Structural Proteomics – From protein stable covalent labeling to chemical cross-linking

Petr Novák

2nd Advanced User School
Institute of Microbiology, Czech Republic, September 28th, 2021

Six Blind Men and the Protein of Unknown Structure

“It was six men of Indostan
To learning much inclined,
Who went to see the Elephant
(Though all of them were blind)
That each by observation
Might satisfy his mind...”
John Godfrey Saxe
(1816-1887)

Structural Mass Spectrometry…

Protein covalent labeling

Chemical cross-linking

H/D exchange

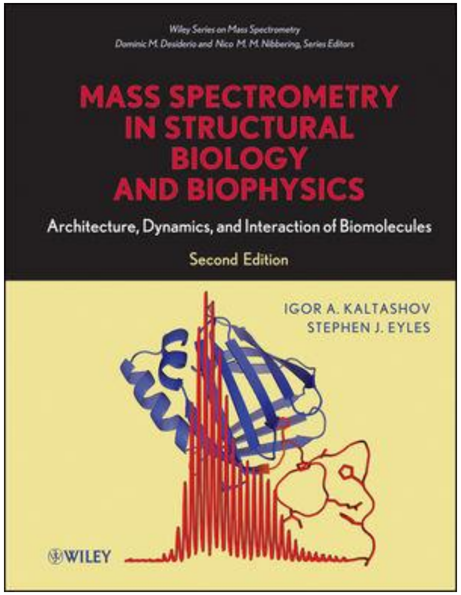
Disulfide bonds mapping

Native mass spectrometry and Ion mobility

Fast photochemical oxidation of proteins

ETD/ECD fragmentation

Limited proteolysis



Wiley Series on Mass Spectrometry
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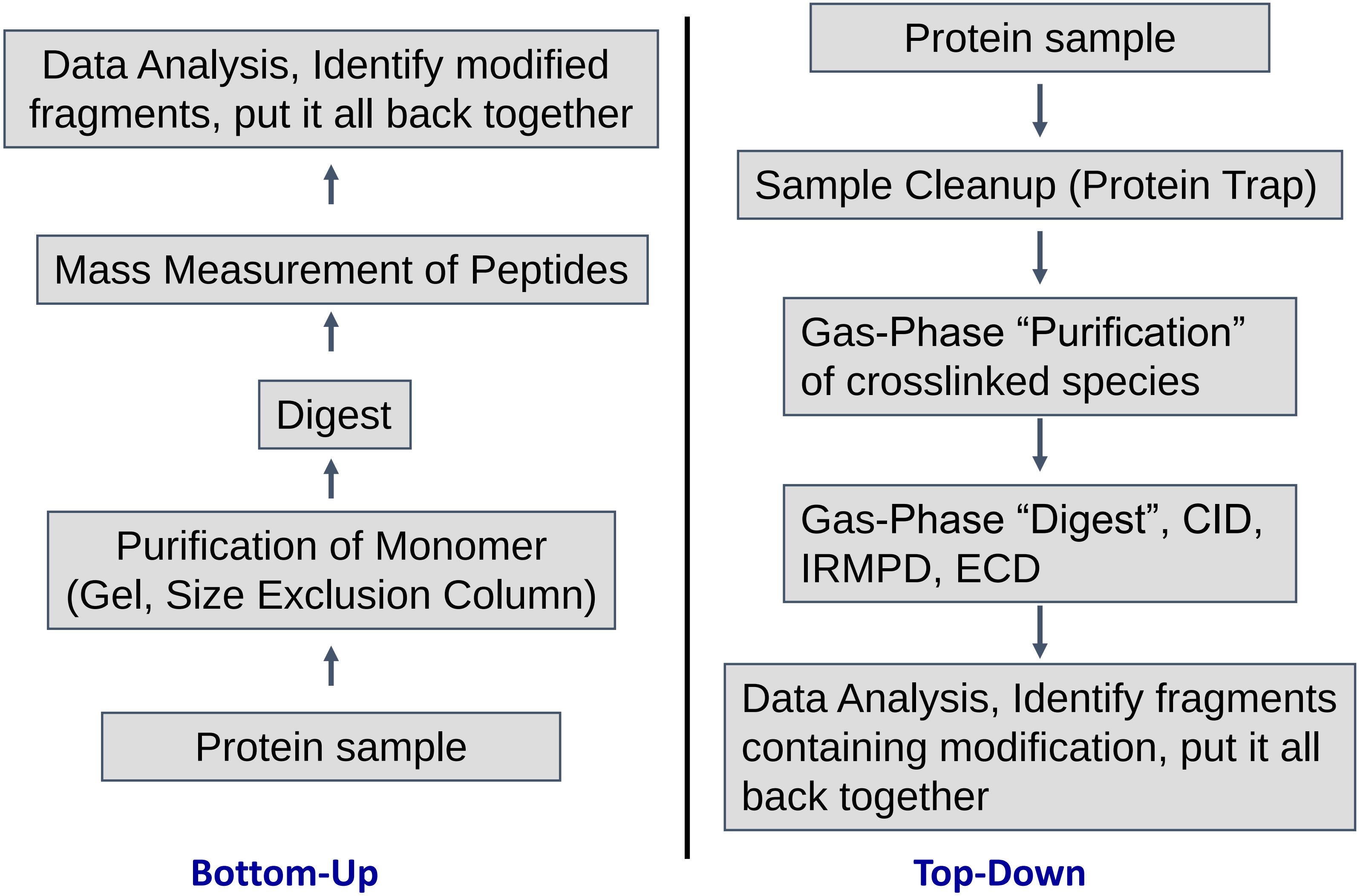
Special Issue on Mass Spectrometry in Structural Biology (2015) Protein Science *24*, 1173-1332

Mass Spectrometry: Goal in Protein Structure Characterization



Data Analysis, Identify modified fragments, put it all back together

↑

Mass Measurement of Peptides

↑

Digest

↑

Purification of Monomer (Gel, Size Exclusion Column)

↑

Protein sample

Bottom-Up

Protein sample

↓

Sample Cleanup (Protein Trap)

↓

Gas-Phase “Purification” of crosslinked species

↓

Gas-Phase “Digest”, CID, IRMPD, ECD

↓

Data Analysis, Identify fragments containing modification, put it all back together

Top-Down

Footprinting

Assay examining higher structures of biomacromolecules by monitoring solvent accessibility of their regions

- Single molecule conformation / Conformational changes
- Ligand binding / biomacromolecular interactions

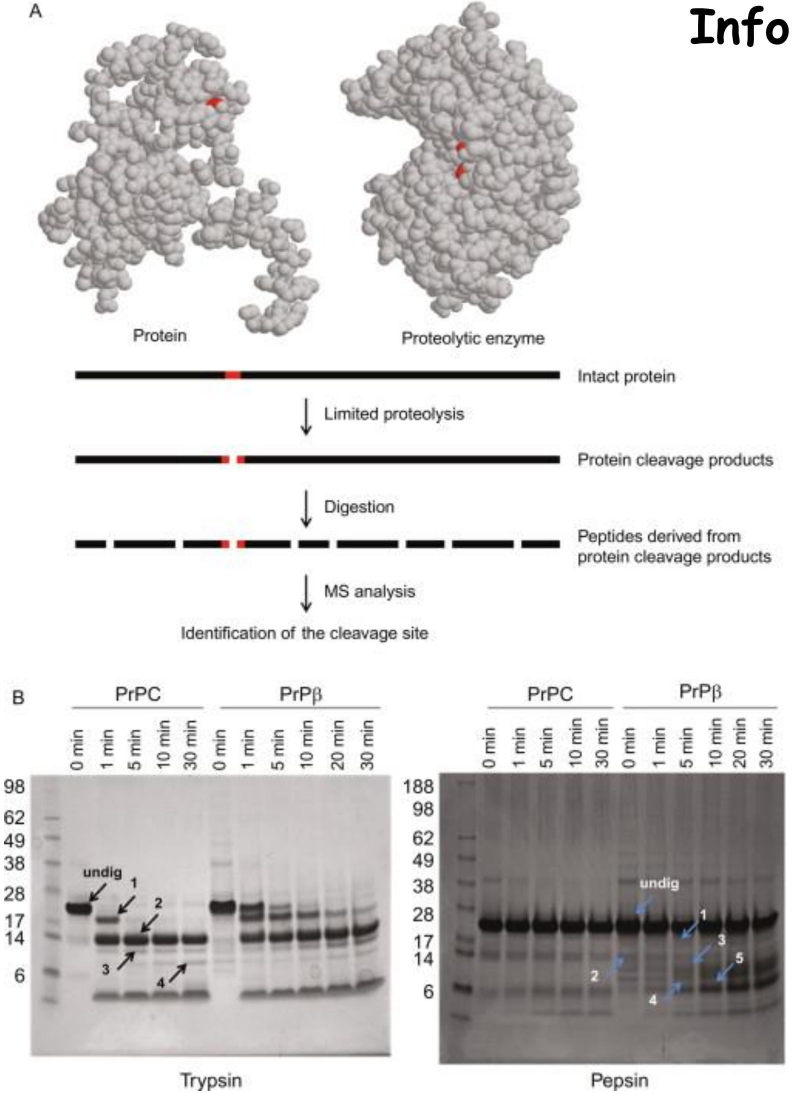
Different techniques

- Enzymatic / chemical cleavage
- Covalent labeling

Covalent labeling

- Hydrogen-deuterium Exchange
- Stable covalent labeling – Chemical or Radical footprinting and cross-linking

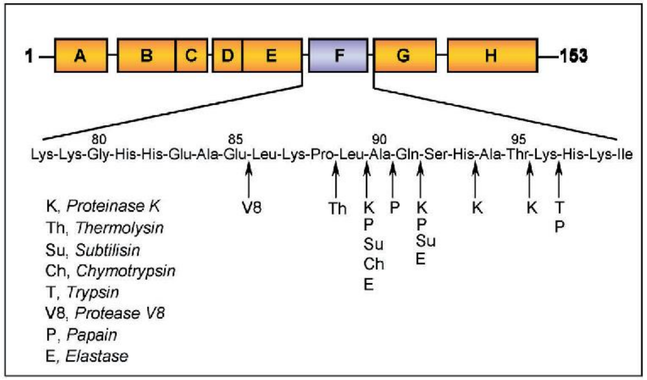
Enzymatic cleavage – Limited proteolysis

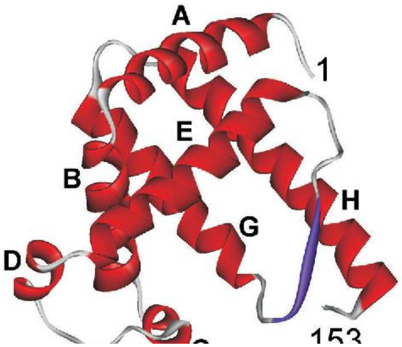


Information about the surface accessible area

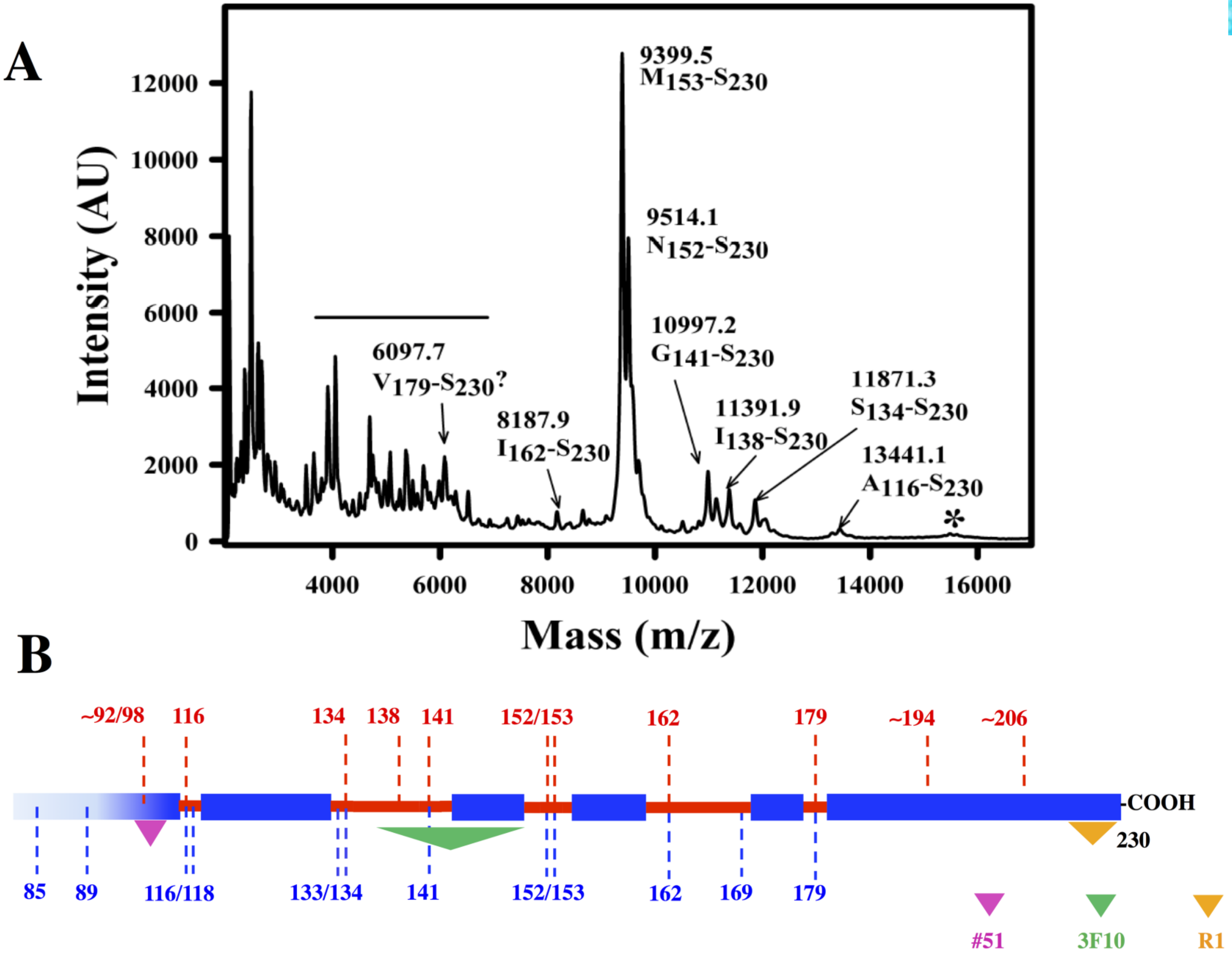
Several available proteases

Native conditions

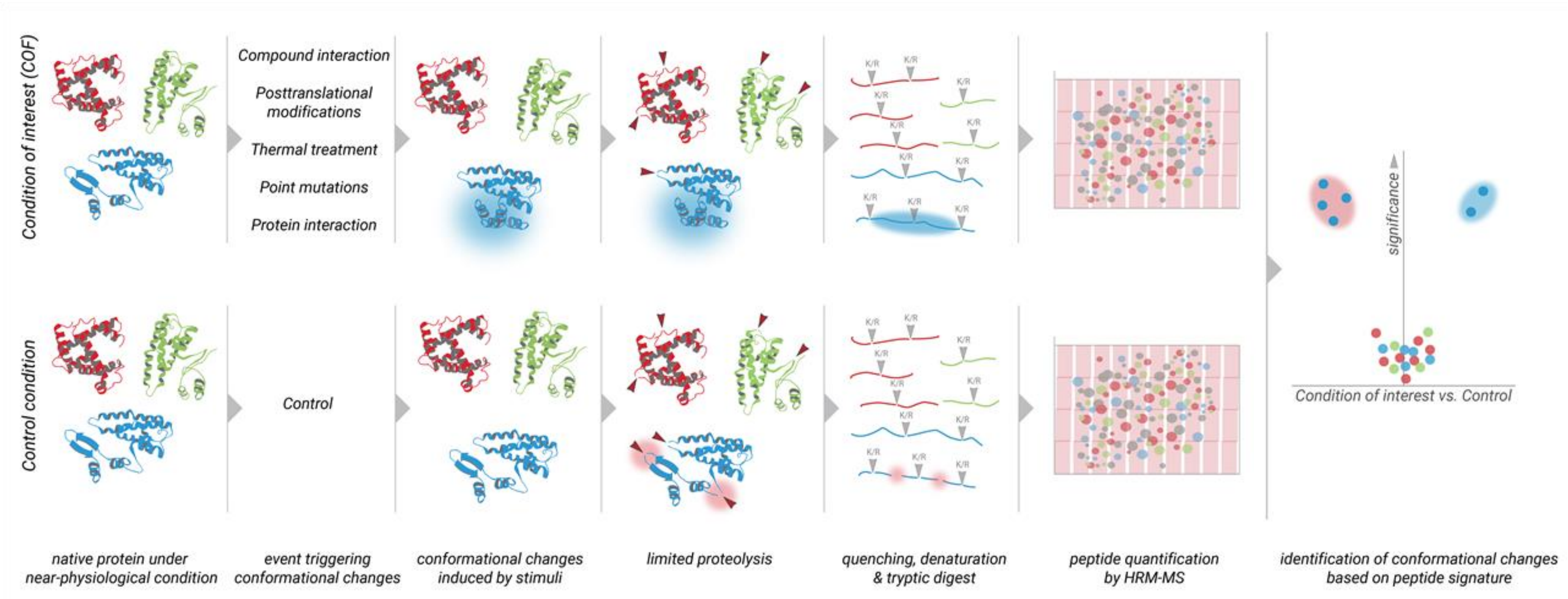




Limited proteolysis – identification of fragments ("Top down")

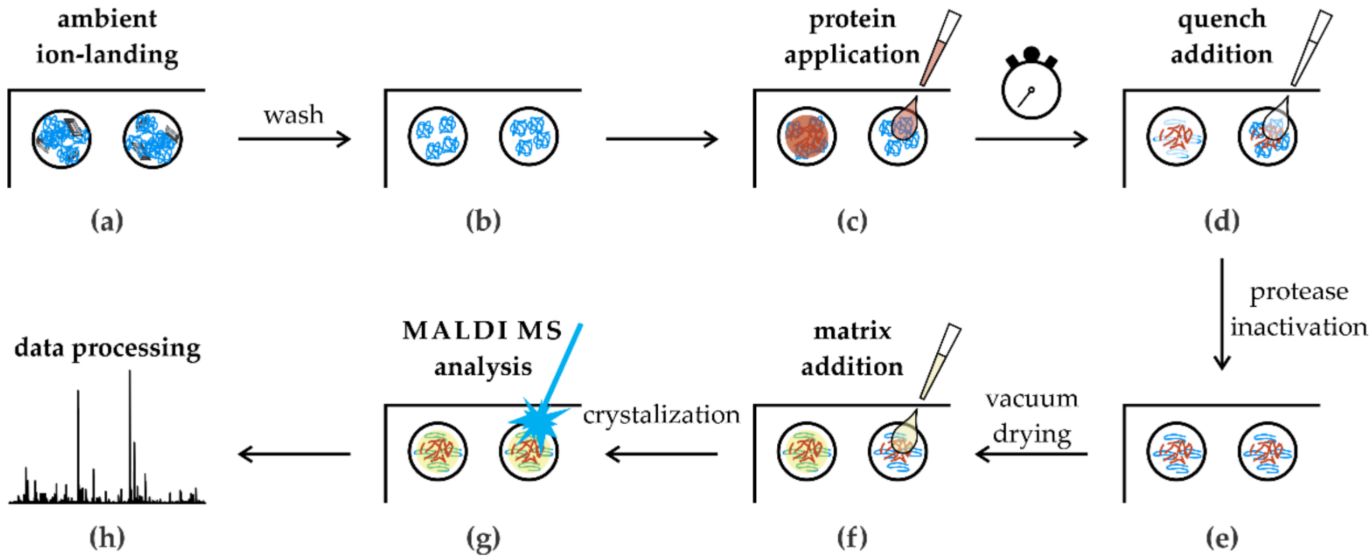


Limited proteolysis – identification of fragments ("Bottom up")



Schopper S, Kahraman A, Leuenberger P, et al. Measuring protein structural changes on a proteome-wide scale using limited proteolysis-coupled mass spectrometry. Nat Protoc. 2017;12(11):2391-2410. doi:10.1038/nprot.2017.100

Limited proteolysis – identification of fragments ("Chip set up")



ambient ion-landing

wash

protein application

quench addition

protease inactivation

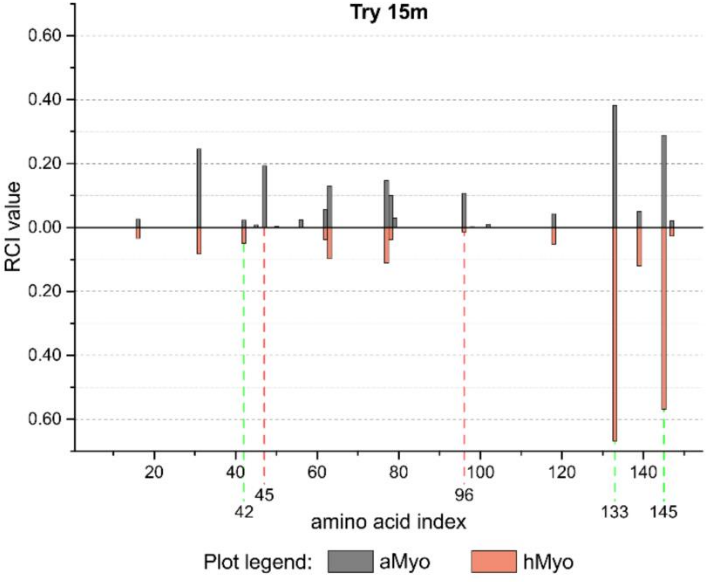
vacuum drying

matrix addition

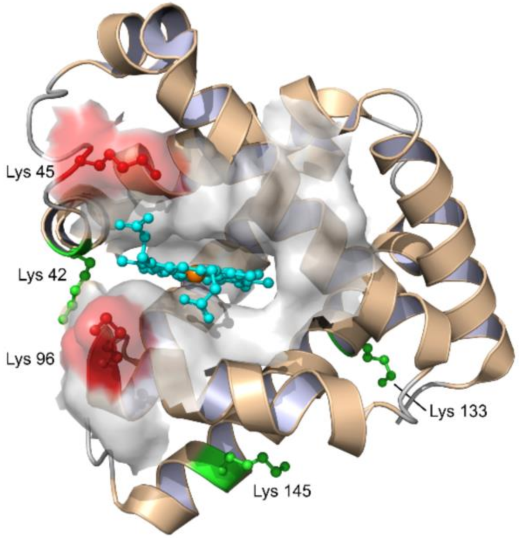
crystalization

MALDI MS analysis

data processing



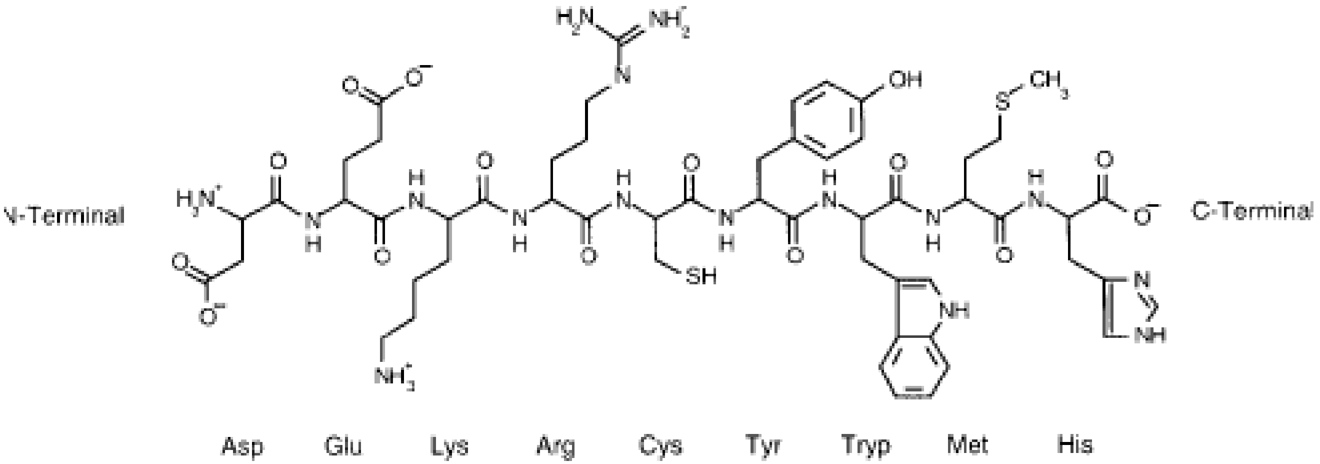
(a)



(b)

Protein covalent labeling and chemical cross-linking

Available amino acid sidechains for covalent modification



Carboxy groups – Asp, Glu, C-term, pKa (3.8, 4.3, 2.3) pH≥7 » deprotonation

Amino groups – Lys, Arg, His, N-term, pKa (9.4, 12, 6.8, 7.8) 7≥pH » protonation

Sulfhydryl groups - Cys . pKa 8.9 pH≥7 » -SH

Aromatic groups– Trp (indol), Tyr (hydroxyfenyl, pKa 9.9)

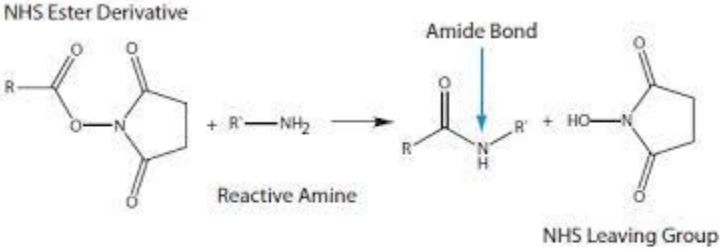
~ 23% of amino acid can be covalently modified

Klapper et. al. Biochem. Biophys. Res. Commun. 1977, 78, 1018.

