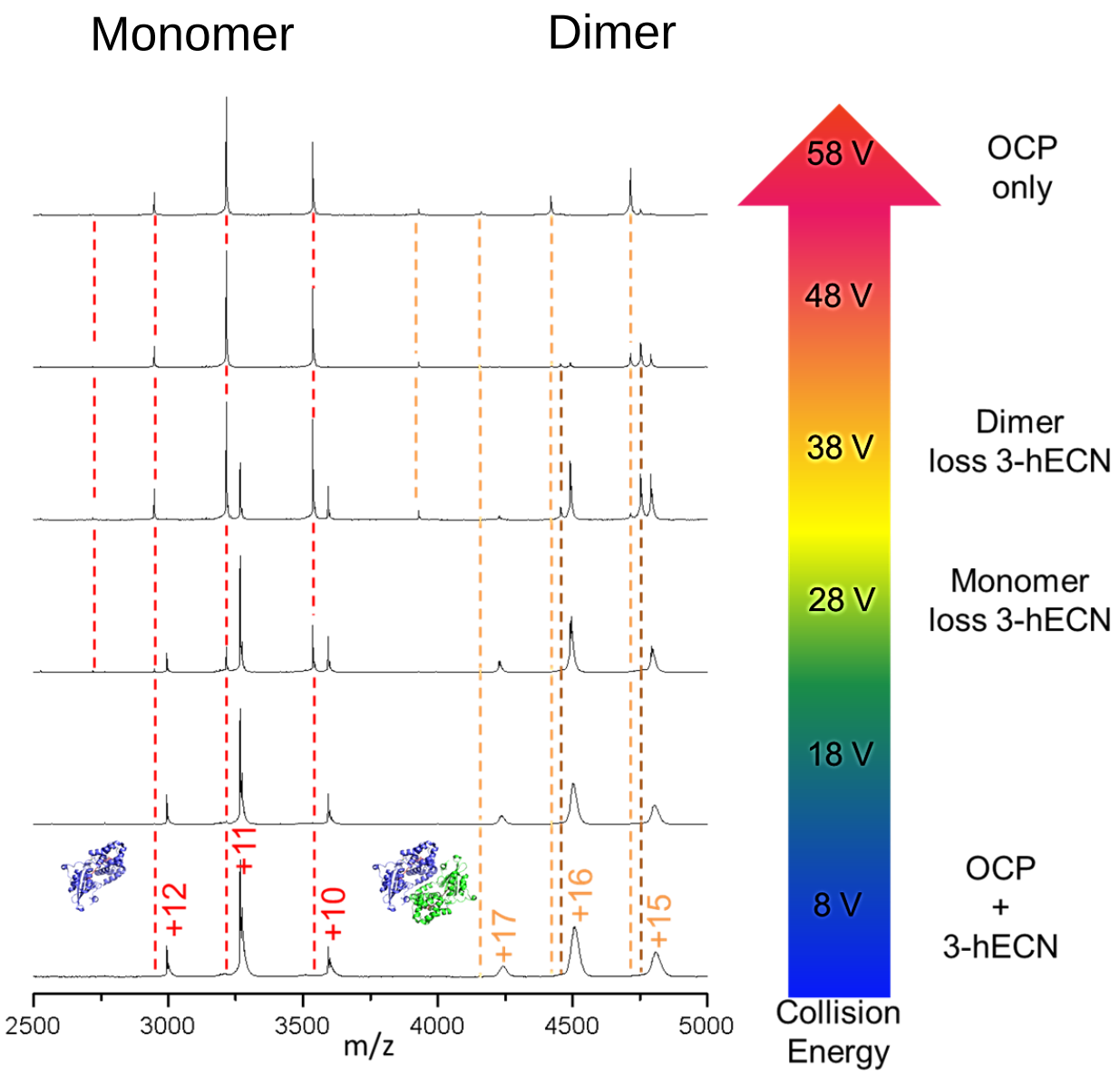

## What we know:

- In dark, **OCP** & **OCP$^{o}$** not attached to phycobilisome (PB).
- With blue-green light, **OCP$^{o}$** → **OCP$^{r}$. OCP$^{r}$** binds to APC trimer at PB core.
- PB protein and chromophore that interact with the **OCP$^{r}$** not identified.
- Trimeric FRP binds to the N-terminal assists **OCP$^{r}$** → **OCP$^{o}$**

