Mass Spectrometric Methods for Predictive Characterization of Lyophilized Products

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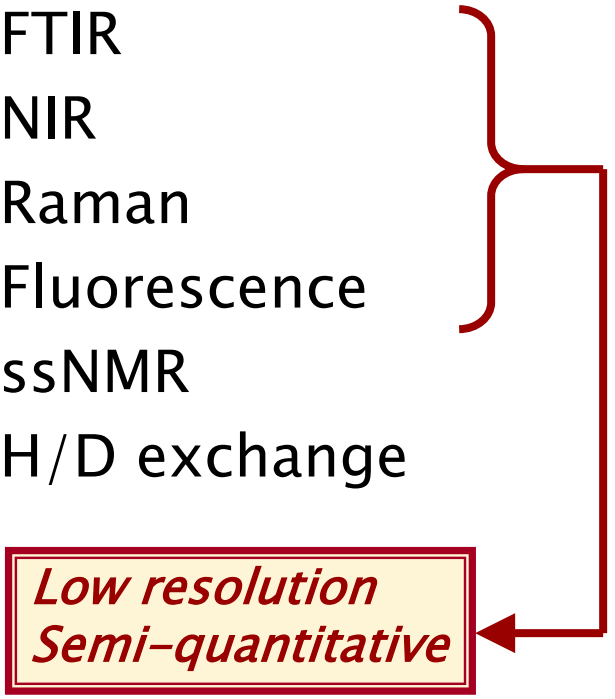
PURDUE UNIVERSITY

Amorphous solids and pharmaceutical biotechnology

- Recombinant protein drugs are often stored as amorphous powders.
  - Bulk API storage
  - Final product
- Of nine protein drug products approved by US FDA in 2011, five are solids.
- Produced by lyophilization or spray drying.
- Degradation can occur during lyophilization and storage.
- Few analytical methods for protein conformation in solids, low resolution, semi-quantitative.

Analytical methods for protein structure

- In solution
  - NMR (2-D)
  - Circular dichroism (CD)
  - Infrared (FTIR)
  - NIR
  - Raman
  - Fluorescence
  - U.V. absorption
  - H/D exchange
  - X-ray crystallography
- In solids
  - FTIR
  - NIR
  - Raman
  - Fluorescence
  - ssNMR
  - H/D exchange



Central hypothesis

Chemical definition of amorphous solids is possible and can be used to rationally design solid formulations of protein drugs.

Current paradigm:

- chemical environment in amorphous solids is unknowable,
- protein conformation in solids can only be measured with low resolution,
- trial-and-error informed by limited physical characterization is the only possible approach to formulation development.

Hydrogen/deuterium exchange (HDX) for proteins in solids

- Conformation and excipient interactions with peptide-level resolution

Y. Li et al., *Biotech. Bioeng.*, 97/6: 1650-1653, 2007
Y. Li et al., *Anal. Biochem.*, 366:18-28, 2007
Y. Li et al. *Pharm. Res.*, 25/2: 259-267, 2008
S. Sinha et al., *Biophys. J.*, 95/12: 5951-5961, 2008
A. Sophocleous et al., *Mol. Pharm.*, 9/4: 718-726, 2012
A. Sophocleous et al., *Mol. Pharm.*, 9/4: 727-733, 2012
B.S. Moorthy et al., *Mol. Pharm.*, 11/6: 1869-1879, 2014
B.S. Moorthy et al., *JoVE*, (98), e52503, 2015

What is HDX?

- A chemical reaction, in which labile hydrogen atoms “exchange” with deuterium.

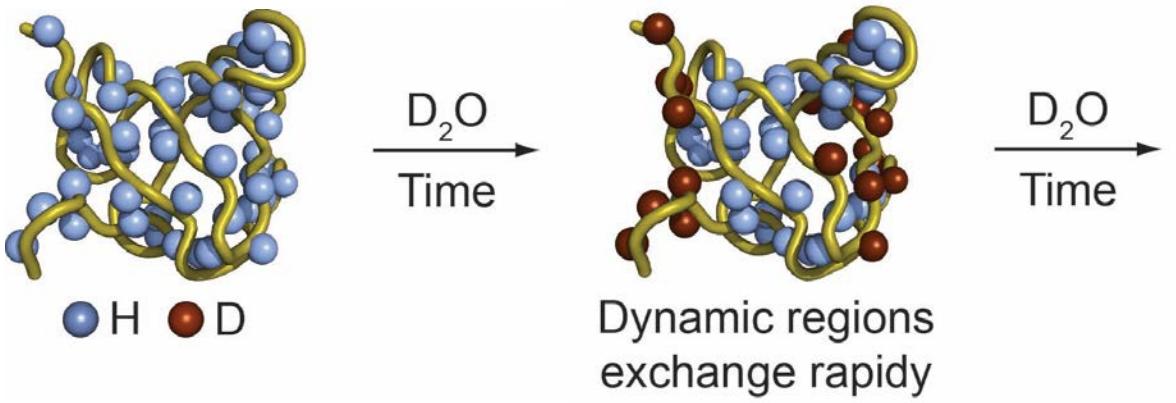
$$2R\text{–}H + D_2O \leftrightarrows 2R\text{–}D + H_2O$$