Covalent modification of amino acid side chains

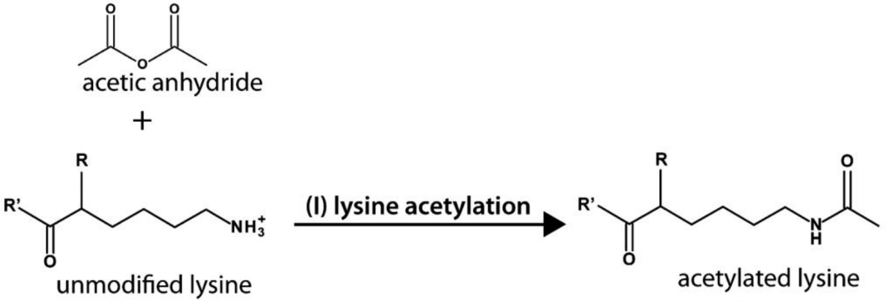
Acetylation

(N-hydroxysuccinimide esters)



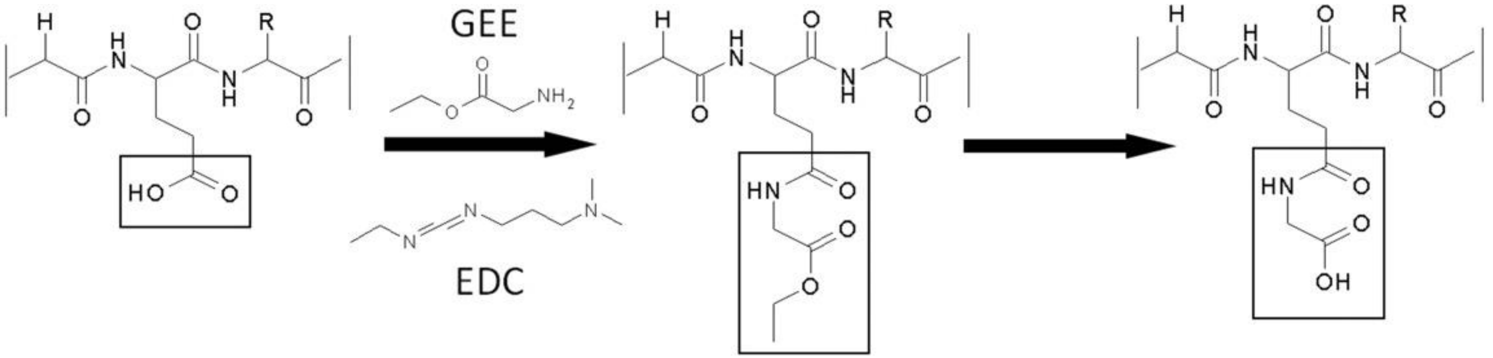
Acetylation

(Anhydrides)



Modification of carboxylic acids

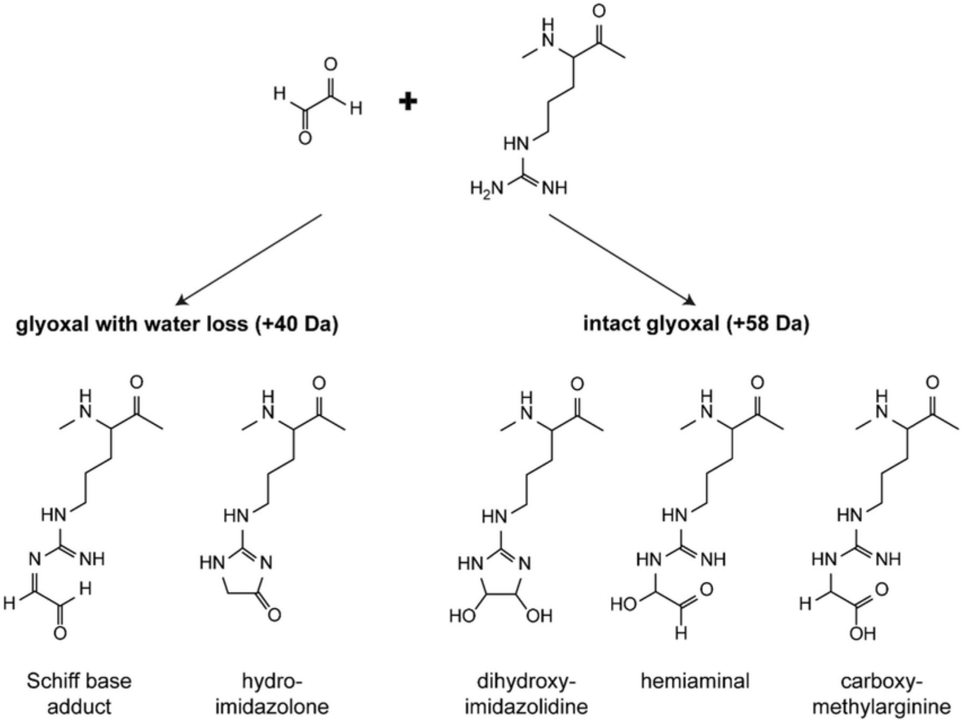
(1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and glycine ethyl ester)



Covalent modification of amino acid side chains

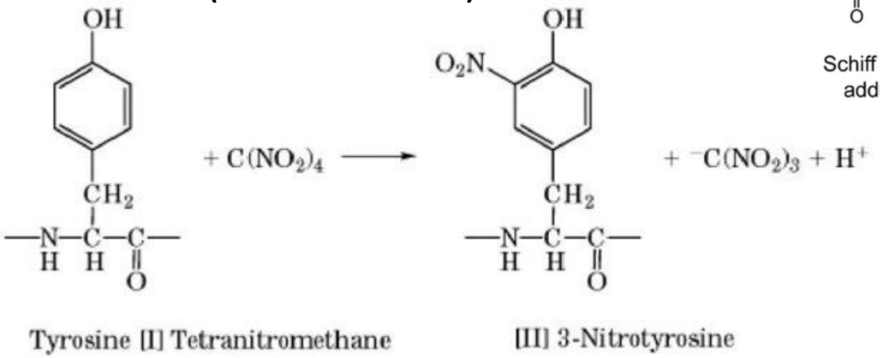
Modification of arginine

(diketones)

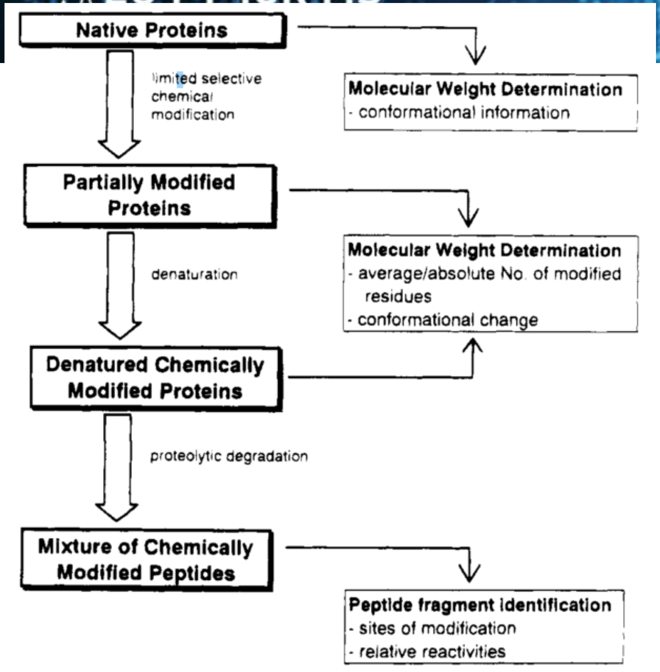


Nitration

(tetranitromethane)



Protein covalent labeling: Lys, Tyr, Arg



Native Proteins

limited selective chemical modification

Molecular Weight Determination
- conformational information

Partially Modified Proteins

denaturation

Molecular Weight Determination
- average/absolute No. of modified residues
- conformational change

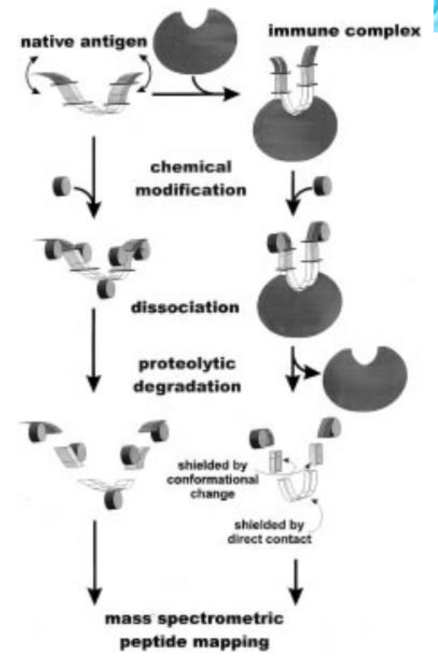
Denatured Chemically Modified Proteins

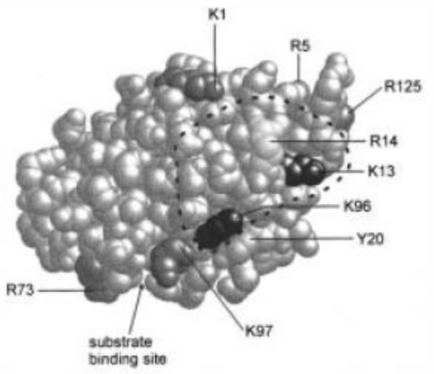
proteolytic degradation

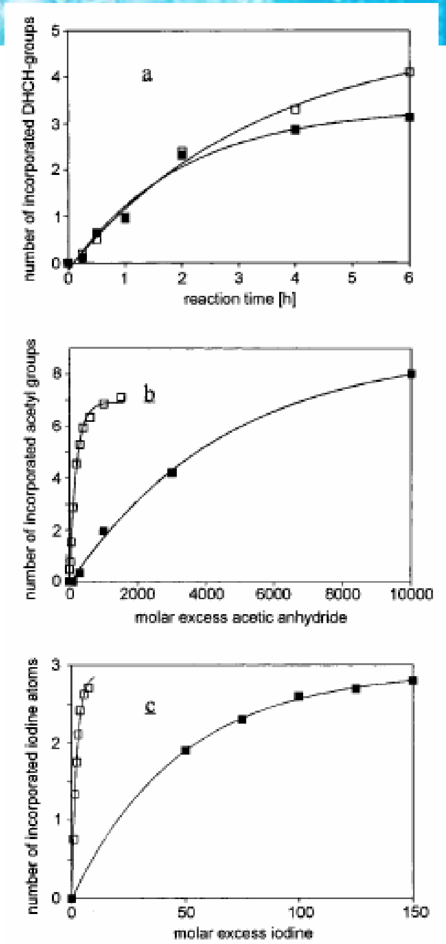
Mixture of Chemically Modified Peptides

Peptide fragment identification
- sites of modification
- relative reactivities

relative reactivity NH_2 groups[a]	lysine residues, ϵ-amino groups		
	RNAse A	HEL	myoglobin
	α-NH_2		α-NH_2
1	41, 104	97, 33 α-NH_2	45, 63, 77, 79, 145, 147
2	1, 7, 37	1	16, 42, 87
3	31, 61, 91	13, 116	56, 50, 62, 78, 102
4	66, 98	96	96, 47, 87, 133,[b] 118[b]



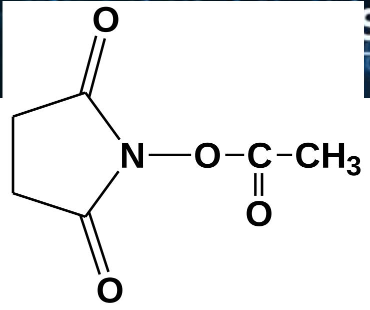


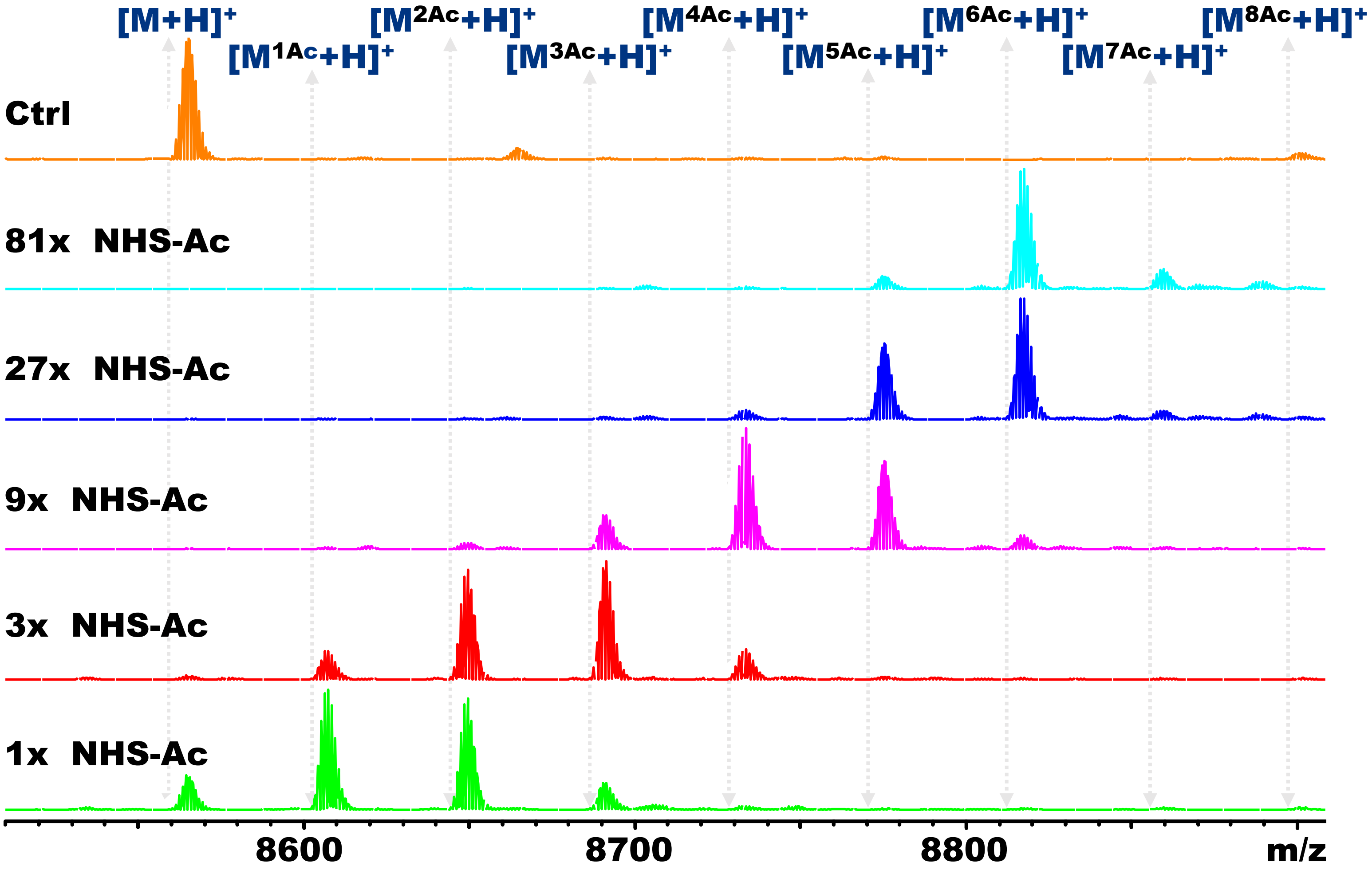


Suckau et. al. *PNAS 1992, 89, 5630*
and ***Glocker et. al.*** *Bioconj. Chem. 1994, 5, 583*

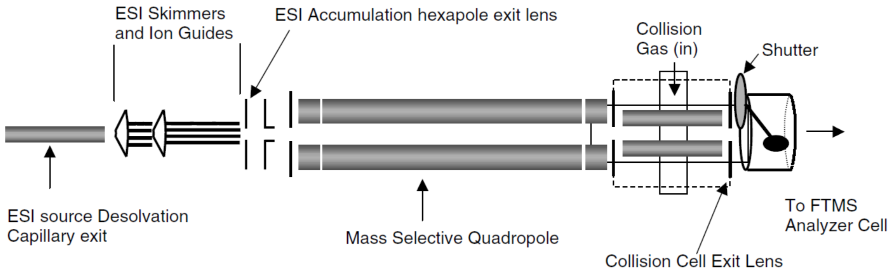
Fiedler et. al. *Bioconj. Chem. 1998, 9, 236*

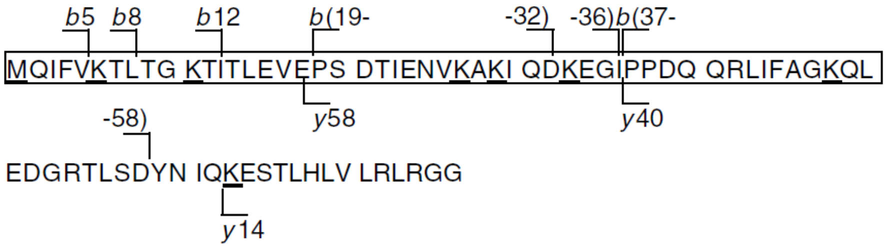
Protein covalent labeling: Top down

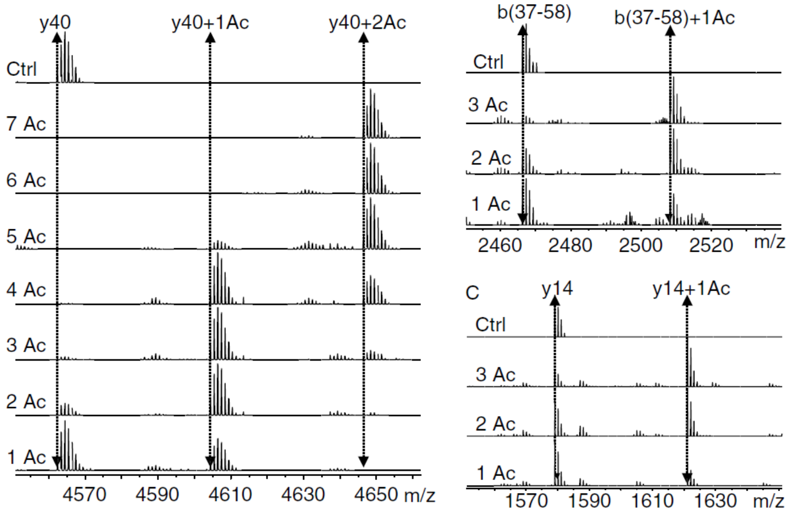


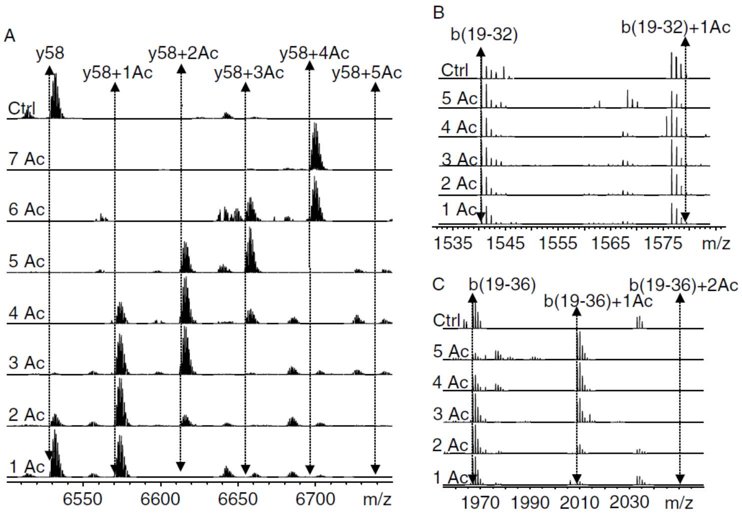


Protein covalent labeling: Top down









Protein covalent labeling: a reactivity of lysine in an issue

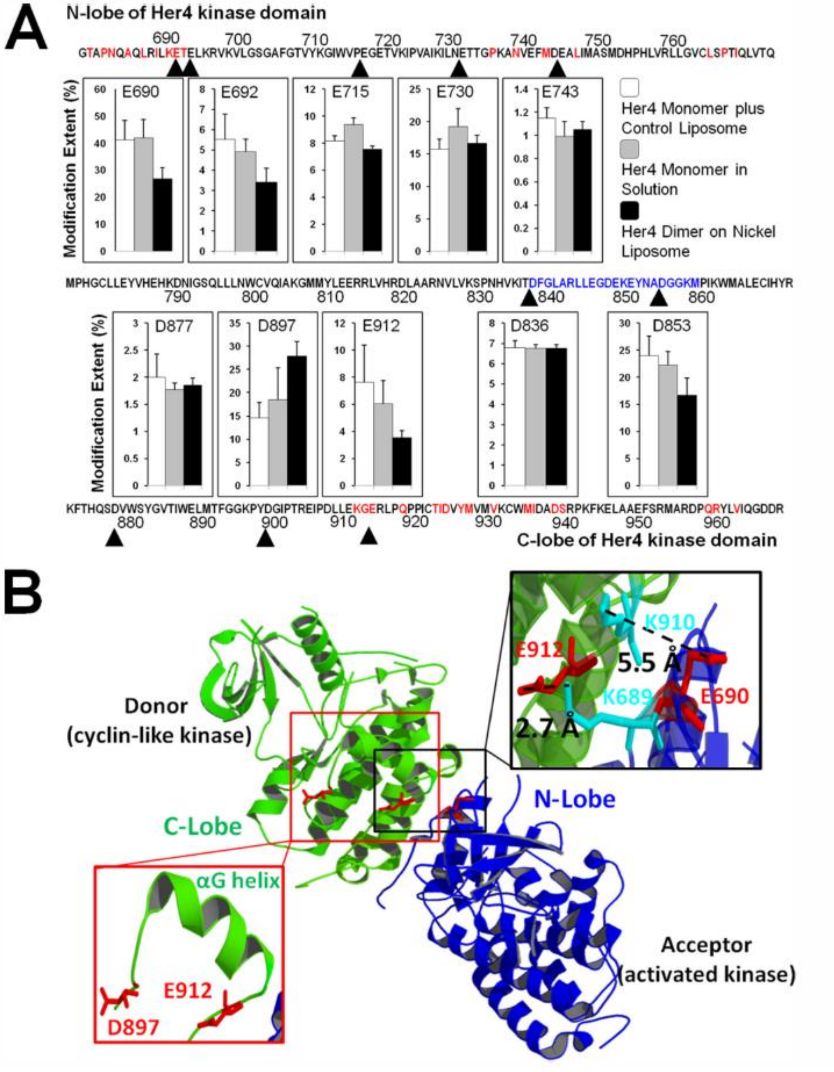
^{1}MQIFVKTLTG ^{11}KTITLEVEPS ^{21}DTIENVKAKI ^{31}QDKEGIPPDQ
^{41}QRLIFAGKQL ^{51}EDGRTLSDYN ^{61}IQKESTLHLV ^{71}LRLRGG

(^{1}M~K6~K48~K63) > K33 > K11 > (K27,K29)

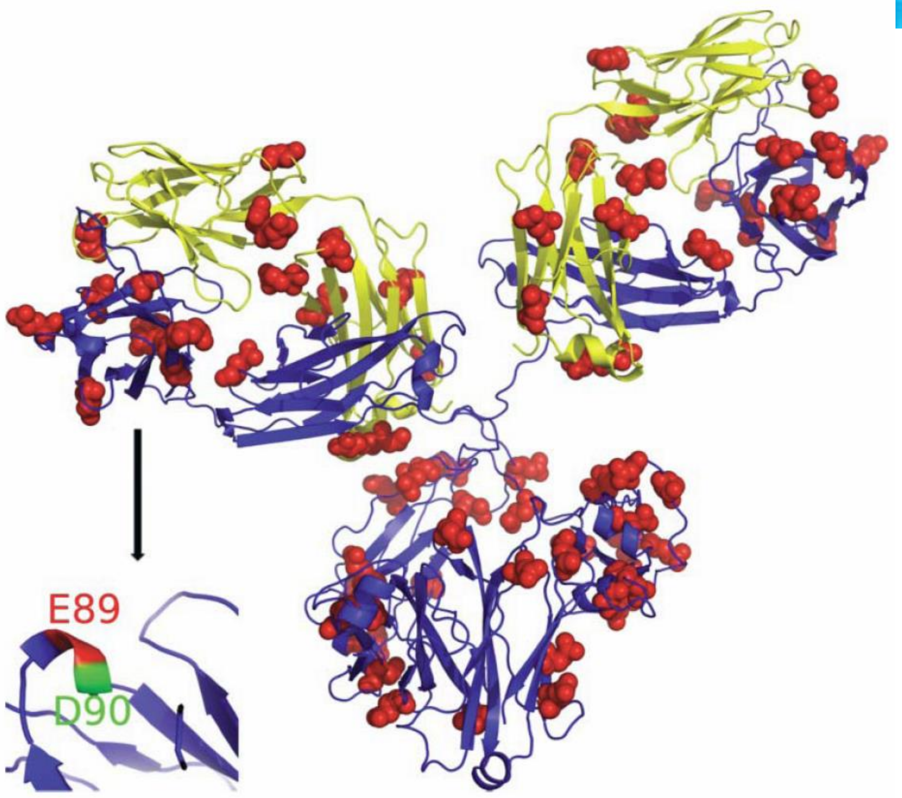
- In agreement with NMR data, which shows.
  - K11 interacts with E34; K29 interacts with D21
- Crystal structure indicates K27 H-bonds to D52.
- More reactive lysines don’t H-bond (K63) or H-bond to backbone carbonyls (K48, K33).
- K48 and K63 participate in formation of polyubiquitin.

Novak et. al. *J. Mass Spectrom. 2004, 39, 322*

Protein covalent labeling: Asp, Glu



Zhang H. et al. MCP 2011



Kaur P. et al. mAb 2015

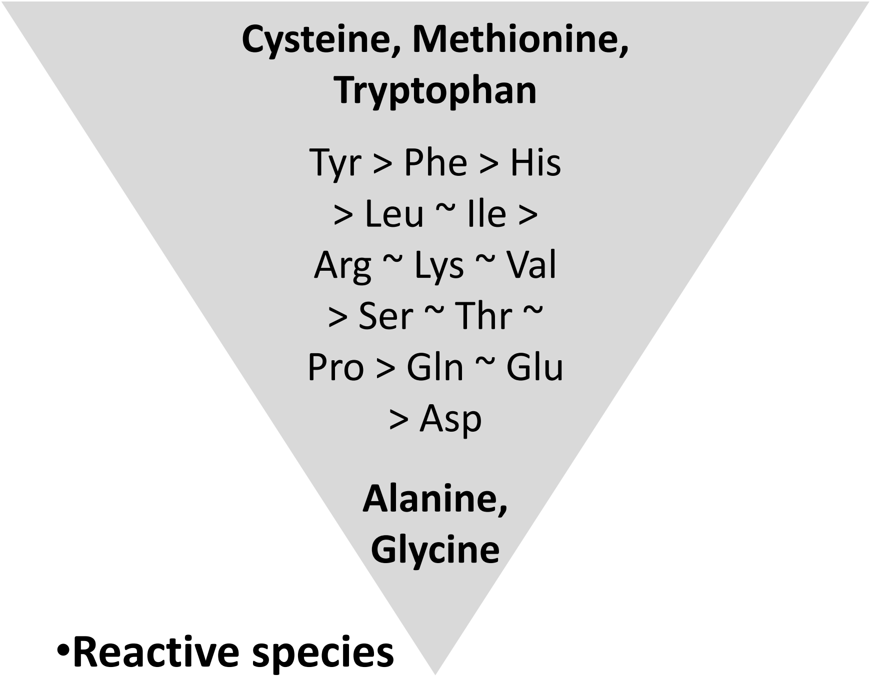
Hydroxyl Radical Footprinting

Products of water or hydrogen peroxide molecule homolytic bond cleavage

Hydroxyl radicals can be generated by various means:

- **Fenton reaction**
- **Irradiation of water by x-rays or electron beams**
- **Photolysis of hydrogen peroxide FPOP (fast photochemical oxidation of proteins)**
- **Other radicals available • OH, • I, • CF3**

The relative reactivity of the amino acid side chains



Cysteine, Methionine, Tryptophan

Tyr > Phe > His > Leu ~ Ile > Arg ~ Lys ~ Val > Ser ~ Thr ~ Pro > Gln ~ Glu > Asp

Alanine, Glycine

- **Reactive species**
- **React efficiently with most AA side chains**
- **Form STABLE oxidation products**

Takamoto K . et al. *Annu Rev Biophys Biomol Struct.* 2006, 35, 251-276

Conditions for radical labeling

- Electron pulse radiolysis:
  - reproducible 1-100 ns pulses; MeV energy range on linear accelerator

- Synchrotron radiolysis:
  - X-ray; 3-30 keV @ beam current ~ 250 mA

- Laser H_2O_2 photolysis:

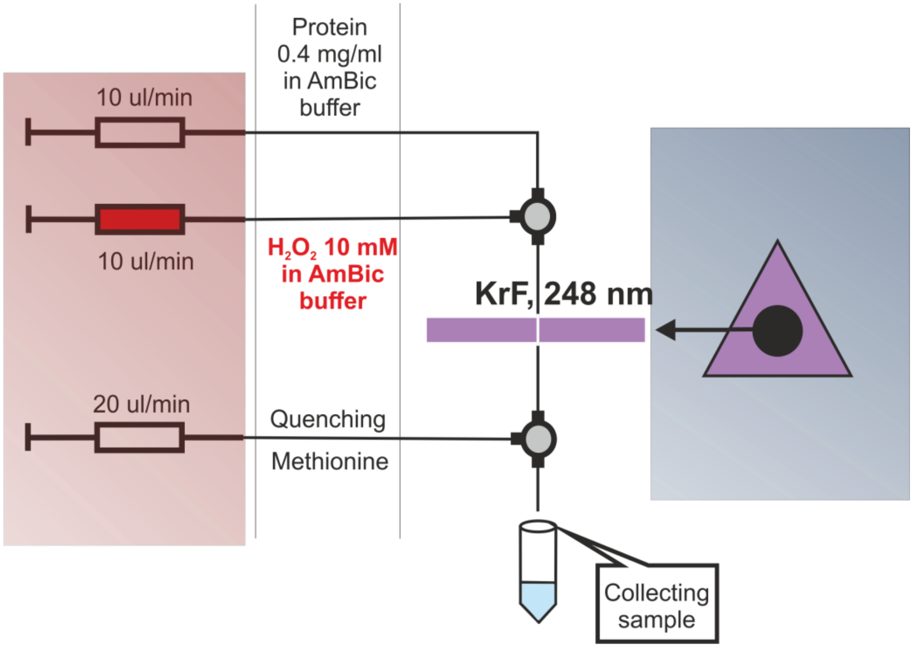
  1% - 0,04% H_2O_2 (mixing by stopped-flow device or just before irradiation); quench and removal of residual peroxide is vital

  - Nd:YAG; 2 mJ/pulse @ 266 nm; 3-5 ns pulse; 1-100 shots
  - 17 ns KrF excimer laser; 50 mJ/pulse @ 248 nm
  - 18 ns KrF excimer laser; 62,5 mJ/pulse @ 248 nm; 16 Hz

Fast photochemical oxidation of proteins

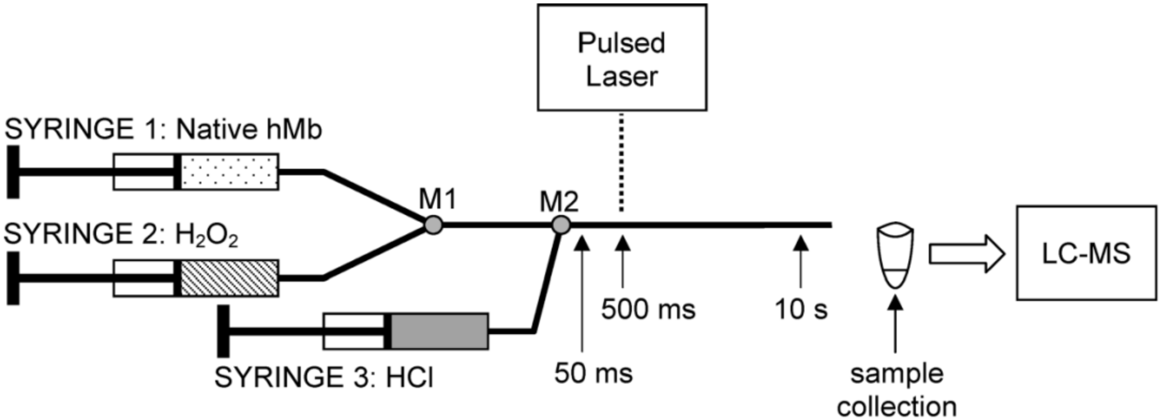
- **Covalent modification preserves the primary sequence of modified residues**

- **High reactivity of •OH the modifications of more than half of amino acid side-chains, providing a higher coverage**



Experimental setup

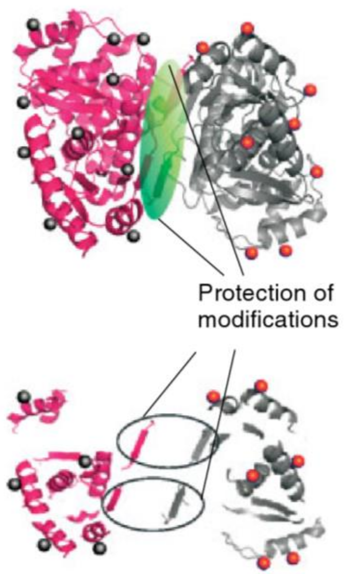
- Sample mixed and irradiated in
    - **µtubes** (sample volume ~ 15 µl) or in
    - **stopped-flow microfluidic mixing device** - essential for folding / kinetic studies (capillary flow ~ 20 µl.min^{-1})



- Short pulses with high energy are needed to create sufficient concentration of radicals on very short (sub-microsecond) timescales to avoid conformational changes of protein during labeling.
- Possible protein conformational changes occur mostly on a longer than milisecond timescale.