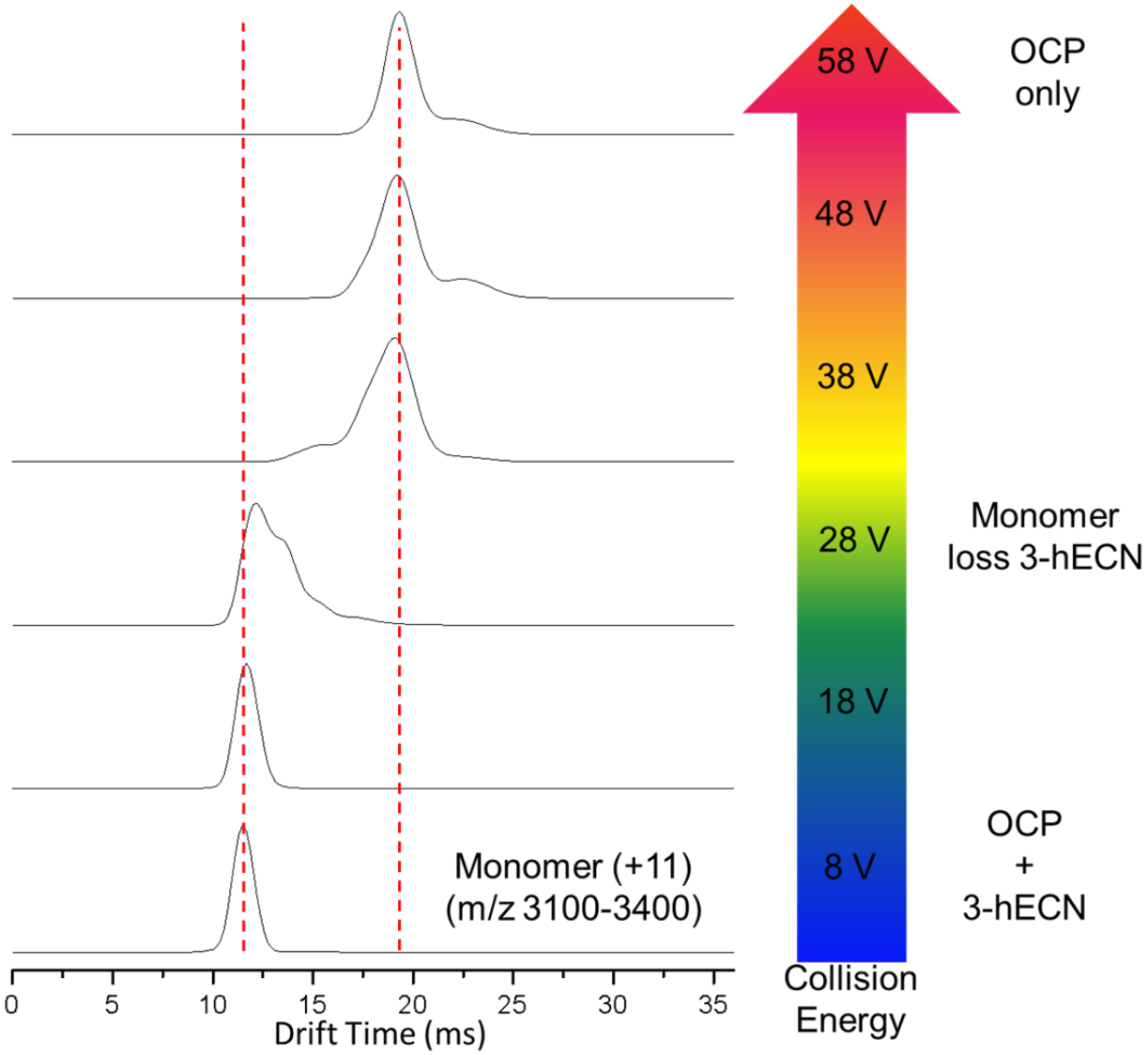
# Native MS of OCP



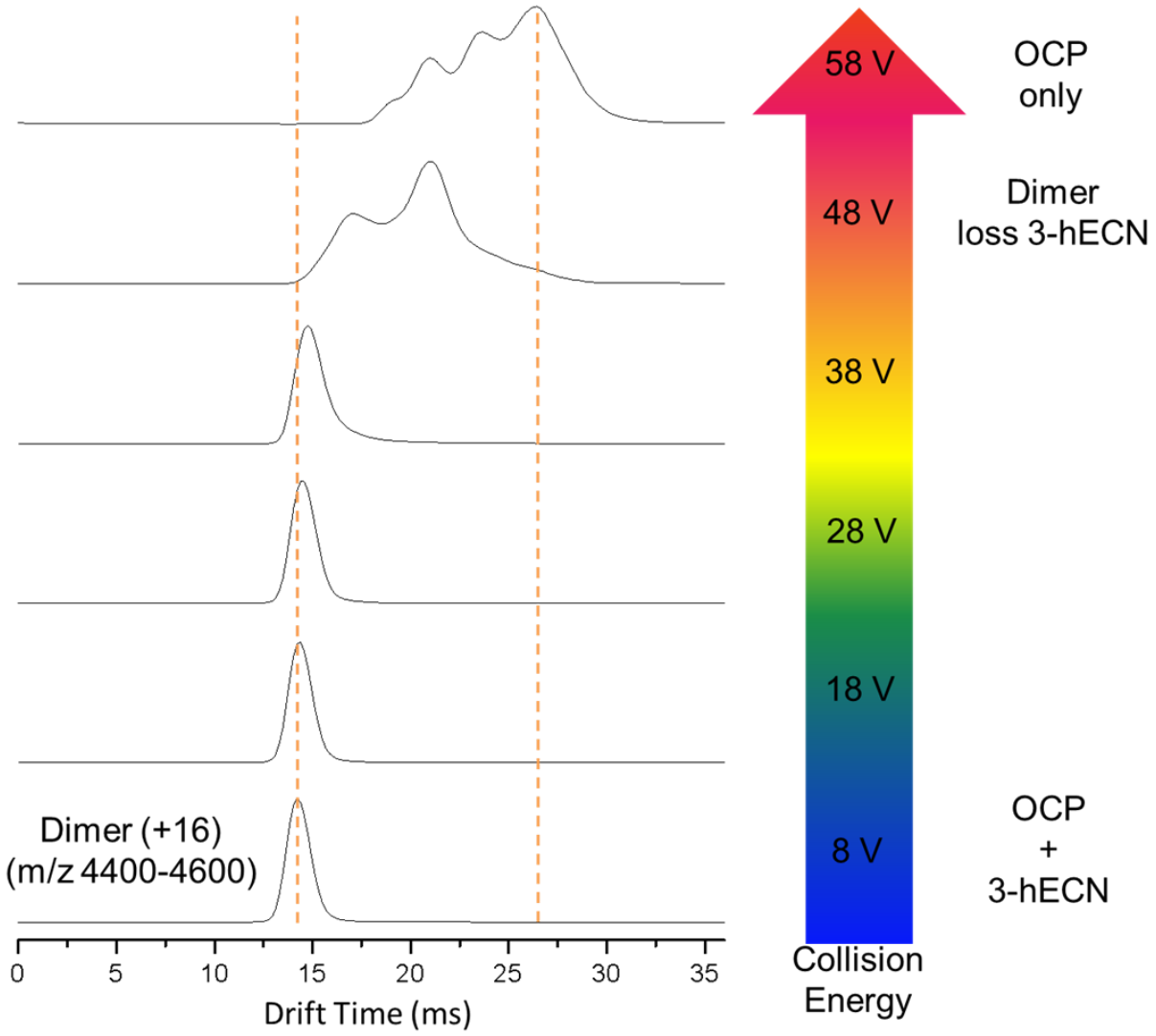
# Native MS of OCP



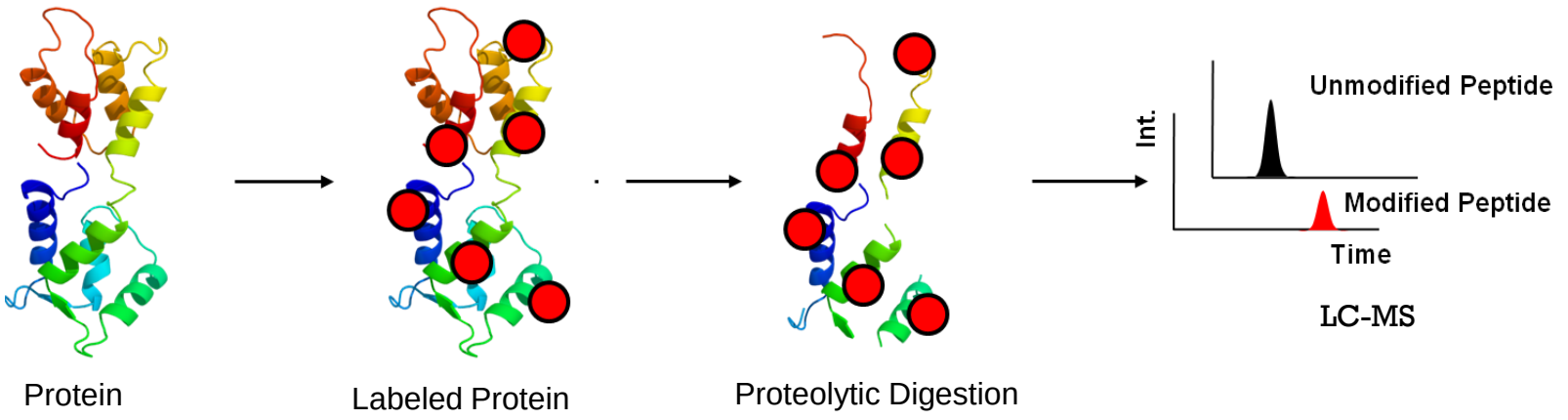
# Native MS and IM of OCP monomer

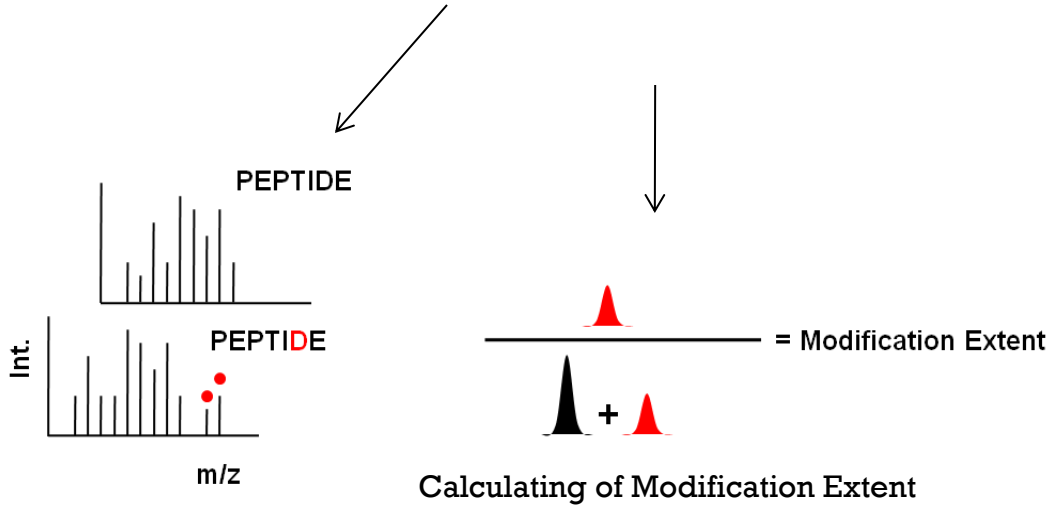


# Native MS and IM of OCP dimer

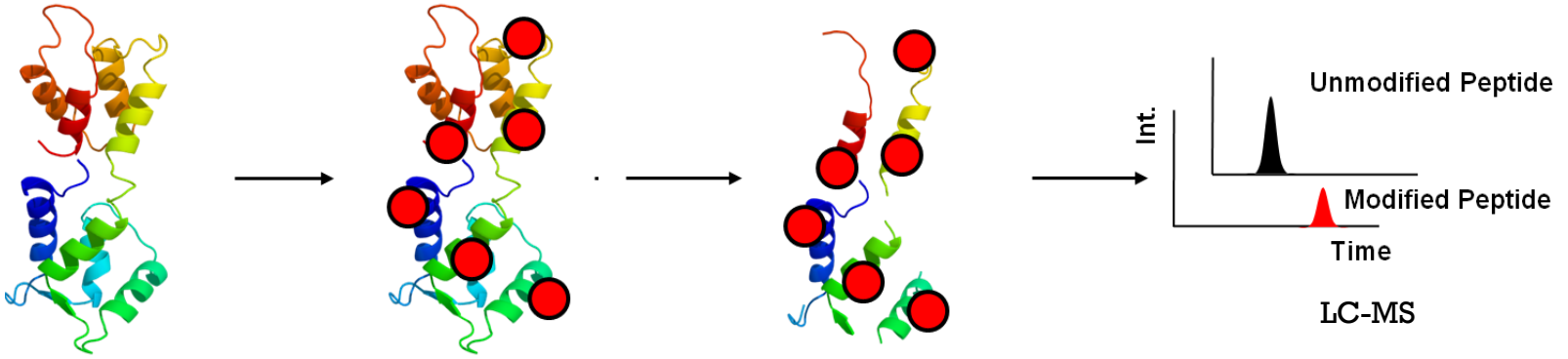


# MS-based protein footprinting



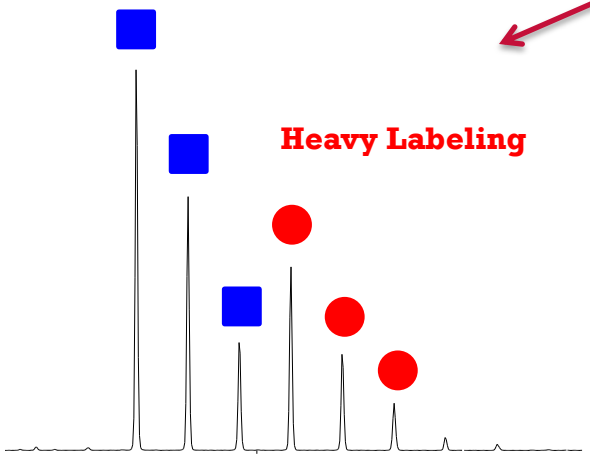



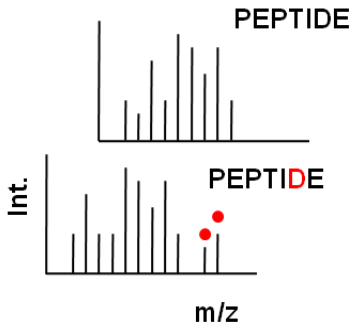
