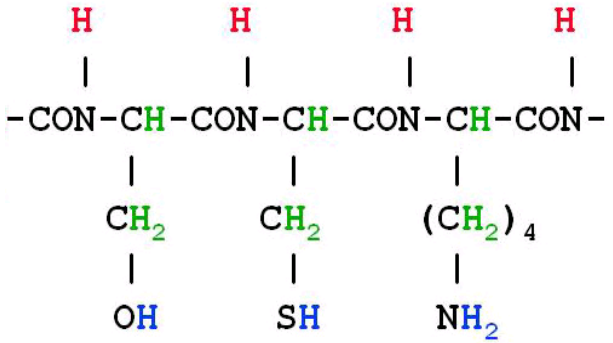
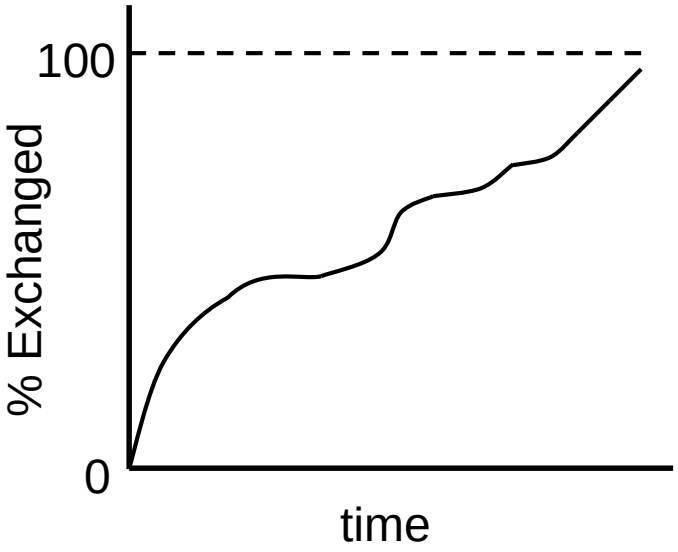
Hydrogen/Deuterium Exchange

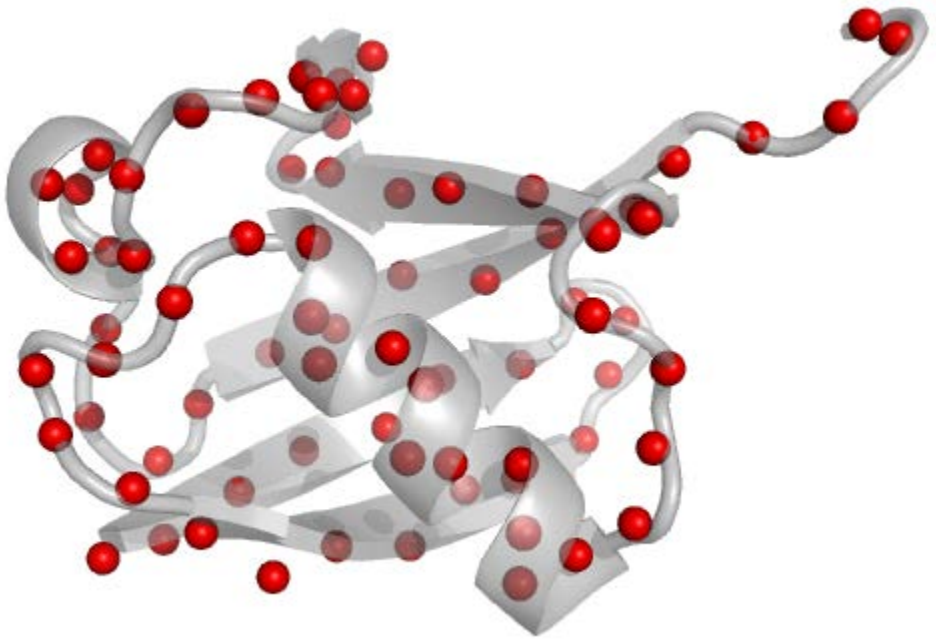
Med Chem 528

Hydrogen/Deuterium exchange probes the accessibility of amide hydrogens





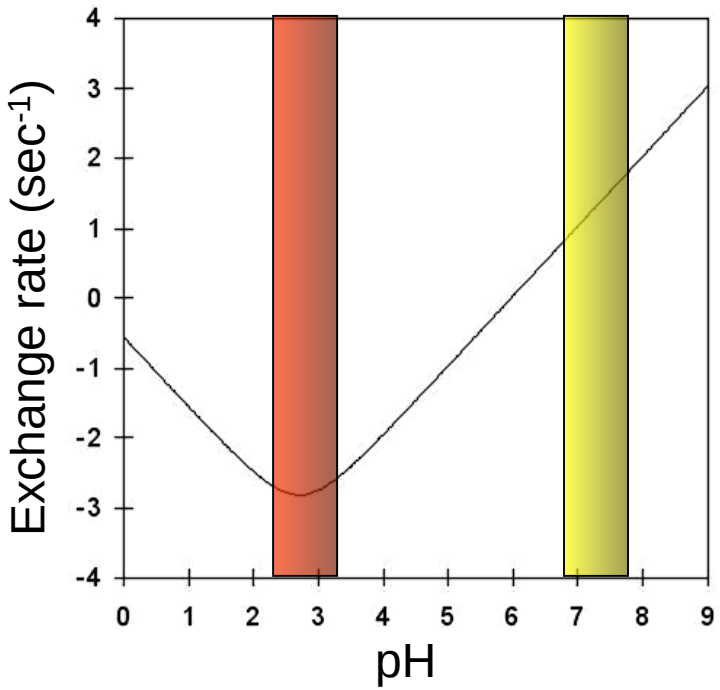
$H_2O \longrightarrow D_2O$



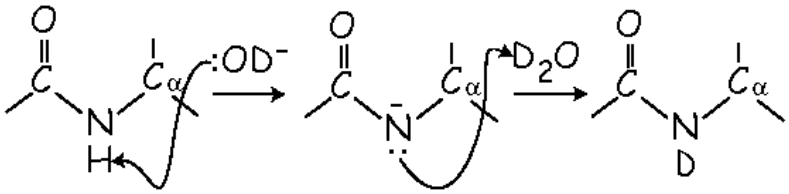
*Labeling doesn't perturb protein structure

Hydrogen exchange catalysis

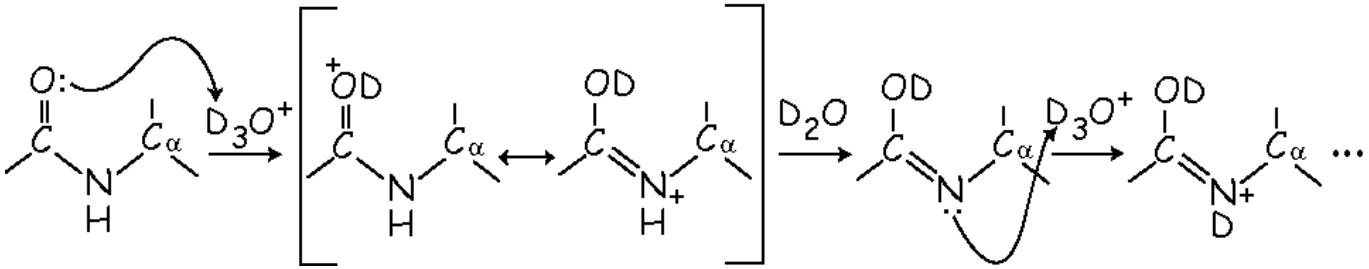
- Two mechanisms of amide proton exchange
  - Slowest at pH 2-3



Base catalysis



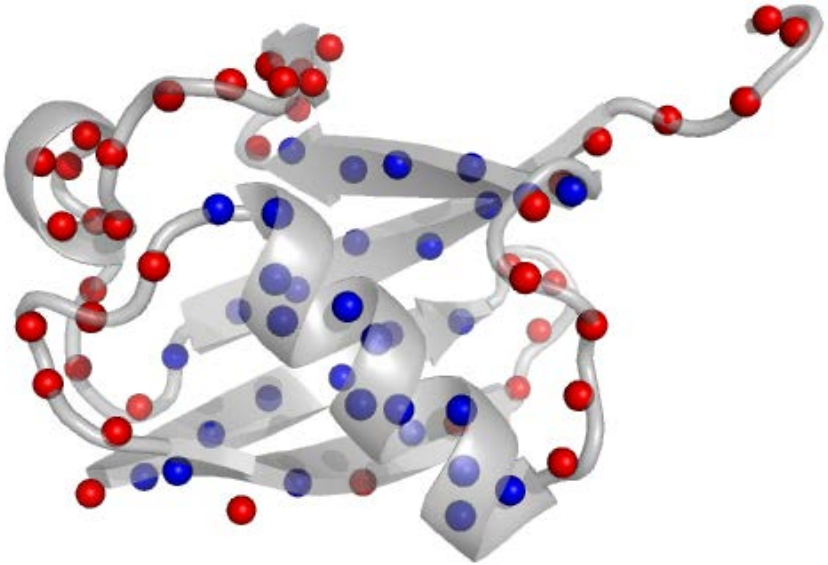
Acid catalysis

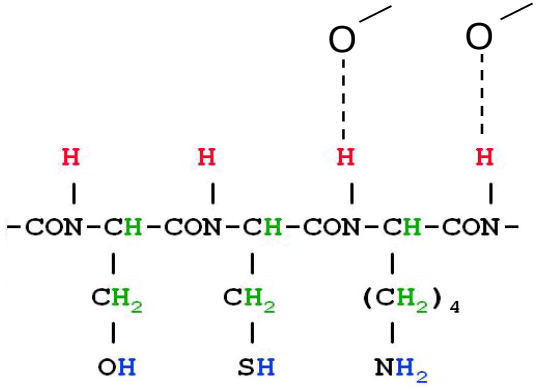


Tuchsen & Woodward, *J. Mol. Biol.* 1985

What governs the rate of H/D exchange in proteins?

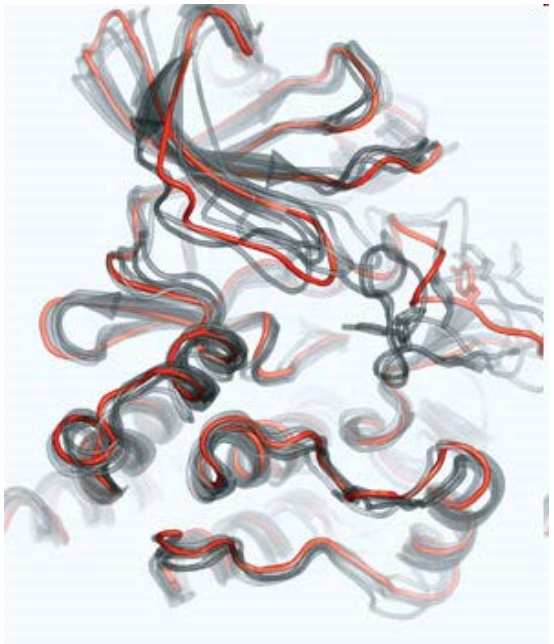
- Exchange not dependent on surface accessibility
  - Englander SW, *J. Am Soc Mass Spec* 2006
- Surface accessibility correlations have been reported
  - Truhlar et al, *J. Am Soc Mass Spec* 2006
- Main “protection factor” is amide hydrogen bonding
  - stable secondary structure
  - secondary effect of solvent accessibility (steric)

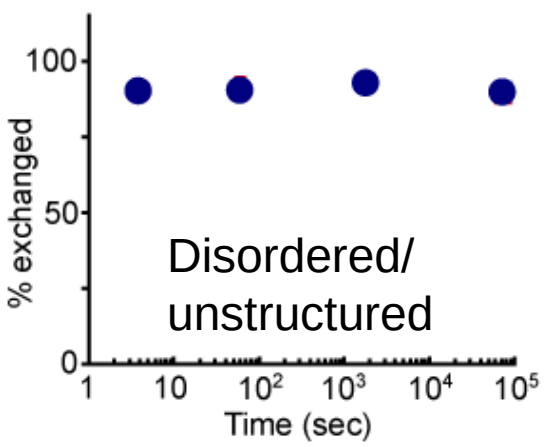


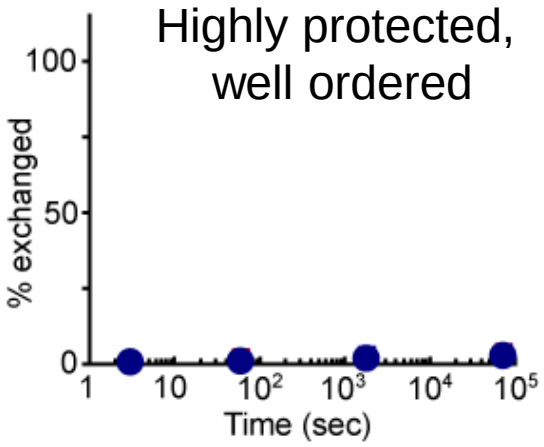


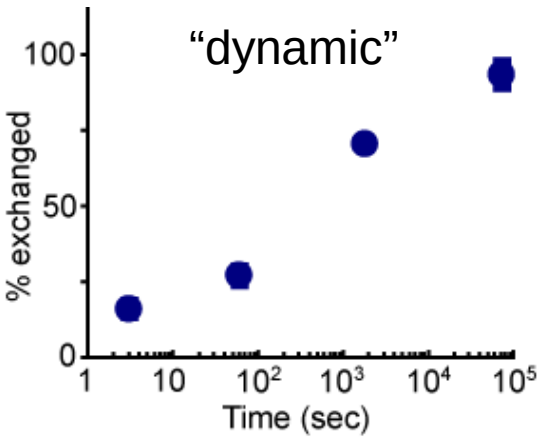
Proteins undergo motions on many time-scales

- H-bonds break and reform as proteins breathe
- Longer deuteration required for more ordered (rigid) sites