- Reaction is quenched at low pH and low temperature.
- The increase in mass (+1 amu) can be detected using mass spectrometry (LC–MS, LC–MS/MS).
- The rate and extent of HDX provides information on protein structure and dynamics in aqueous solution.
- Due to back exchange, only deuteration of backbone amide groups is detected.

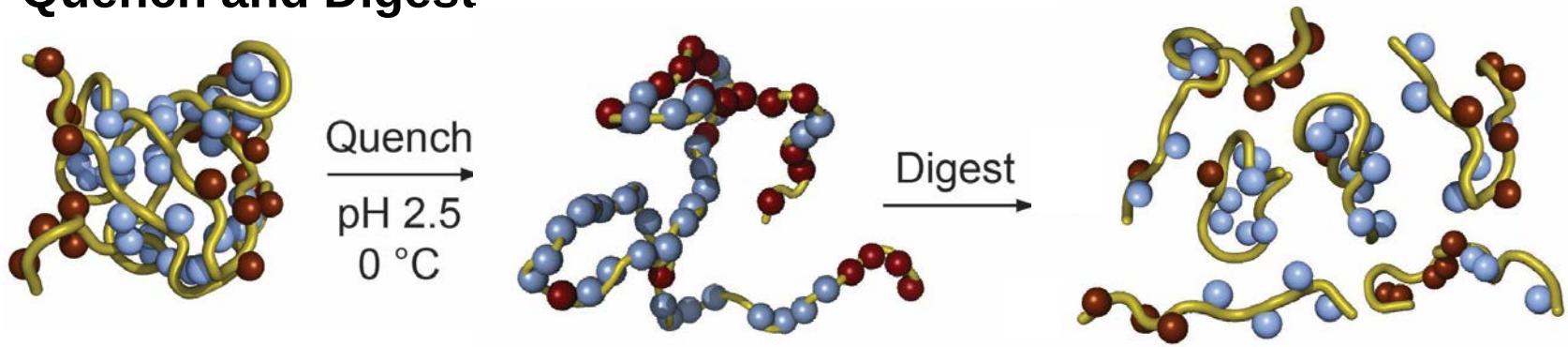
Basic principles of HDX

MS analysis

H/D Exchange



Quench and Digest

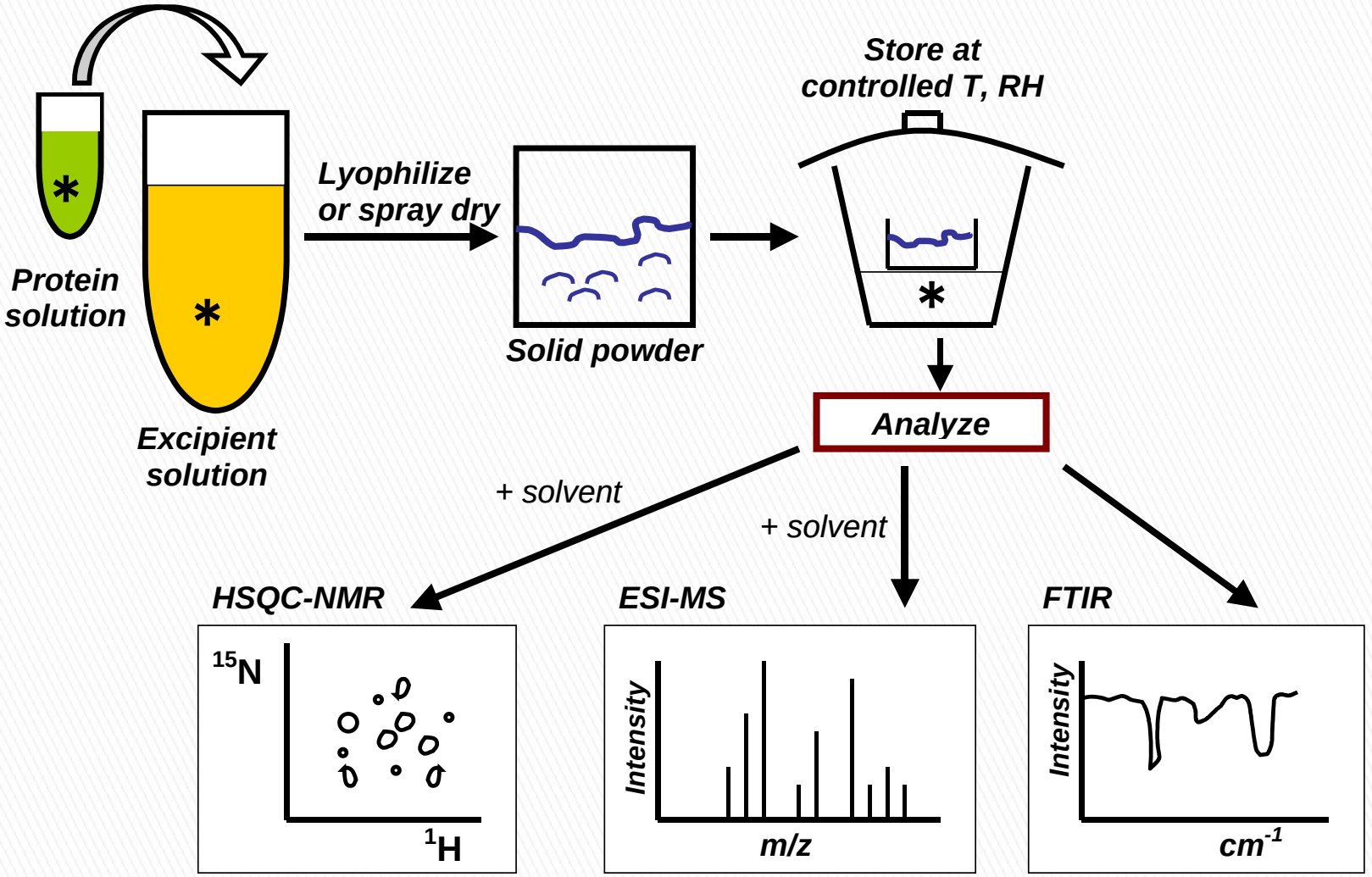


Quenching locks in deuterium and unfolds the protein

Digestion localizes the information

HDX for lyophilized solids (ssHDX)

Expose powder to D_2O vapor



Potential benefits of ssHDX

…and some unknowns

POTENTIAL BENEFITS

- Information on protein structure, with peptide-level resolution.
- Screen formulations and/or processes for effects on protein structure.
- Obtain fundamental information on protein structure and dynamics in amorphous solids.
- Obtain fundamental information on amorphous solids (spatial, dynamic heterogeneity) with protein as a reporter.