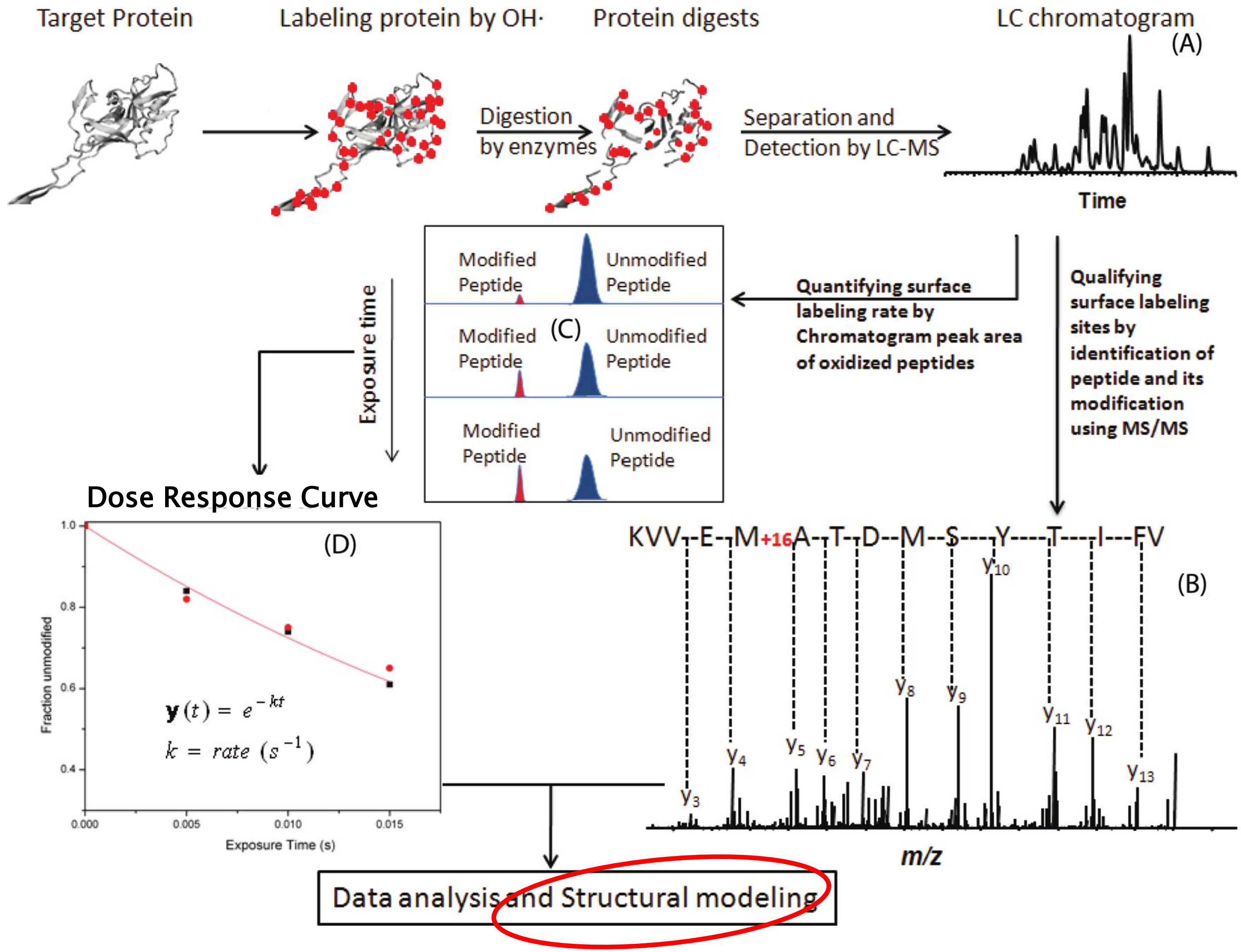
Hydroxyl radical footprinting – work-flow

Protein 1-2 Complex



Protection of modifications

OR



Target Protein

Labeling protein by OH·

Digestion by enzymes

Protein digests

Separation and Detection by LC-MS

LC chromatogram

Qualifying surface labeling sites by identification of peptide and its modification using MS/MS

Quantifying surface labeling rate by Chromatogram peak area of oxidized peptides

Dose Response Curve

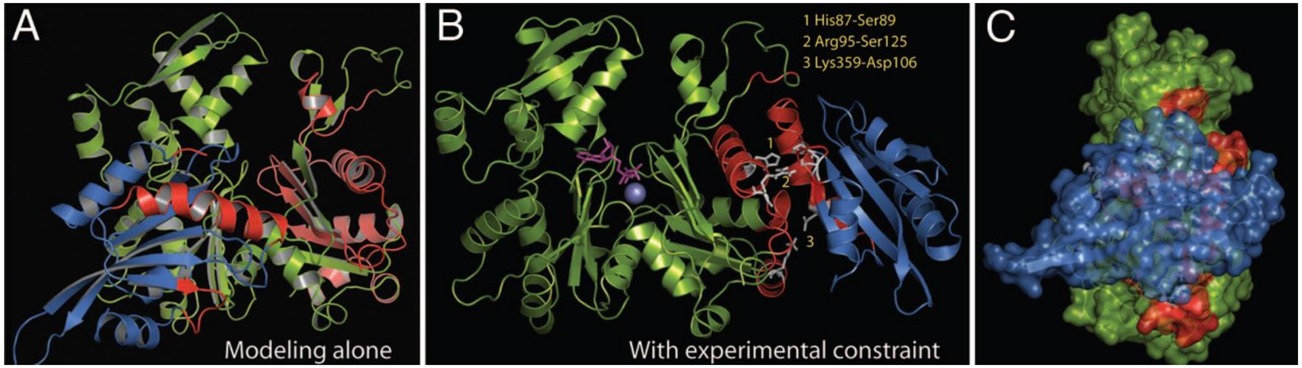
$$\mathbf{y}(t) = e^{-kt}$$

$$k = rate\ (s^{-1})$$

Data analysis and Structural modeling

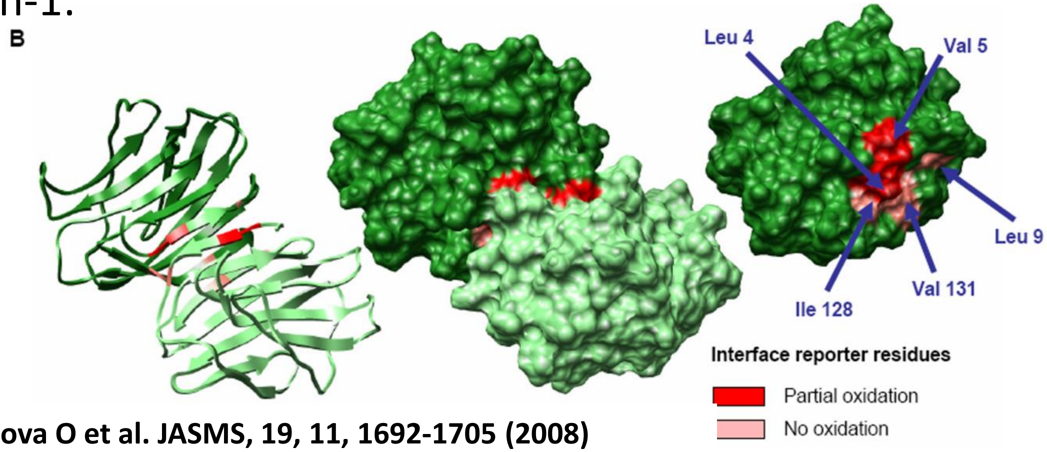
Some (recent) results

- Protein-protein interaction
  - Actin – coffilin interaction:



Kamal JK et al. PNAS, 104, 19, 7910-7915 (2007)

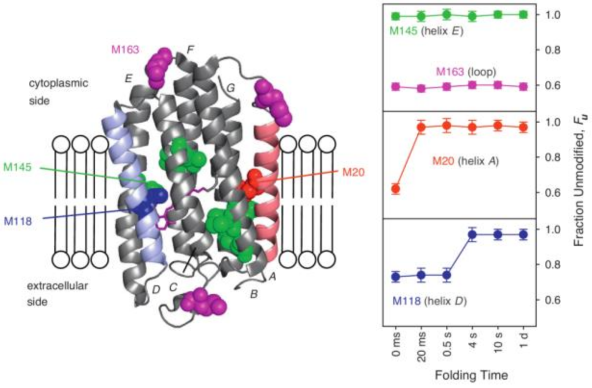
  - Dimerization interface of galectin-1:



Charvatova O et al. JASMS, 19, 11, 1692-1705 (2008)

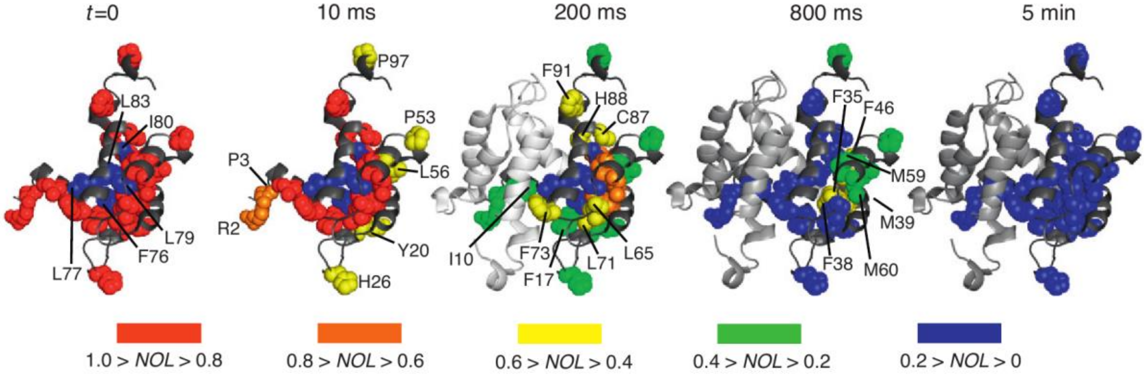
More recent results

- Protein structure
    - Integral membrane protein (BR) structure in its natural lipid environment



Pan Y et al. J Mol Biol, 410, 146-158 (2011)

- Protein folding kinetics
    - Time-resolved folding and dimerization changes in Ca-binding protein



Stocks BB et al. J Mol Biol, 409, 669-679 (2011)

Fast Photochemical Iodination and Top down

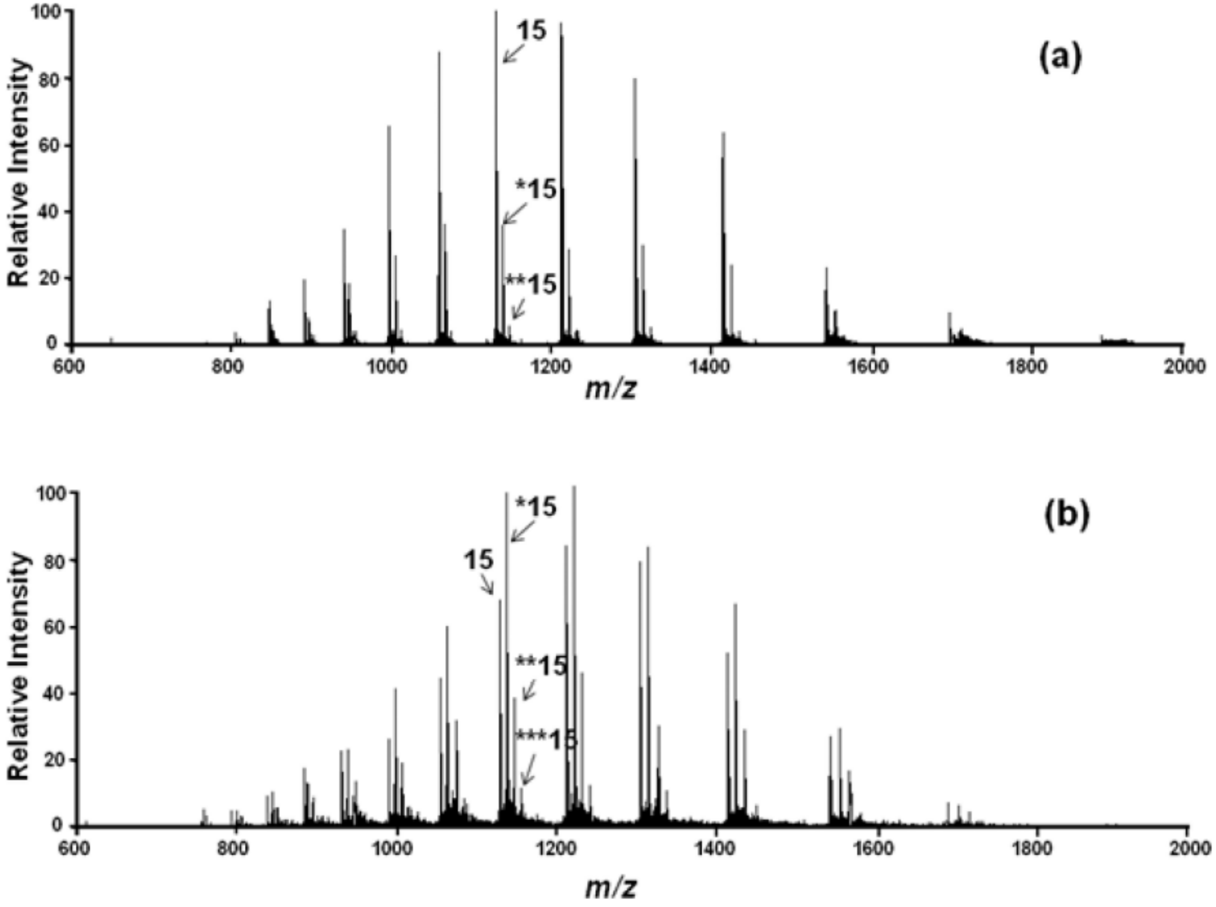
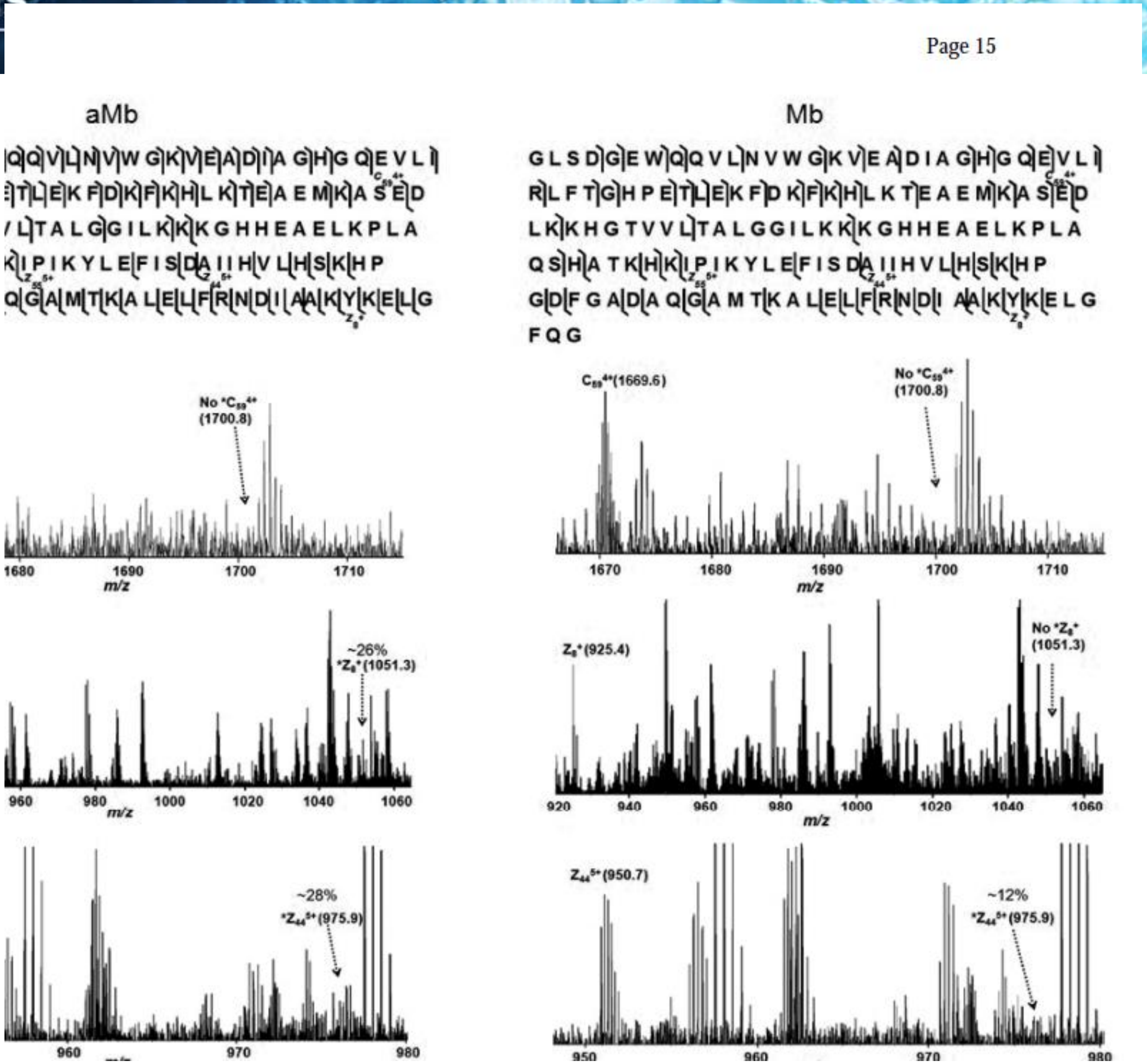


Figure 1.
(a) Full ESI mass spectrum of iodinated myoglobin (Mb). **(b)** Full ESI mass spectrum of iodinated apomyoglobin (aMb). The unmodified, mono-, di- and tri-iodinated species of the 15th charge state are indicated by the number of stars.

Chen J, Cui W, Giblin D, Gross ML. New protein footprinting: fast photochemical iodination combined with top-down and bottom-up mass spectrometry. J Am Soc Mass Spectrom. 2012;23(8):1306-1318. doi:10.1007/s13361-012-0403-1

Fast Photochemical Iodination and Top down



Chen J, Cui W, Giblin D, Gross ML. New protein footprinting: fast photochemical iodination combined with top-down and bottom-up mass spectrometry. J Am Soc Mass Spectrom. 2012;23(8):1306-1318. doi:10.1007/s13361-012-0403-1

Fast Photochemical Iodination and Bottom up

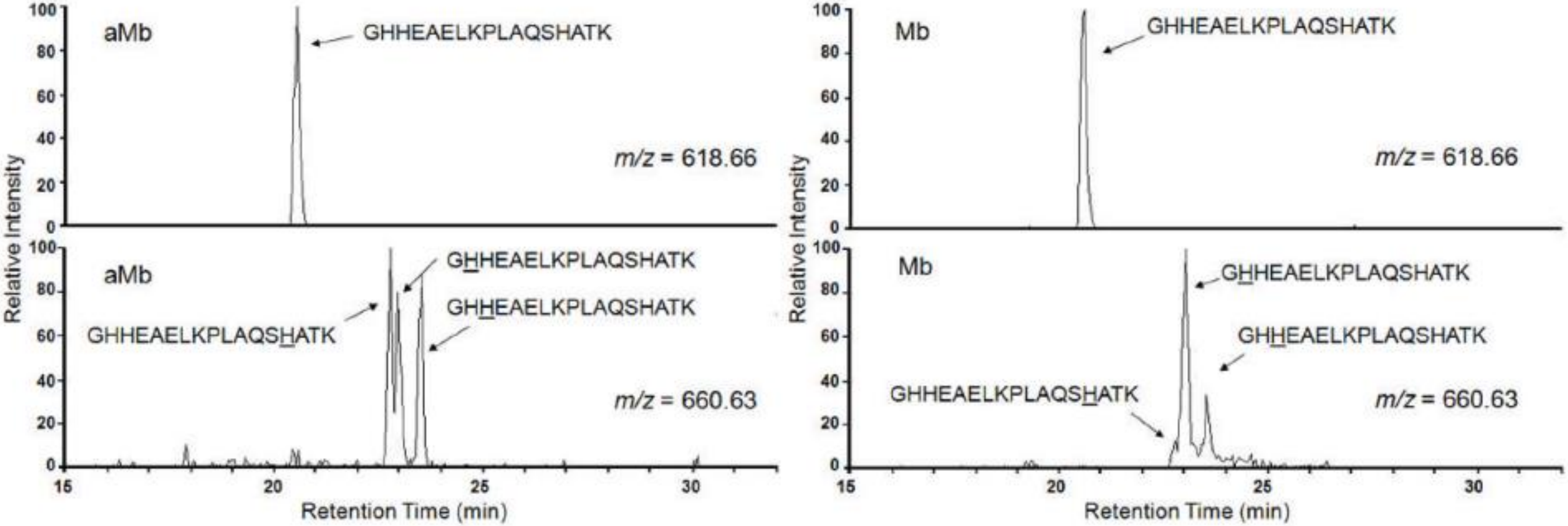
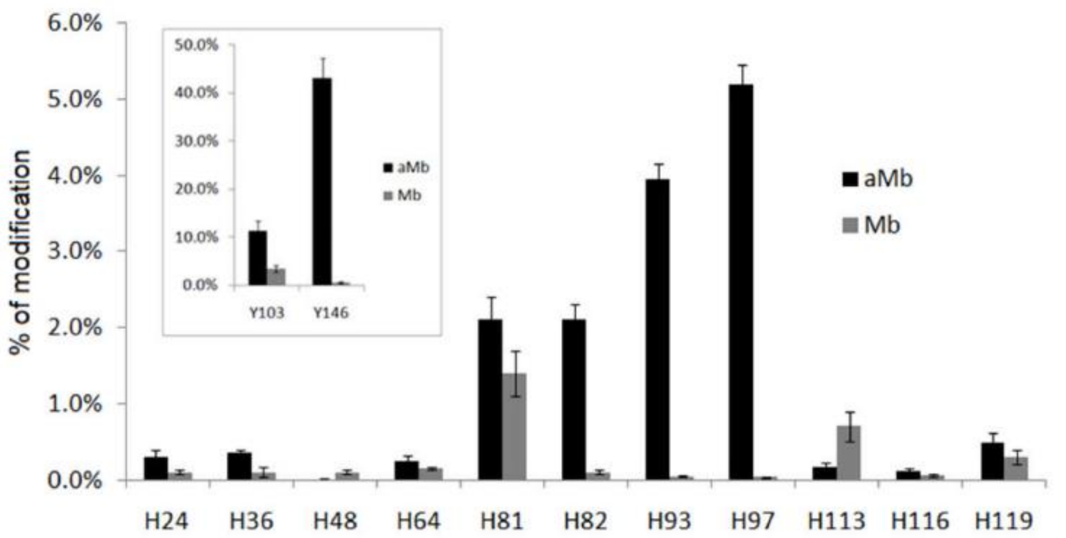
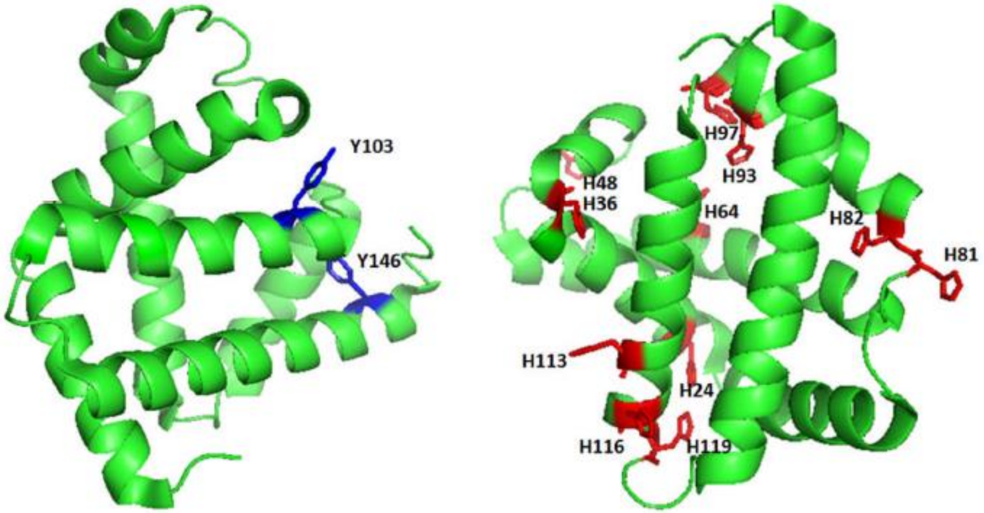


Figure 3.
Extracted ion chromatograms of doubly charged peptide 80-96 of aMb (top left) and Mb (top right), monoiodinated peptide 80-96 of aMb (bottom left) and aMb (bottom right). The assigned modification sites are underlined.