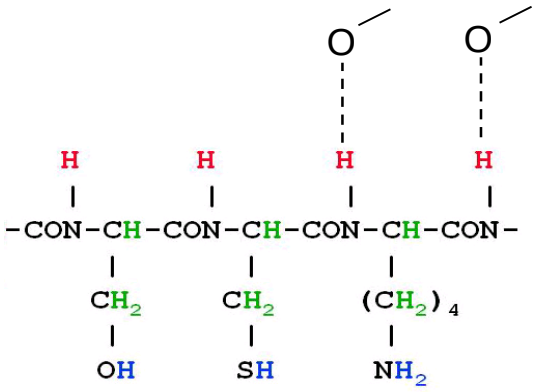


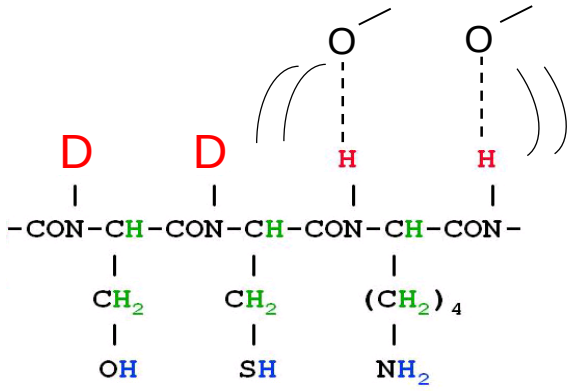


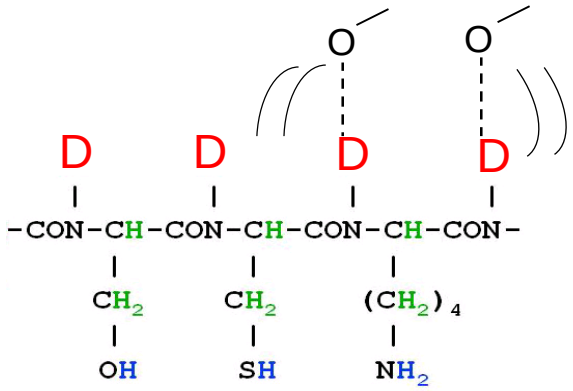


Exchange regimes

- Small & fast local structural fluctuations (“EX2”)
  - Brief exposure of amides for exchange

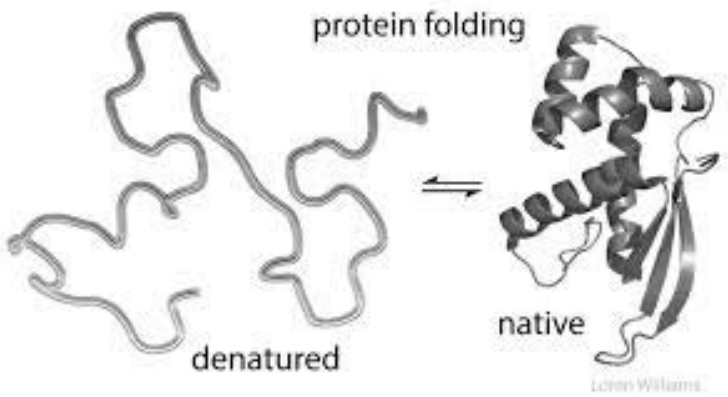






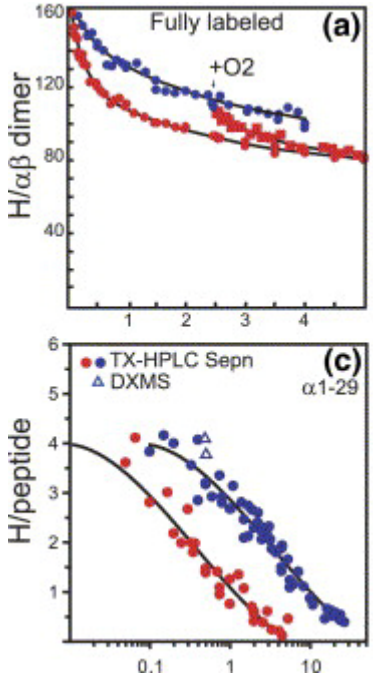
Exchange regimes

- Small & fast local structural fluctuations (“EX2”)
  - Brief exposure of amides for exchange
- Large & slow unfolding events (“EX1”)
  - Exposes amides for a relatively long period



Measuring H/D kinetics

- 1950's - Ultra-precision densitometry
- 1960-70s Tritium exchange with scintillation counting
  - HPLC to remove residual ^{3}H



Measuring H/D kinetics

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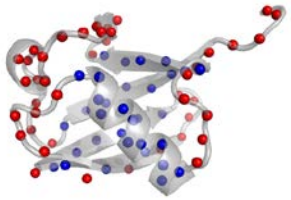
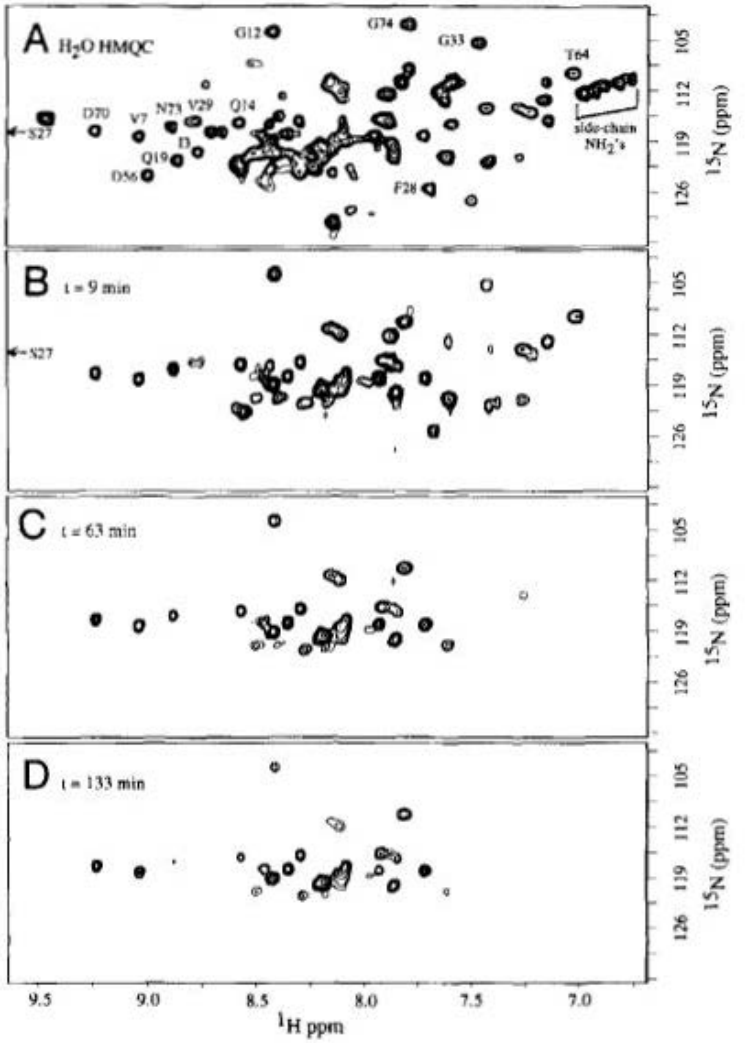
  - HPLC to remove residual ^{3}H
- Late 1980s modern NMR to detect deuteration of amides
  - 2D ^{1}H-^{15}N HSQC to monitor amides as they disappear

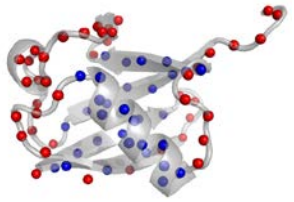


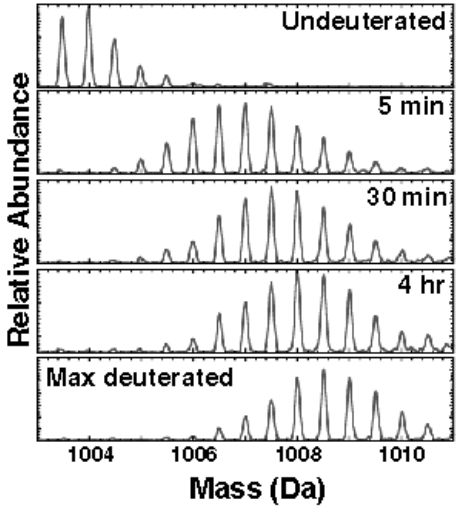


Protein Science (1995), 4:983-993.

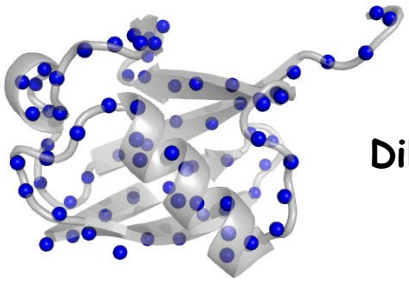
Measuring H/D kinetics

- 1950's - Ultra-precision densitometry
- 1960-70s Tritium exchange with scintillation counting
  - HPLC to remove residual ^{3}H
- Late 1980s modern NMR to detect deuteration of amides
  - 2D ^{1}H-^{15}N HSQC to monitor amides as they disappear
- 1990s Mass spectrometry
  - D is 1 Da heavier than H
  - Fast & sensitive





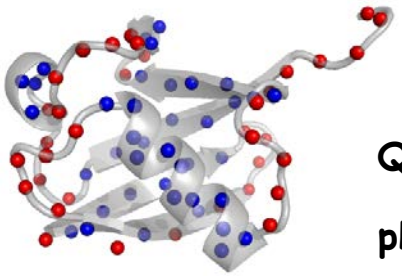
HDX-MS experimental setup



Stock protein
(in H_2O)

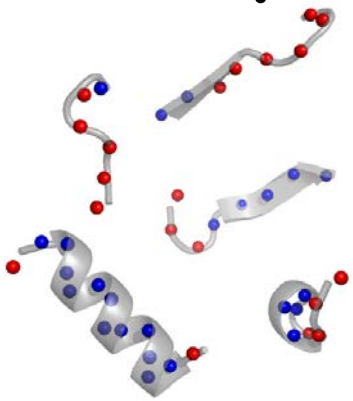
Dilute into D_2O

(sec-
hours)
pH 7.4



Quench
pH 2.5
0°C

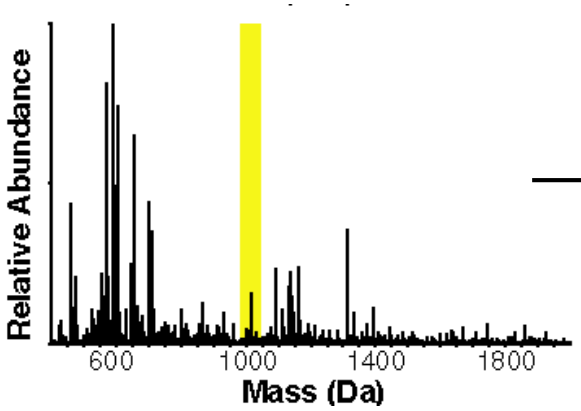
Pepsin
5 min

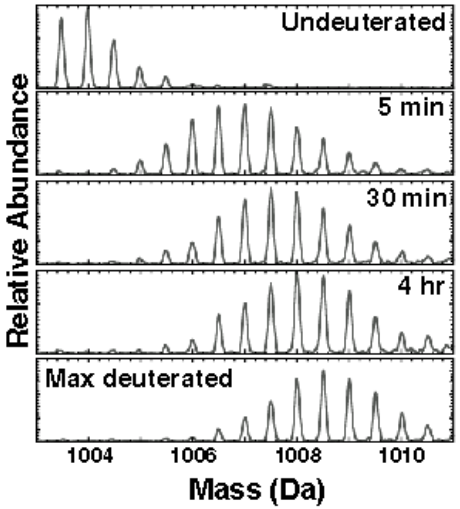


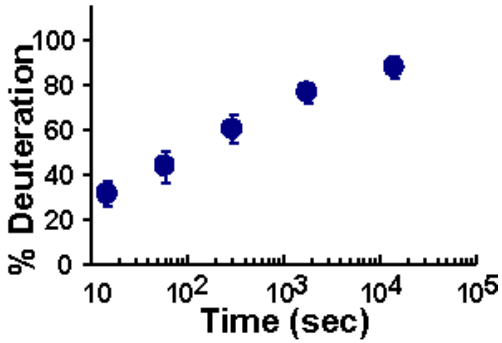
Peptides

Freeze in liquid N_2
store at -80°C

LC-MS







*Each D increases the
peptide mass by 1 Da

Why is H/D exchange so popular?

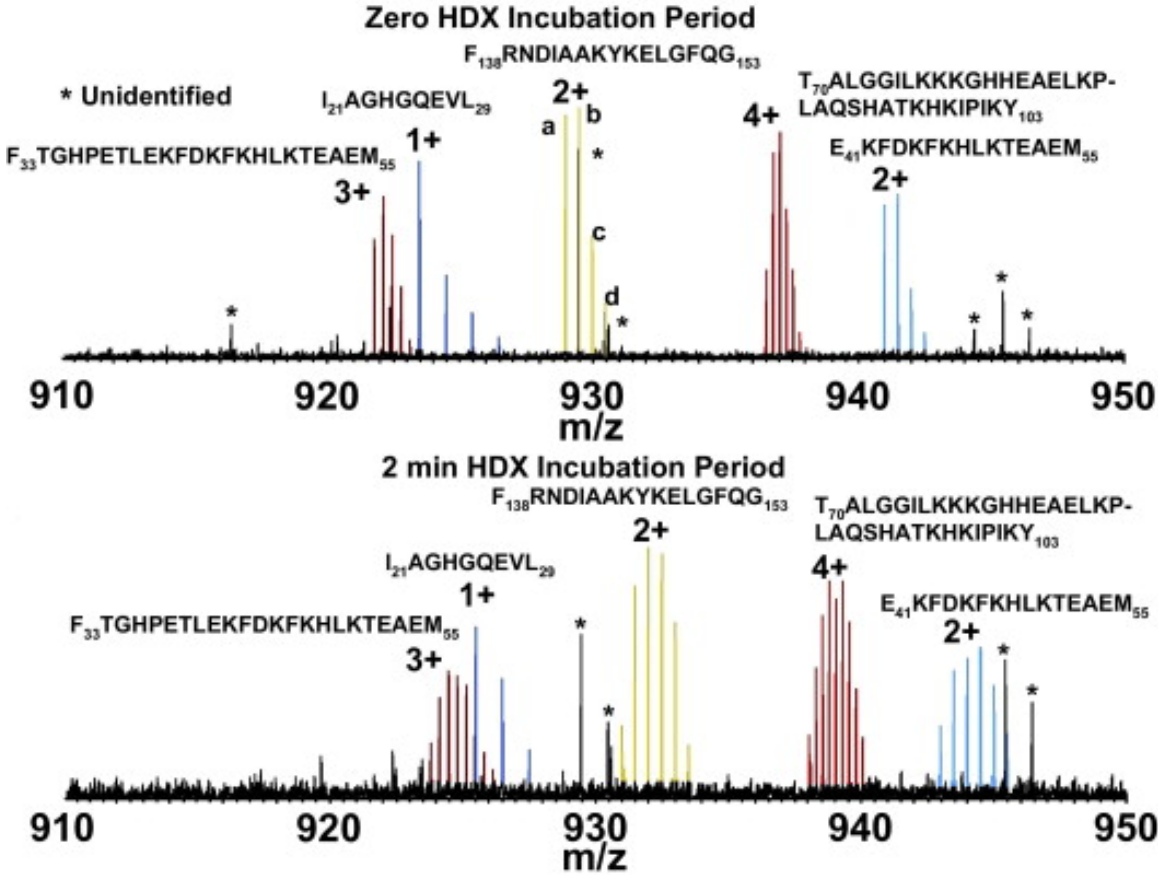
- Measures local amide accessibility
- Probe transient conformational states
- Mapping protein-ligand interfaces
  - (epitope mapping)
- Biopharmaceutical characterization

- Probes the solution state of a protein
- Requires very little sample (~10's of mgs)
- Relatively fast
  - Weeks
- Applicable to just about any system
  - Large complexes
  - Membrane proteins
  - Glycoproteins
  - Impure samples

What's the protein size limit?