# MS-based protein footprinting



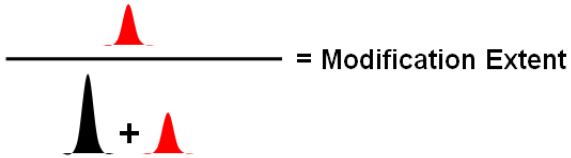

New approach

Light Labeling



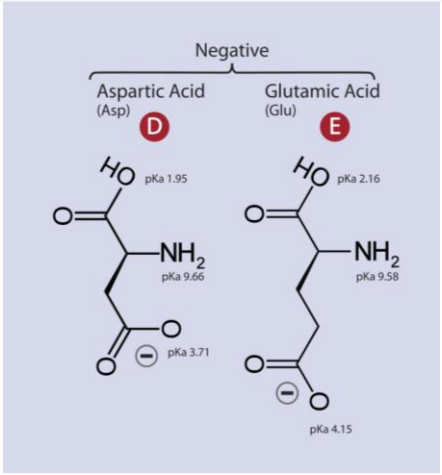

Locating Modified Site

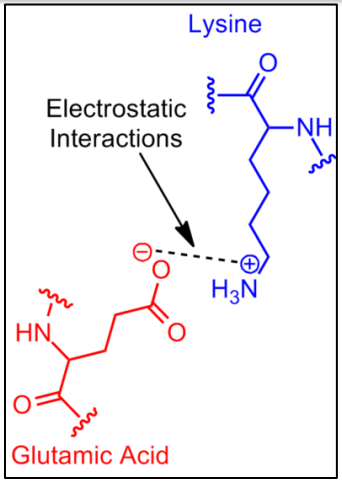


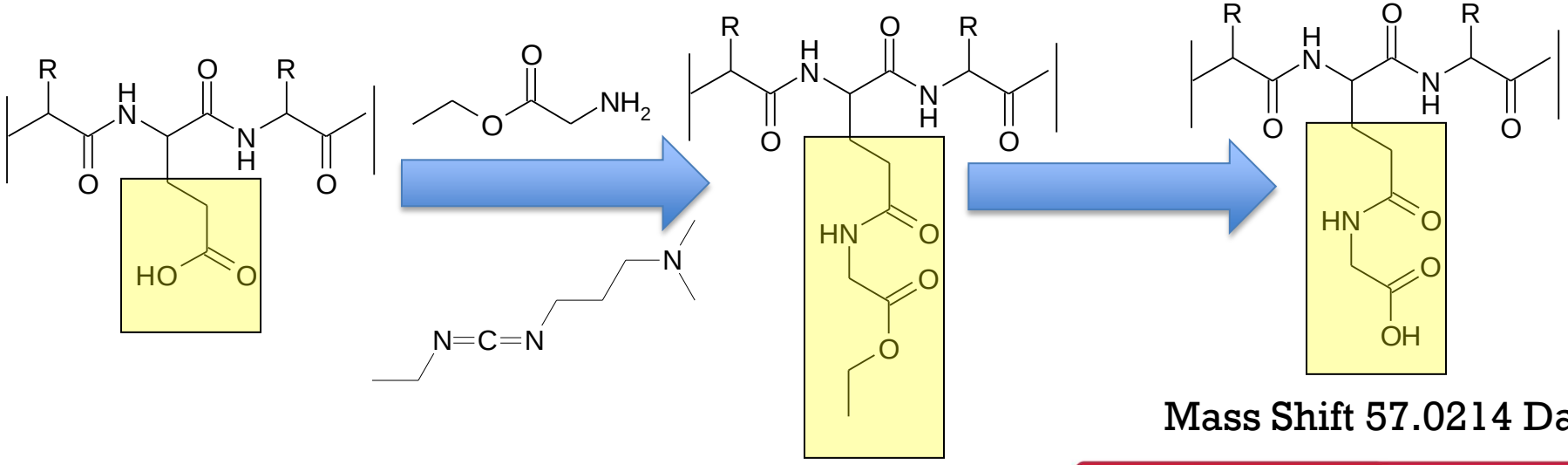

Calculating of Modification Extent

Washington University in St.Louis

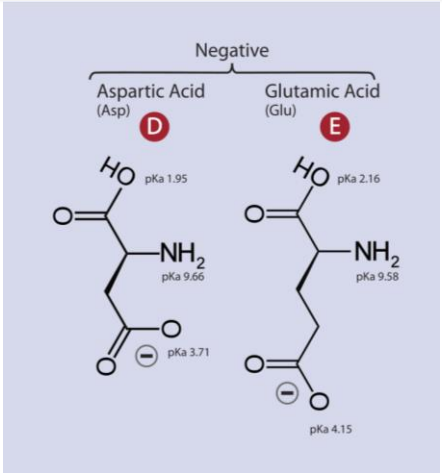
# Carboxyl-Group Labeling (GEE)