Chen J, Cui W, Giblin D, Gross ML. New protein footprinting: fast photochemical iodination combined with top-down and bottom-up mass spectrometry. J Am Soc Mass Spectrom. 2012;23(8):1306-1318. doi:10.1007/s13361-012-0403-1

Fast Photochemical Iodination: Data visualization

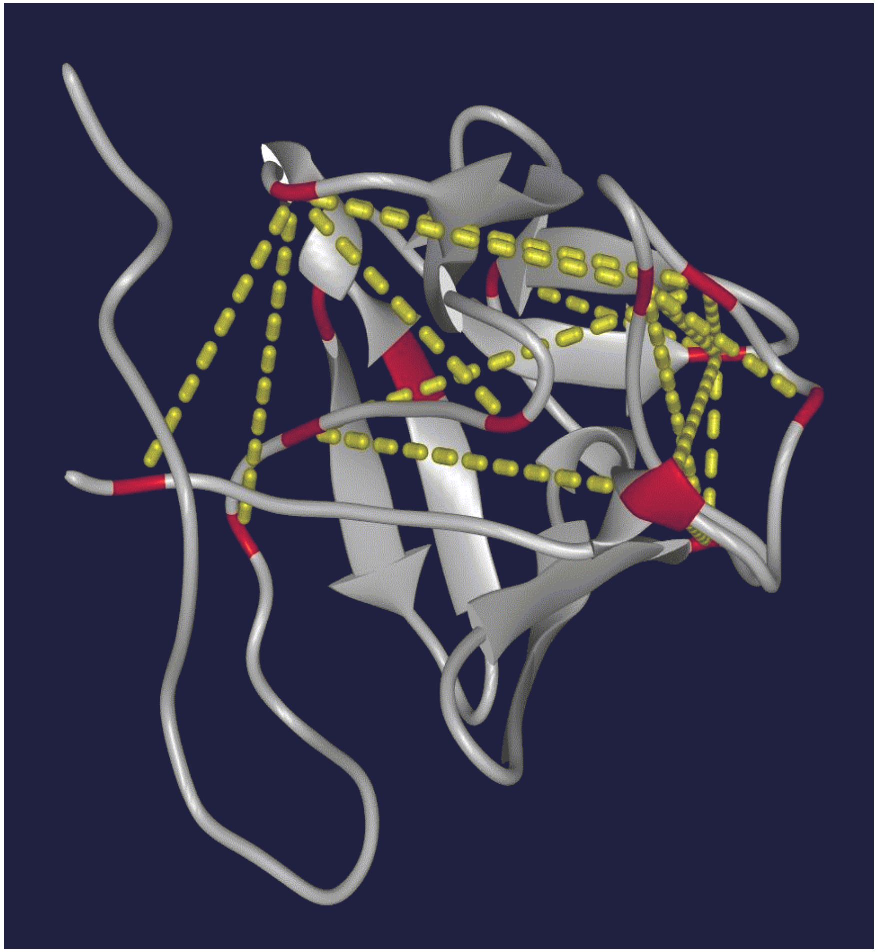




Chen J, Cui W, Giblin D, Gross ML. New protein footprinting: fast photochemical iodination combined with top-down and bottom-up mass spectrometry. J Am Soc Mass Spectrom. 2012;23(8):1306-1318. doi:10.1007/s13361-012-0403-1

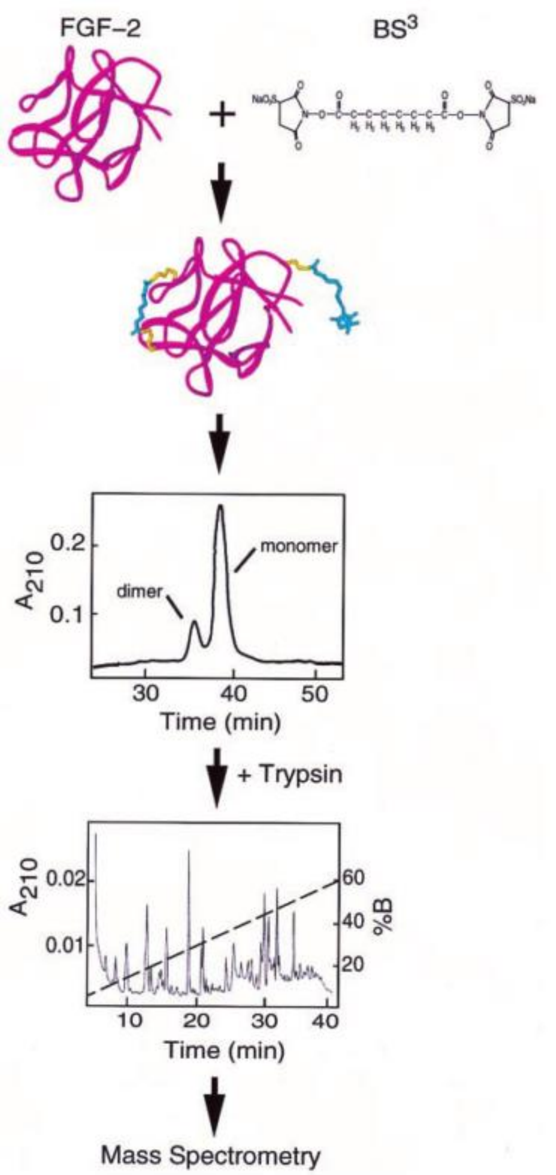
Chemical cross-linking

Chemical cross-linking combined with MS has been proposed as a method to obtain distance constraints (MS3D)

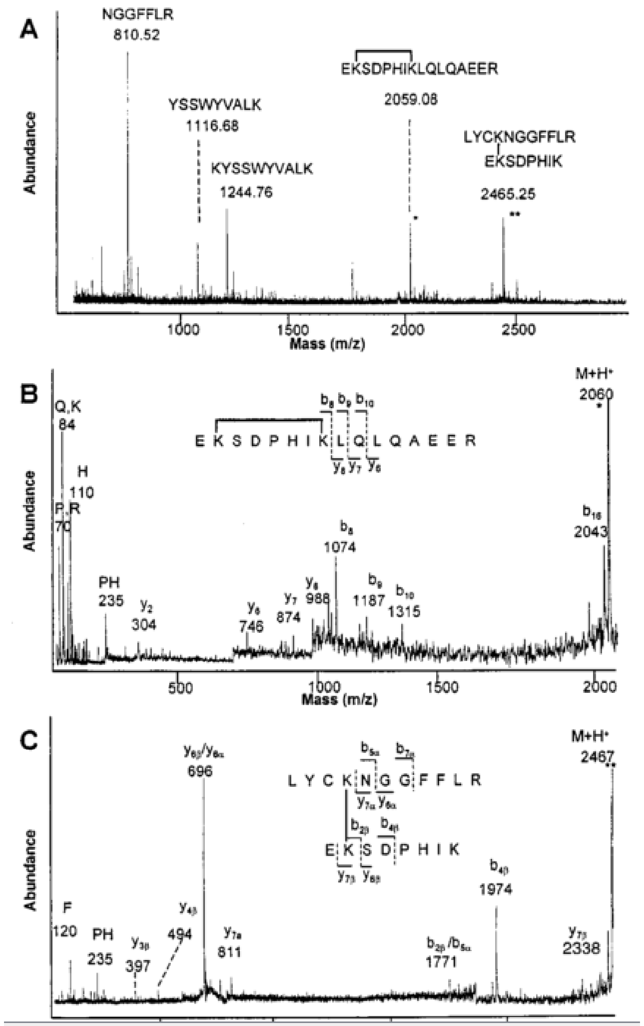


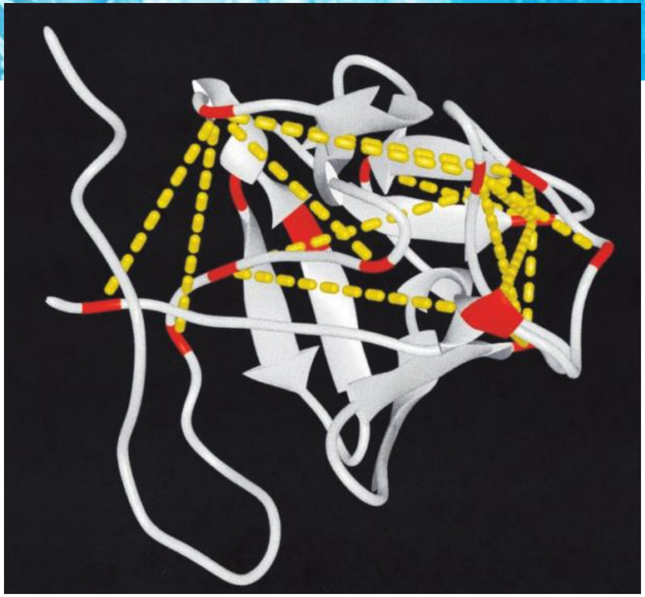
Young, Tang et al. *PNAS* 97(11):5802-6. 2000.

Chemical cross-linking: the first 3-D structure

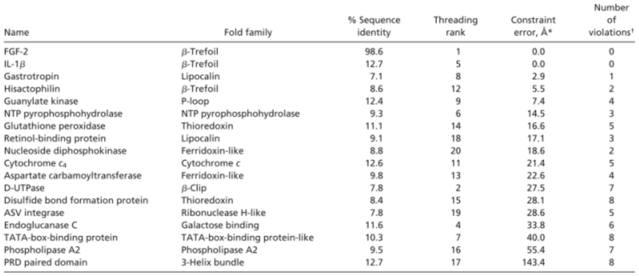


Fibroblast grow factor 2





Top 20 threading models ranked by constraint error



Name	Fold family	% Sequence identity	Threading rank	Constraint error, Å*	Number of violations†
FGF-2	β-Trefoil	98.6	1	0.0	0
IL-1β	β-Trefoil	12.7	5	0.0	0
Gastrotropin	Lipocalin	7.1	8	2.9	1
Hisactophilin	β-Trefoil	8.6	12	5.5	2
Guanylate kinase	P-loop	12.4	9	7.4	4
NTP pyrophosphohydrolase	NTP pyrophosphohydrolase	9.3	6	14.5	3
Glutathione peroxidase	Thioredoxin	11.1	14	16.6	5
Retinol-binding protein	Lipocalin	9.1	18	17.1	3
Nucleoside diphosphokinase	Ferridoxin-like	8.8	20	18.6	2
Cytochrome c_4	Cytochrome *c*	12.6	11	21.4	5
Aspartate carbamoyltransferase	Ferridoxin-like	9.8	13	22.6	4
D-UTPase	β-Clip	7.8	2	27.5	7
Disulfide bond formation protein	Thioredoxin	8.4	15	28.1	8
ASV integrase	Ribonuclease H-like	7.8	19	28.6	5
Endoglucanase C	Galactose binding	11.6	4	33.8	6
TATA-box-binding protein	TATA-box-binding protein-like	10.3	7	40.0	8
Phospholipase A2	Phospholipase A2	9.5	16	55.4	7
PRD paired domain	3-Helix bundle	12.7	17	143.4	8

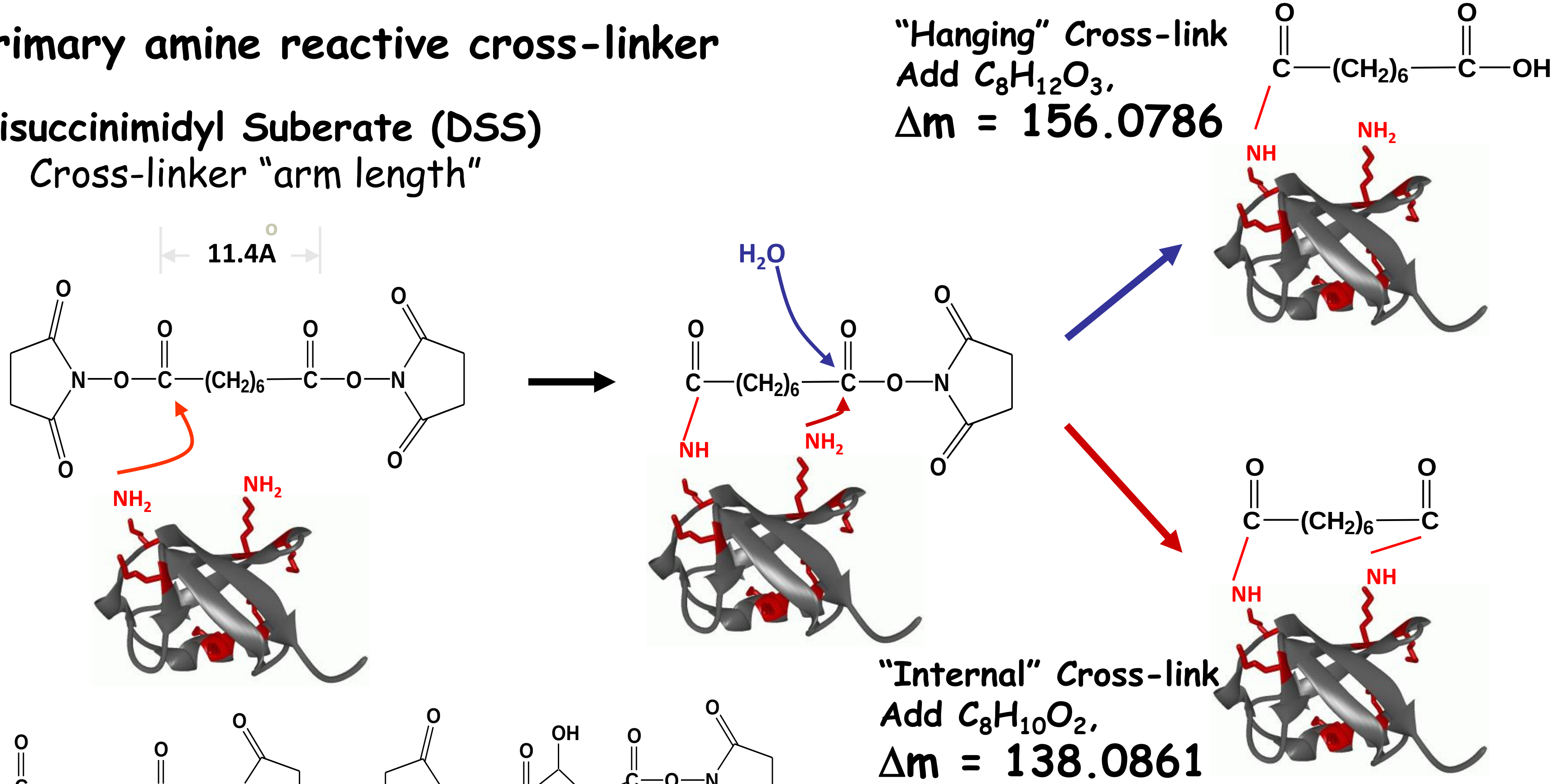
***Young et. al.** PNAS 2000, 97, 5802*

Chemical cross-linking: the chemistry behind…

Primary amine reactive cross-linker

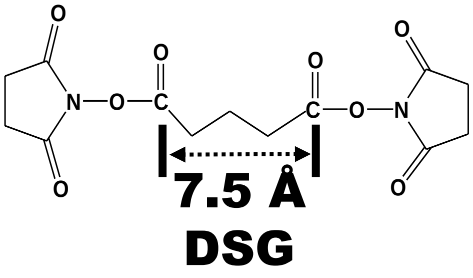
Disuccinimidyl Suberate (DSS)

Cross-linker "arm length"



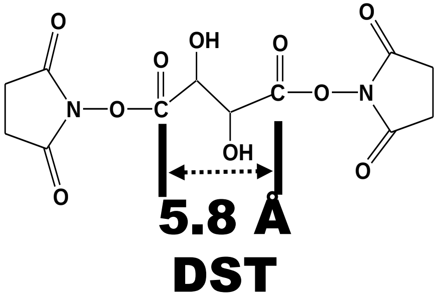
"Hanging" Cross-link
Add $C_8H_{12}O_3$,
Δm = 156.0786

"Internal" Cross-link
Add $C_8H_{10}O_2$,
Δm = 138.0861



7.5 Å

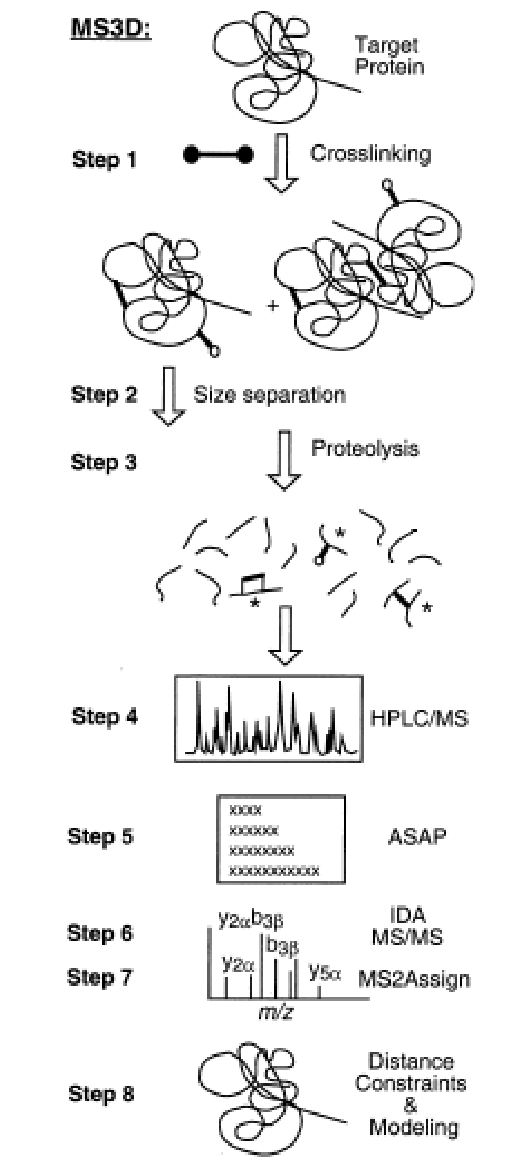
DSG

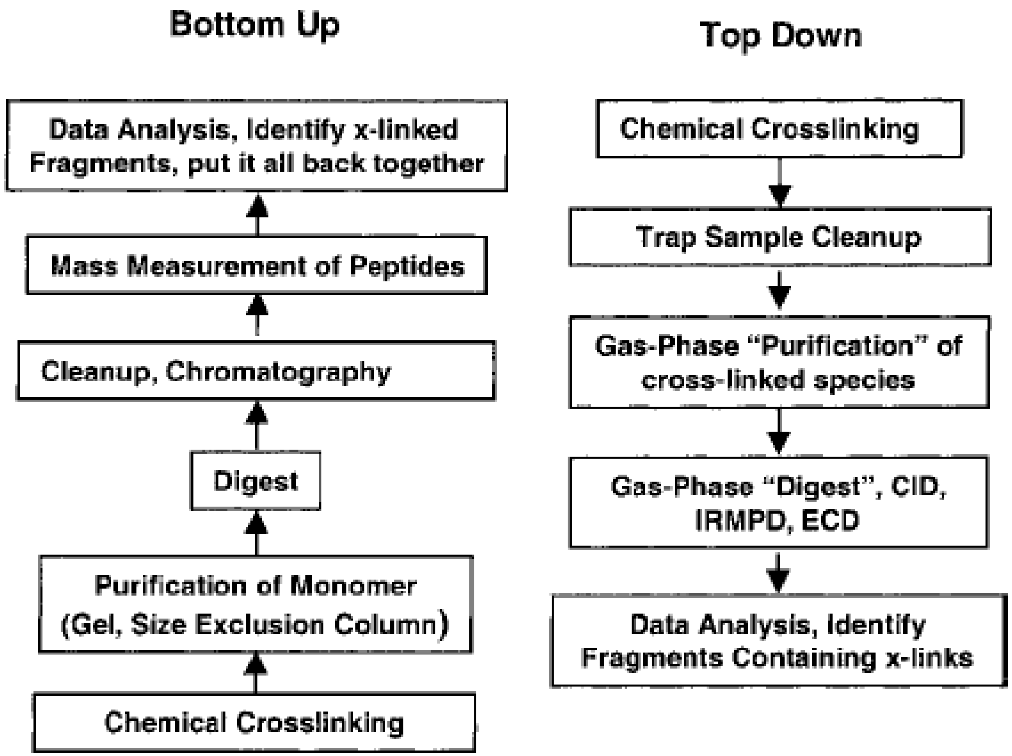


5.8 Å

DST

CXMS experiment





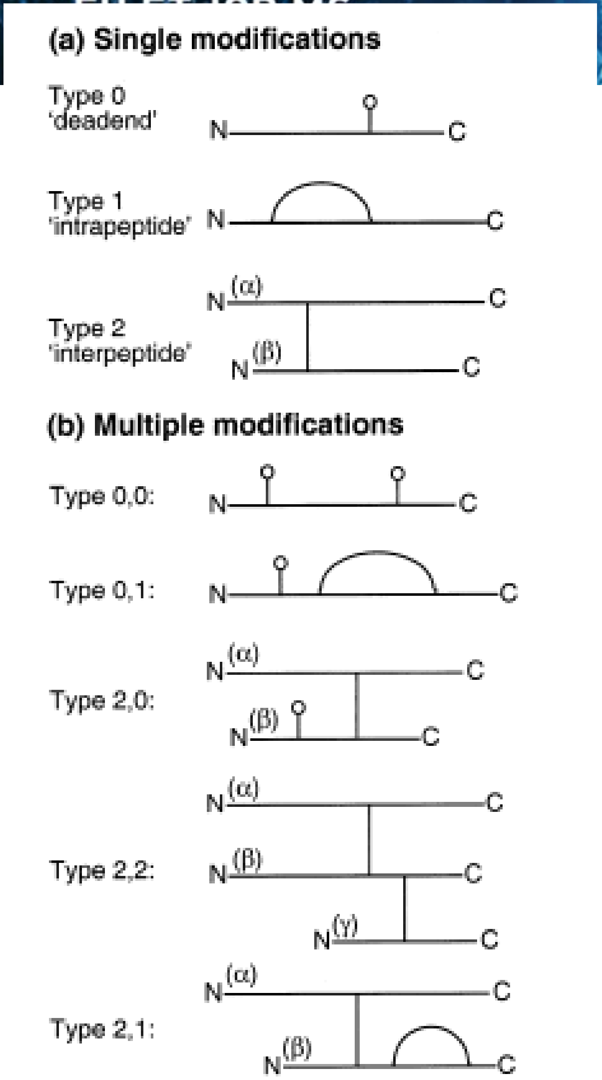
Bottom Up

- Data Analysis, Identify x-linked Fragments, put it all back together
- Mass Measurement of Peptides
- Cleanup, Chromatography
- Digest
- Purification of Monomer (Gel, Size Exclusion Column)
- Chemical Crosslinking

Top Down

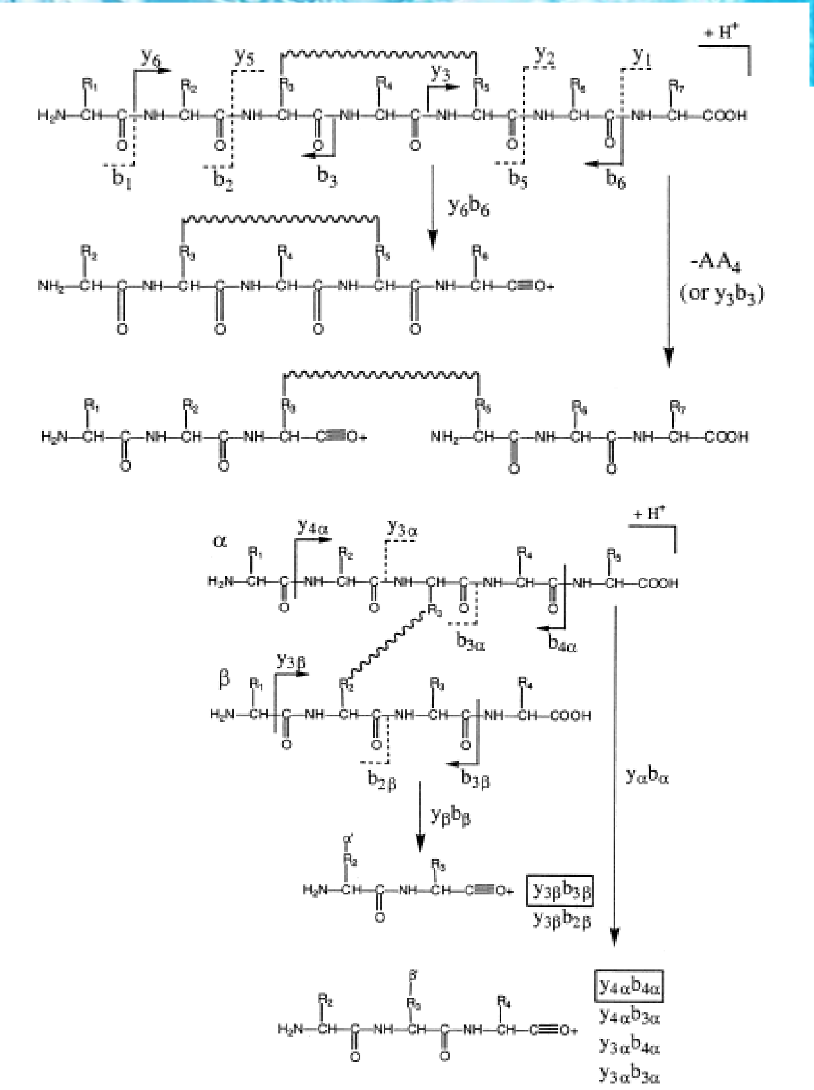
- Chemical Crosslinking
- Trap Sample Cleanup
- Gas-Phase “Purification” of cross-linked species
- Gas-Phase “Digest”, CID, IRMPD, ECD
- Data Analysis, Identify Fragments Containing x-links

Nomenclature of peptide cross-linked fragments



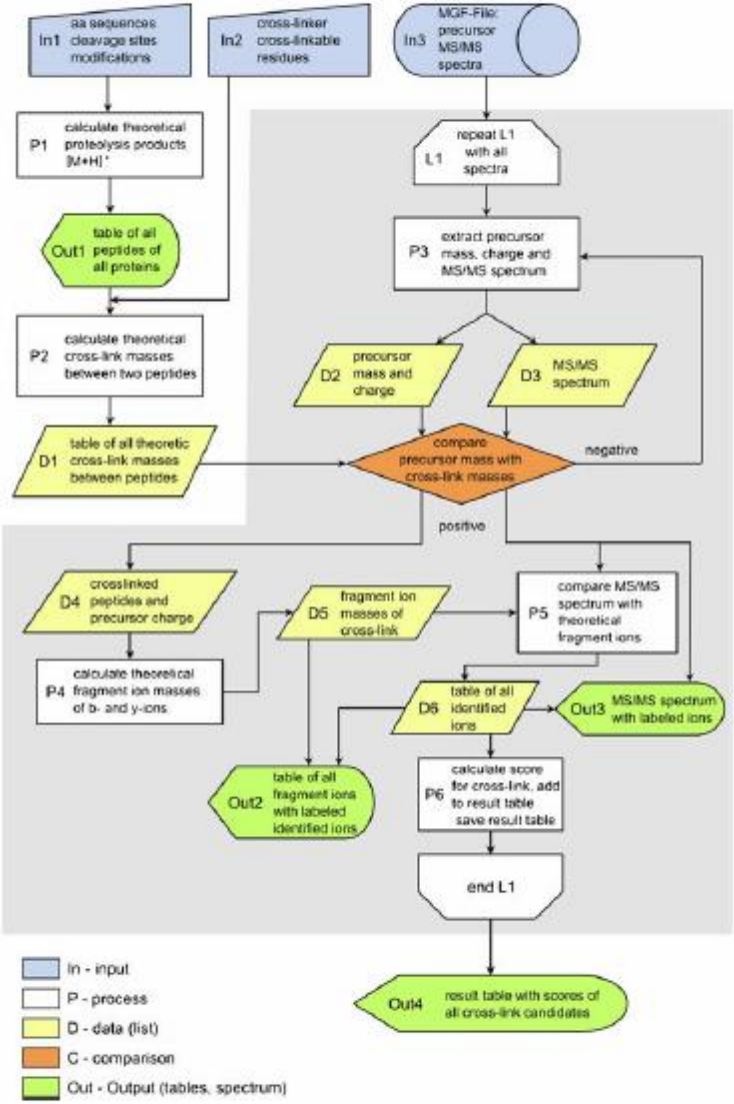
Type 1

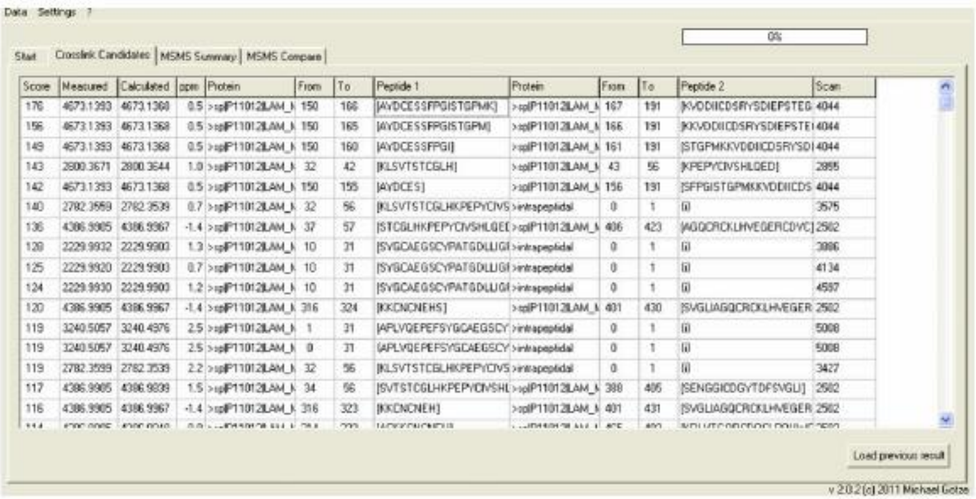
Type 2



Schilling B, Row RH, Gibson BW, Guo X, Young MM. MS2Assign, automated assignment and nomenclature of tandem mass spectra of chemically crosslinked peptides. J Am Soc Mass Spectrom. 2003;14(8):834-850.