- Complexity of mixtures is the limiting factor (spectral overlap)
- MS

  Resolution helps



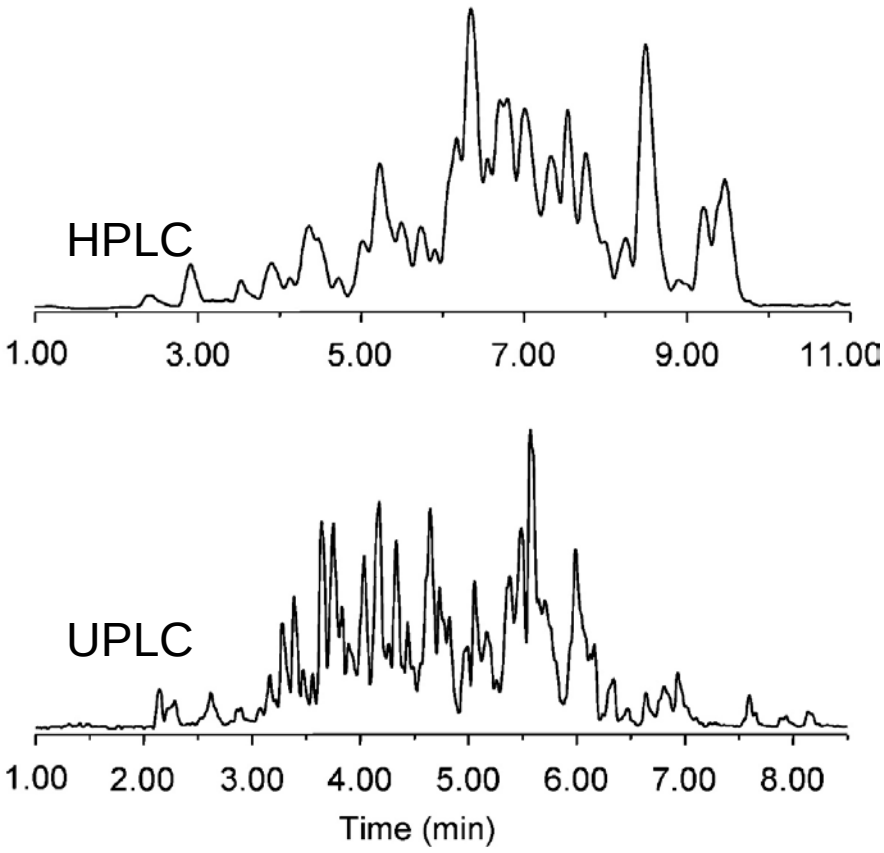
Kazazic et al., 2009 *JASMS*

What's the protein size limit?

- Complexity of mixtures is the limiting factor (spectral overlap)
- MS

  Resolution helps
- LC

  UPLC helps



Wales et al., 2008 *Anal Chem*

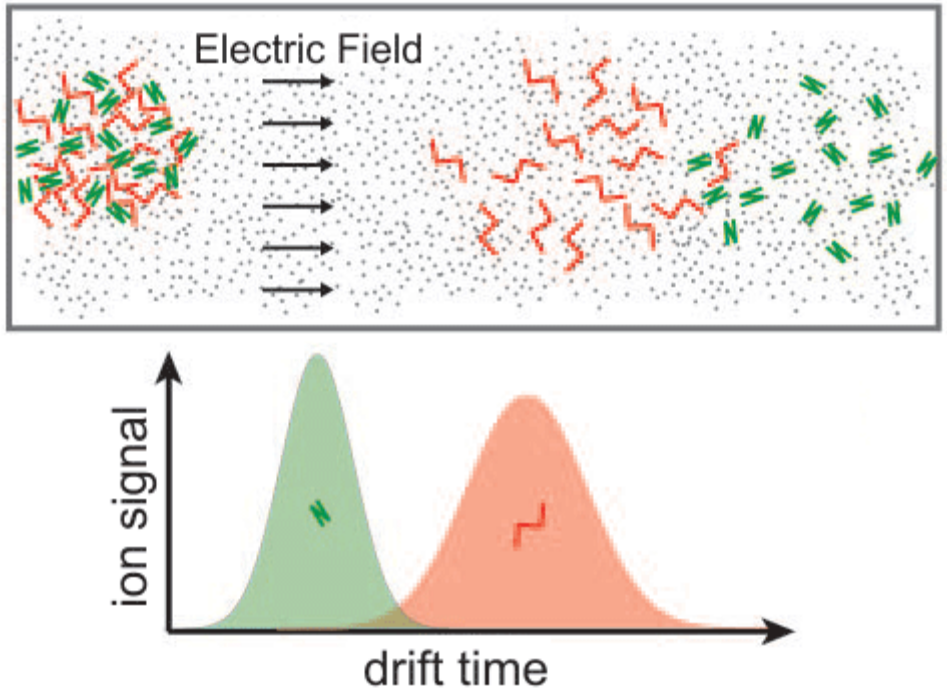
What's the protein size limit?

- Complexity of mixtures is the limiting factor (spectral overlap)
- MS

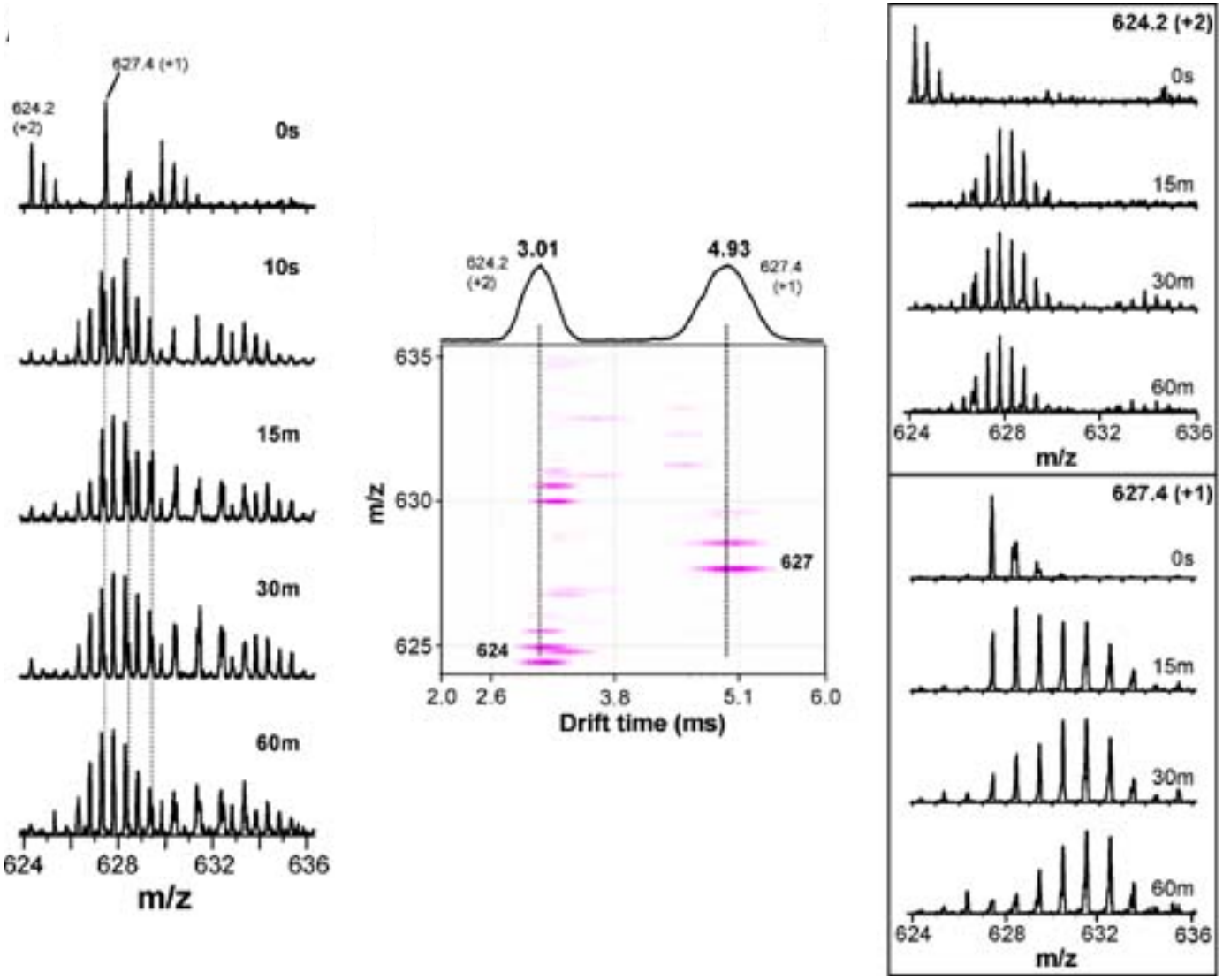
  Resolution helps
- LC

  UPLC helps
- Ion mobility

  Additional dimension



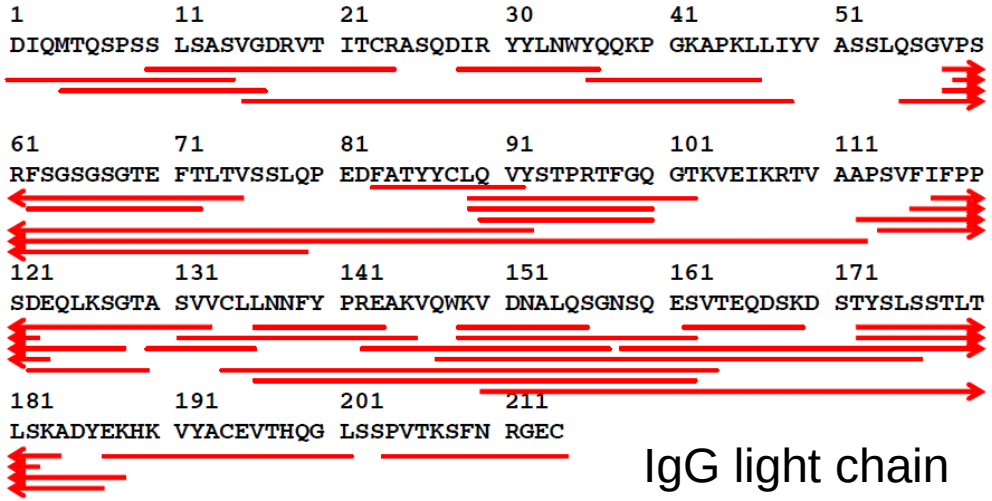
Ion mobility helps resolve peptides for HDX-MS



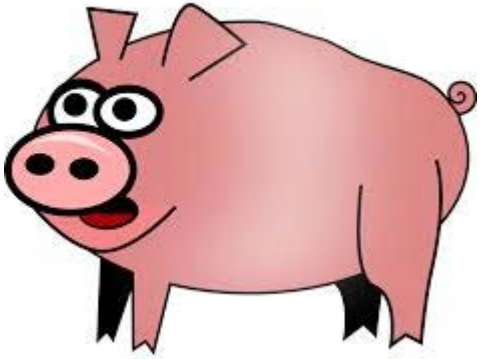
Iacob, Murphy & Engen (2008) *Rapid Comm Mass Spectrom*

What's the sequence resolution?

- Limited by available proteases
  - pH 2.5, 0°C, Gnd/Urea
  - Pepsin, Aspergillopepsin, Rhizopus protease, *Nepenthesin* protease
    - Non-specific



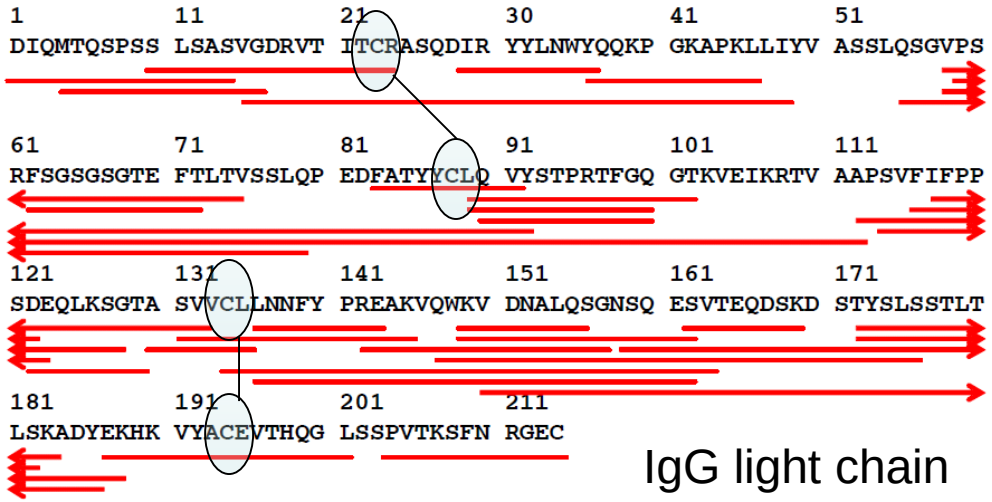
IgG light chain



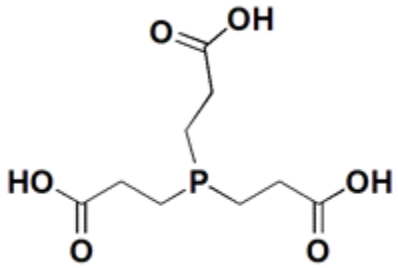
Houde et al, *Anal Chem* 2009

Sequence coverage is limited by the available proteases

- Limited by available proteases
  - pH 2.5, 0°C, Gnd/Urea
  - Pepsin, Aspergillopepsin, Rhizopus protease, *Nepenthesin* protease
    - Non-specific
- Reduction of disulfides with TCEP
  - Tris(2-Carboxyethyl) phosphine
  - Works at low pH