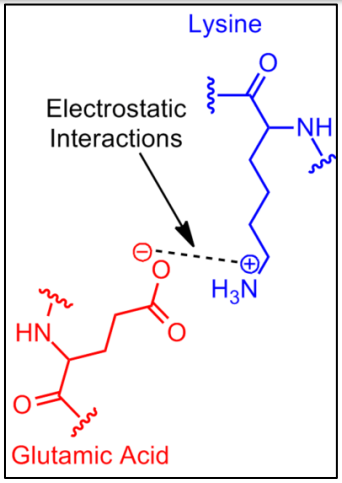


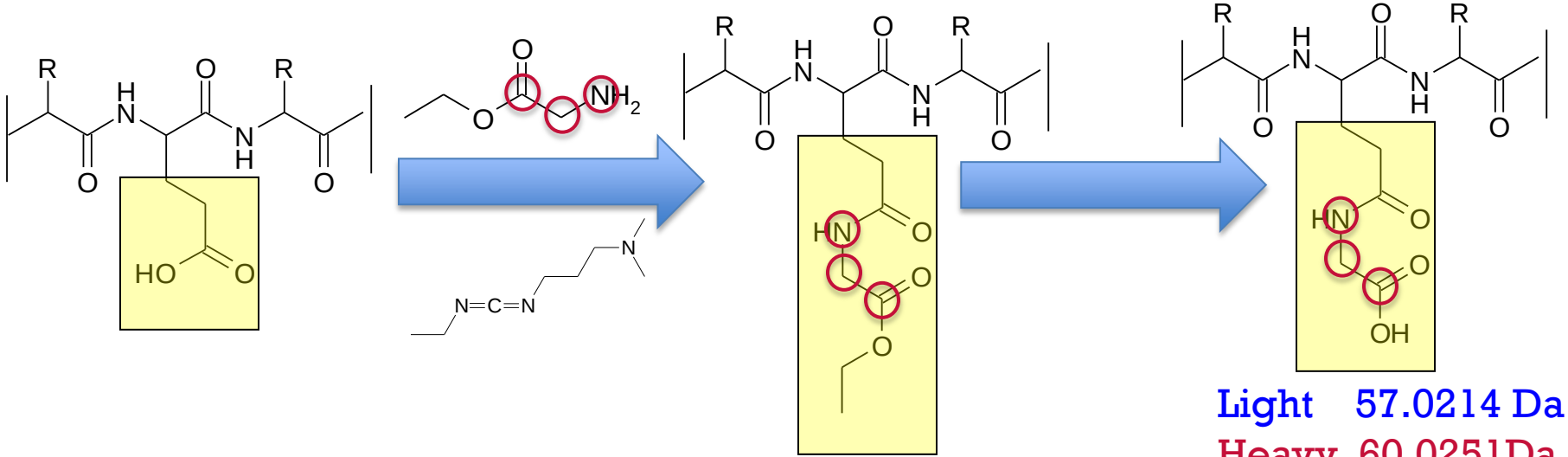



# Carboxyl-Group Labeling (i-GEE)



Light 85.0527 Da
Heavy 88.0564 Da

Light 57.0214 Da
Heavy 60.0251Da

# Experimental Design

Dark

Pulsed light

Continuous light

5 min

30 min

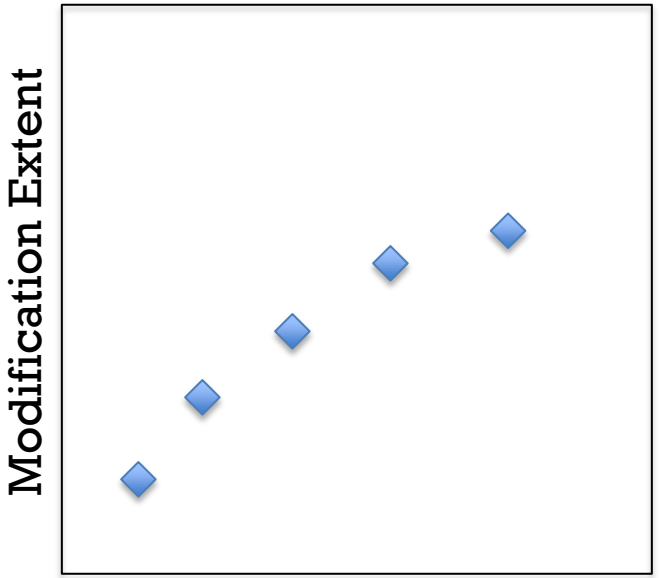
60 min

90 min

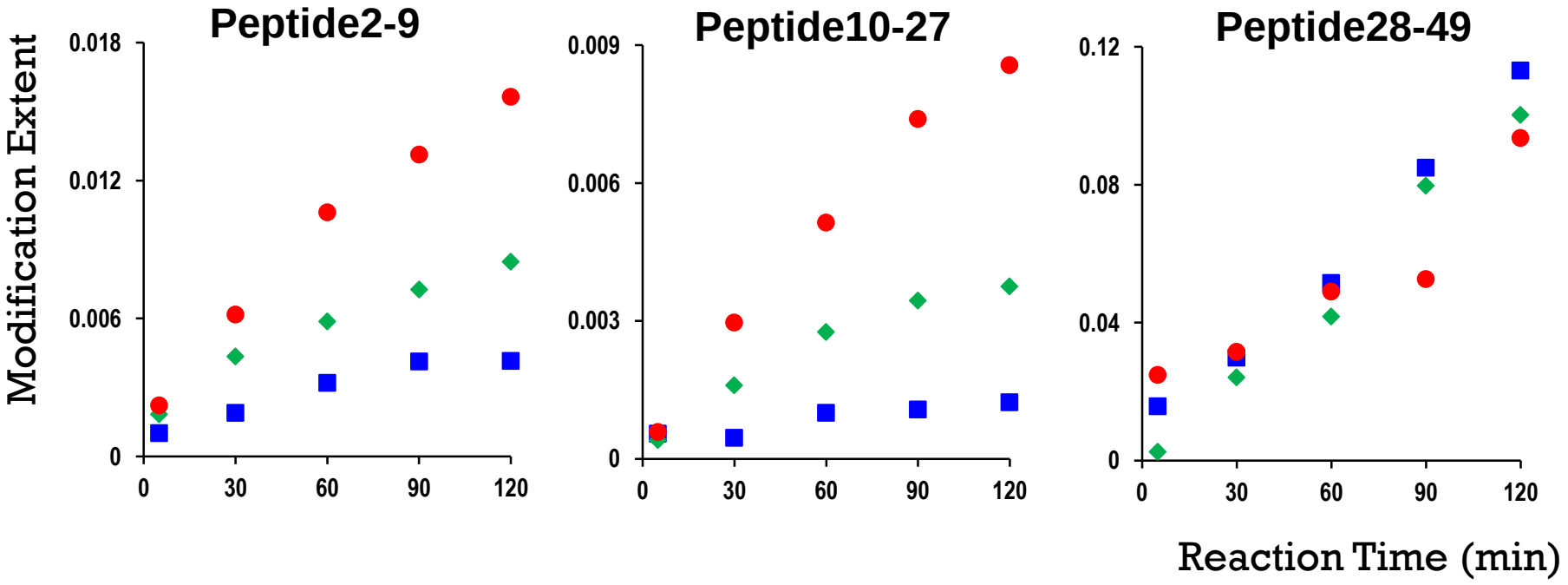