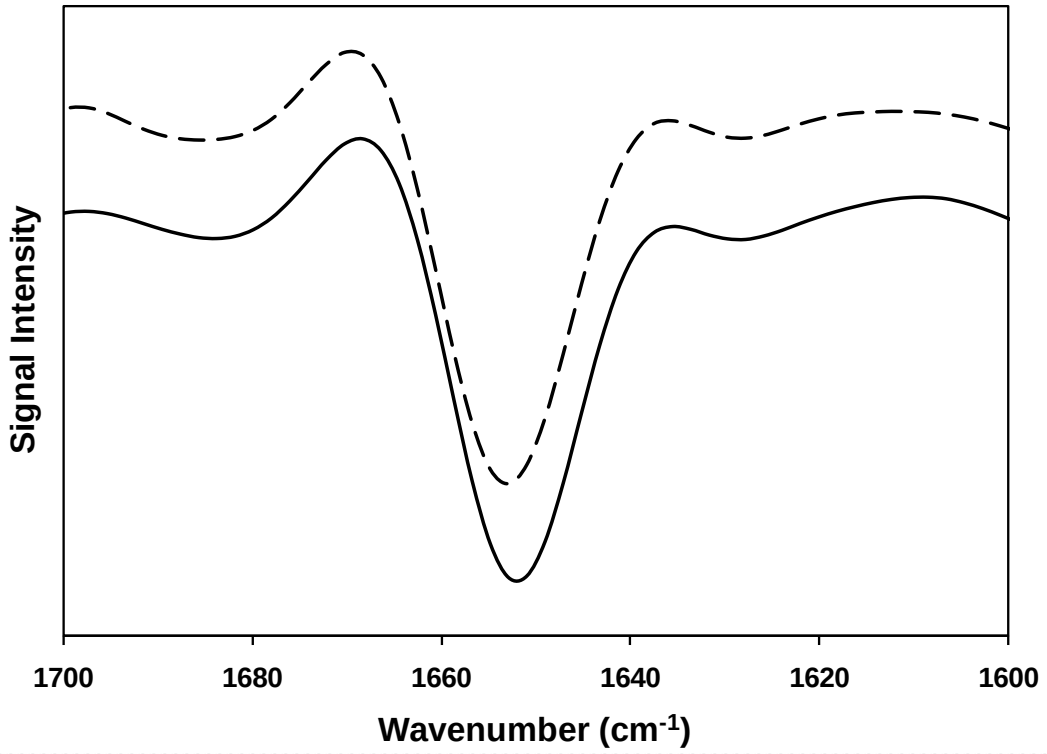
UNKNOWNS

- Will it work?
- Will $D_2O(g)$ sorption kinetics affect the results?
- Are HDX results predictive of storage stability?
- How should the results be interpreted?

Myoglobin (holo)

http://emedicine.medscape.com/article/1007814-overview

No detectable secondary structural differences observed post-lyophilization by FTIR

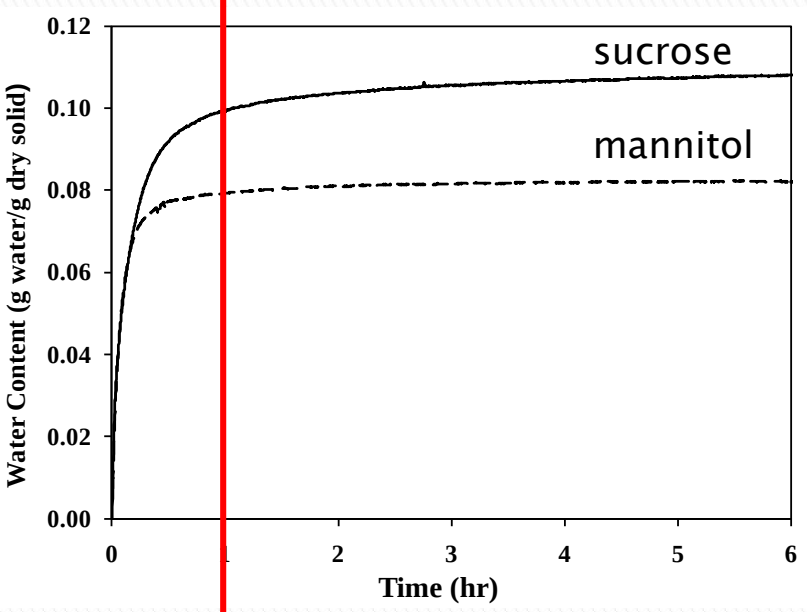


native-like α-helix peak at 1655 cm^{-1}

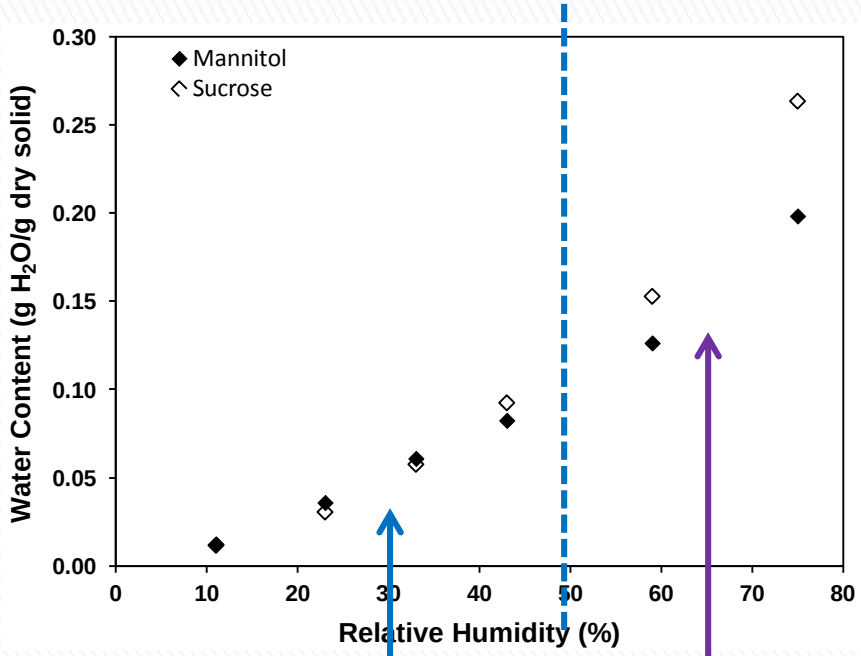
Second-derivative FTIR spectra of Mb in lyophilized solids containing mannitol (solid line) or sucrose (dashed line).