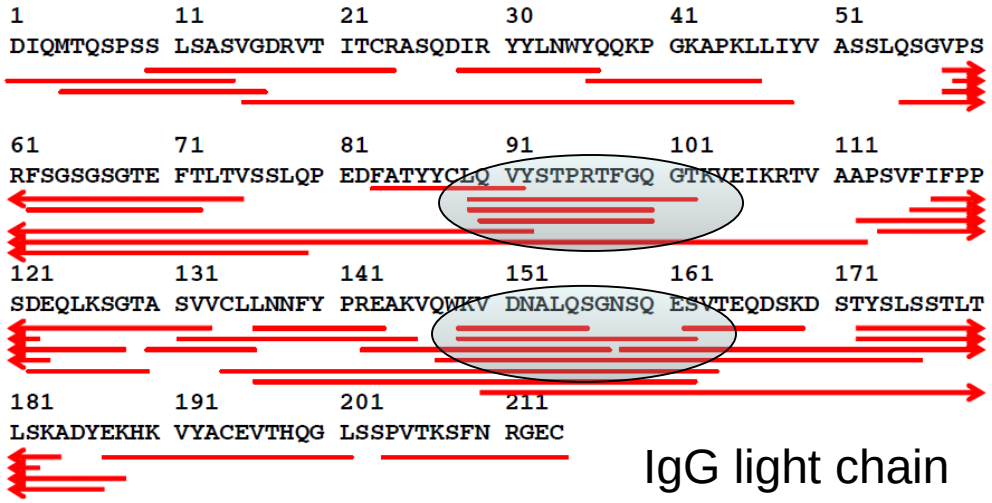


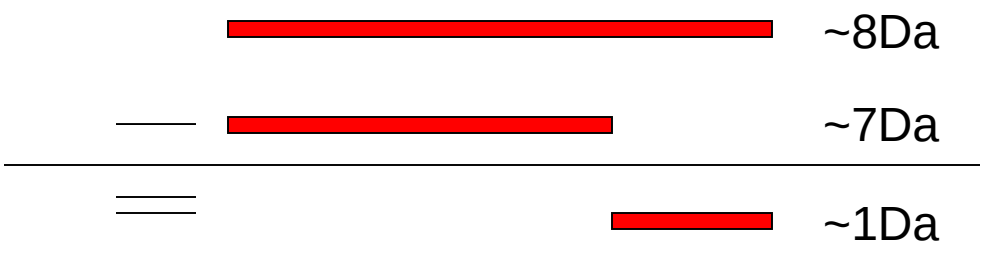
IgG light chain



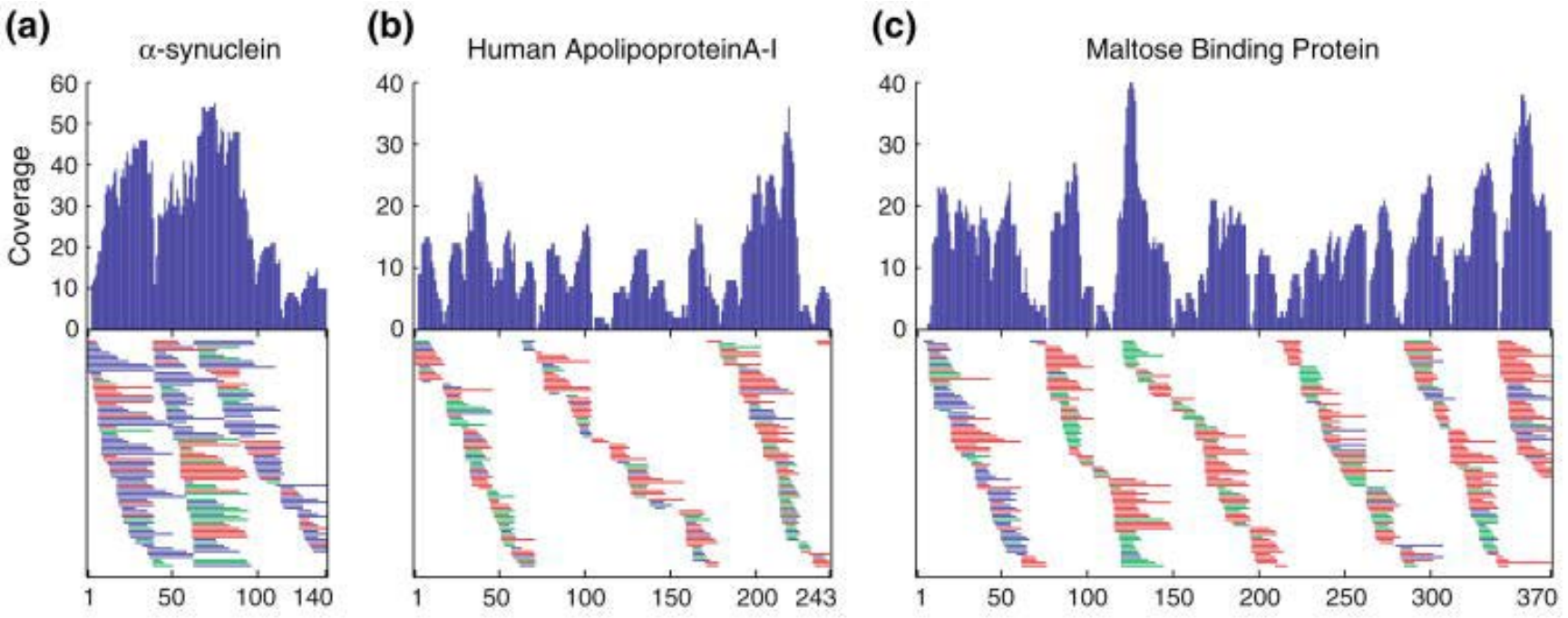
Overlapping fragments give higher resolution

- Overlapping peptides are commonly generated with pepsin
- Deuteration of smaller fragments can be calculated





More peptides = higher resolution

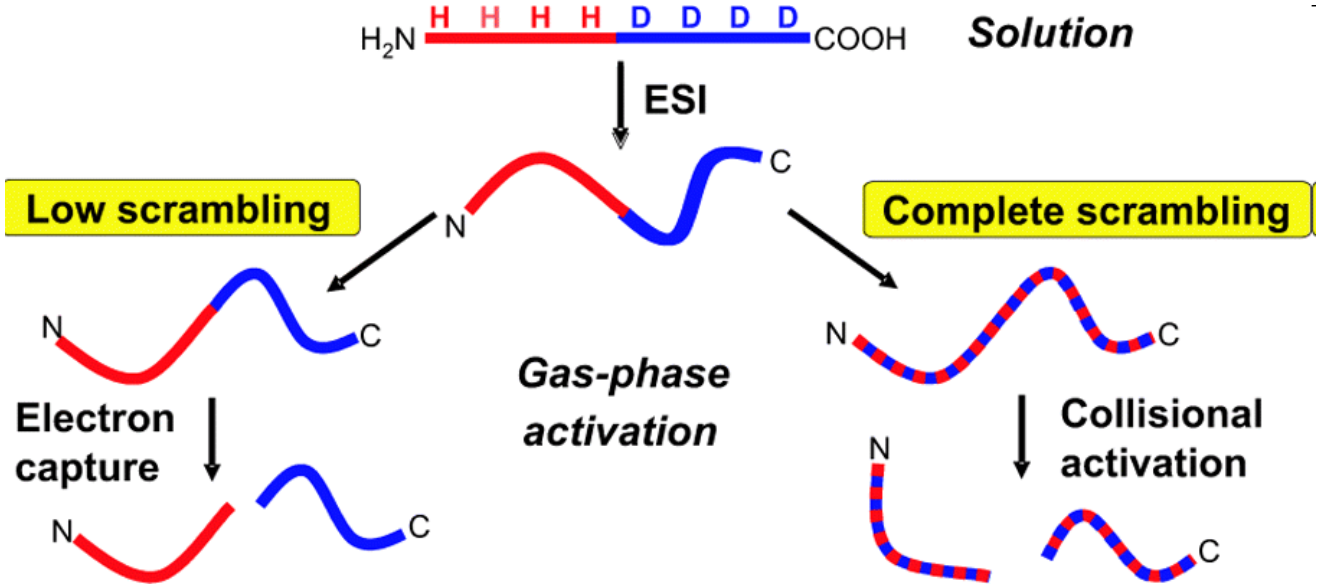


- The more peptides you can track the more precisely you can localize deuterium exchange kinetics.
- Another good reason to have optimal LC and MS resolution (observe lower abundance peptides).

Mayne et al. *JASMS.*, 2011

Why not simply do MS/MS to localize deuterium?

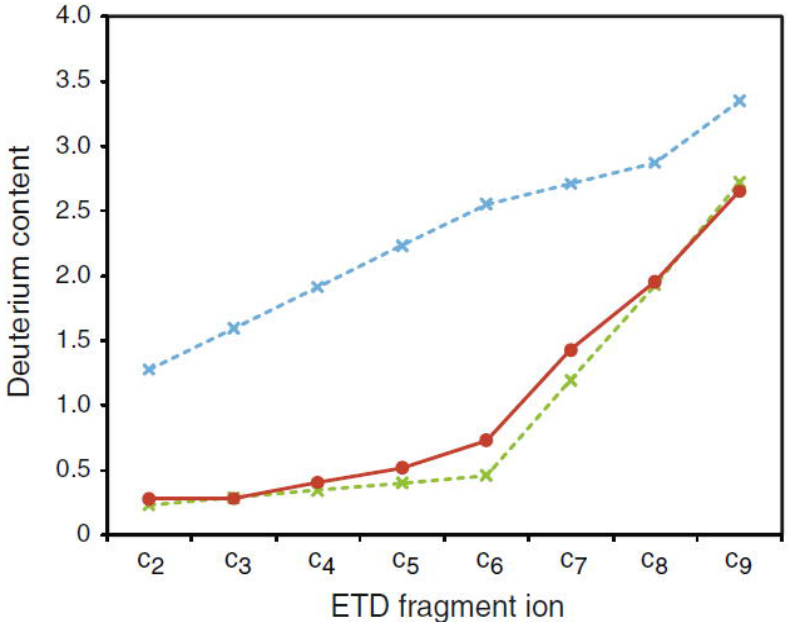
- **Collision activated dissociation (CID)**
- **electron capture dissociation (ECD/ETD)**
  - Gentle fragmentation through radical chemistry

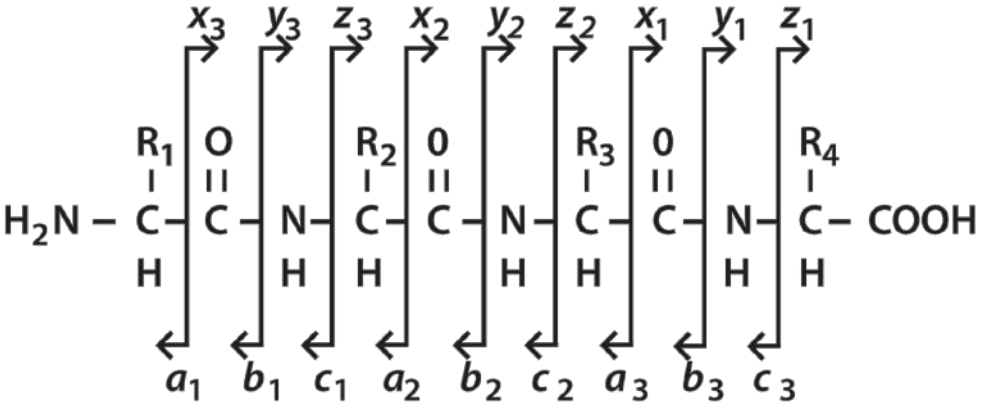


Rand et al. *J. Am. Chem. Soc.*, 2008

Site-specific resolution by ETD

- Mass shifts with c/z ion series (ETD) can localize deuterium to specific amides (red)
- CID based fragment ions (b/y) have lost all relevant deuterium exchange information.

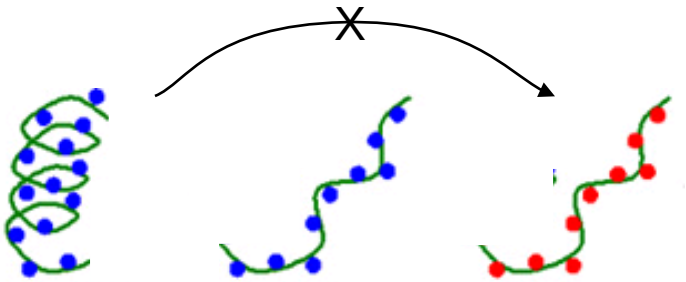




Rand et al. *JASMS*, 2011

Quantitative measure of Dynamics

- Probing the rates protein motions



$F_H \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U_H \xrightarrow[D_2O]{k_2} U_D$

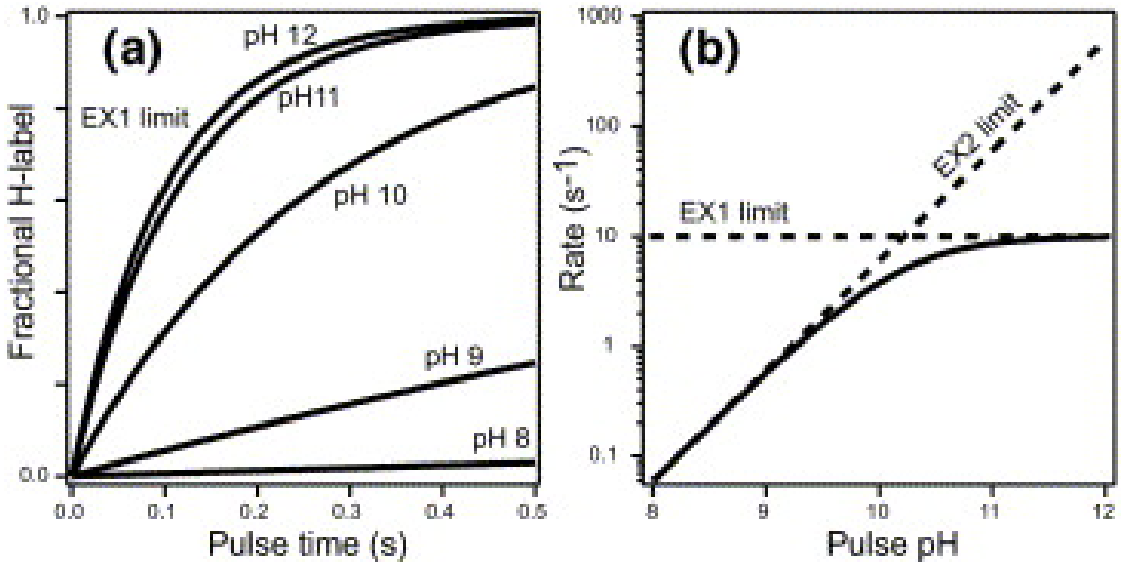
k_1 : opening rate
k_{-1} : closing rate
k_2 : intrinsic exchange rate