120 min

GEE labeling

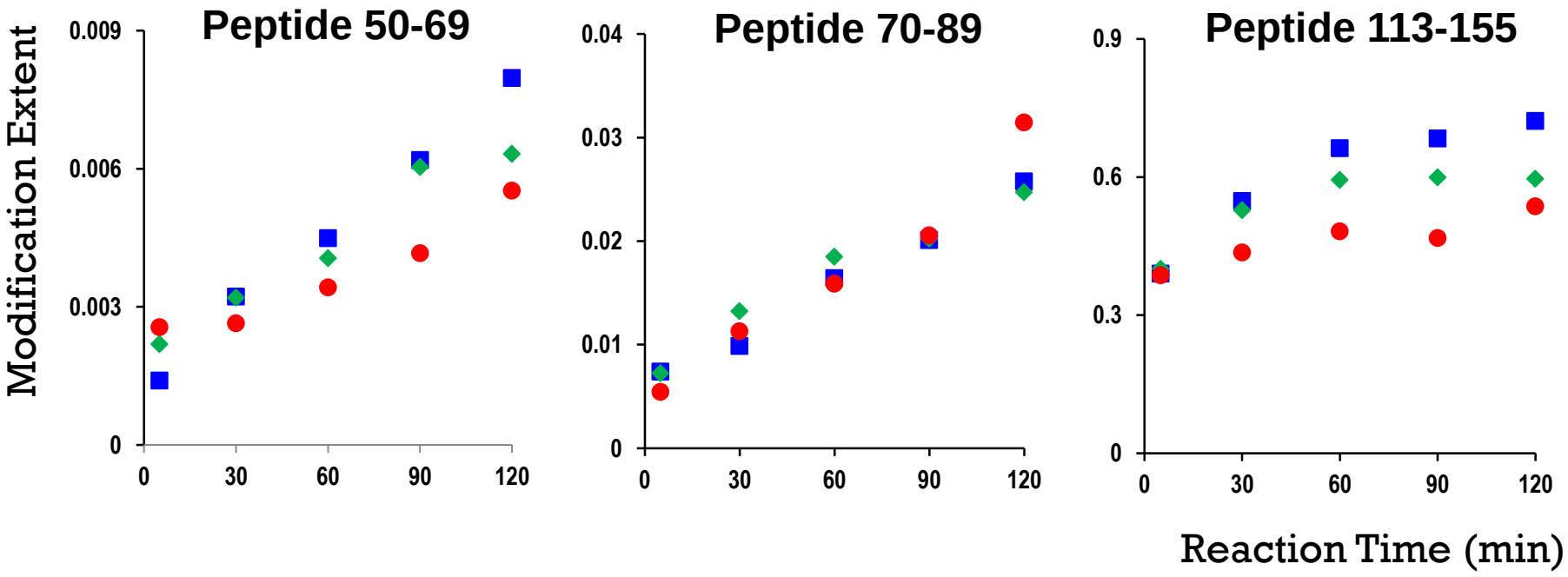
## GEE labeling curve



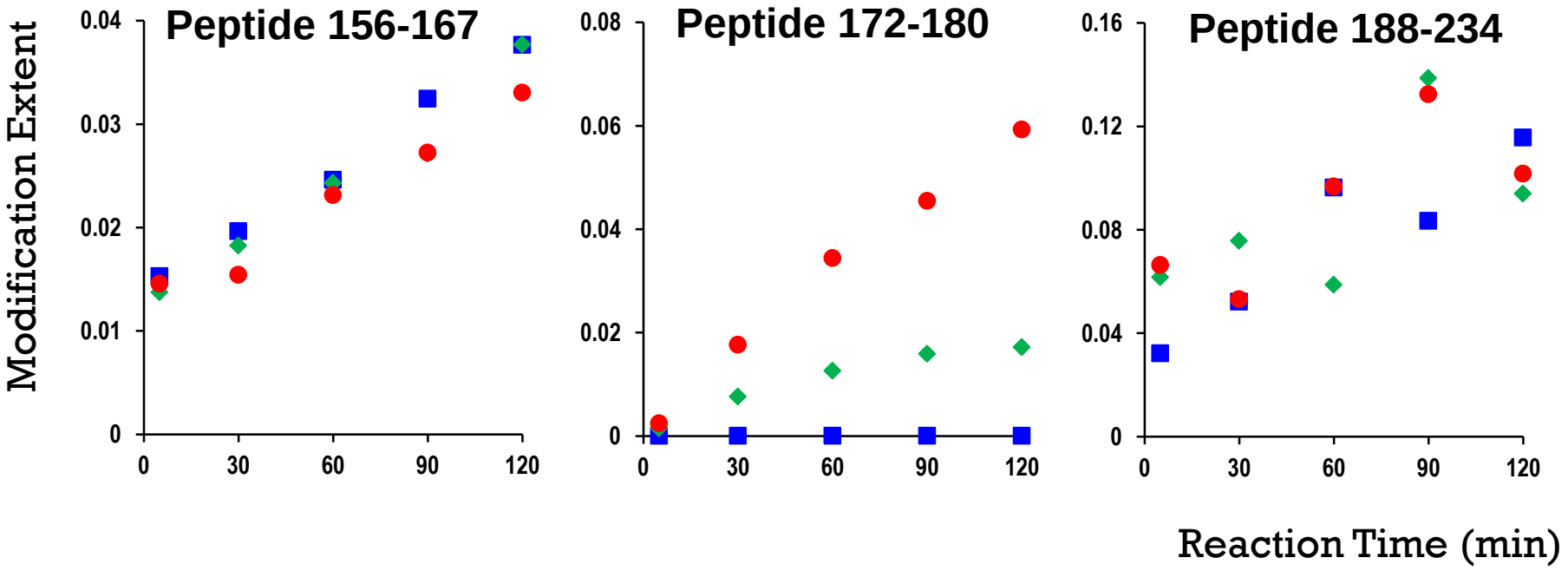
# Protein footprinting results (GEE)



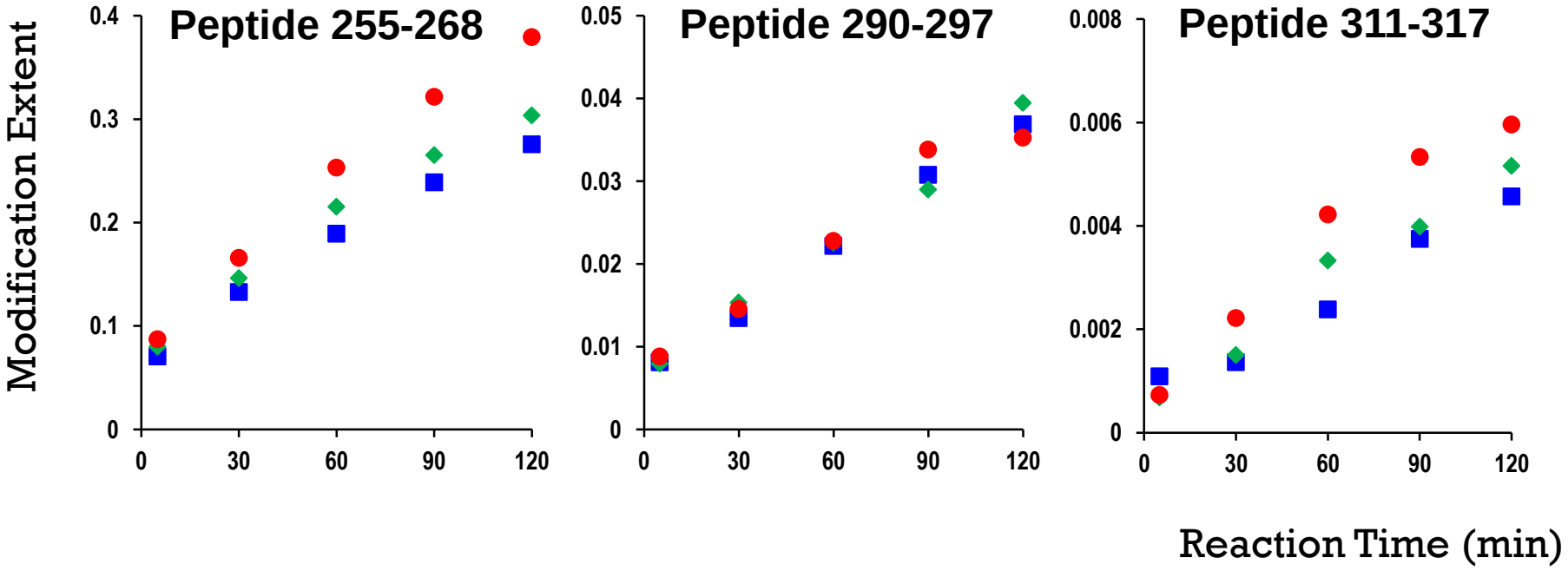
# Protein footprinting results (GEE)



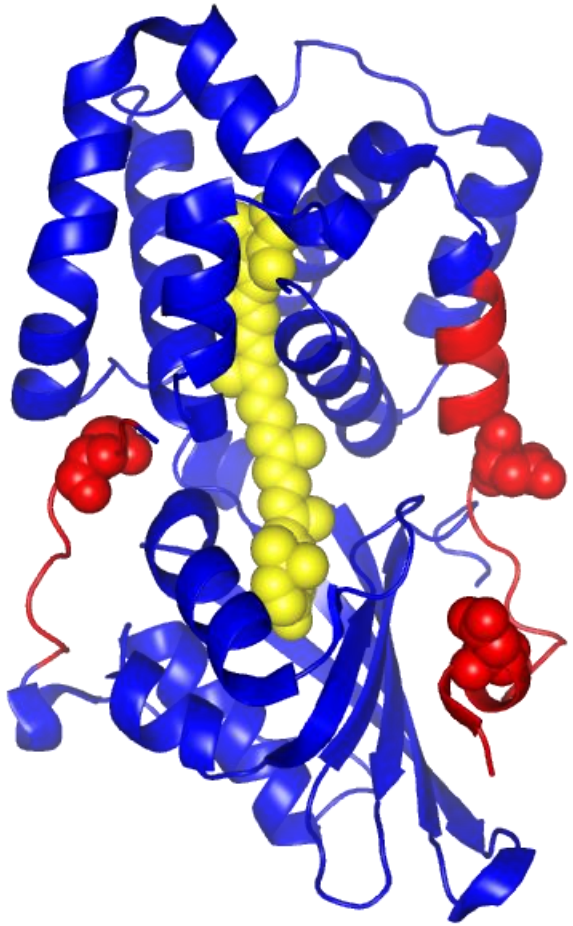
# Protein footprinting results (GEE)