Data analysis: STAVROX

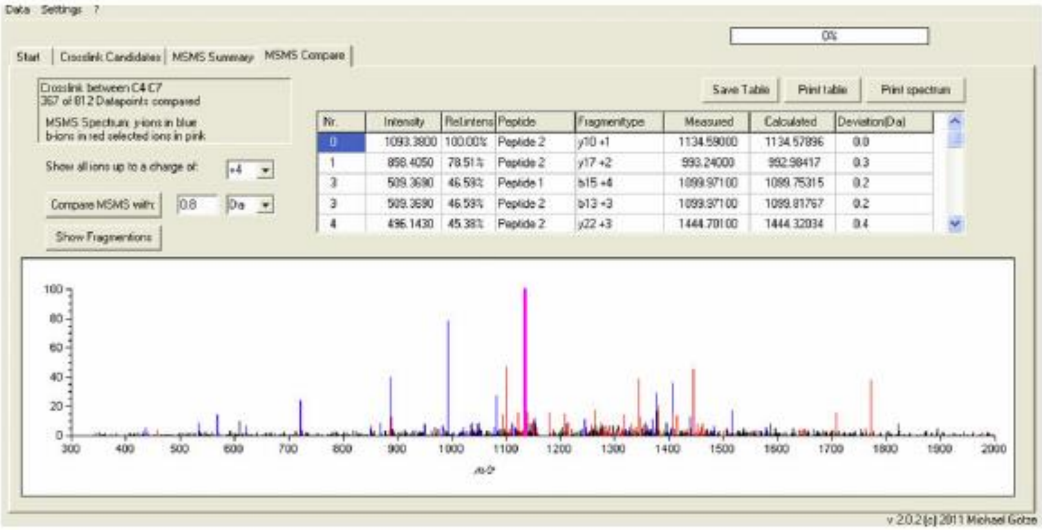




Score	Measured	Calculated	ppm	Protein	From	To	Peptide 1	Protein	From	To	Peptide 2	Scan
176	4673.1393	4673.1368	0.5	>sp|P11012|LAM_	150	166	[AYDCESSFPGISTGPMK]	>sp|P11012|LAM_	167	191	[KVDDIICDSRYSDIEPSTEG	4044
156	4673.1393	4673.1368	0.5	>sp|P11012|LAM_	150	165	[AYDCESSFPGISTGPM]	>sp|P11012|LAM_	166	191	[KKVDDIICDSRYSDIEPSTE	4044
149	4673.1393	4673.1368	0.5	>sp|P11012|LAM_	150	160	[AYDCESSFPGI]	>sp|P11012|LAM_	161	191	[STGPMKKVDDIICDSRYSD	4044
143	2800.3671	2800.3644	1.0	>sp|P11012|LAM_	32	42	[KLSVTSTCGLH]	>sp|P11012|LAM_	43	56	[KPEPYCIVSHLQED]	2895
142	4673.1393	4673.1368	0.5	>sp|P11012|LAM_	150	155	[AYDCES]	>sp|P11012|LAM_	156	191	[SFPGISTGPMKKVDDIICDS	4044
140	2782.3599	2782.3539	0.7	>sp|P11012|LAM_	32	56	[KLSVTSTCGLHKPEPYCIVS	>intrapeptidal	0	1	[i]	3575
136	4386.9905	4386.9967	-1.4	>sp|P11012|LAM_	37	57	[STCGLHKPEPYCIVSHLQED	>sp|P11012|LAM_	406	423	[AGQCRCKLHVEGERCDVC]	2502
128	2229.9932	2229.9900	1.3	>sp|P11012|LAM_	10	31	[SYGCAEGSCYPATGDLLIGI	>intrapeptidal	0	1	[i]	3886
125	2229.9920	2229.9900	0.7	>sp|P11012|LAM_	10	31	[SYGCAEGSCYPATGDLLIGI	>intrapeptidal	0	1	[i]	4134
124	2229.9930	2229.9900	1.2	>sp|P11012|LAM_	10	31	[SYGCAEGSCYPATGDLLIGI	>intrapeptidal	0	1	[i]	4597
120	4386.9905	4386.9967	-1.4	>sp|P11012|LAM_	316	324	[KKCNCNEHS]	>sp|P11012|LAM_	401	430	[SVGLIAGQCRCKLHVEGER	2502
119	3240.5057	3240.4976	2.5	>sp|P11012|LAM_	1	31	[APLVQEPEFSYGCAEGSCY	>intrapeptidal	0	1	[i]	5008
119	3240.5057	3240.4976	2.5	>sp|P11012|LAM_	0	31	(APLVQEPEFSYGCAEGSCY	>intrapeptidal	0	1	[i]	5008
119	2782.3599	2782.3539	2.2	>sp|P11012|LAM_	32	56	[KLSVTSTCGLHKPEPYCIVS	>intrapeptidal	0	1	[i]	3427
117	4386.9905	4386.9839	1.5	>sp|P11012|LAM_	34	56	[SVTSTCGLHKPEPYCIVSHL	>sp|P11012|LAM_	388	405	[SENGGICDGYTDFSVGLI]	2502
116	4386.9905	4386.9967	-1.4	>sp|P11012|LAM_	316	323	[KKCNCNEH]	>sp|P11012|LAM_	401	431	[SVGLIAGQCRCKLHVEGER	2502

v 2.0.2 (c) 2011 Michael Gotze

↓



Crosslink between C4 C7
367 of 812 Datapoints compared

MSMS Spectrum: y-ions in blue
b-ions in red selected ions in pink

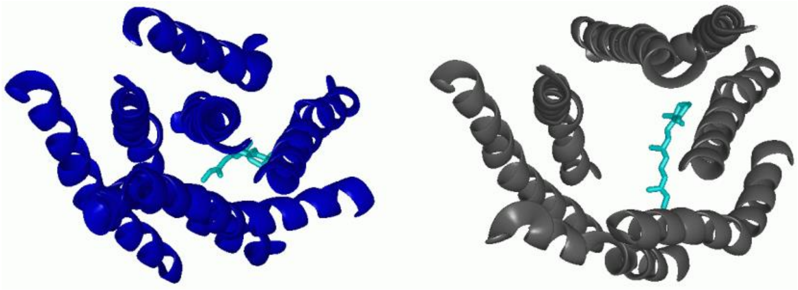
Nr.	Intensity	Rel.intens	Peptide	Fragmenttype	Measured	Calculated	Deviation(Da)
0	1093.3800	100.00%	Peptide 2	y10 +1	1134.59000	1134.57896	0.0
1	858.4050	78.51%	Peptide 2	y17 +2	993.24000	992.98417	0.3
3	509.3690	46.59%	Peptide 1	b15 +4	1099.97100	1099.75315	0.2
3	509.3690	46.59%	Peptide 2	b13 +3	1099.97100	1099.81767	0.2
4	496.1430	45.38%	Peptide 2	y22 +3	1444.70100	1444.32034	0.4

v 2.0.2 (c) 2011 Michael Gotze

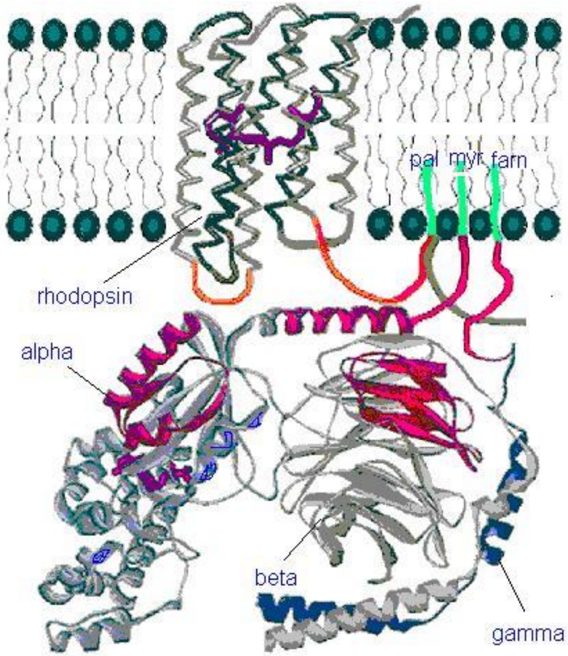
Gotze M. et al. JASMS 2012

Middle down - *Rhodopsin*

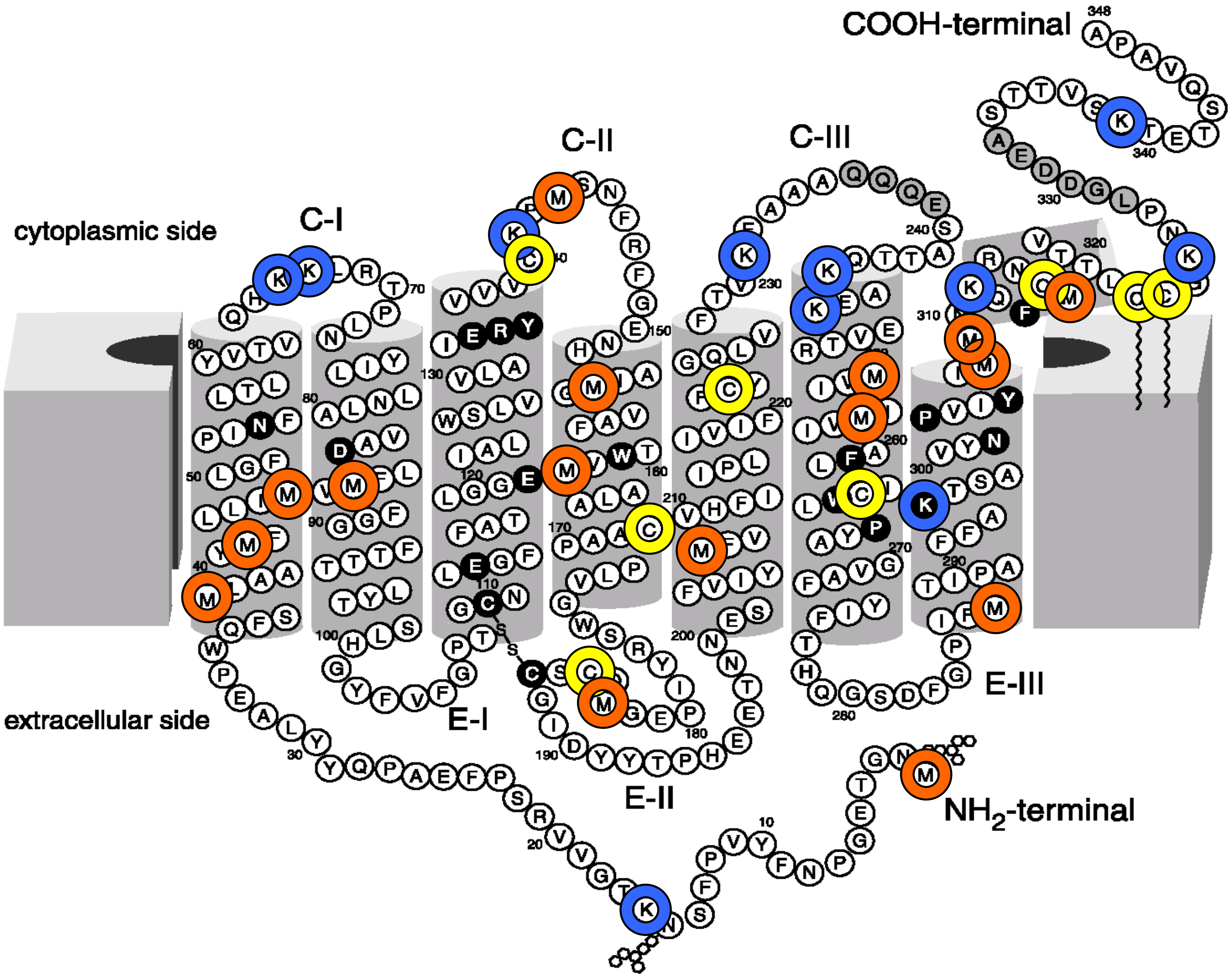
Rhodopsin has open structure/function questions



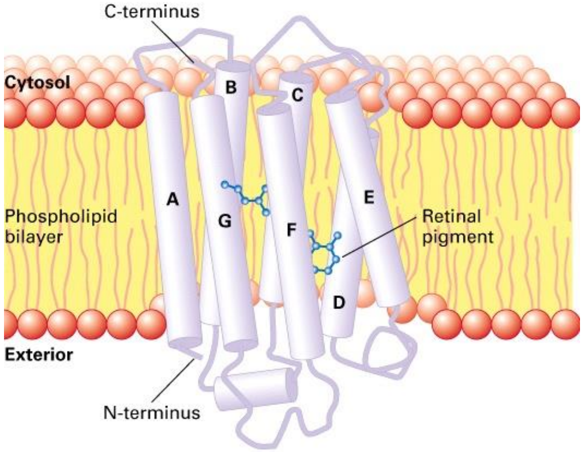
- **What is the conformational change that occurs upon light activation?**
- **What is the configuration of loops involved in Gi binding (not visible on X-ray)?**



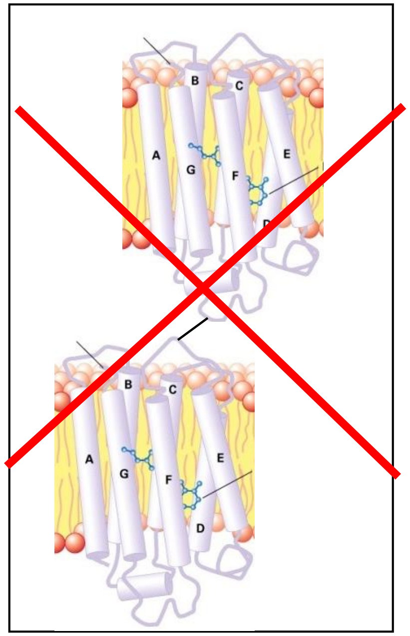
Rhodopsin Has Many Potential Targets for Cross-linking



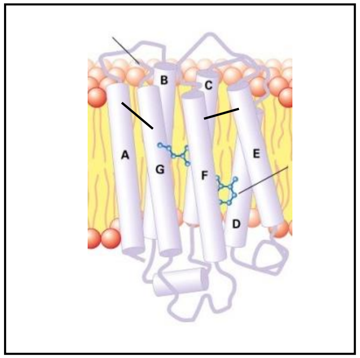
Proteoliposomes/Detergent



Crosslink Protein



Purify Monomer

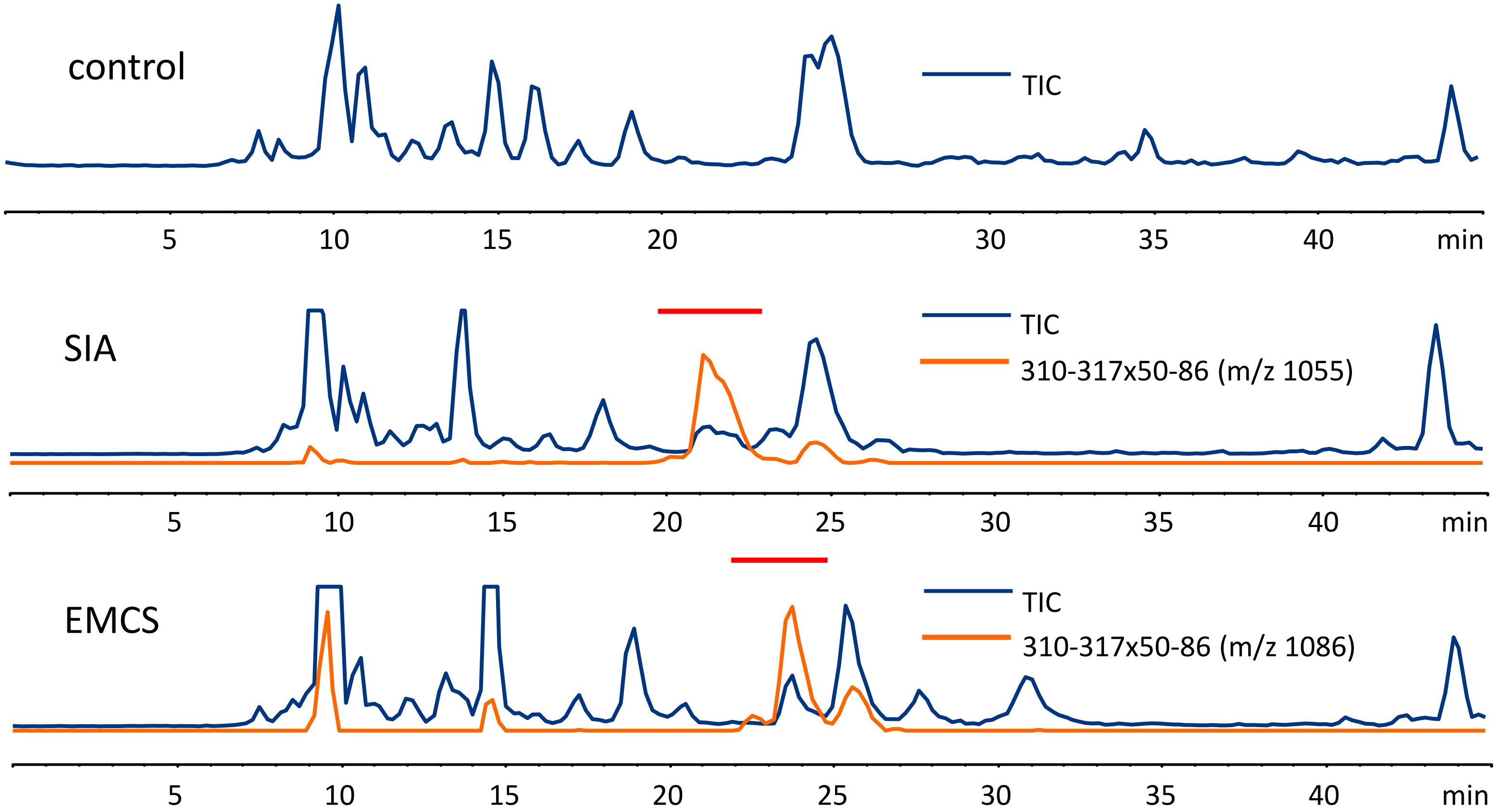


Protein Digestion

LC/MS Analysis

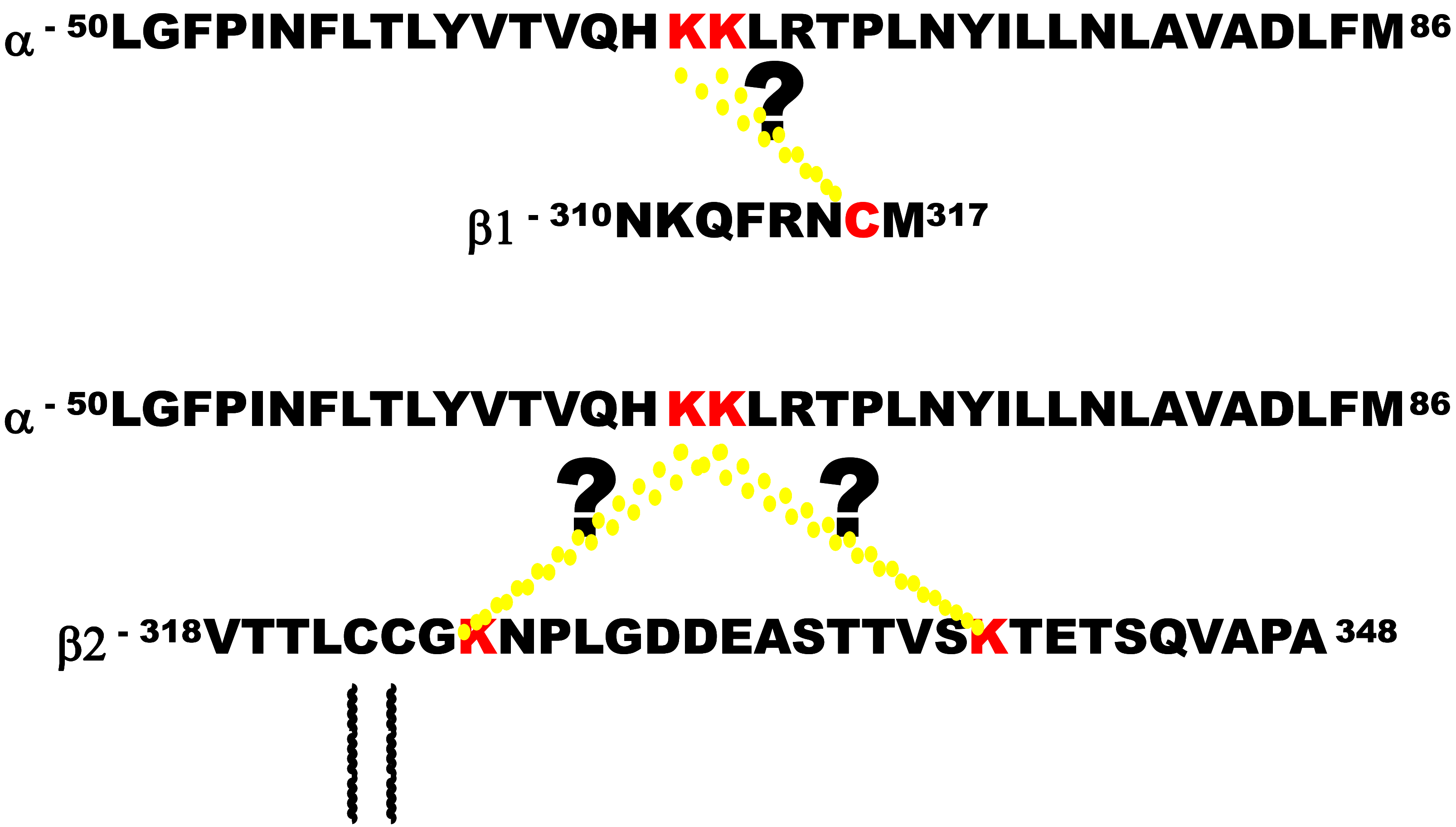
Data Analysis

LCMS analysis of Rhodopsine CNBr digest



Red line corresponds to extracted ion chromatograms of selected cross-linked peptides.

What's wrong? Too many possibilities….



α - ^{50}LGFPINFLTLYVTVQH KKLRTPLNYILLNLAVADLFM^{86}

?

$\beta1$ - ^{310}NKQFRNCM^{317}

α - ^{50}LGFPINFLTLYVTVQH KKLRTPLNYILLNLAVADLFM^{86}

? ?

$\beta2$ - ^{318}VTTLCCGKNPLGDDEASTTVSKTETSQVAPA^{348}

EU FT-ICR MS

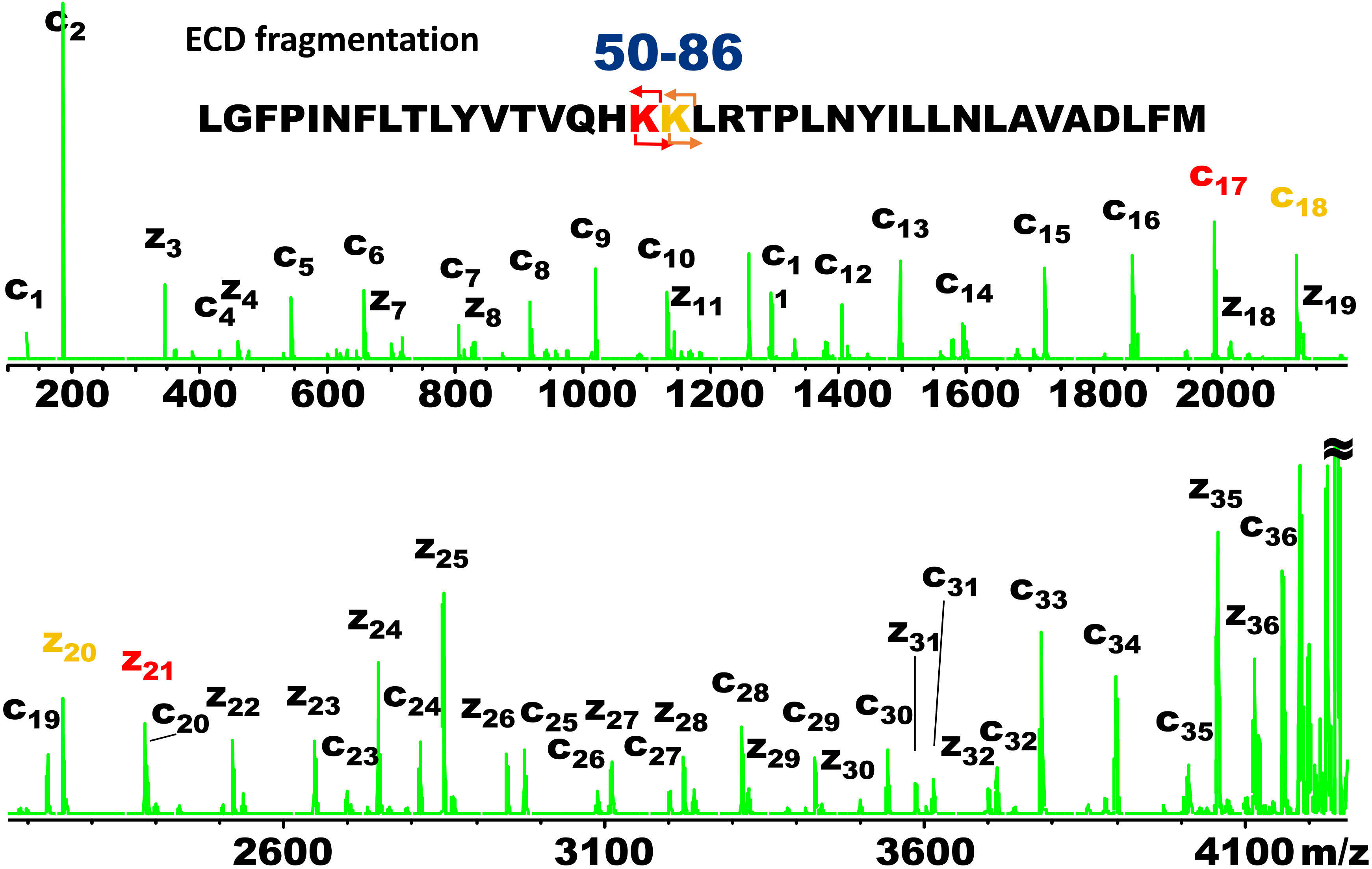
HORIZON 2020

Can We Resolve the Cross-link at K66/K67?