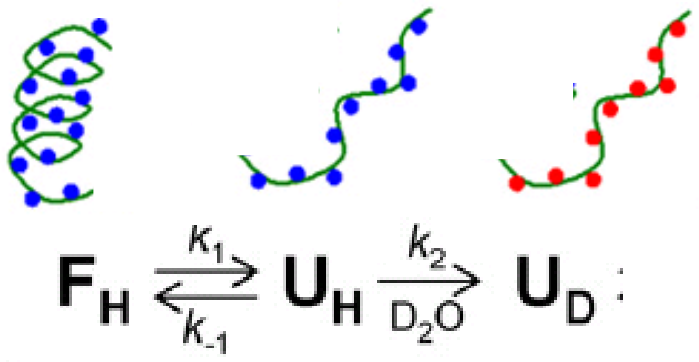
- Intrinsic exchange rate (k_2)
  - Well known for all amino acids at various temperatures/pHs

Englander S.W., *J. Am Soc Mass Spec* 2006

Consequences of pH dependence

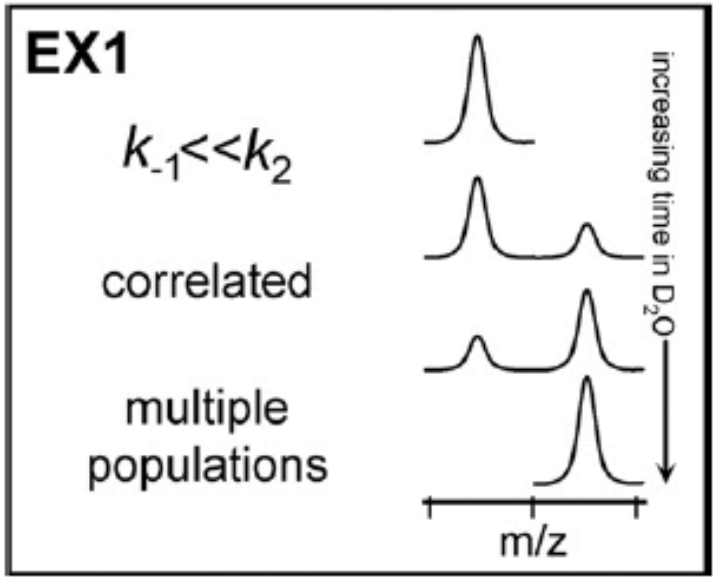
- k_2 is pH dependent and k_{obs} can plateau at high pH
  - EX1 limit
- Going higher in pH doesn't accelerate exchange since its limited by protein unfolding rate
  - k_1 limited

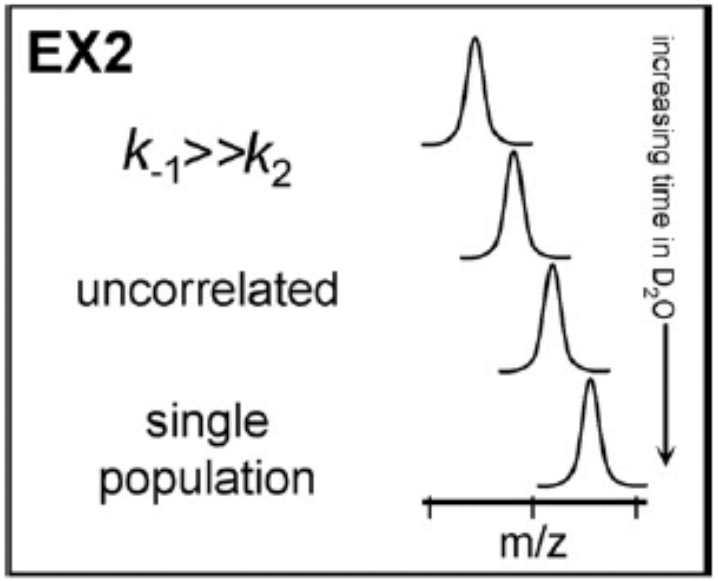




Two realms of exchange kinetics

$$F_H \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U_H \xrightarrow[D_2O]{k_2} U_D$$

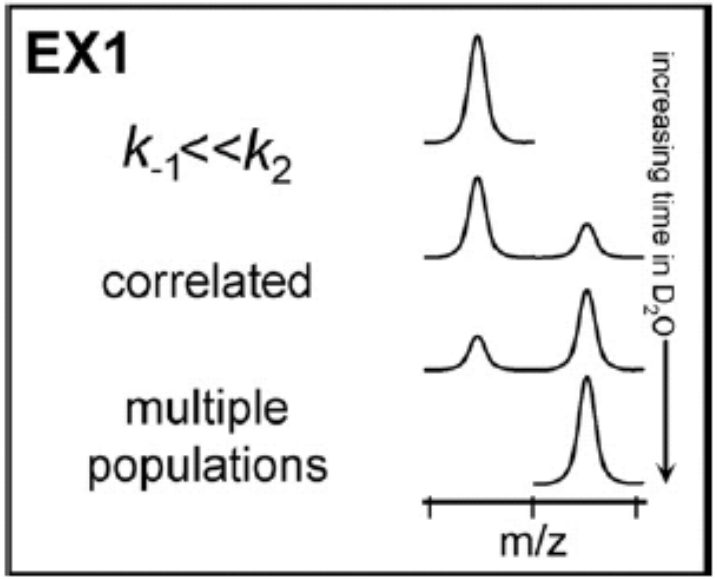




*pH/temperature of deuteration can change type of observed kinetics (by changing k_2)

EX1 exchange reveals local unfolding rates

$$F_H \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U_H \xrightarrow[D_2O]{k_2} U_D$$



- A direct measure of the opening rate (k_1) at a specific site in the protein

EX2 exchange reveals local stability

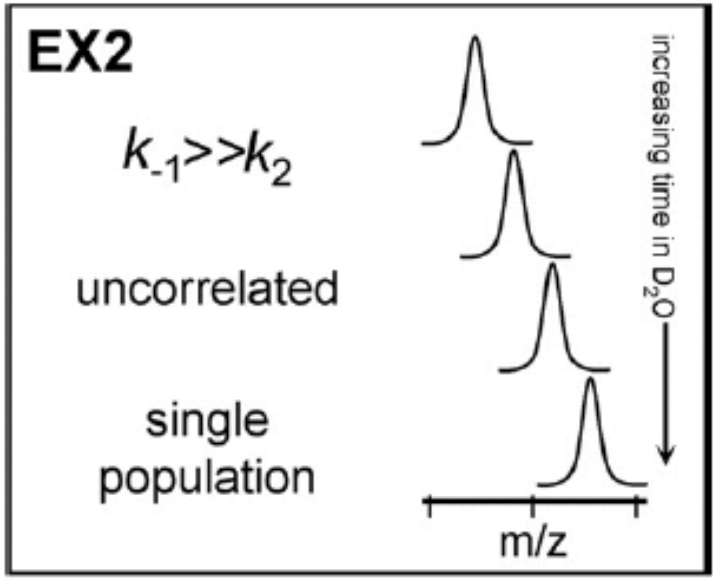
$$F_H \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U_H \xrightarrow[D_2O]{k_2} U_D$$

- Compare measured rate (k_{obs}) to intrinsic rates (k_2)
  - "protection factor"

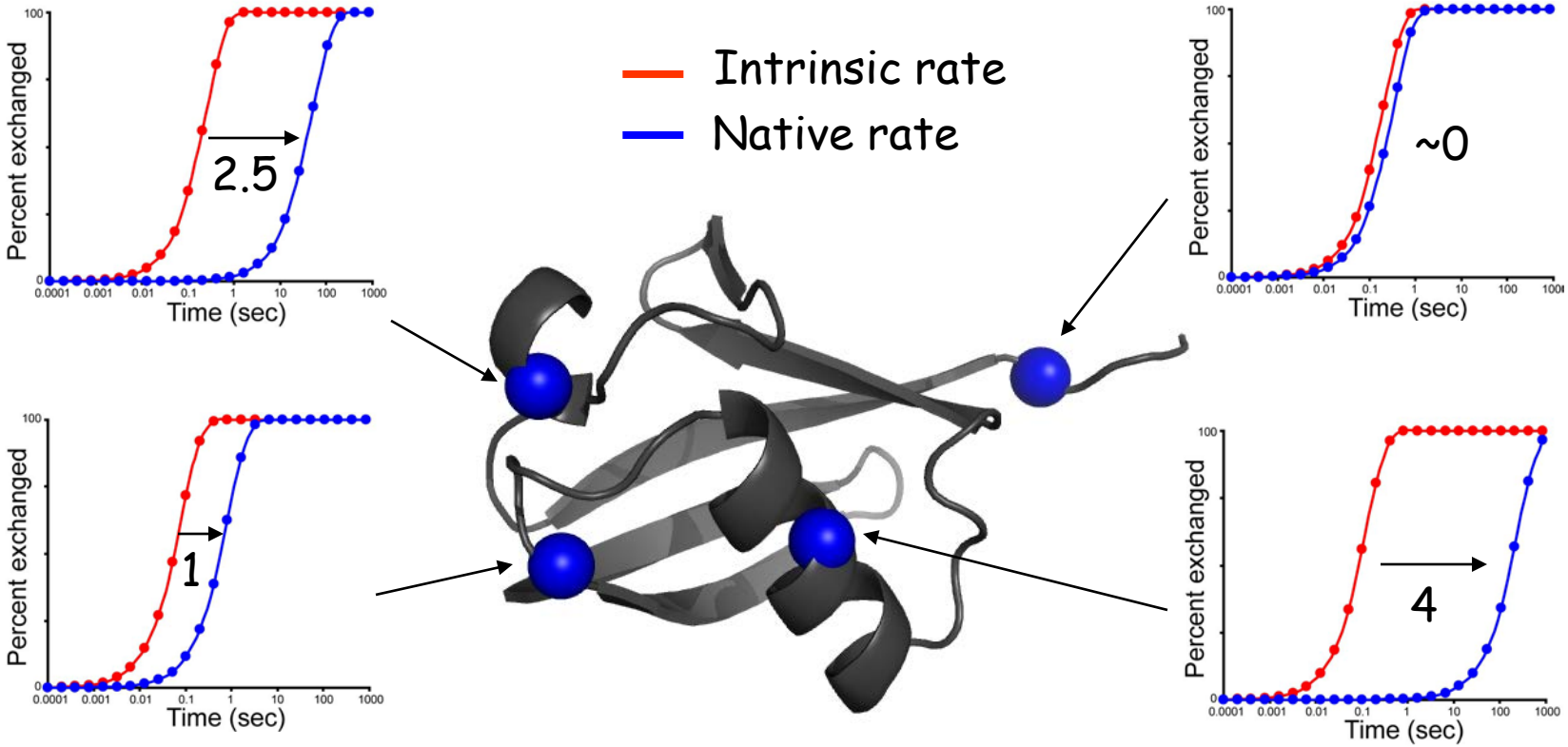
$$k_{obs} = (k_1 / k_{-1})\, k_2 = K_{op}\, k_2$$

$$\Delta G = -RT \ln K_{op}$$

- Estimate local stability



Exchange rates & protection factors



$$\text{Protection factor (PF)} = \text{Log}\frac{k_{obs}}{k_2}$$

Uses of HXMS

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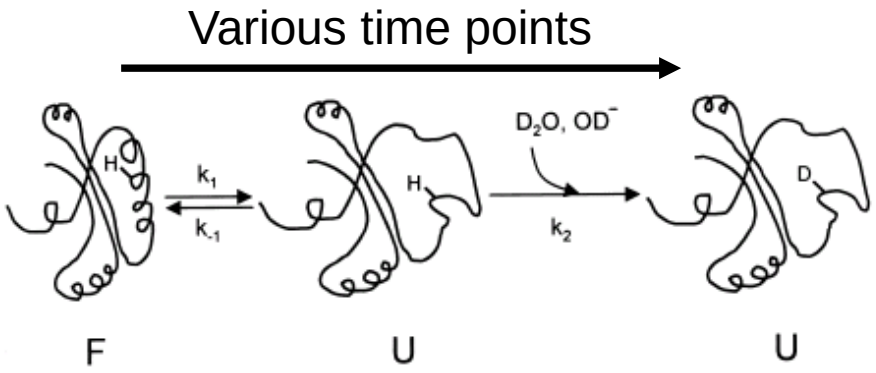
Continuous vs. pulsed labeling

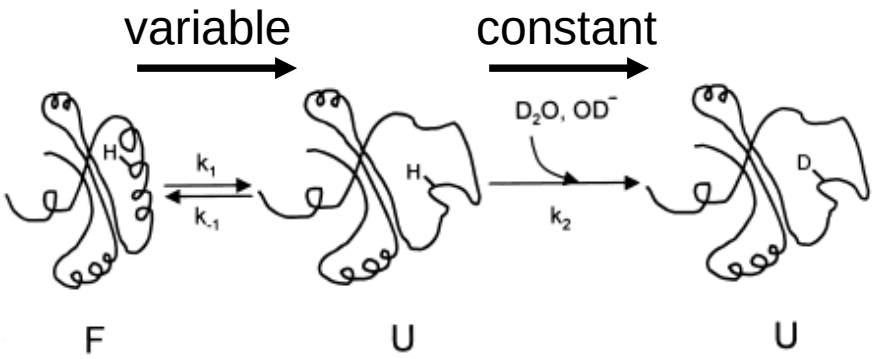
Continuous

- Protein in D_2O constantly getting labeled as sites become accessible
- Measures deuterium uptake as a function of deuteration time
  - Provides kinetic or thermodynamic information