Water sorption and water content

Myoglobin / mannitol or sucrose 1:1, 5 °C



Water vapor sorption is essentially complete in ~ 1 h.

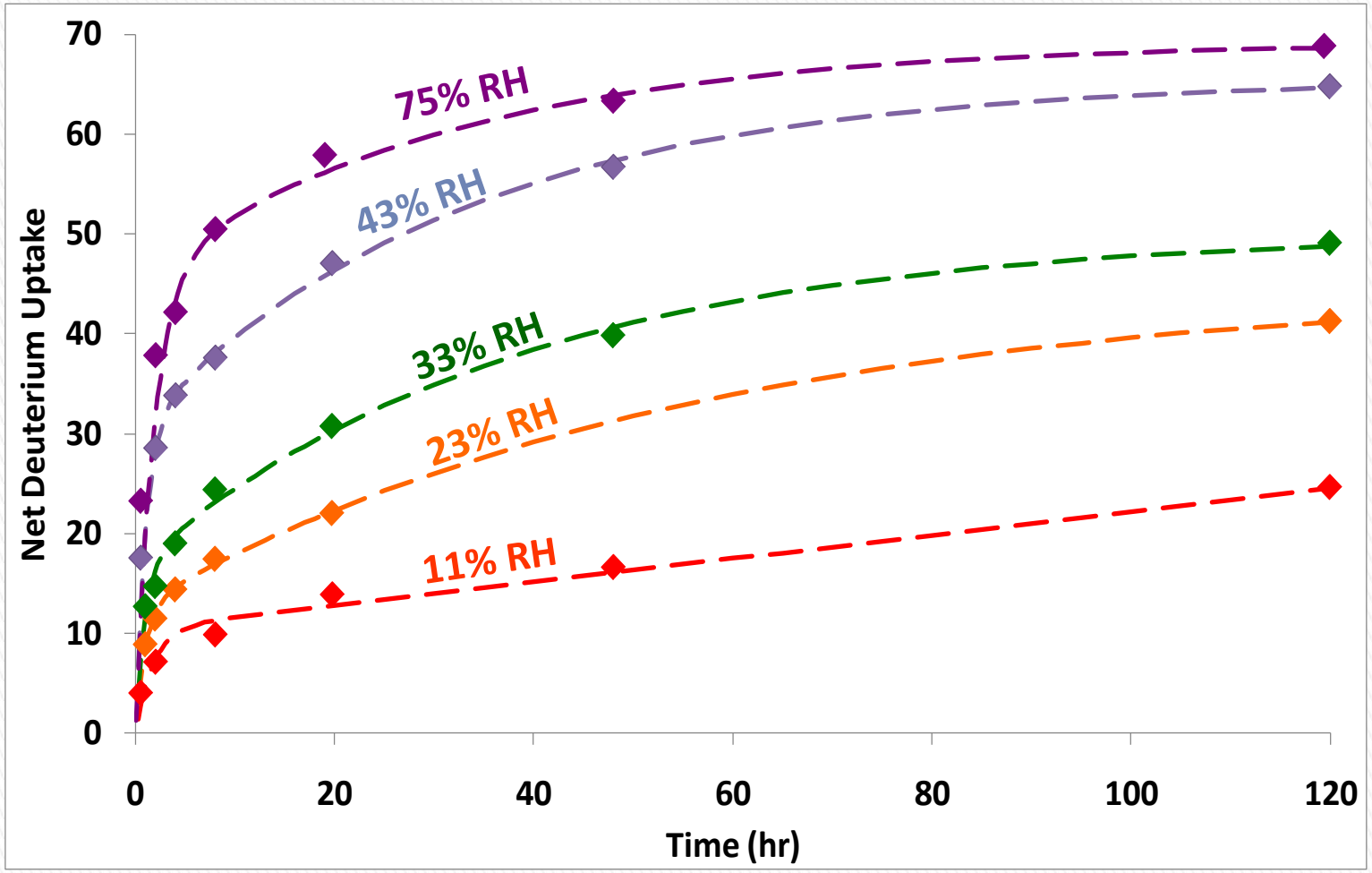


At low RH, water content of mannitol and sucrose formulations are equal.