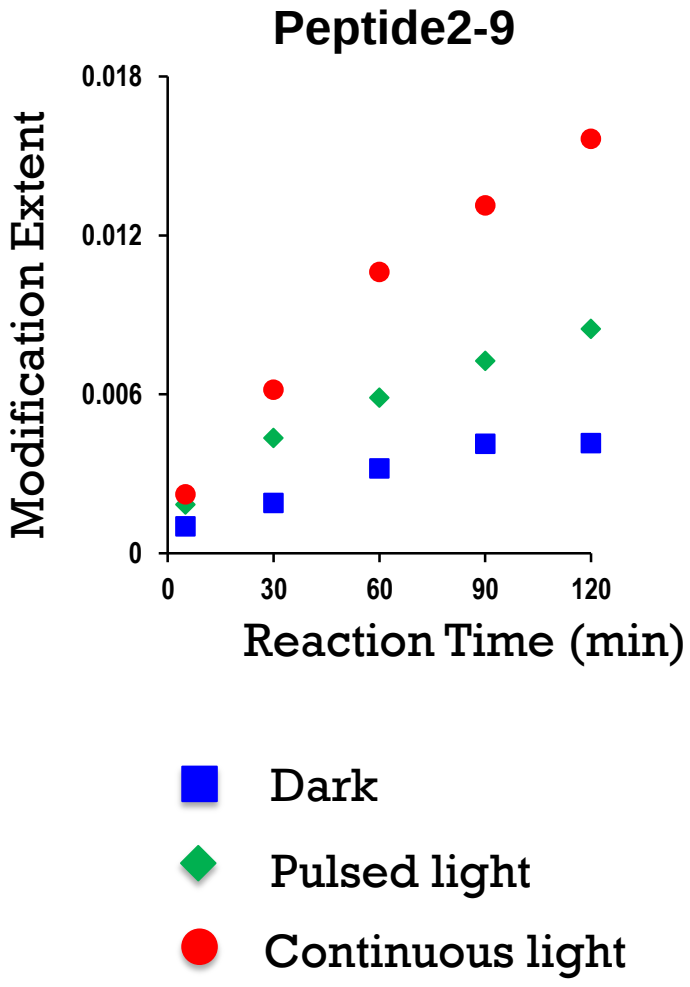


# Protein footprinting results (GEE)

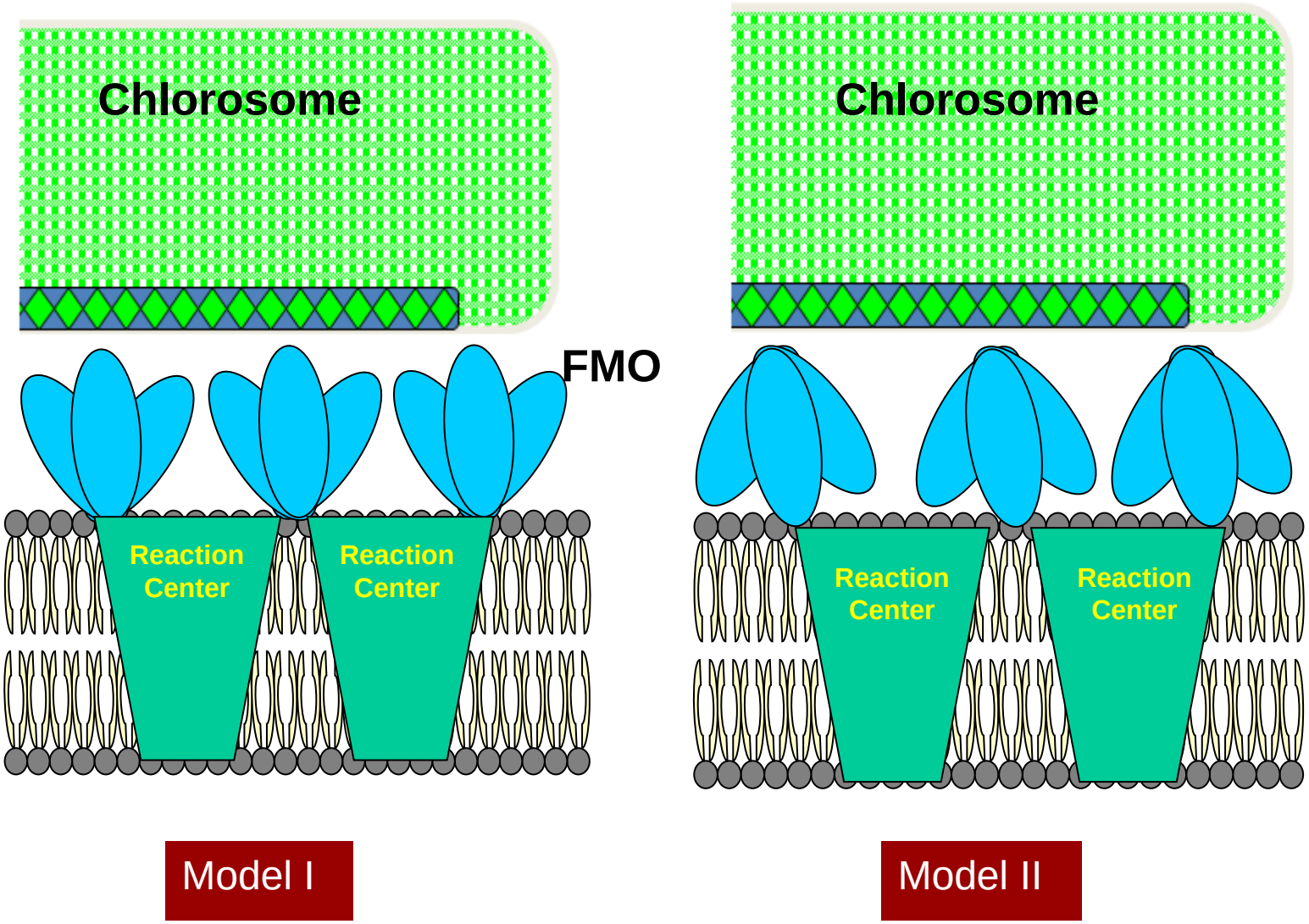


# Locating the region involved in conformational changes





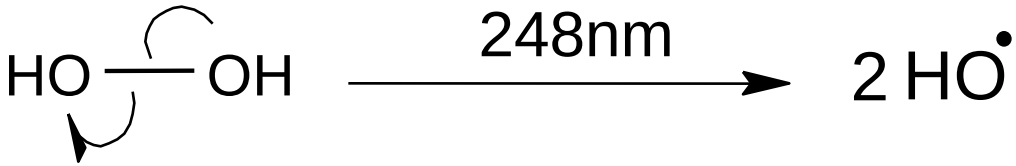
# First application of GEE Footprinting

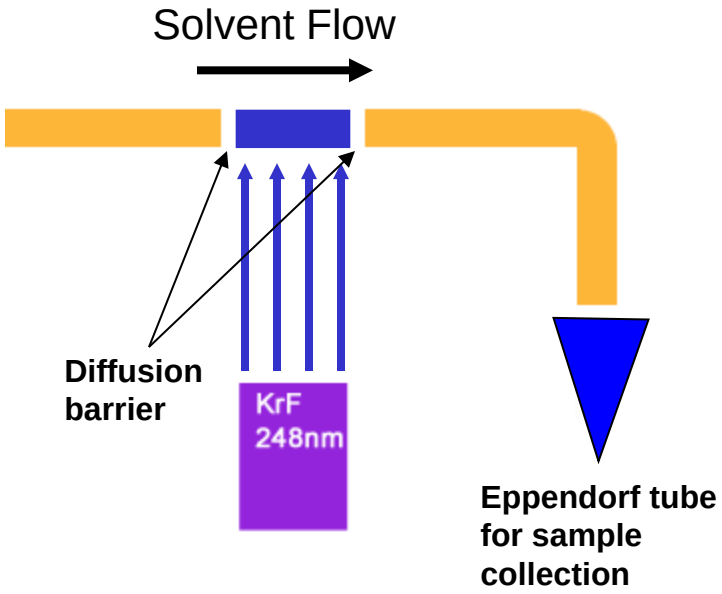



Gross, Blankenship, PNAS, 2009

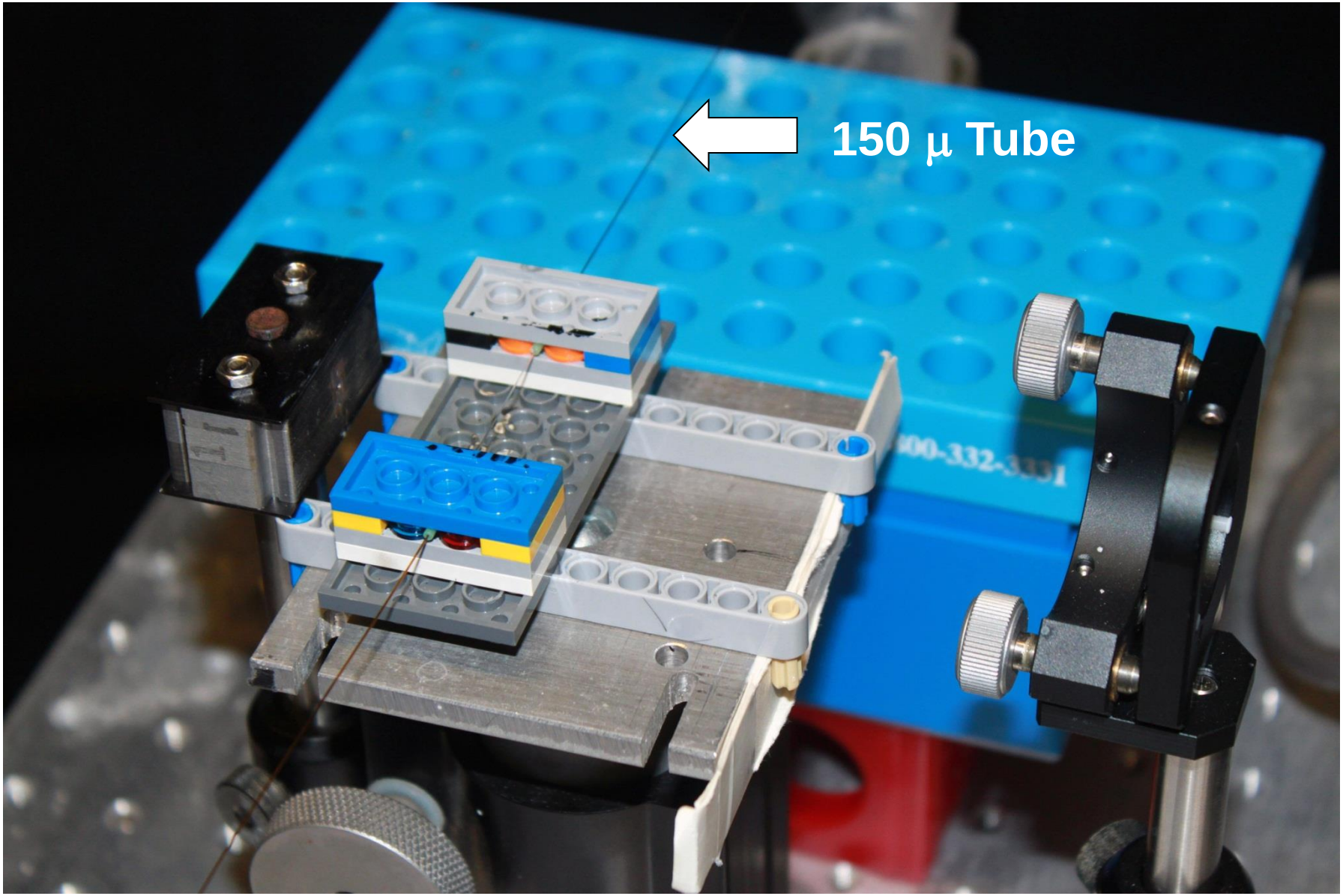
# Another approach to footprinting

## Hydroxyl and other Radical Footprinting: Fast Photochemical Oxidation of Proteins (FPOP)

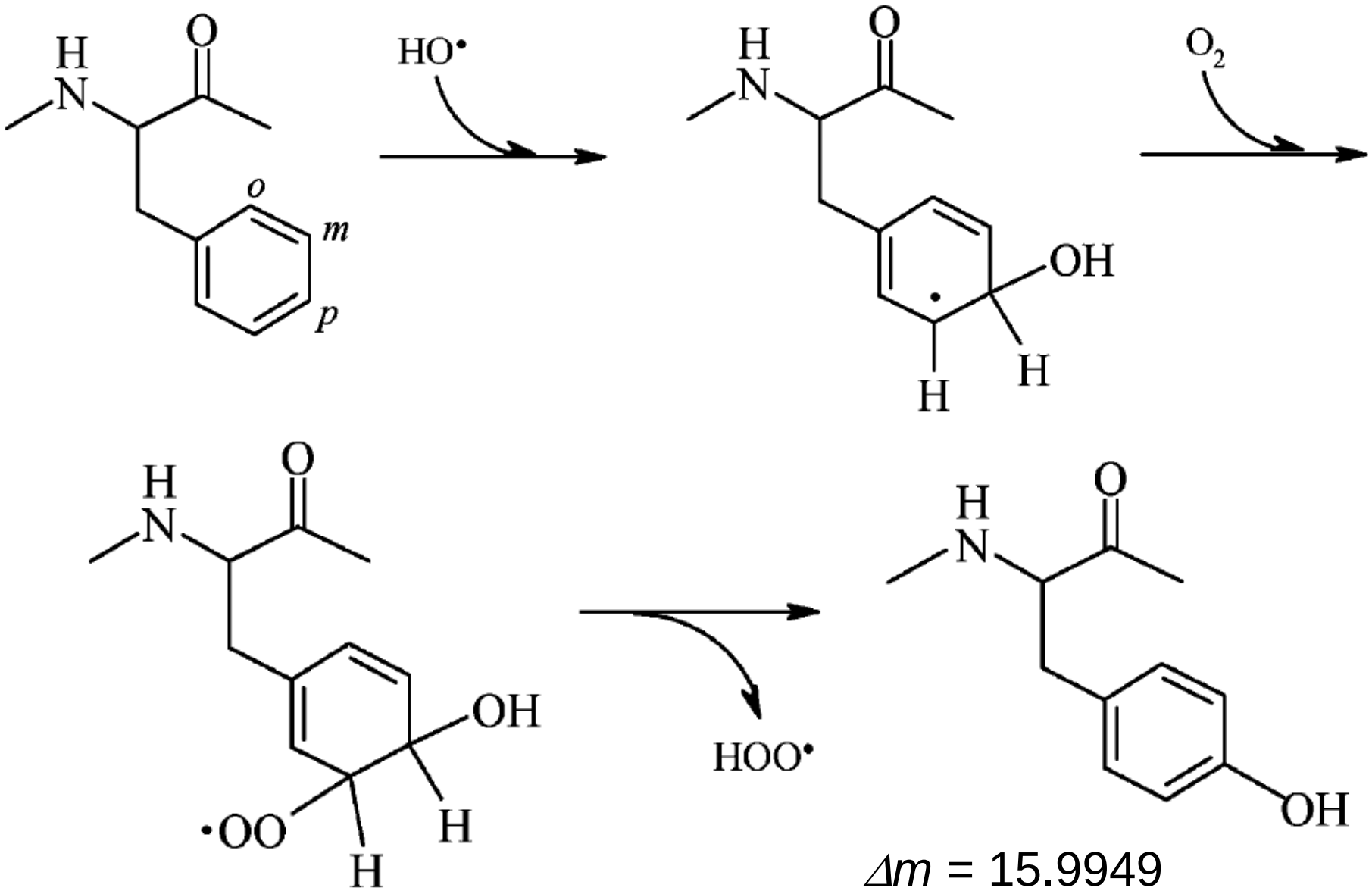


# FPOP set up:

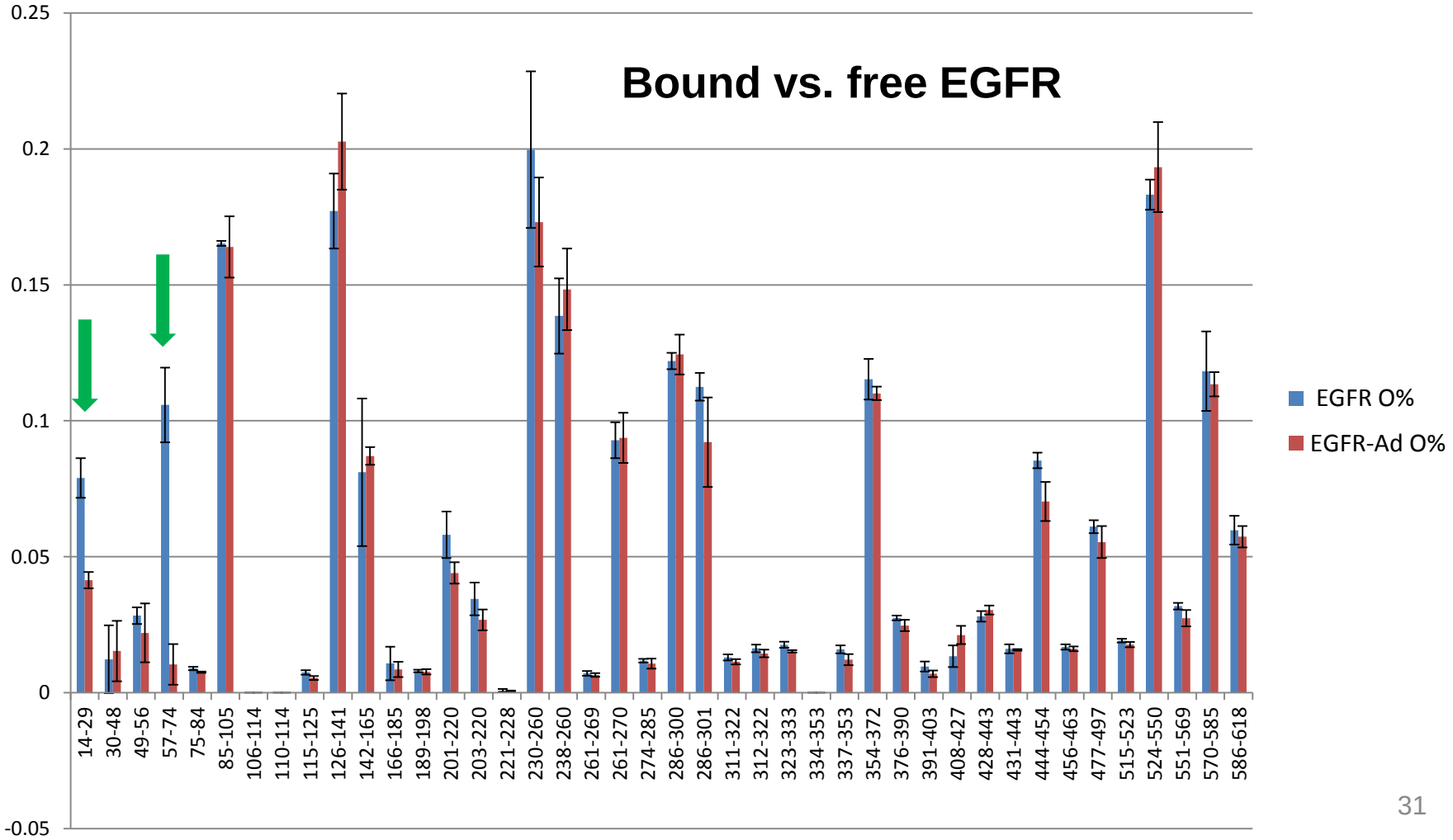


# Example Modification of a Protein

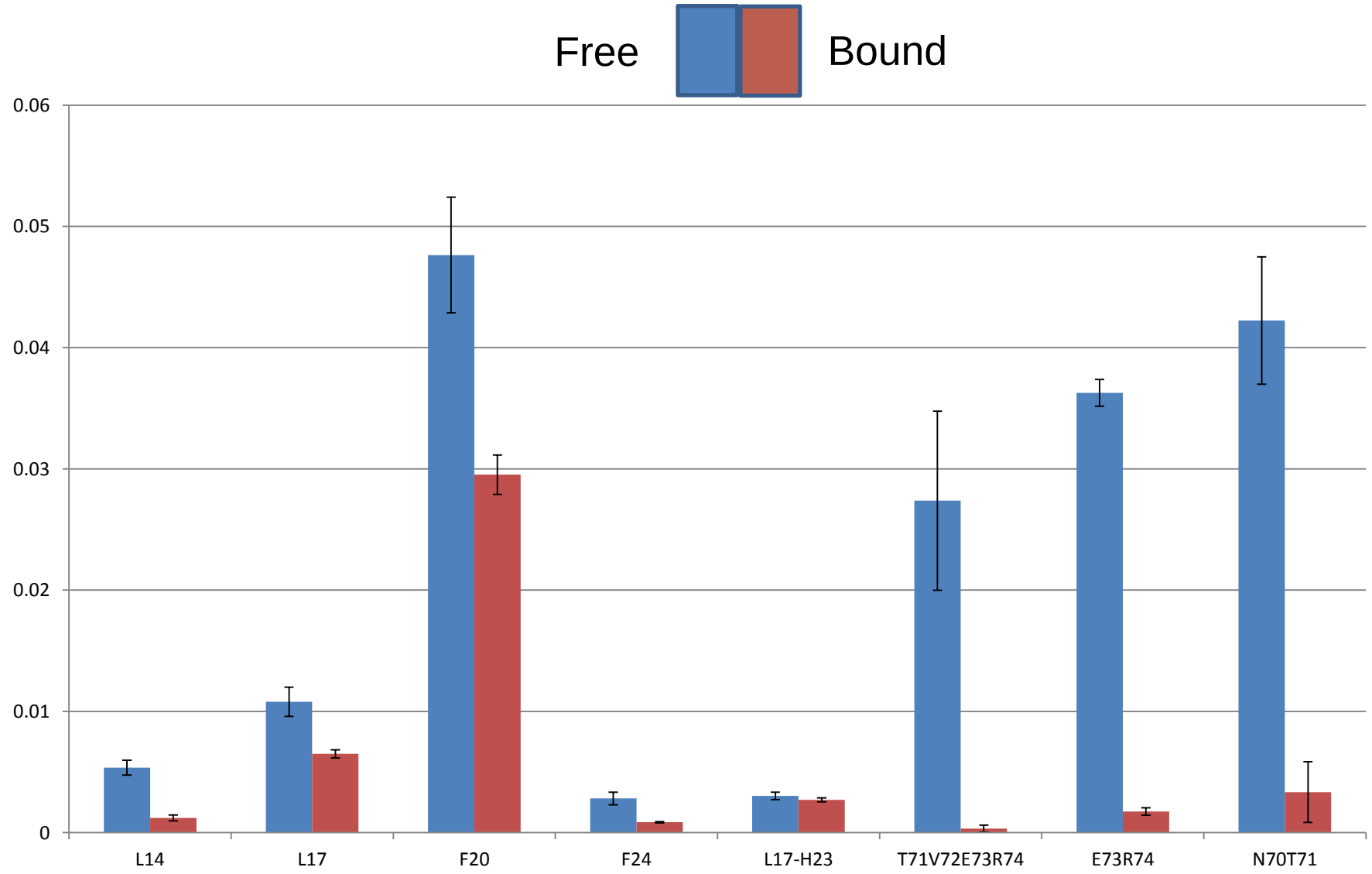



$\Delta m$ = 15.9949

# Epidermal Growth Factor-Therapeutic Protein Binding Interface: Peptide level



# Residue-level oxidative modification differences for free and bound EGFR



analytical chemistry

ARTICLE

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# Fast Photochemical Oxidation of Proteins for Epitope Mapping

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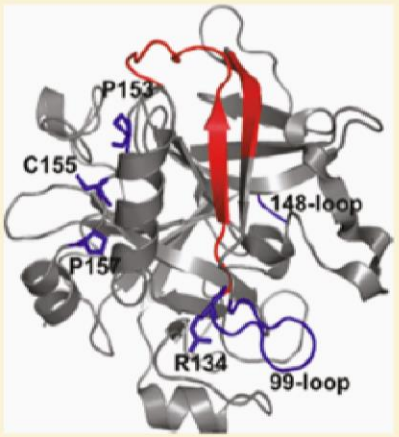
Supporting Information

**ABSTRACT:** The growing use of monoclonal antibodies as therapeutics underscores the importance of epitope mapping as an essential step in characterizing antibody−antigen complexes. The use of protein footprinting coupled with mass spectrometry, which is emerging as a tool in structural biology, offers opportunities to map antibody-binding regions of antigens. We report here the use of footprinting via fast photochemical oxidation of proteins (FPOP) with OH radicals to characterize the epitope of the serine protease thrombin. The data correlate well with previously published results that determined the epitope of thrombin. This study marks the first time oxidative labeling has been used for epitope mapping.




Anal. Chem., 2011, 7657