Pulsed

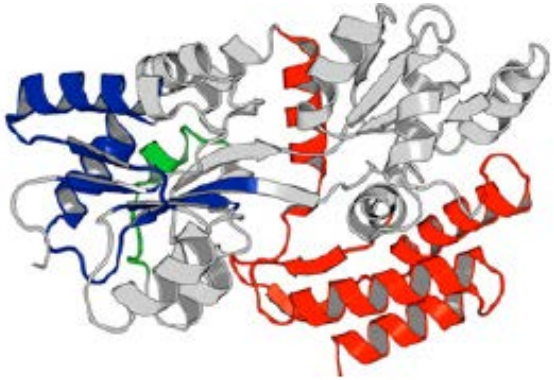
- Protein incubated with perturbant (Urea/Gnd, acid, etc.) for set time
- Rapid (high pH) pulse used to label accessible sites
  - Provides a rapid snapshot of a protein's structure in solution

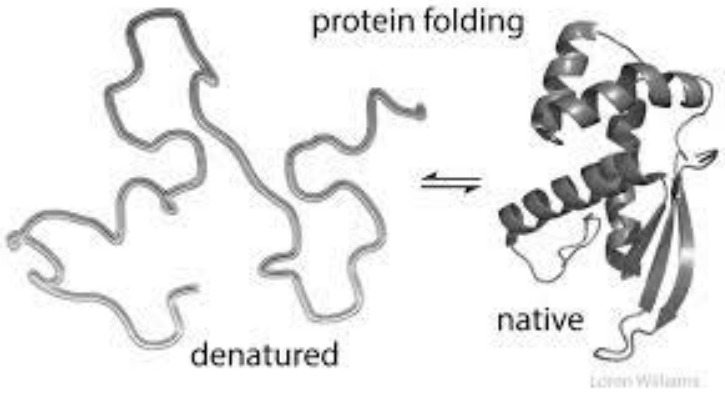




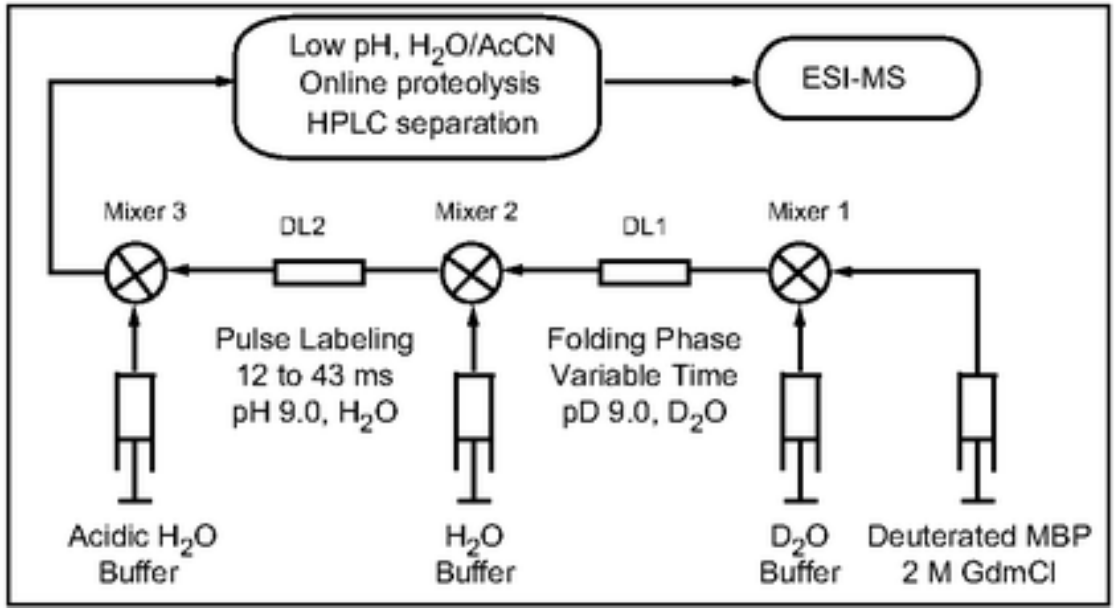
Pulse labeling to track protein folding processes

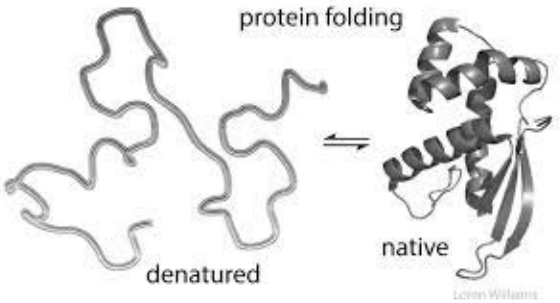
- How do proteins fold?
- Nucleation events
  - Certain secondary structure forms first
  - Anchor for the rest of the polypeptide to fold





Millisecond pulse HDX labeling

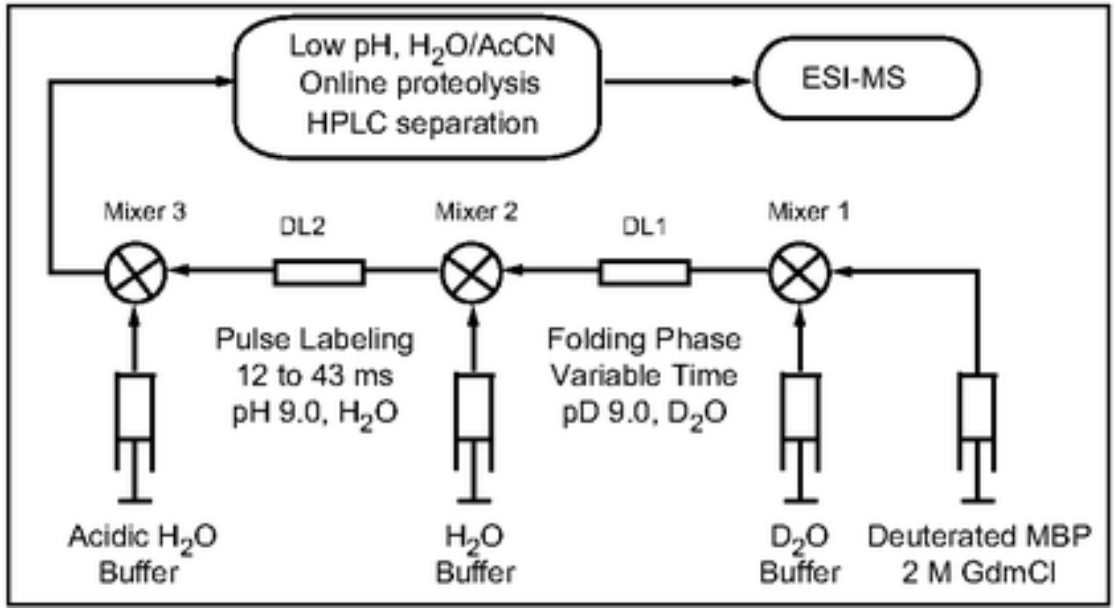


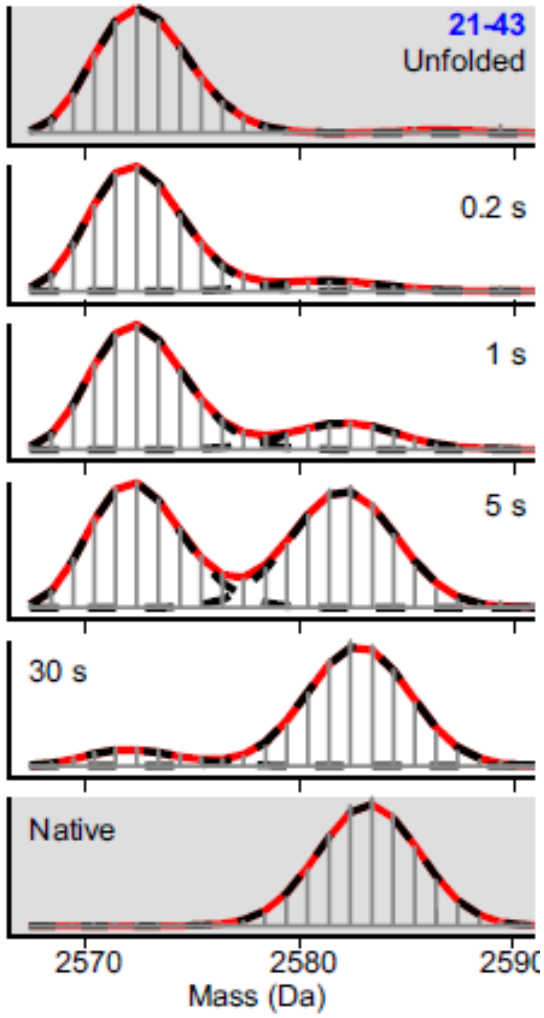


- Denatured protein (D_2O 2M GndHCl)
- Refolding (D_2O pH 9): 50 ms – 3 min
- Pulse (H_2O pH 9)
- Quench

Walters et al, *PNAS* 2013

Millisecond pulse HDX labeling

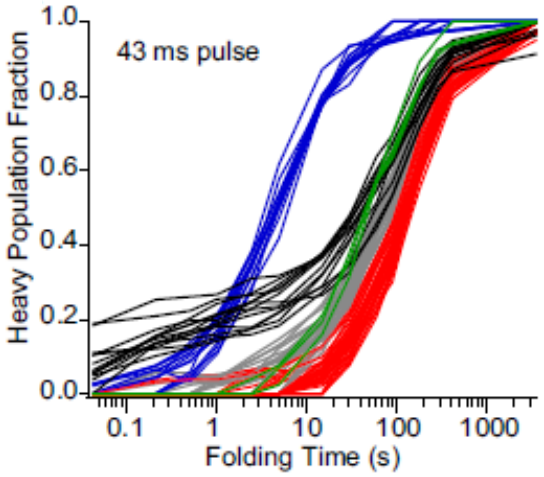


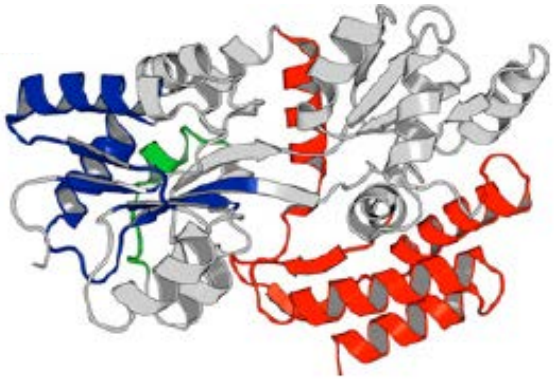


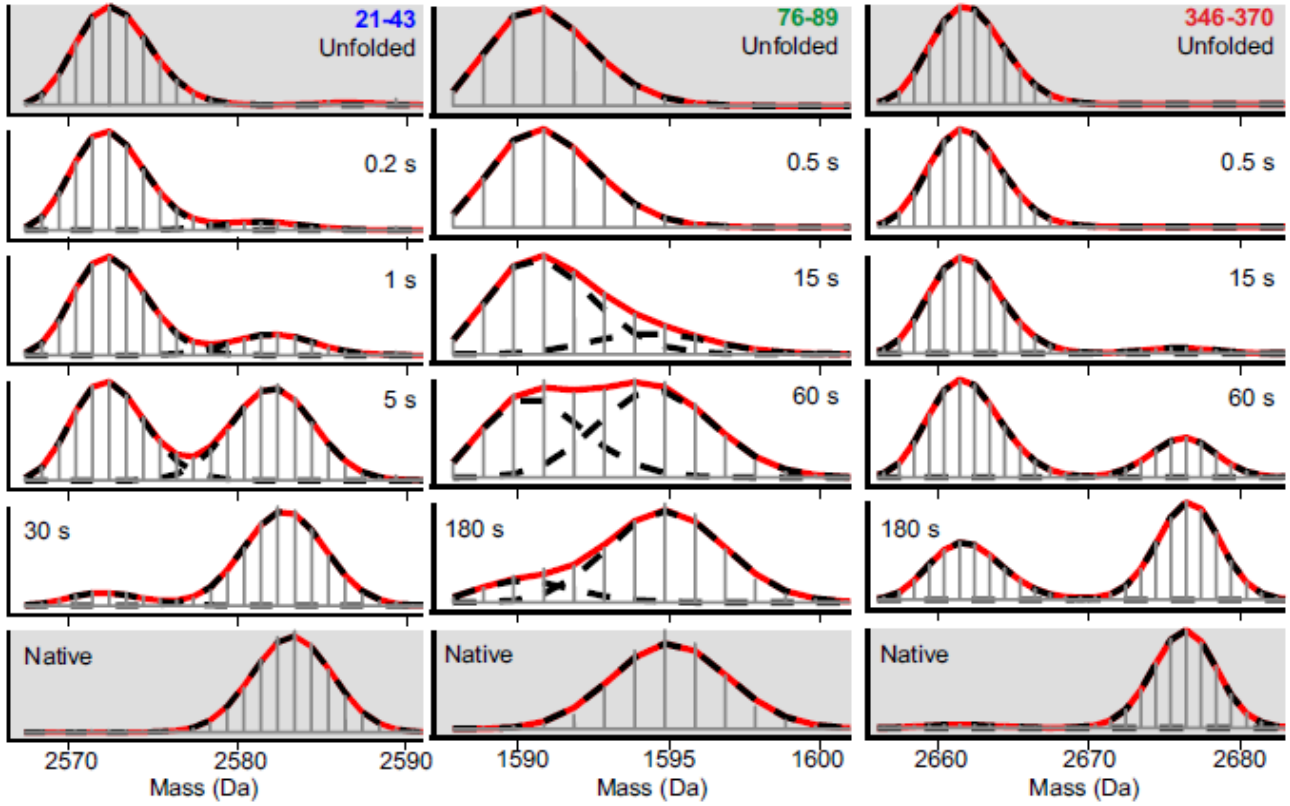
- Denatured protein (D_2O 2M GndHCl)
- Refolding (D_2O pH 9): 50 ms – 3 min
- Pulse (H_2O pH 9)
- Quench