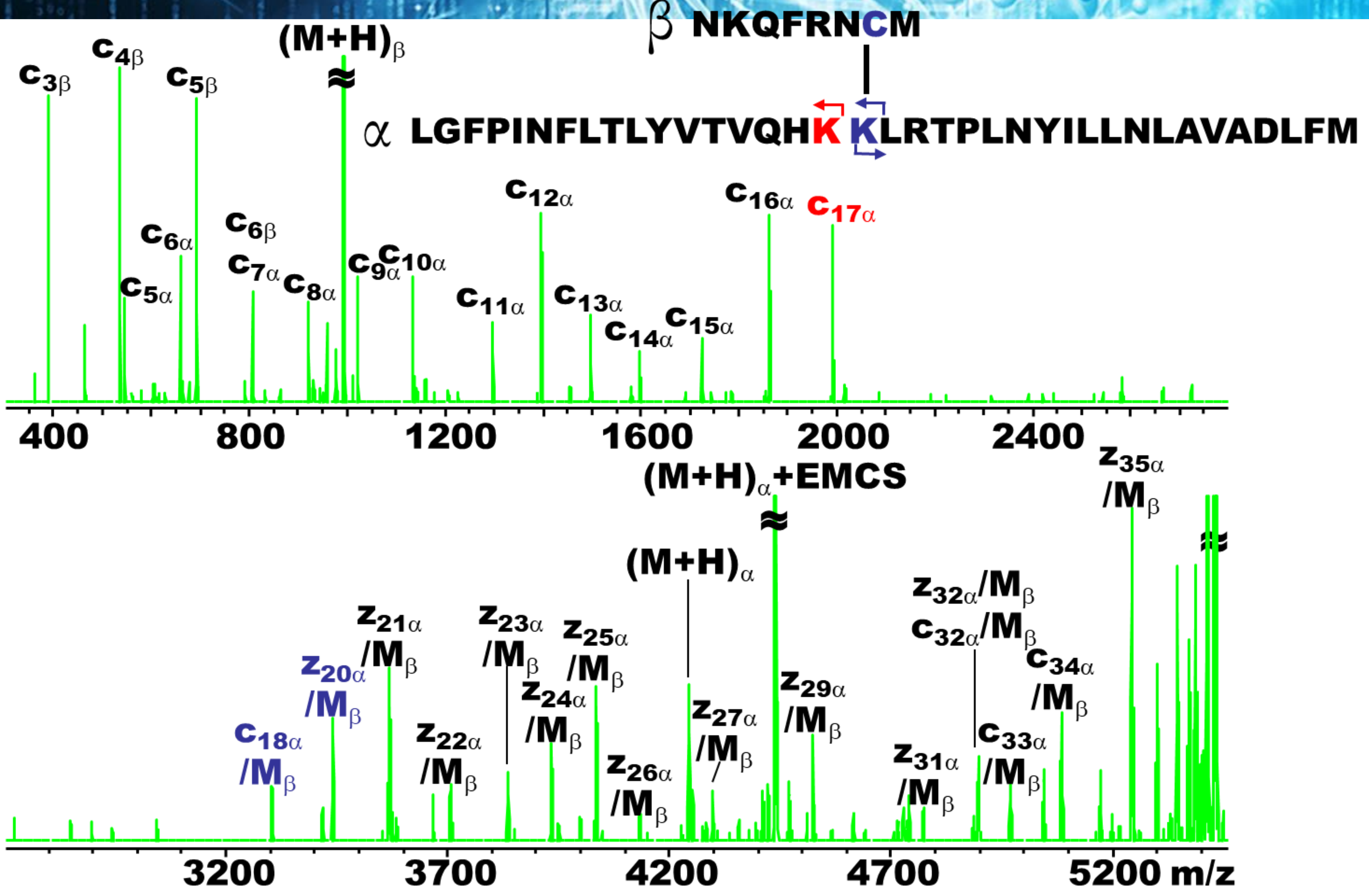
ECD fragmentation

50-86

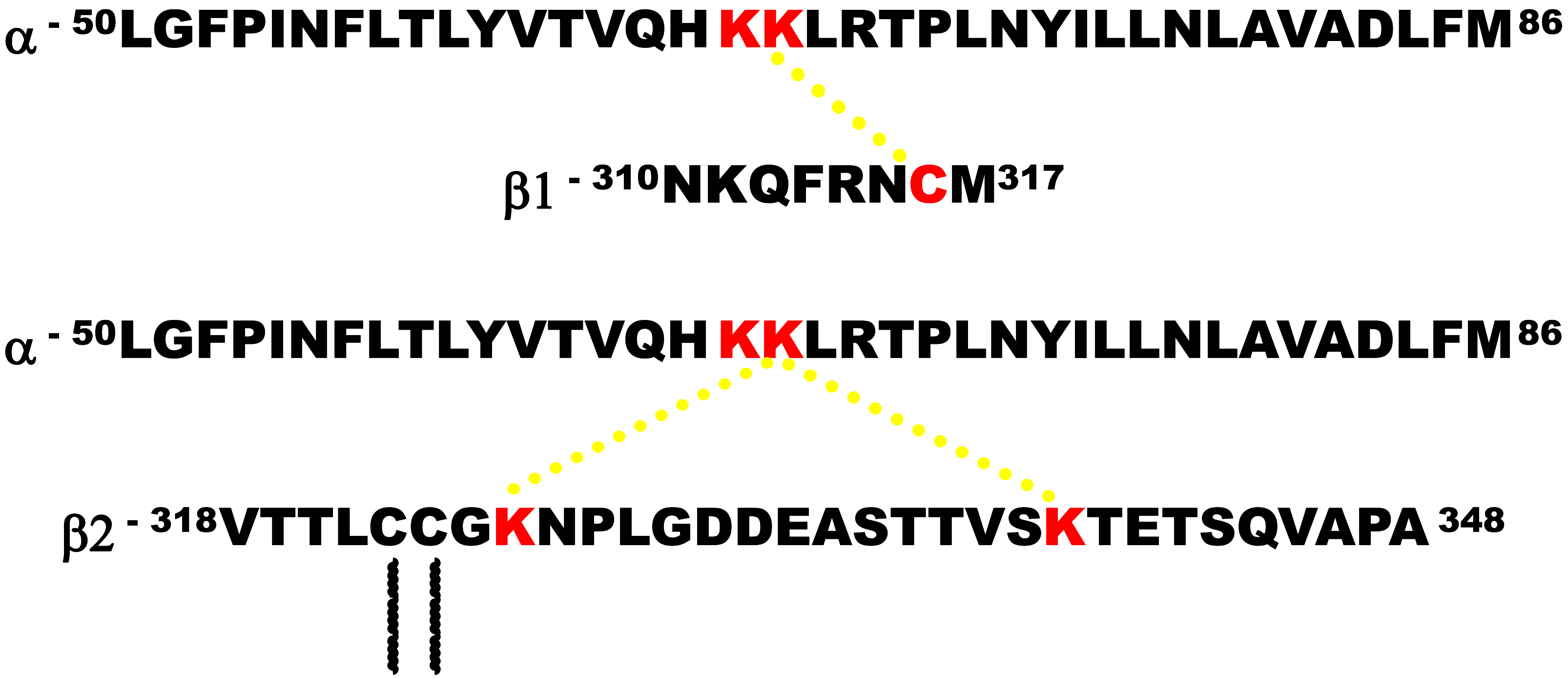
LGFPINFLTLYVTVQHKKLRTPLNYILLNLAVADLFM



ECD fragmentation of cross-linked peptides



ECD enables single residue resolution!



α - ^{50}LGFPINFLTLYVTVQHKKLRTPLNYILLNLAVADLFM^{86}

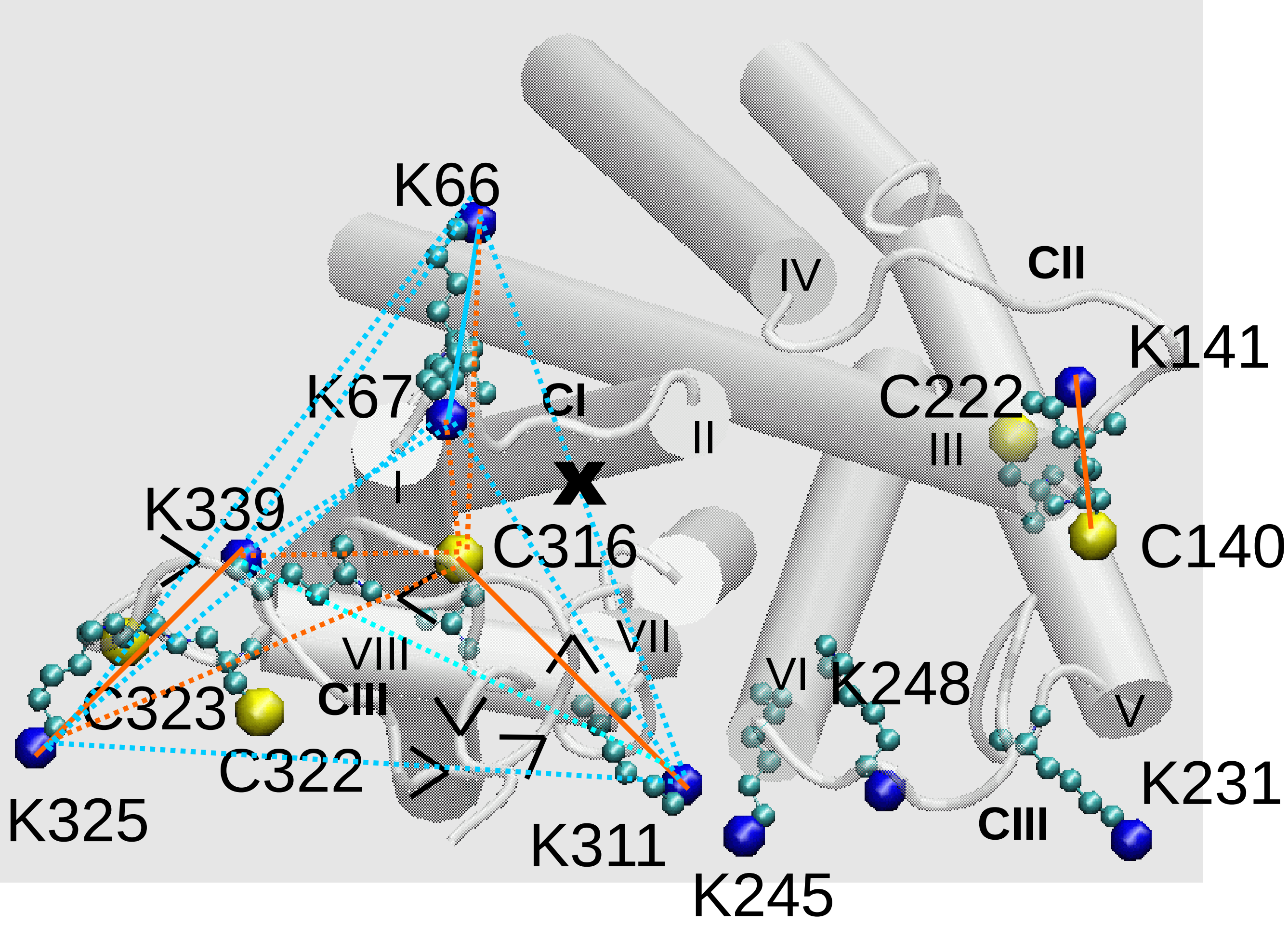
β1 - ^{310}NKQFRNCM^{317}

α - ^{50}LGFPINFLTLYVTVQHKKLRTPLNYILLNLAVADLFM^{86}

β2 - ^{318}VTTLCCGKNPLGDDEASTTVSKTETSQVAPA^{348}

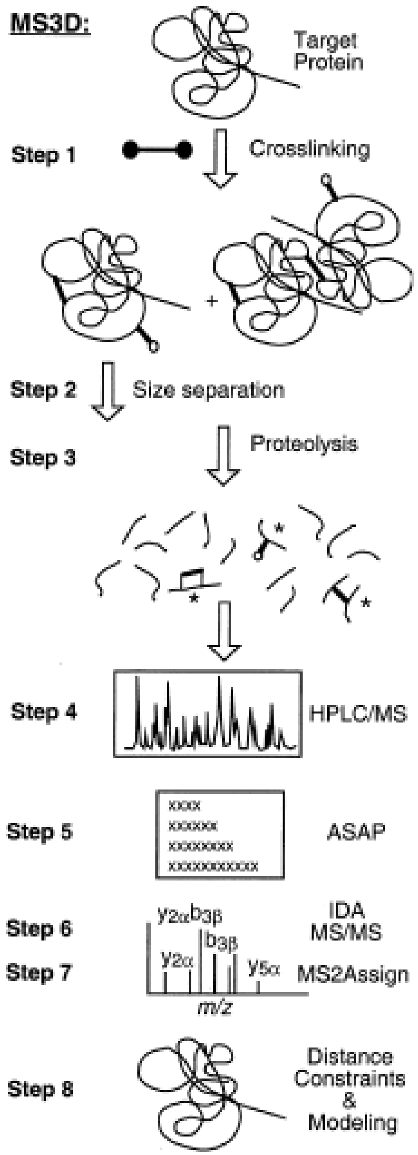
Novak P, Haskins WE, Ayson MJ, et al. Unambiguous assignment of intramolecular chemical cross-links in modified mammalian membrane proteins by Fourier transform-tandem mass spectrometry. Anal Chem. 2005;77(16):5101-5106. doi:10.1021/ac040194r

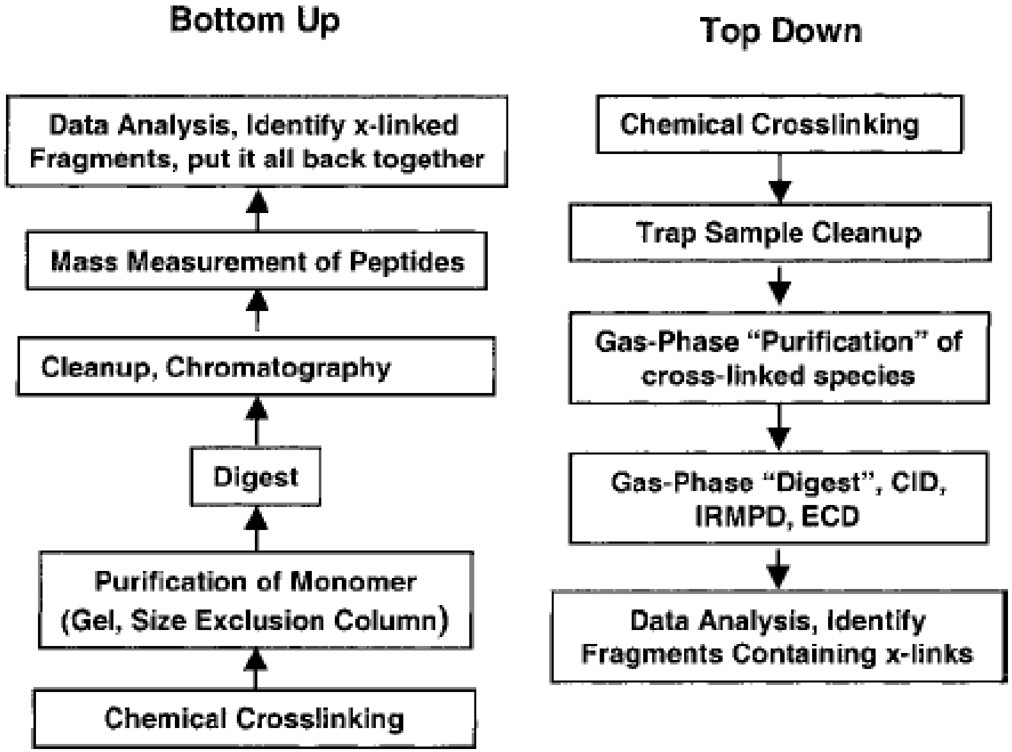
The Cytoplasmic Face of Rhodopsin



Jacobsen RB, Sale KL, Ayson MJ, et al. Structure and dynamics of dark-state bovine rhodopsin revealed by chemical cross-linking and high-resolution mass spectrometry. Protein Sci. 2006;15(6):1303-1317. doi:10.1110/ps.052040406

CXMS experiment: Topdown approach





Bottom Up

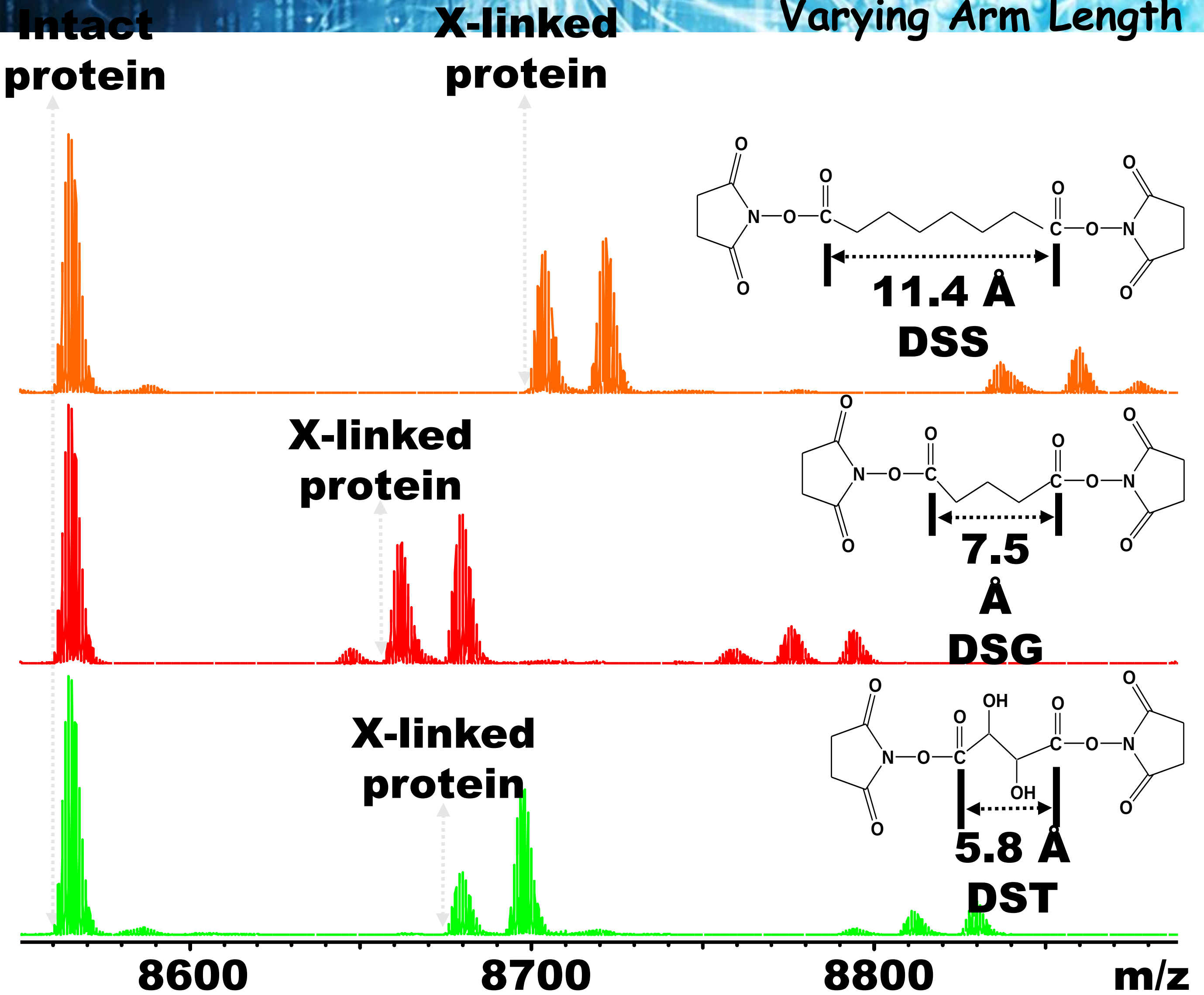
- Data Analysis, Identify x-linked Fragments, put it all back together
- Mass Measurement of Peptides
- Cleanup, Chromatography
- Digest
- Purification of Monomer (Gel, Size Exclusion Column)
- Chemical Crosslinking

Top Down

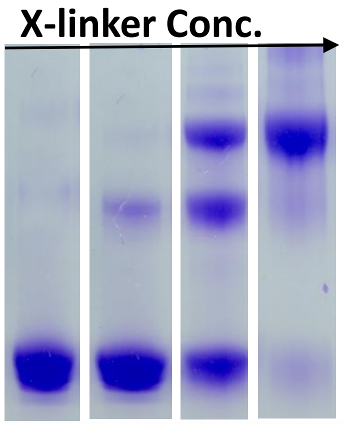
- Chemical Crosslinking
- Trap Sample Cleanup
- Gas-Phase "Purification" of cross-linked species
- Gas-Phase "Digest", CID, IRMPD, ECD
- Data Analysis, Identify Fragments Containing x-links

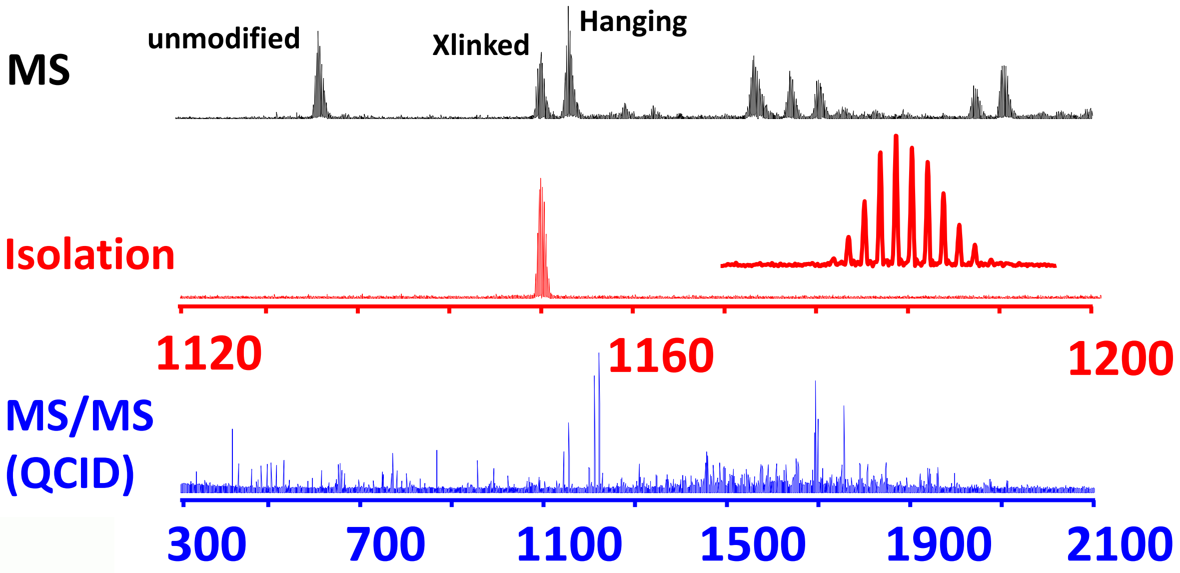
Top down: Cross-linked Ubiquitin with a Series of Cross-linkers

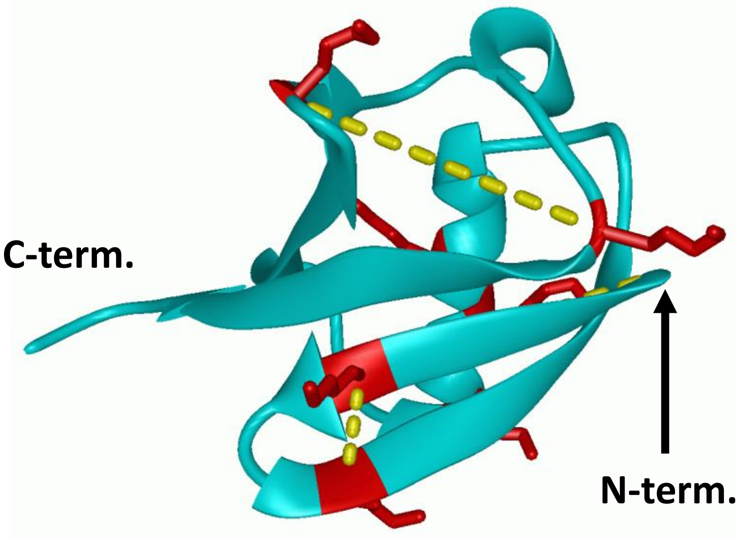
Varying Arm Length



Chemical cross-linking: the identification of cross-link







Cross-Link	DSS	DSG	DST	Constraint $(N_e\text{-}N_e)$
M1 – K6	Yes	Yes	Yes	Distance < 5.8 Å
K6-K11	Yes	Yes	Yes	Distance < 5.8 Å
K48-K63	Yes	Yes	No	5.8 Å < Distance < 7.5 Å

Novak et. al. *Eur. J. Mass Spectrom. 2003, 9, 623*

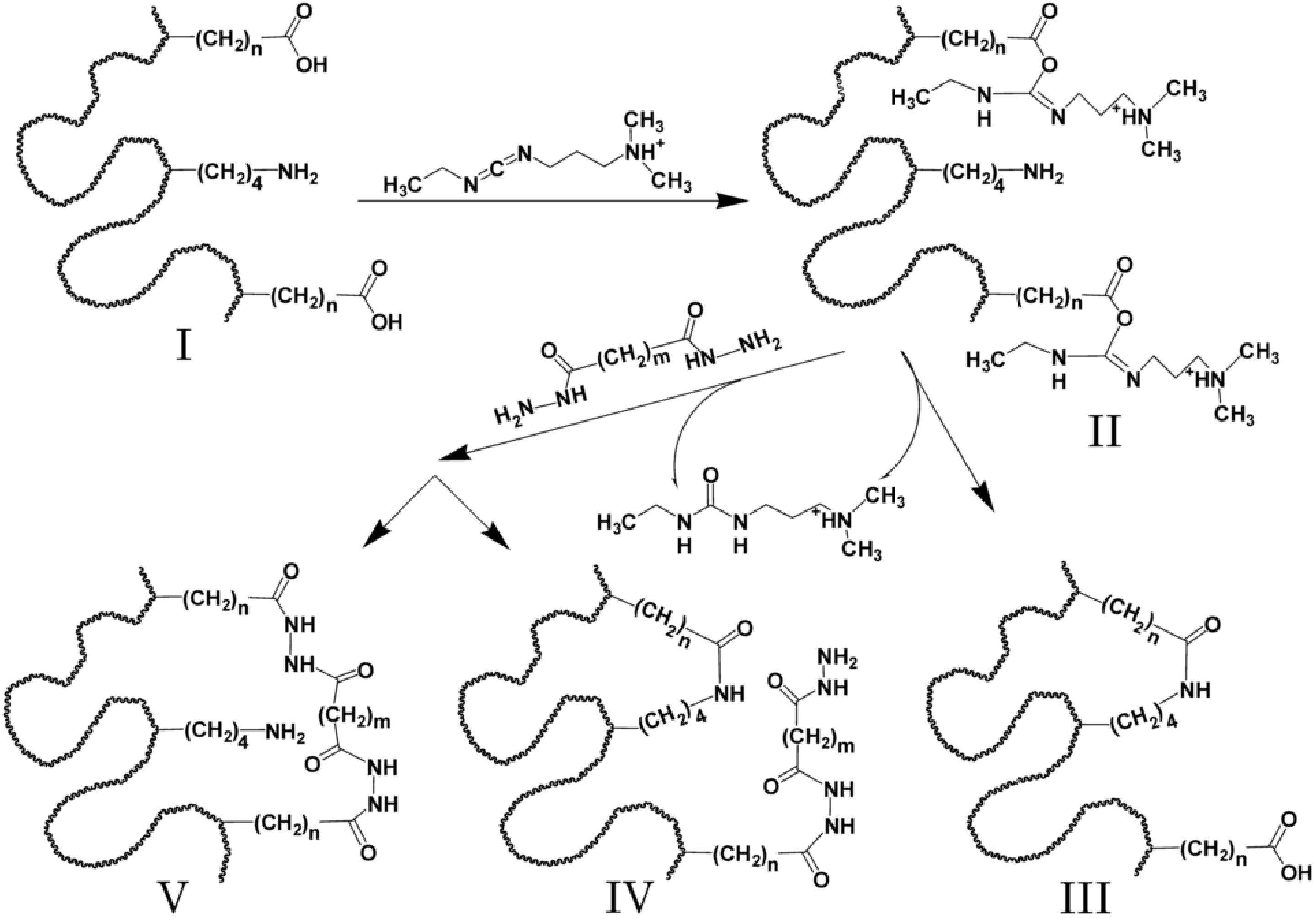
Zero-Length and Carboxy-Carboxy Cross-linking