J. Am. Soc. Mass Spectrom. (2013)
DOI: 10.1007/s13361-013-0582-4

RESEARCH ARTICLE

# Complementary MS Methods Assist Conformational Characterization of Antibodies with Altered S–S Bonding Networks

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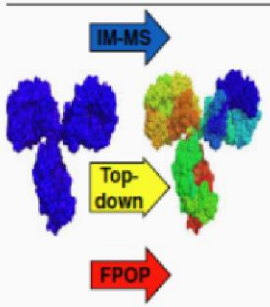
[2]Analytical Research and Development, Biotherapeutics Pharmaceutical Sciences, Pfizer, Inc., Chesterfield, MO 63017, USA




**Abstract.** As therapeutic monoclonal antibodies (mAbs) become a major focus in biotechnology and a source of the next-generation drugs, new analytical methods or combination methods are needed for monitoring changes in higher order structure and effects of post-translational modifications. The complexity of these molecules and their vulnerability to structural change provide a serious challenge. We describe here the use of complementary mass spectrometry methods that not only characterize mutant mAbs but also may provide a general framework for characterizing higher order structure of other protein therapeutics and biosimilars. To frame the challenge, we selected members of the IgG2 subclass that have distinct disulfide isomeric structures as a model to evaluate an overall approach that uses ion mobility, top-down MS sequencing, and protein footprinting in the form of fast photochemical oxidation of proteins (FPOP). These three methods are rapid, sensitive, respond to subtle changes in conformation of Cys→Ser mutants of an IgG2, each representing a single disulfide isoform, and may be used in series to probe higher order structure. The outcome suggests that this approach of using various methods in combination can assist the development and quality control of protein therapeutics.

**Key words:** Antibody characterization, Protein structure, Fast photochemical oxidation of proteins (FPOP), Top-down, Ion mobility, Antibody mutants, IgG2

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Review