Walters et al, *PNAS* 2013

Measuring protein folding with pulse labeled HDX







Walters et al, *PNAS* 2013

Uses of HXMS

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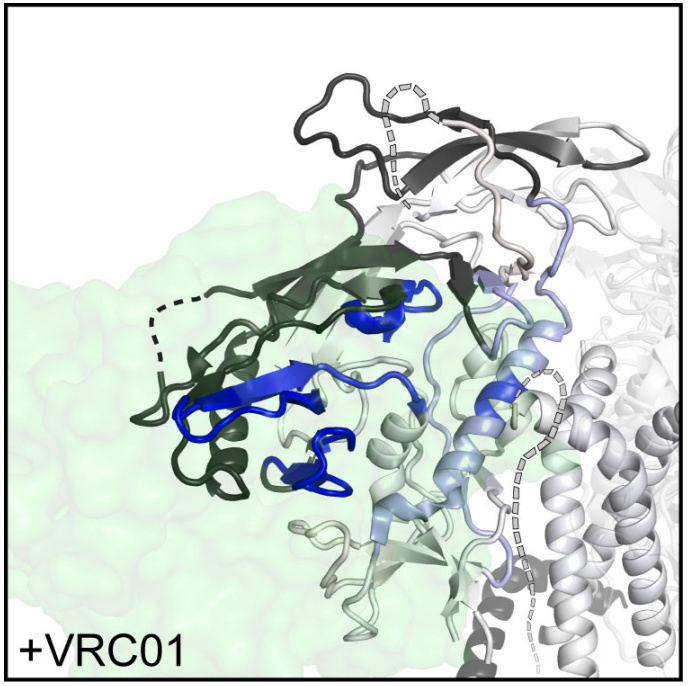
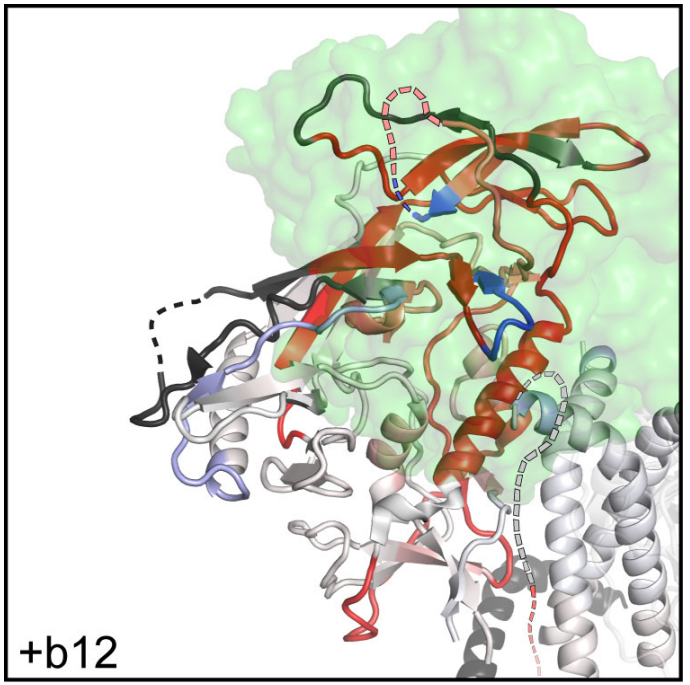
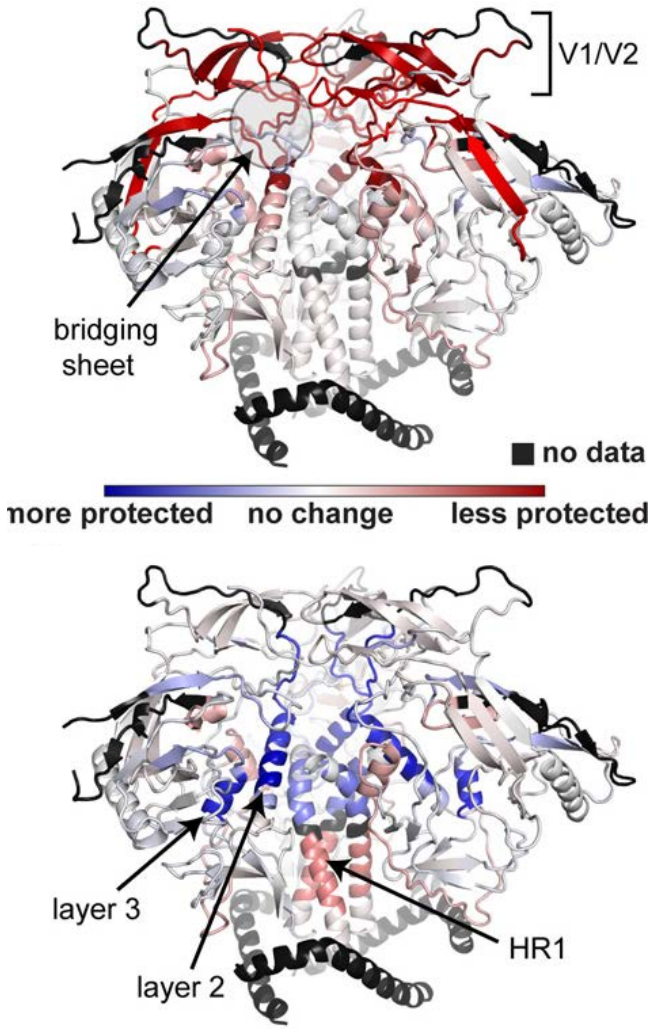
Interface mapping

- Comparison of free and bound HDX profiles can reveal interactions sites
- Allosteric effects!
  - Caveat if structure is unknown
  - Benefit if structure is known





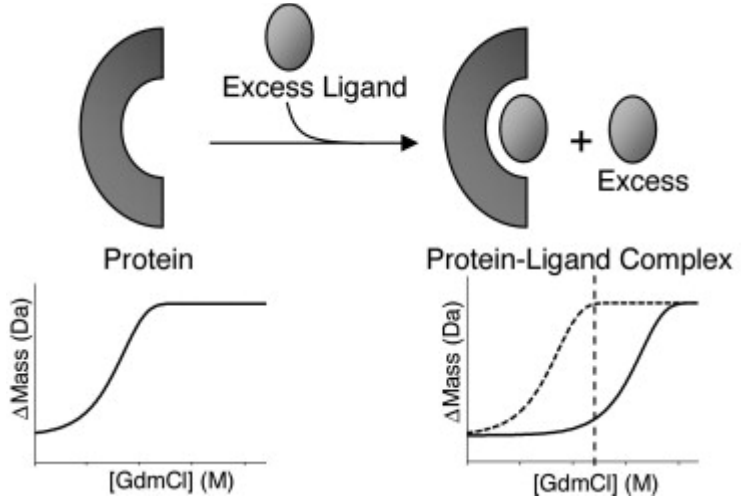
Example of extensive allosteric changes with ligand binding

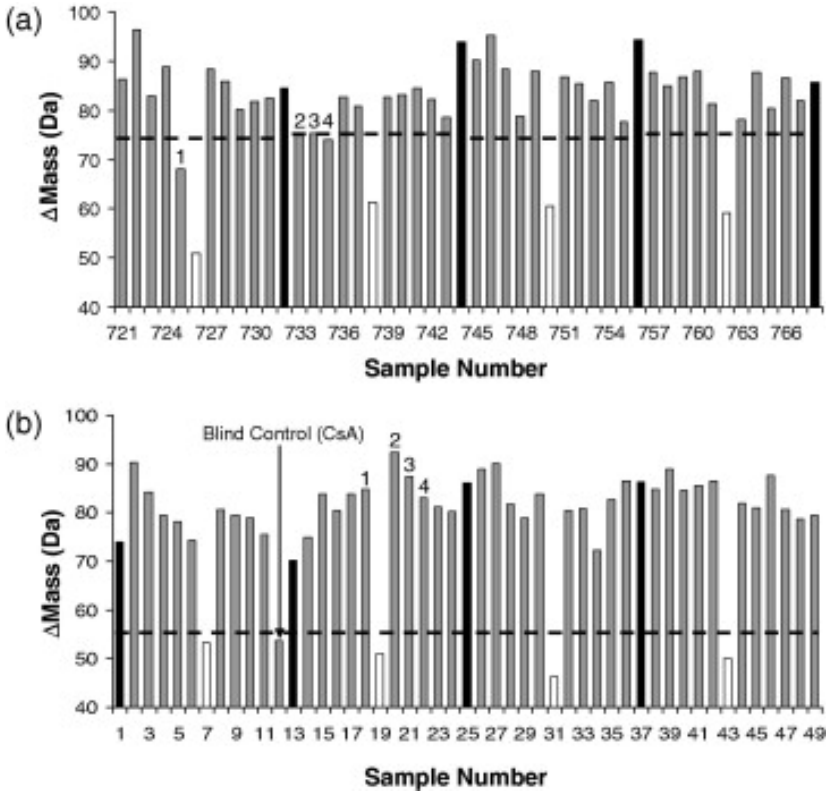


- Two drugs bind nearly the same site on the proteins
- Yet have dramatically different effects on the exchange profiles
- Excellent probe to monitor allosteric effects

Guttman et al. 2014 *Structure*

Screening for protein-ligand interactions





- Intact protein HDX with rapid MALDI
- 3 min/ligand

Hopper et al., 2008 *JASMS*

Uses of HXMS

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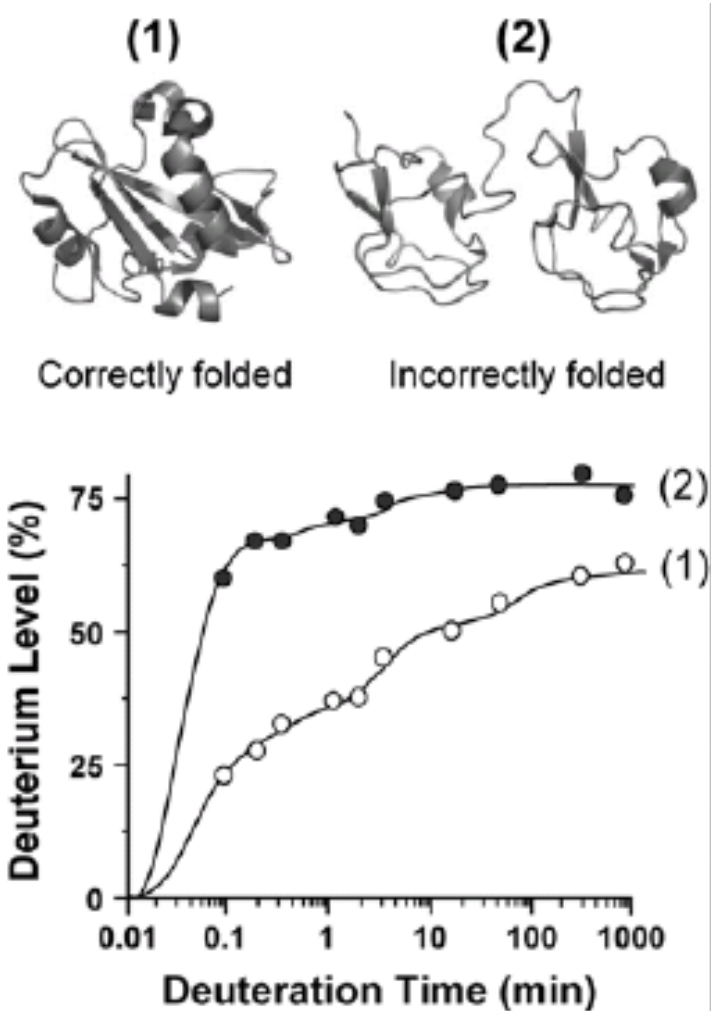
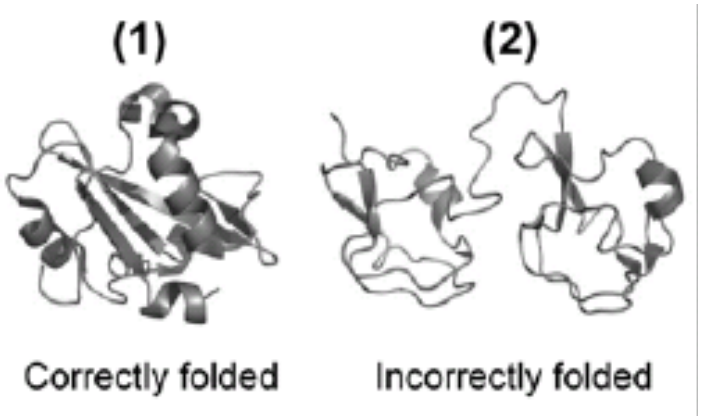
Biopharmaceuticals

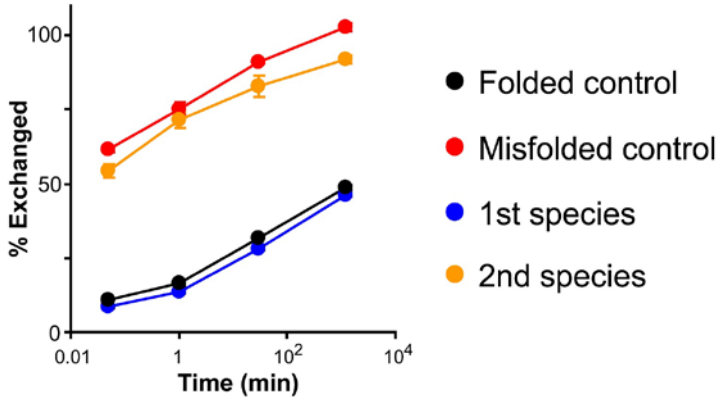


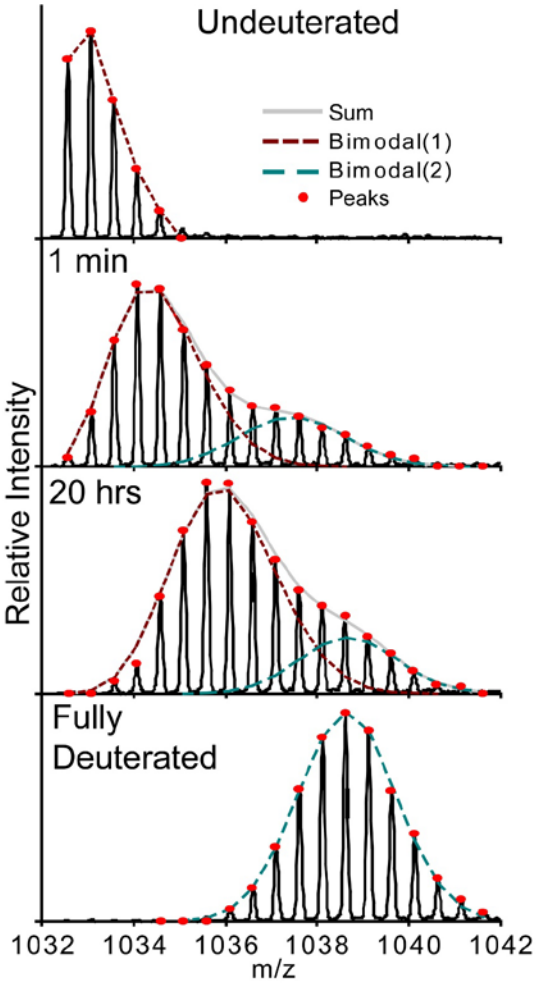
- Used to ensure proper folding of large molecule drugs
  - **Higher order structure (HOS)**
  - Especially useful if no activity assay is available
- Formulation
  - Identify optimal buffers/formulation and sites of aggregation
  - Solid state HDX of lyophilized material