- “Zero-Length” Cross-linking
    - **No cross-linker used.**
    - **Activate carboxylic acid groups with EDC.**
    - **Activated acid side-chains react with primary amine side-chains (DEO-XK).**
    - **Cross-link formed via new amide linkage.**

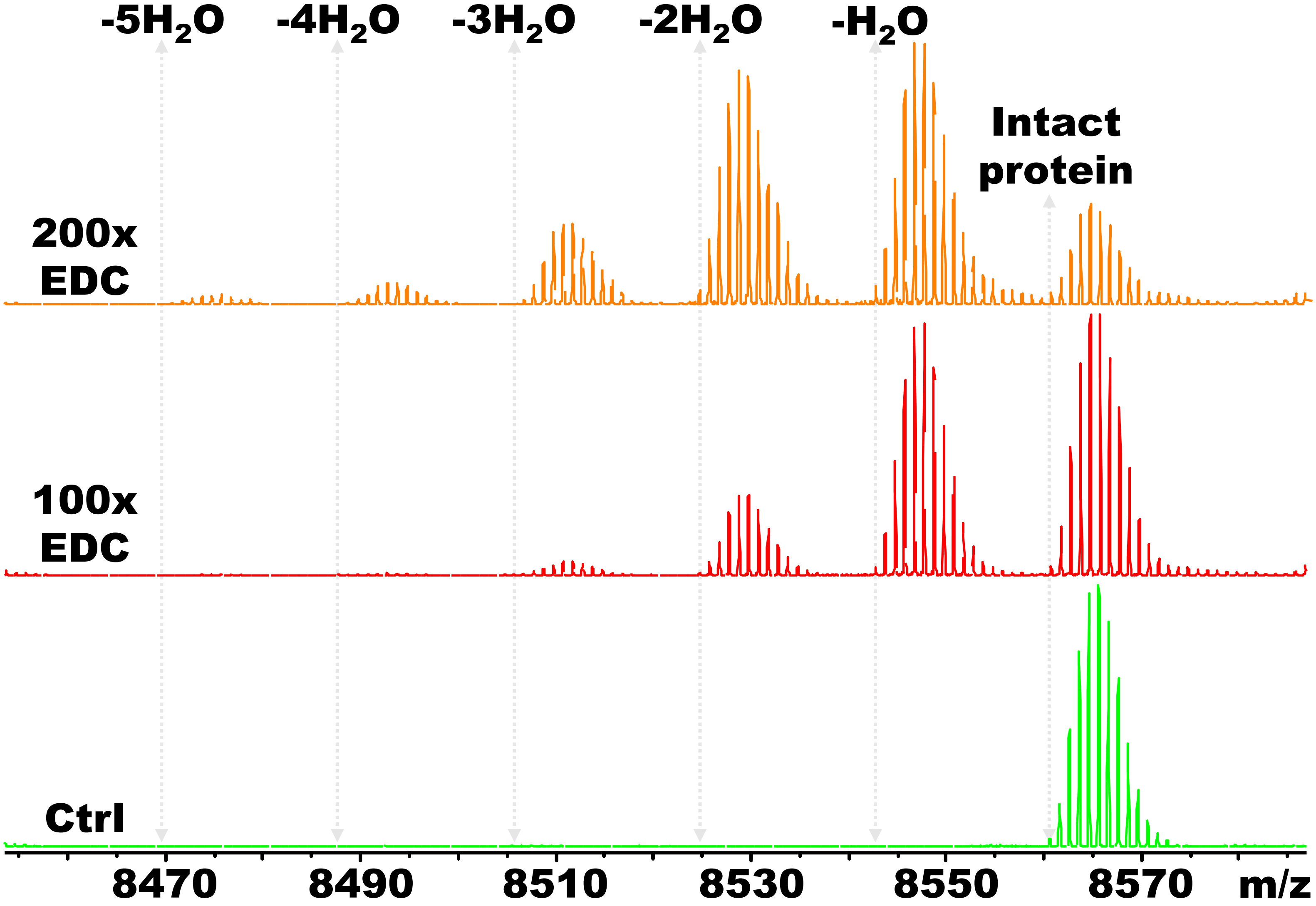
- EDC activation can also be used to cross-link acidic side-chains to each other (DEO-DEO)
    - **Use dihydrazides as the cross-linking reagent.**

Chemical cross-linking: an alternative chemistry

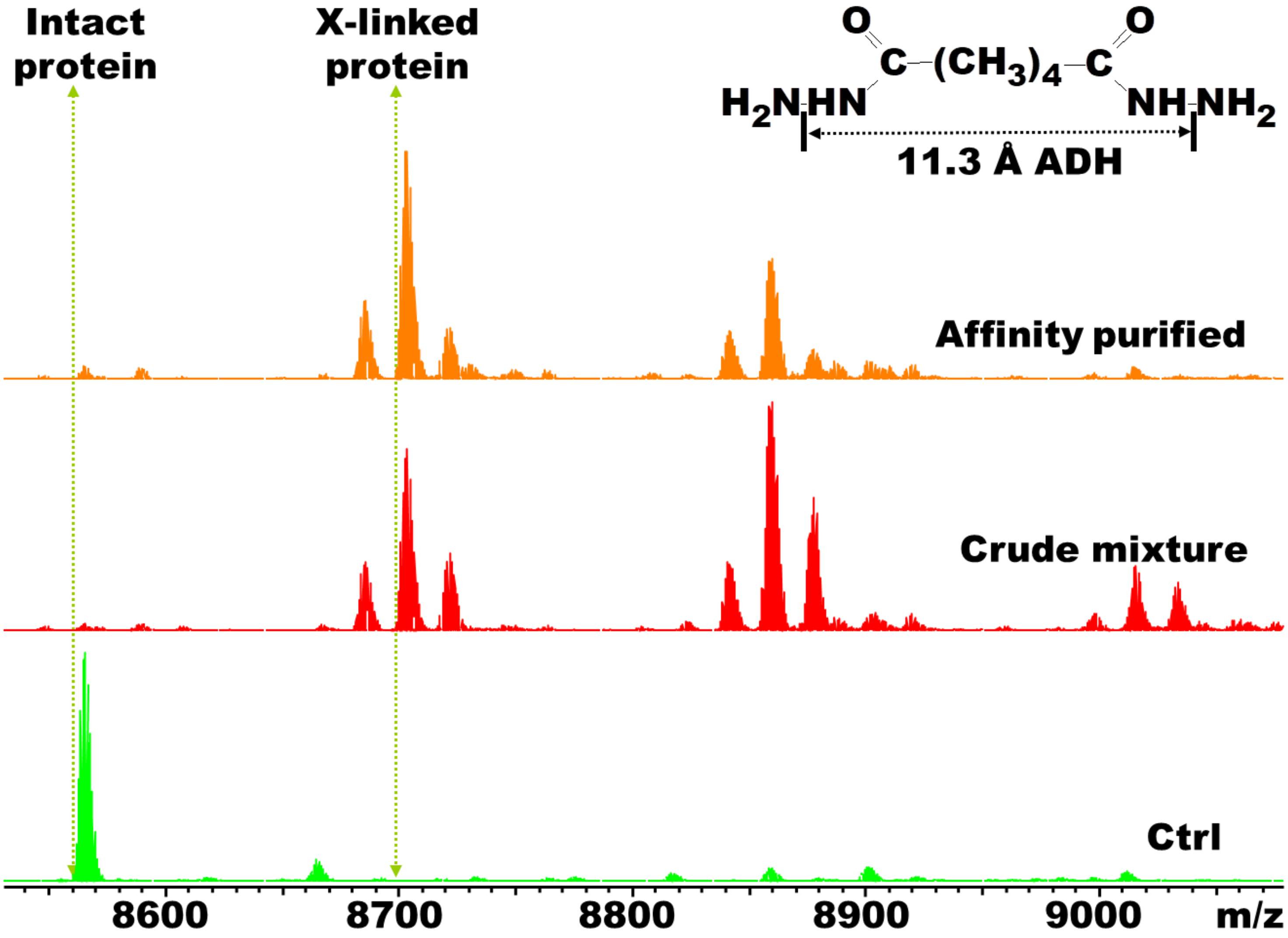
Carboxylic group reactive cross-linkers



Zero-Length Cross-linking

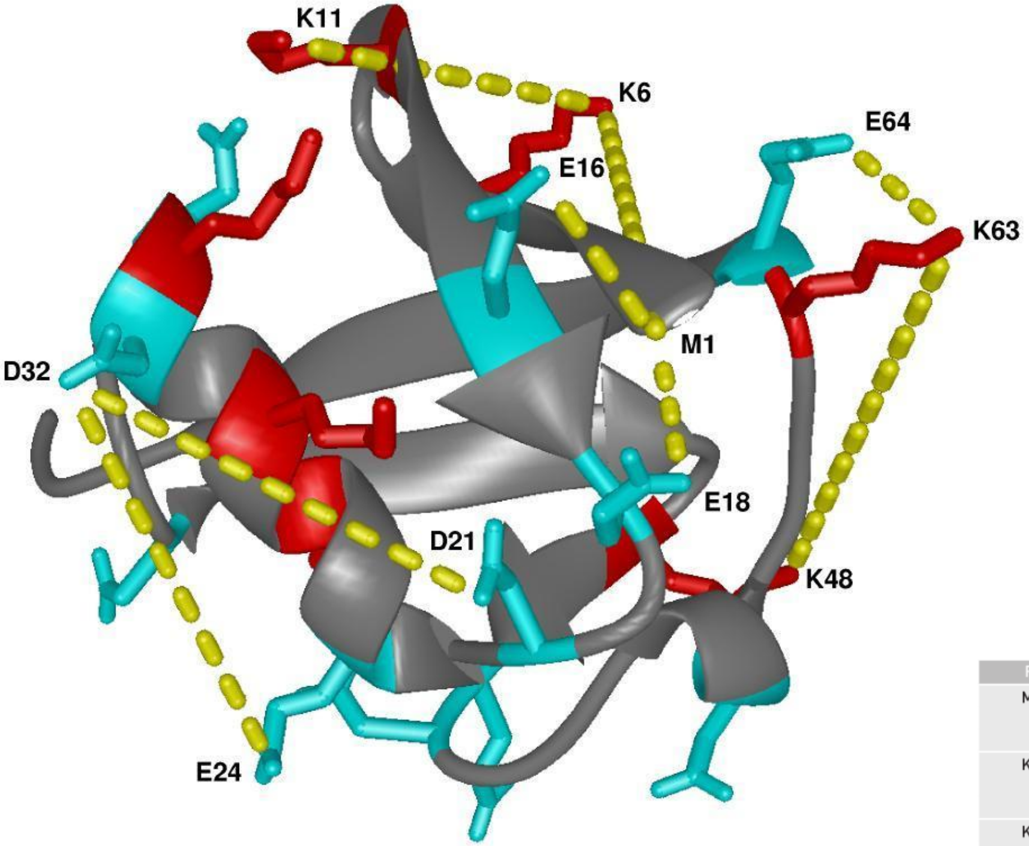


Carboxy-Carboxy Cross-linking



$H_2N\text{-}HN\text{-}C(=O)\text{-}(CH_3)_4\text{-}C(=O)\text{-}NH\text{-}NH_2$

Chemical cross-linking: an alternative chemistry



Residue	Cross-linker	Cross-link	Constraint (X-ray constraint)	Type
M1–K6	DSS	Yes	5.8Å < Distance < 7.5Å (20.0Å)	N_α–N_ε
	DSG	Yes		
	DST	No		
K6–K11	DSS	Yes	Distance < 5.8Å (14.0Å)	N_ε–N_ε
	DSG	Yes		
	DST	Yes		
K48–K63	DSS	Yes	5.8Å < Distance < 7.5Å (19.8Å)	N_ε–N_ε
	DSG	Yes		
	DST	No		
M1–E16	EDC	Yes	Distance < 1.5Å (6.2Å)	N_α–C_δ
M1–E18	EDC	Yes	Distance < 1.5Å (4.4Å)	N_α–C_δ
K63–E64	EDC	Yes	Distance < 1.5Å (4.8Å)	N_ε–C_δ
D21–D32	ADH	Yes	5.8Å < Distance < 7.5Å (12.9Å)	C_γ–C_γ
	SDH	No		
E24–D32	ADH	Yes	5.8Å < Distance < 7.5Å (14.0Å)	C_δ–C_γ
	SDH	No		

Novak et. al. *Eur. J. Mass Spectrom. 2008, 14, 355*

Bottom up/Top down

TOP DOWN MS3D WORKS WELL FOR:

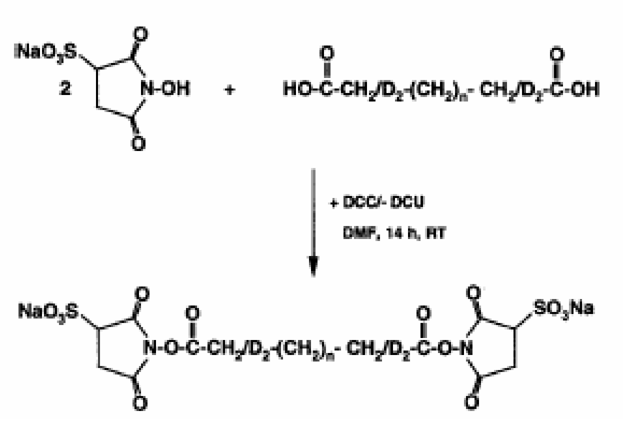
- Characterizing new cross-linking chemistry on small model proteins (< 20 kDa).
- Small proteins with unknown or partially known structures.
- Reactivity studies.

BOTTOM UP APPROACH WITH DIGEST FOR LARGER PROTEINS

- Localizing cross-links with digests alone is difficult
- Combined bottom up/top down approach
  - Digest protein with a single protease
  - Analyze the large cross-linked fragments with top down methods

Introduction of isotopically labeled probes

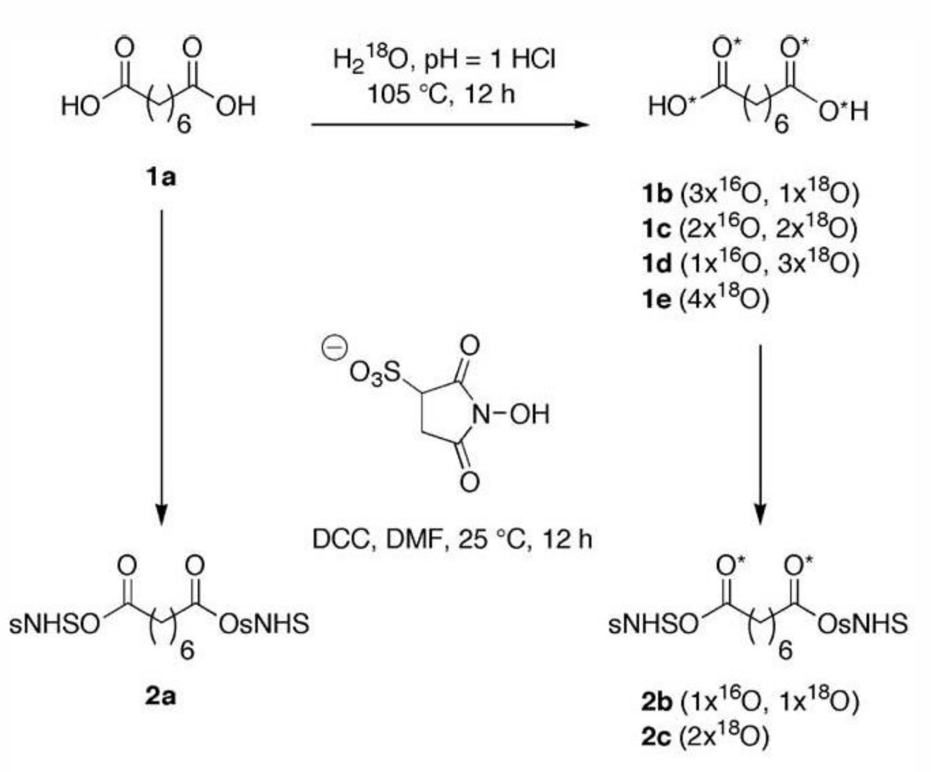
Incorporation of stable isotopes (deuterium) to the linker



^{13}C available as well

Muller DR. et al. Anal. Chem. 2001

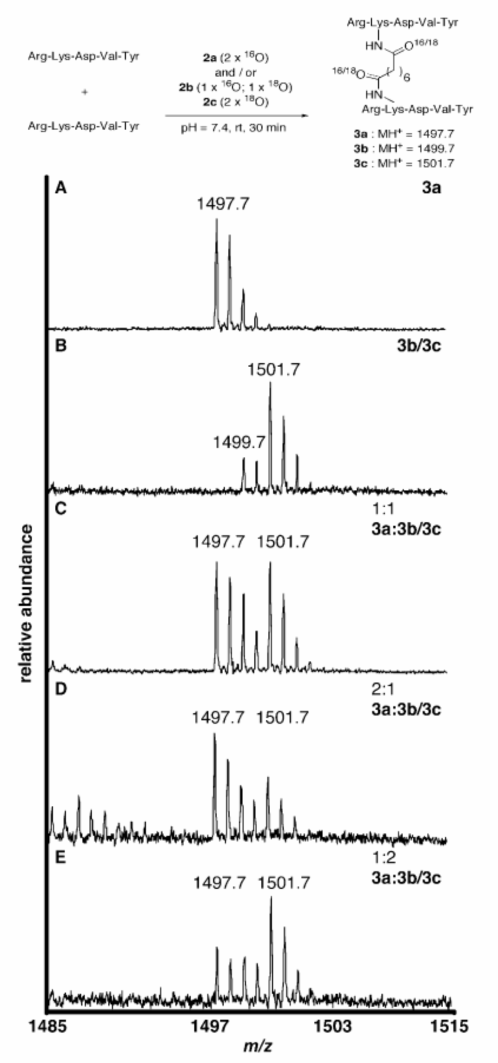
Incorporation of stable isotopes (oxygen, ^{18}O) to the linker



Collins CJ. et al. Bioorg. Med. Chem. Lett. 2003

Introduction of isotopically labeled probes

Simplifies data analysis



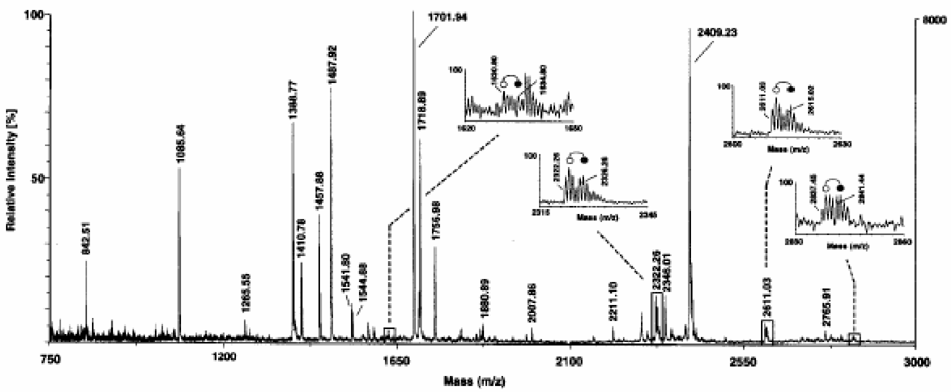
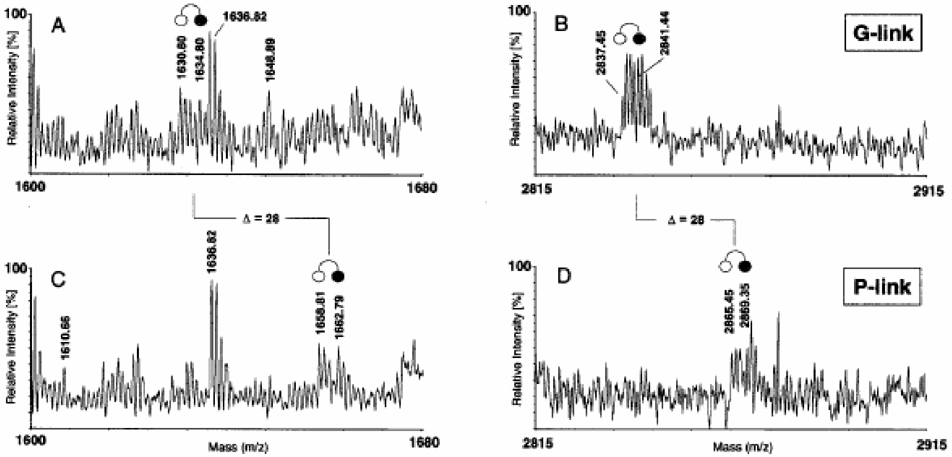


Figure 4. MALDI mass map obtained from G-linked Op18−tubulin complexes with doublet regions expanded in insets.