# Mass spectrometry for the biophysical characterization of therapeutic monoclonal antibodies

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ABSTRACT

**Monoclonal antibodies (mAbs) are powerful therapeutics, and their characterization has drawn considerable attention and urgency. Unlike small-molecule drugs (150–600 Da) that have rigid structures, mAbs (~150 kDa) are engineered proteins that undergo complicated folding and can exist in a number of low-energy structures, posing a challenge for traditional methods in structural biology. Mass spectrometry (MS)-based biophysical characterization approaches can provide structural information, bringing high sensitivity, fast turnaround, and small sample consumption. This review outlines various MS-based strategies for protein biophysical characterization and then reviews how these strategies provide structural information of mAbs at the protein level (intact or top-down approaches), peptide, and residue level (bottom-up approaches), affording information on higher order structure, aggregation, and the nature of antibody complexes.**

# Summary: Native Spray, ECD, IM, Footprinting

- ECD fragments protein assemblies (and antibodies) in flexible regions rather than dissociating to constituent proteins.
- Native MSProvides count of number of pigment molecules in antenna and PS reaction-center complexes.
- Native MS admits protein assemblies to gas phase. OCP accommodates excess light by dimer dissociation.
- Footprinting determines interface and mechanism for monomer-dimer equilibrium in OCP
- Footprinting maps epitopes in antibody and antibody similar interactions with antigens