Engen J.R., *Anal Chem* 2009

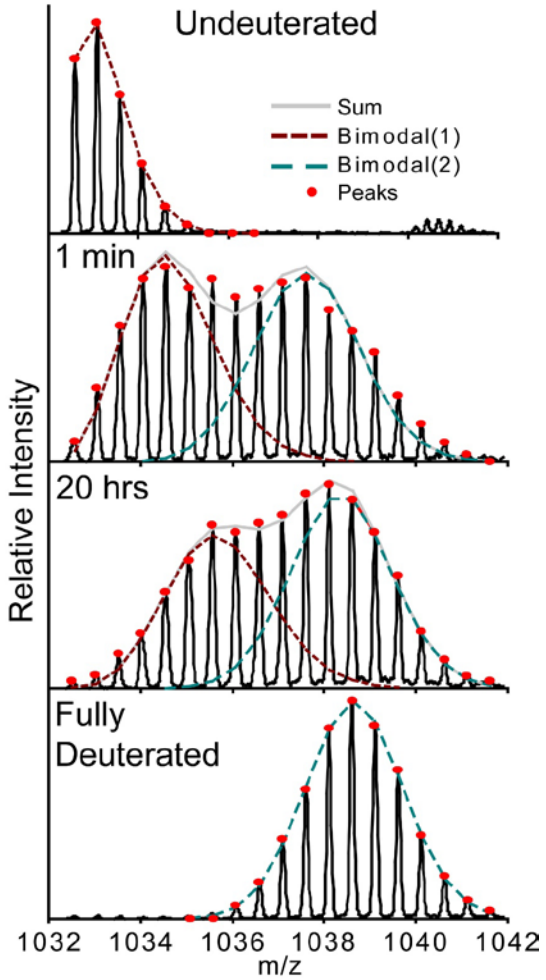
Conformational Purity from HDX







~80% pure



~50% pure

HX MS UPLC System

nanoAcquity UltraPerformance LC® HD-Exchange

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LEAP Technologies

Anal. Chem. **2008**, *80*, 6815–6820

High-Speed and High-Resolution UPLC Separation at Zero Degrees Celsius

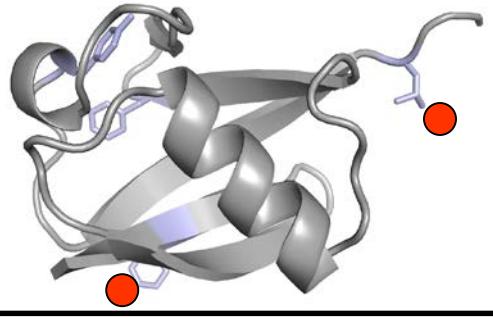
Thomas E. Wales,[†] Keith E. Fadgen,[‡] Geoff C. Gerhardt,[‡] and John R. Engen*[,†,§]

The Barnett Institute and the Department of Chemistry & Chemical Biology, Northeastern University, Boston, Massachusetts 02115, and Waters Corporation, Milford, Massachusetts 01757

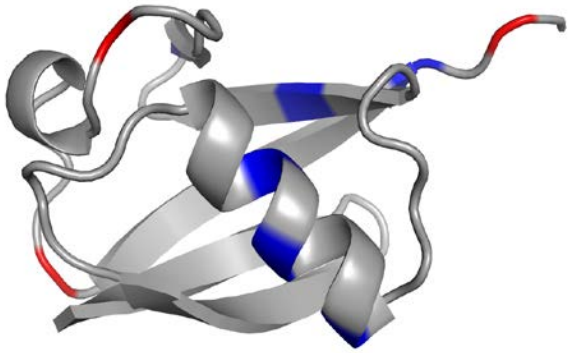
Protein structural information from a mass measurement

Covalent modifications

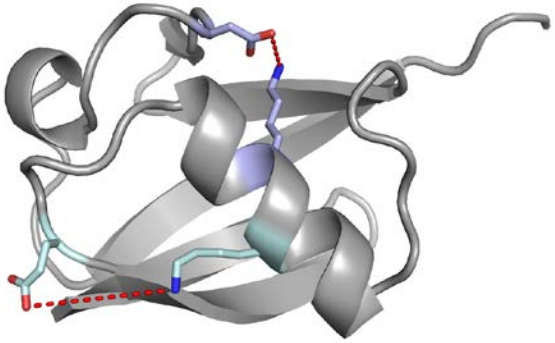
- Side chain specific reagents
- Oxidative labeling



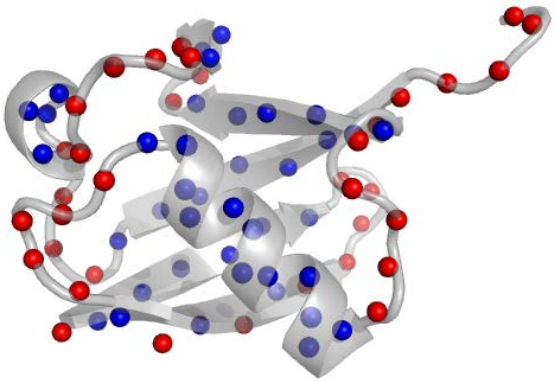
Proteolytic susceptibility



Cross-linking



Hydrogen/Deuterium exchange

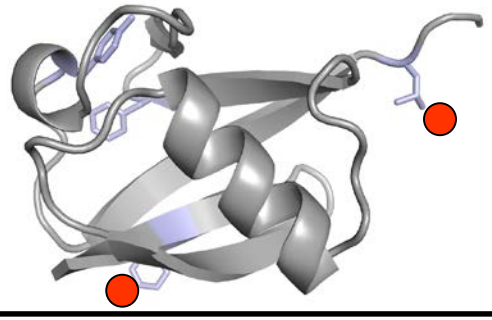


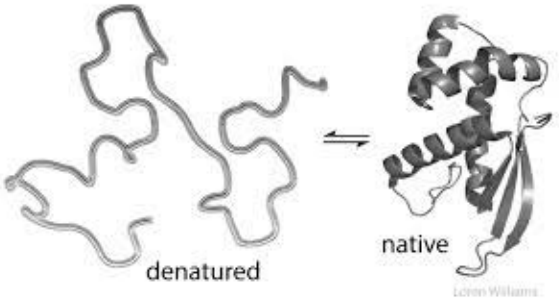
Covalent foot printing with MS

- Stable covalent modification
  - Oxidative labeling (hydroxyl radicals)
- Exposed sidechains are more reactive than buried ones
- Protein is perturbed by labeling!
  - Fast labeling is key (ms or faster)

Covalent modifications

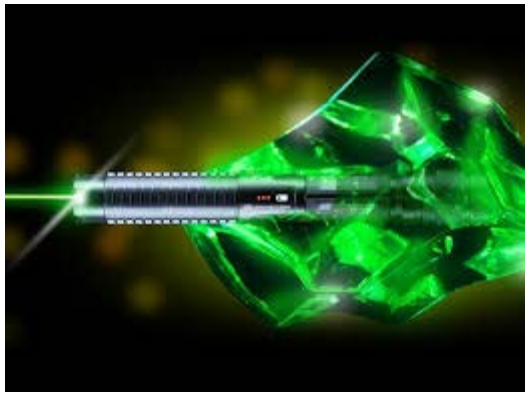
- Side chain specific reagents
- Oxidative labeling





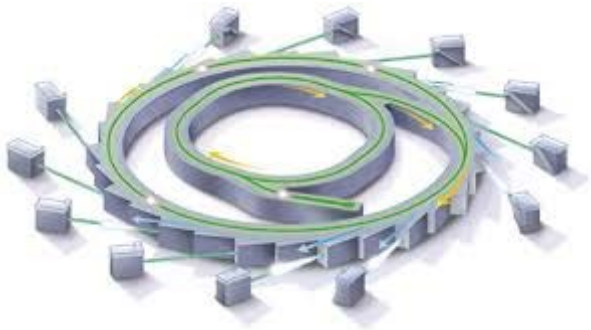
Fast protein oxidation via radicals

- Fast photochemical oxidation of proteins (FPOP)
  - UV laser splits hydrogen peroxide to form radicals

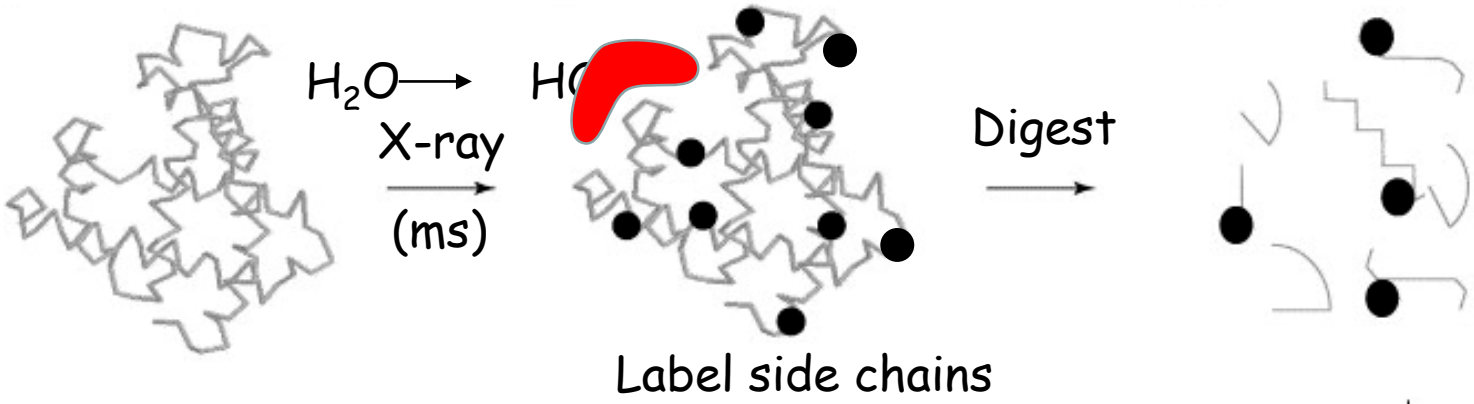


$$H_2O_2 \xrightarrow{h\nu} 2\,HO^{\bullet}$$

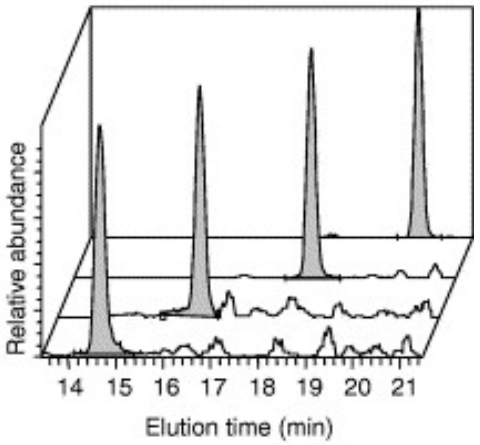
- X-ray foot printing
- Synchrotron radiation
  - X-rays split water to form radicals



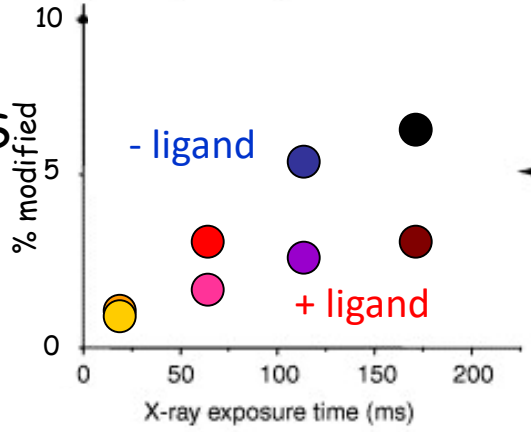
X-ray Footprinting (XF-MS)



LC-MS

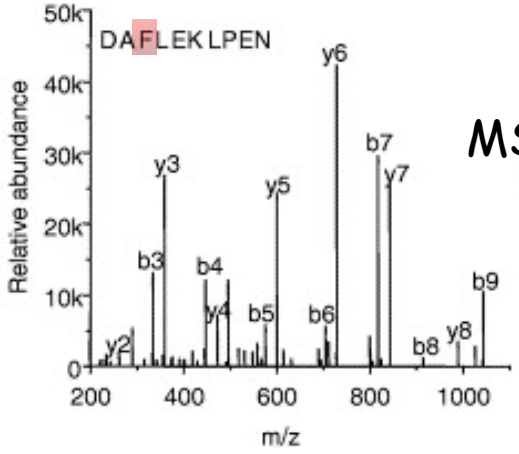


Dose response



MS/MS

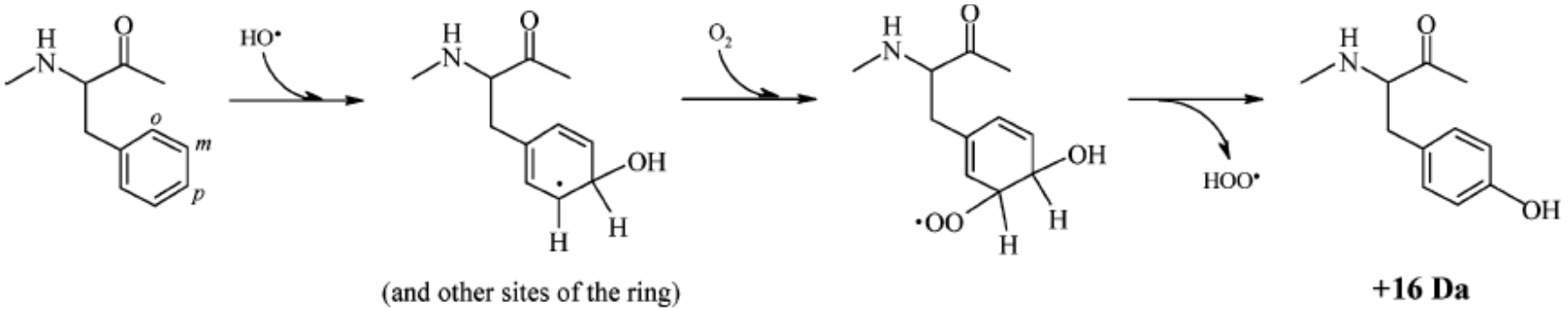
Site identification



Guan & Chance Trends in Biochemical sciences 2005

Chemistry of side chain oxidation

- Most reactive sites commonly yield +16 Da species
  - Met, Cys, Phe, Tyr, Trp*



Xu & Chance, *Chem. Rev.* 2007