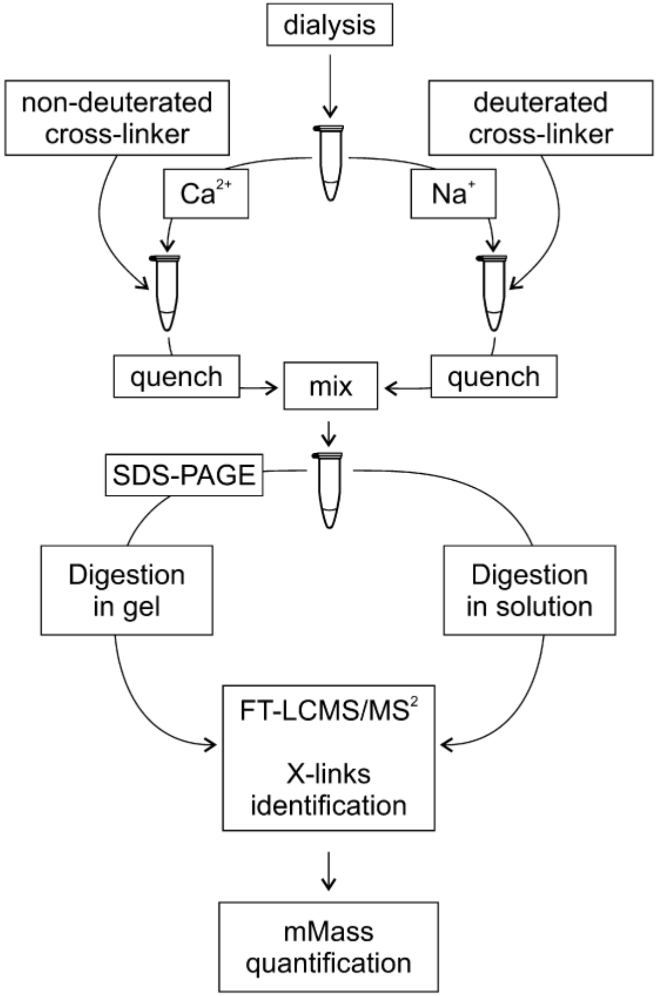


Collins CJ. et al. Bioorg. Med. Chem. Lett. 2003

Muller DR. et al. Anal. Chem. 2001

Quantitative chemical cross-linking

Protein dynamics and conformationa


dialysis

non-deuterated cross-linker

deuterated cross-linker

Ca^{2+}

Na^{+}

quench

mix

quench

SDS-PAGE

Digestion in gel

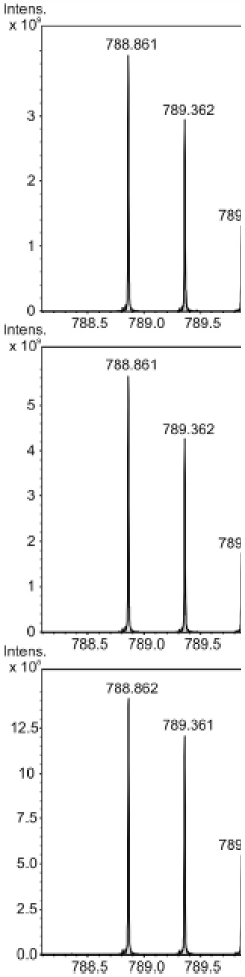
Digestion in solution

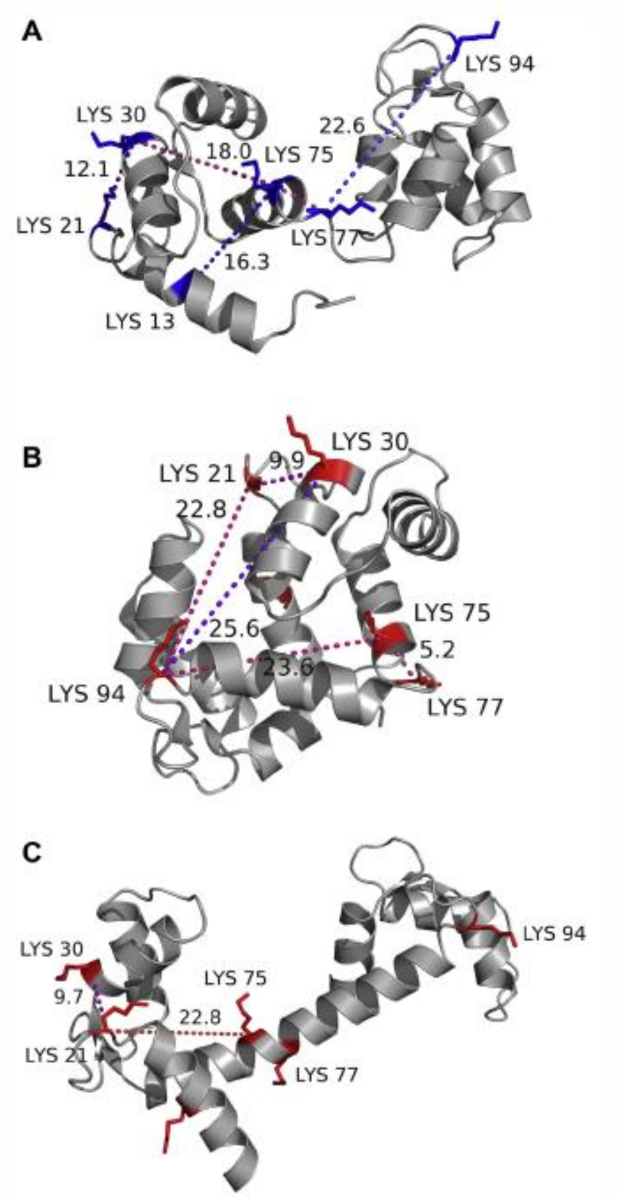
$FT\text{-}LCMS/MS^2$

X-links identification

mMass quantification

Kukacka Z. et al. Methods 2015





er

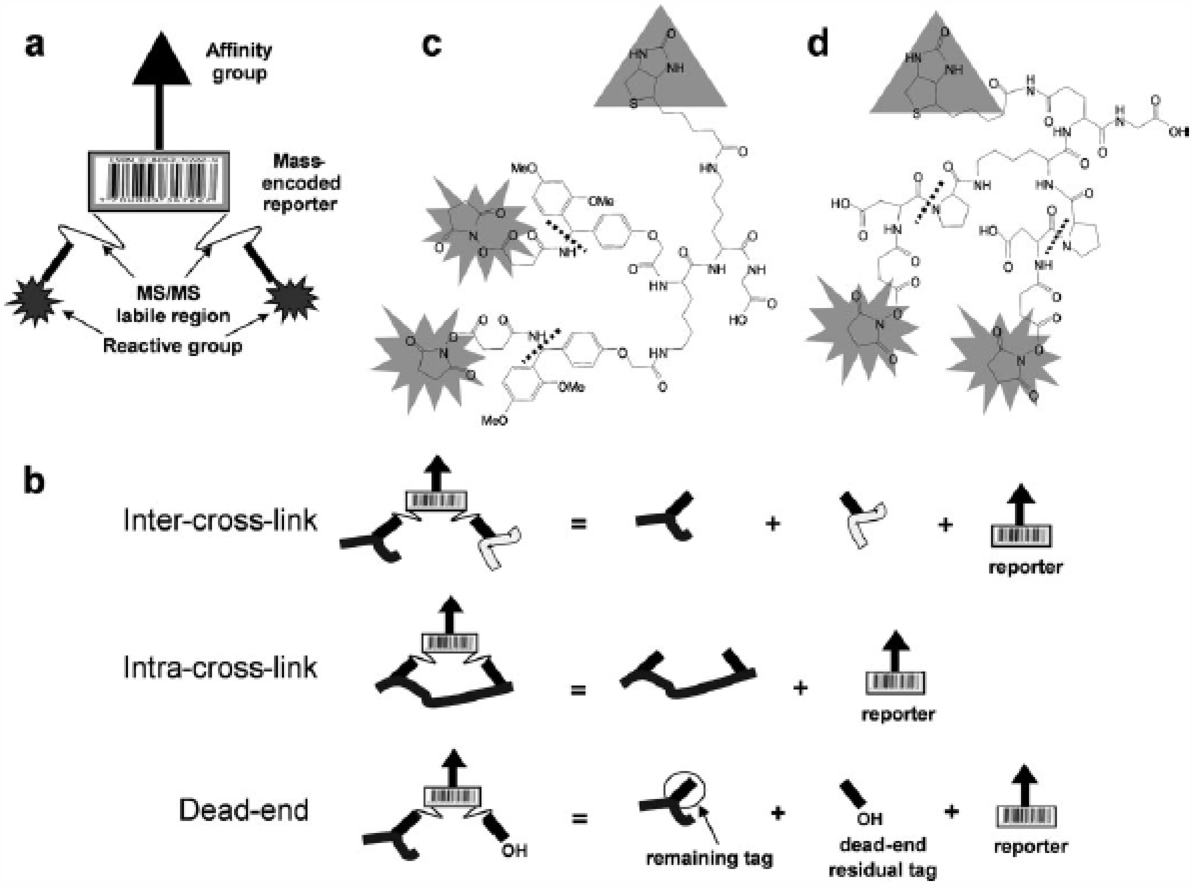
a^+

rs

er

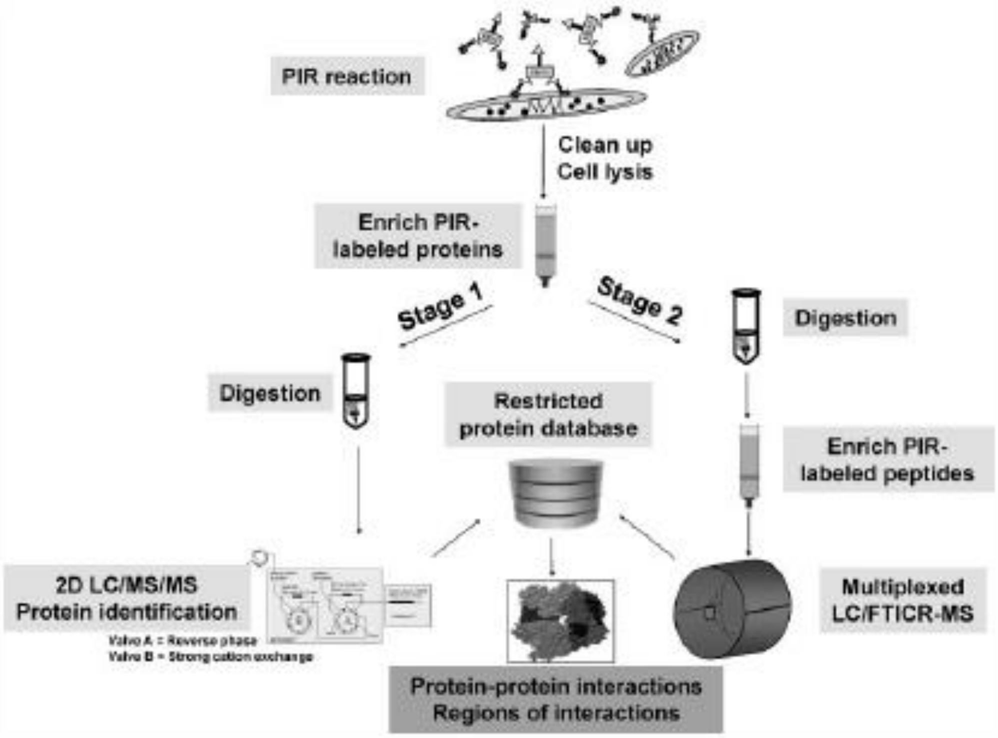
Novel cross-linker design

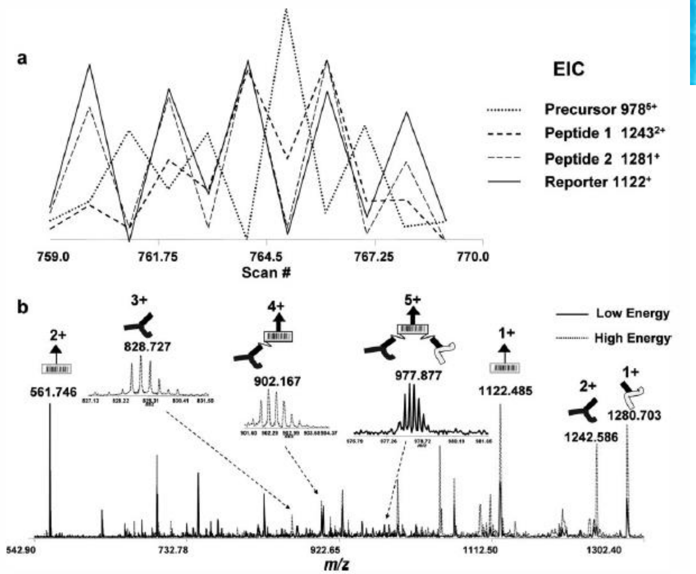
- Affinity tag for purification
- Gas phase cleavable linker for easy data analysis

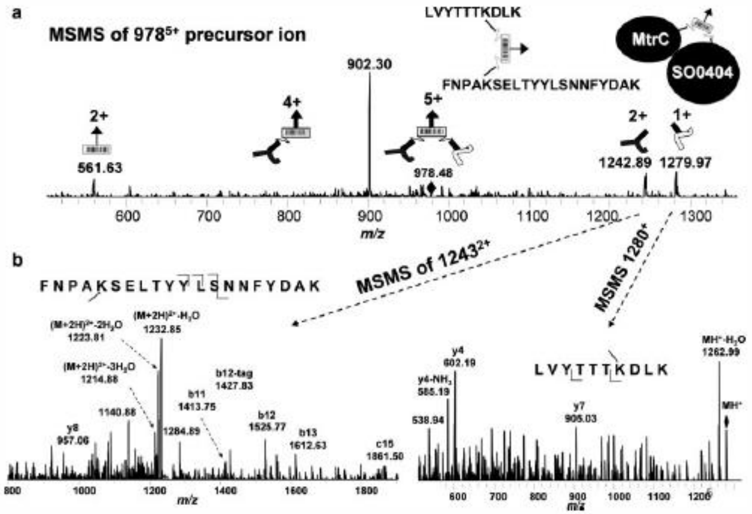


Tang X. et al. Anal. Chem. 2005

Smart design enables in vivo cross-linking

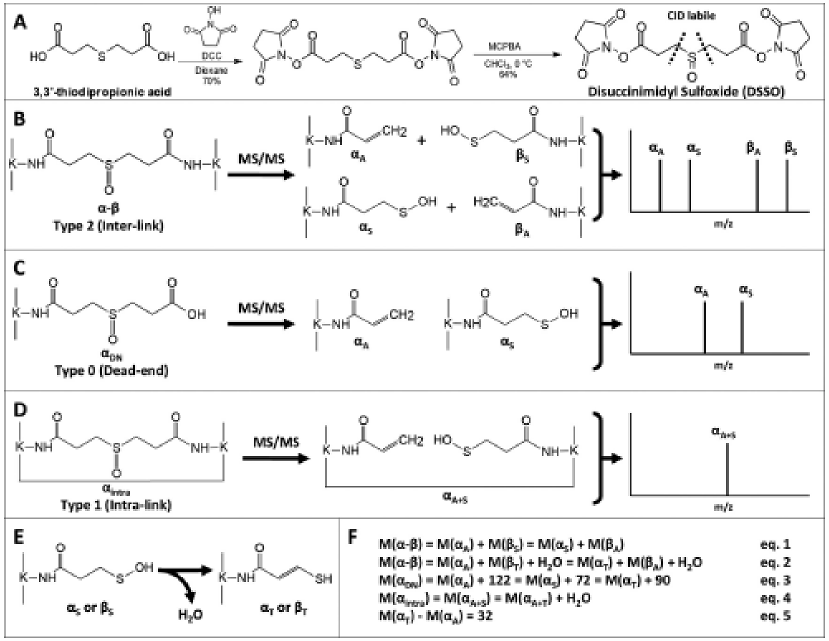






New generation of cleavable cross-linkers

DSSO

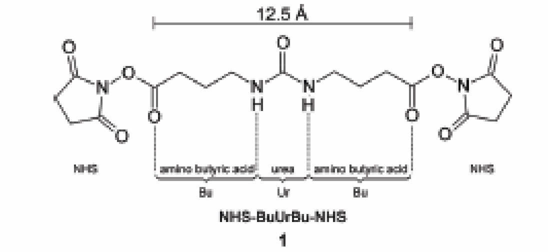


$$M(\alpha\text{-}\beta) = M(\alpha_A) + M(\beta_S) = M(\alpha_S) + M(\beta_A) \qquad \text{eq. 1}$$
$$M(\alpha\text{-}\beta) = M(\alpha_A) + M(\beta_T) + H_2O = M(\alpha_T) + M(\beta_A) + H_2O \qquad \text{eq. 2}$$
$$M(\alpha_{DN}) = M(\alpha_A) + 122 = M(\alpha_S) + 72 = M(\alpha_T) + 90 \qquad \text{eq. 3}$$
$$M(\alpha_{intra}) = M(\alpha_{A+S}) = M(\alpha_{A+T}) + H_2O \qquad \text{eq. 4}$$
$$M(\alpha_T) - M(\alpha_A) = 32 \qquad \text{eq. 5}$$

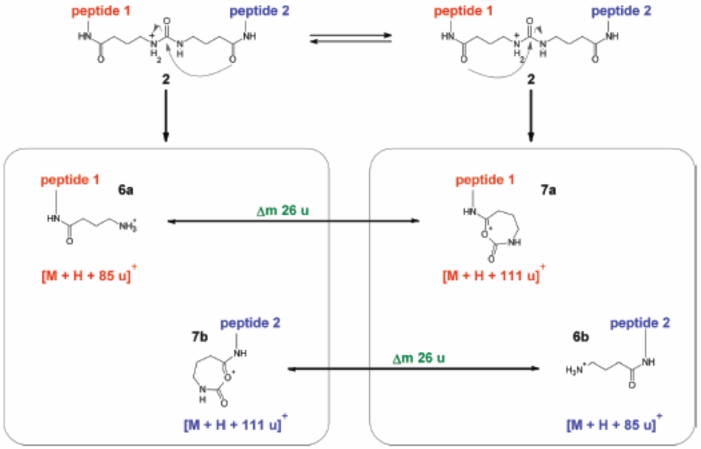
Kao MQ. et al. Mol. Cell Prot. 2010

DSSBu

Scheme 1. Structure of the Symmetric NHS-BuUrBu-NHS Compound (1) for Chemical Cross-Linking

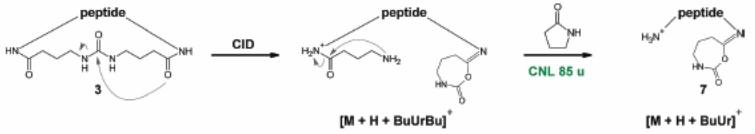


Scheme 3. Fragmentation Mechanism of Protonated 2 upon CID, Delivering Two Complementary Doublets of 26 u Mass Shifted Product Ions[a]



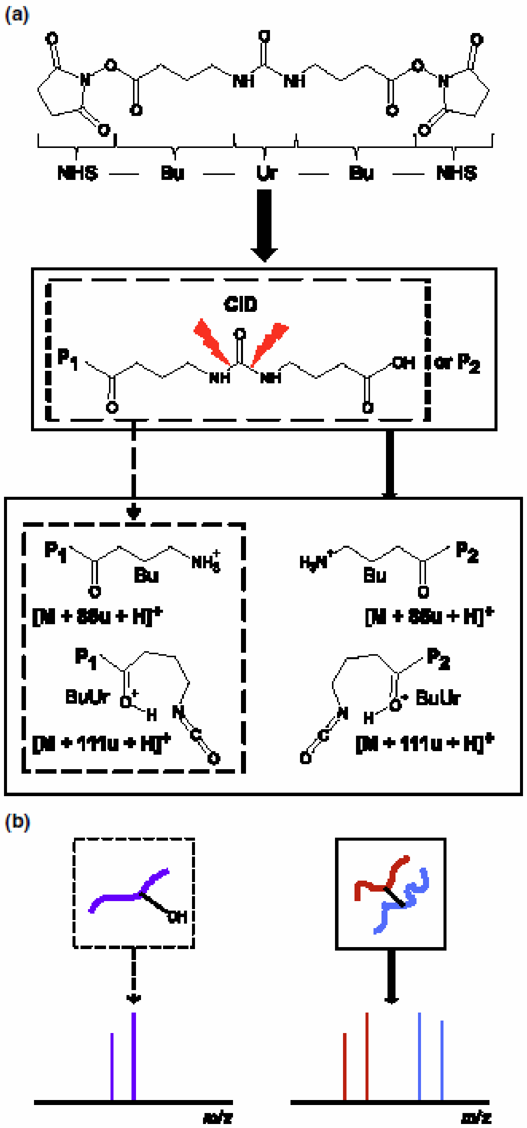
[a] Product ions of peptide 1 are **6a** and **7a**, and product ions of peptide 2 are **6b** and **7b**.

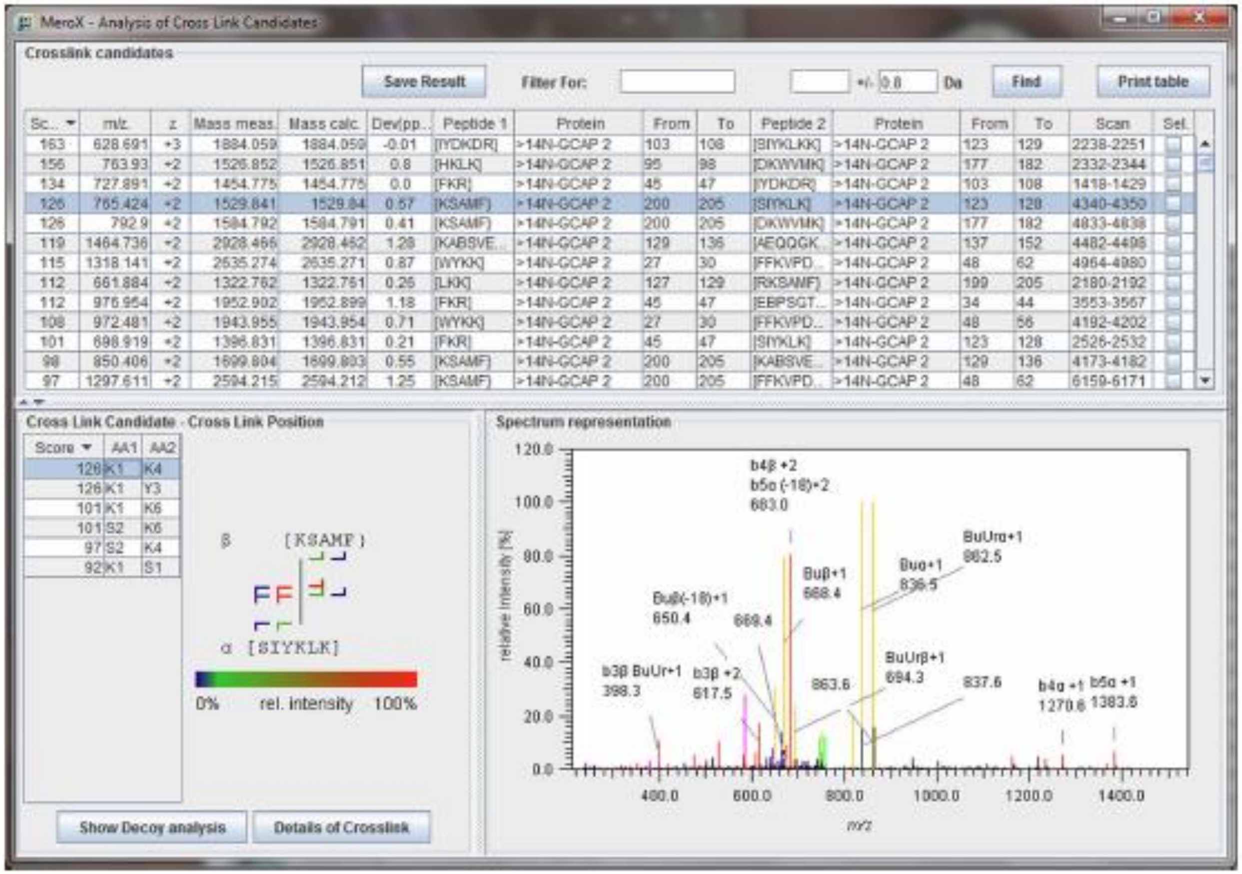
Scheme 4. Fragmentation Mechanism of a Protonated Type 1 Modified Peptide (3) upon CID, Delivering a Product Ion That Is Modified with BuUr $[M + H + BuUr]^+$ (7) by a CNL of Pyrolidinone (85 u)



Muller A. et al. Anal. Chem. 2011

Data analysis: MEROX





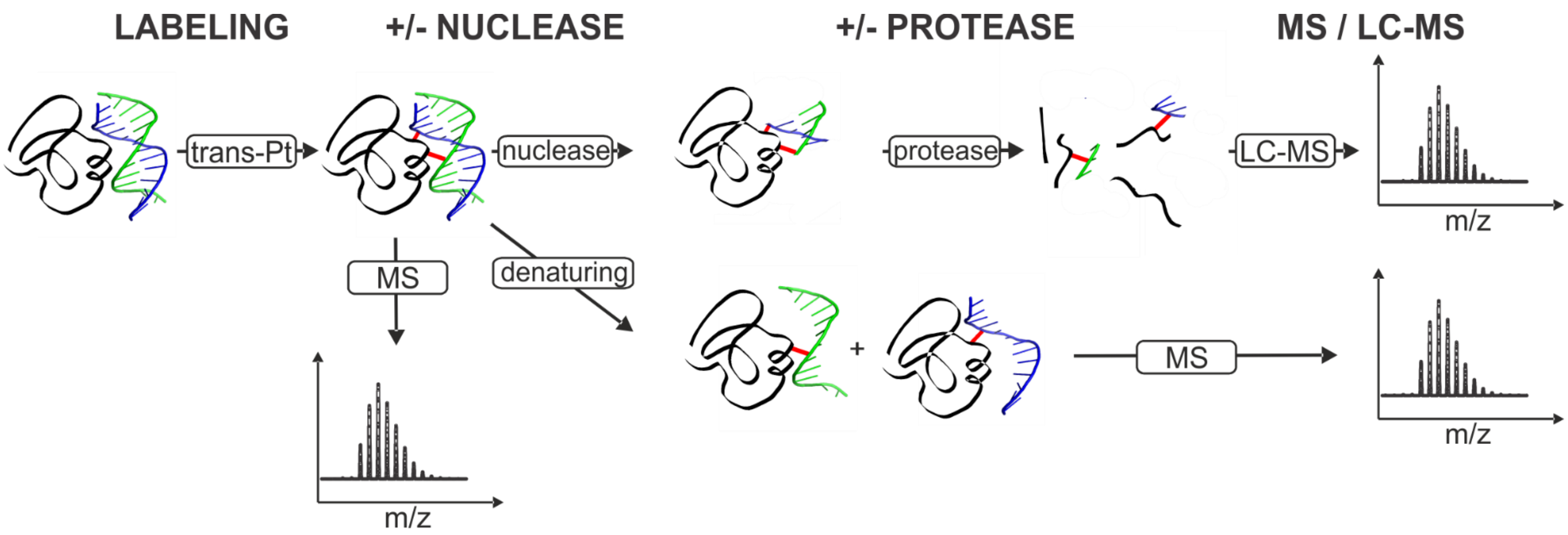
Sc...	m/z	z	Mass meas.	Mass calc.	Dev[pp...	Peptide 1	Protein	From	To	Peptide 2	Protein	From	To	Scan	Sel.
163	628.691	+3	1884.059	1884.059	-0.01	[IYDKDR]	>14N-GCAP 2	103	108	[SIYKLKK]	>14N-GCAP 2	123	129	2238-2251	☐
156	763.93	+2	1526.852	1526.851	0.8	[HKLK]	>14N-GCAP 2	95	98	[DKWVMK]	>14N-GCAP 2	177	182	2332-2344	☐
134	727.891	+2	1454.775	1454.775	0.0	[FKR]	>14N-GCAP 2	45	47	[IYDKDR]	>14N-GCAP 2	103	108	1418-1429	☐
126	765.424	+2	1529.841	1529.84	0.57	[KSAMF]	>14N-GCAP 2	200	205	[SIYKLK]	>14N-GCAP 2	123	128	4340-4350	☐
126	792.9	+2	1584.792	1584.791	0.41	[KSAMF]	>14N-GCAP 2	200	205	[DKWVMK]	>14N-GCAP 2	177	182	4833-4838	☐
119	1464.736	+2	2928.466	2928.462	1.28	[KABSVE...	>14N-GCAP 2	129	136	[AEQQGK...	>14N-GCAP 2	137	152	4482-4498	☐
115	1318.141	+2	2635.274	2635.271	0.87	[WYKK]	>14N-GCAP 2	27	30	[FFKVPD...	>14N-GCAP 2	48	62	4964-4980	☐
112	661.884	+2	1322.762	1322.761	0.26	[LKK]	>14N-GCAP 2	127	129	[RKSAMF]	>14N-GCAP 2	199	205	2180-2192	☐
112	976.954	+2	1952.902	1952.899	1.18	[FKR]	>14N-GCAP 2	45	47	[EBPSGT...	>14N-GCAP 2	34	44	3553-3567	☐
108	972.481	+2	1943.955	1943.954	0.71	[WYKK]	>14N-GCAP 2	27	30	[FFKVPD...	>14N-GCAP 2	48	56	4192-4202	☐
101	698.919	+2	1396.831	1396.831	0.21	[FKR]	>14N-GCAP 2	45	47	[SIYKLK]	>14N-GCAP 2	123	128	2526-2532	☐
98	850.406	+2	1699.804	1699.803	0.55	[KSAMF]	>14N-GCAP 2	200	205	[KABSVE...	>14N-GCAP 2	129	136	4173-4182	☐
97	1297.611	+2	2594.215	2594.212	1.25	[KSAMF]	>14N-GCAP 2	200	205	[FFKVPD...	>14N-GCAP 2	48	62	6159-6171	☐

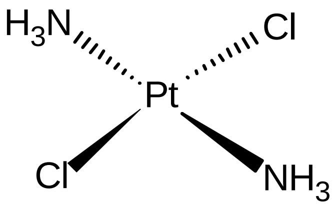
Cross Link Candidate - Cross Link Position

Score	AA1	AA2
126	K1	K4
126	K1	Y3
101	K1	K6
101	S2	K6
97	S2	K4
92	K1	S1

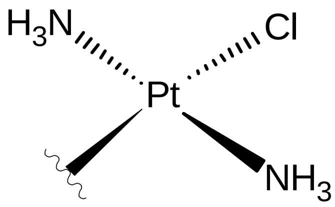
Gotze M. et al. JASMS 2015

Protein-nucleic acid cross-linking

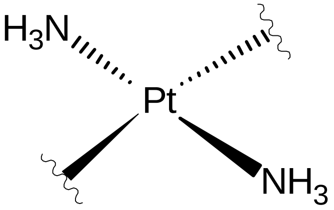




Trans-platinum



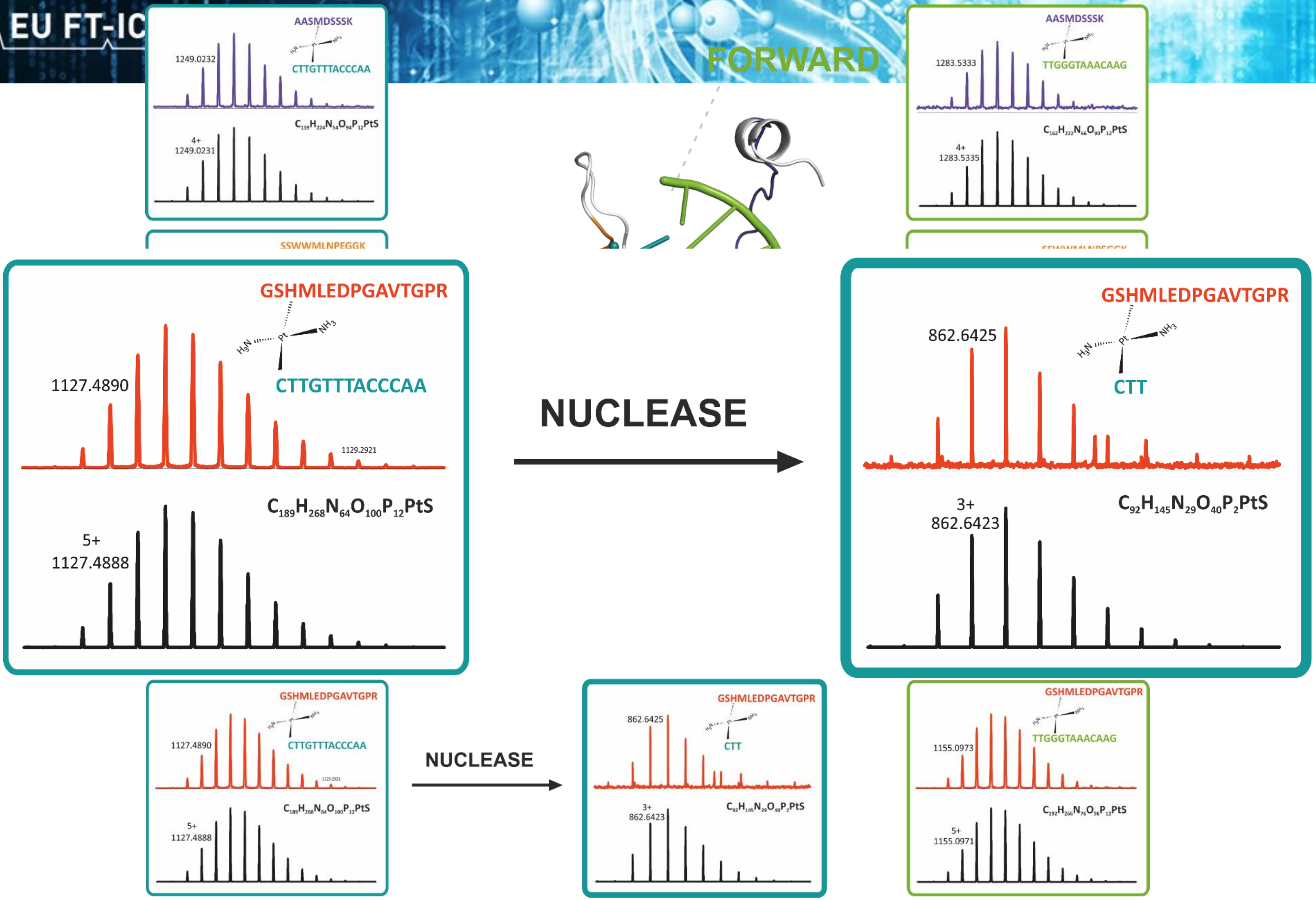
Trans-platinum (monofunctional)



Trans-platinum (bifunctional)

Slavata, L. et al Biomolecules 2019, 9(10), 535

Protein – DNA cross-links



Kukačka Z et al J Proteome Res. 2021 Apr 2;20(4):2021-2027

Thanks for your attention!

EU FT-ICR MS

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