At high RH, water content in sucrose formulation is greater than in mannitol.

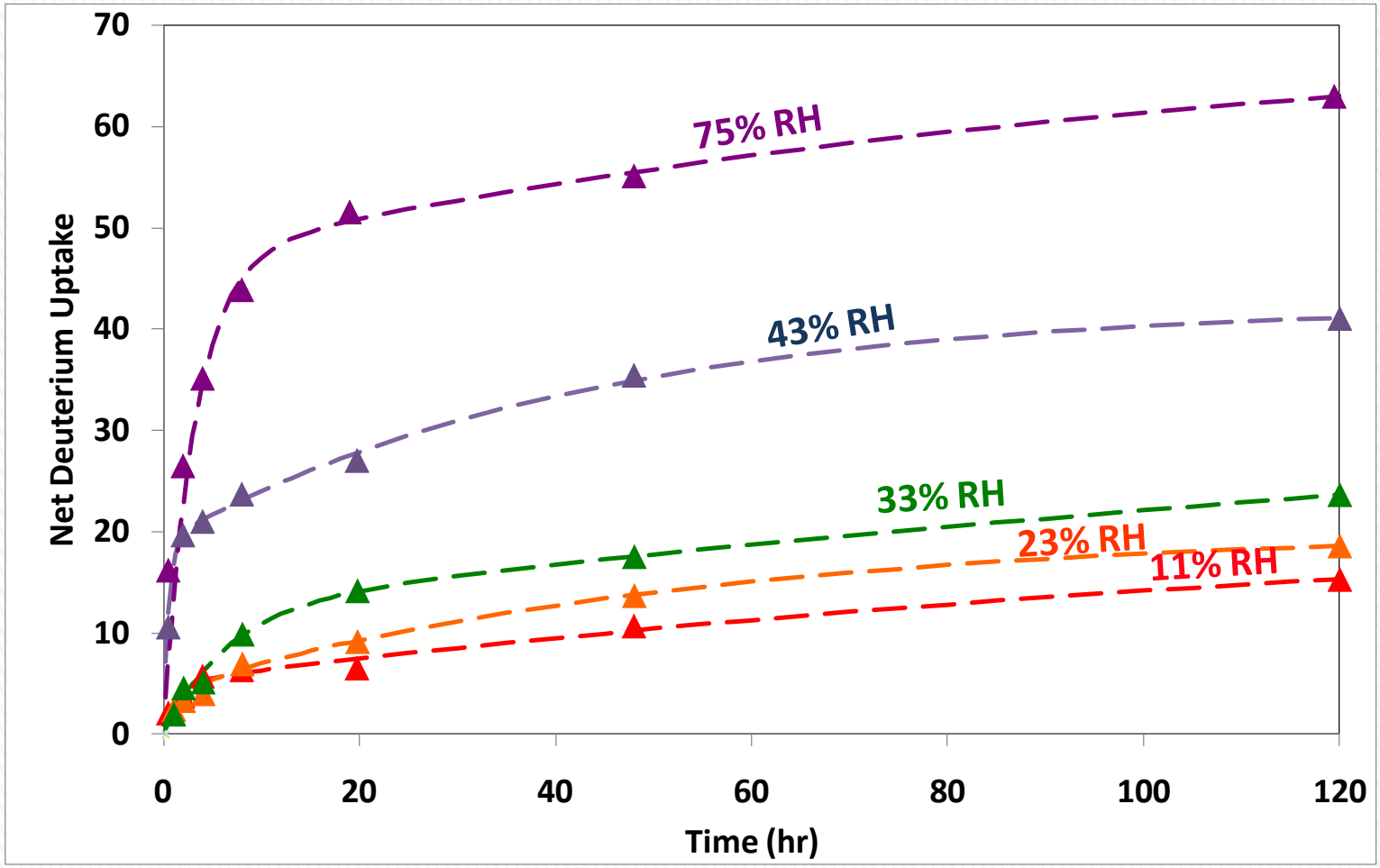
Effect of RH on deuterium uptake

Intact protein: *Myoglobin / mannitol 1:1, 5 °C*



Effect of RH on deuterium uptake

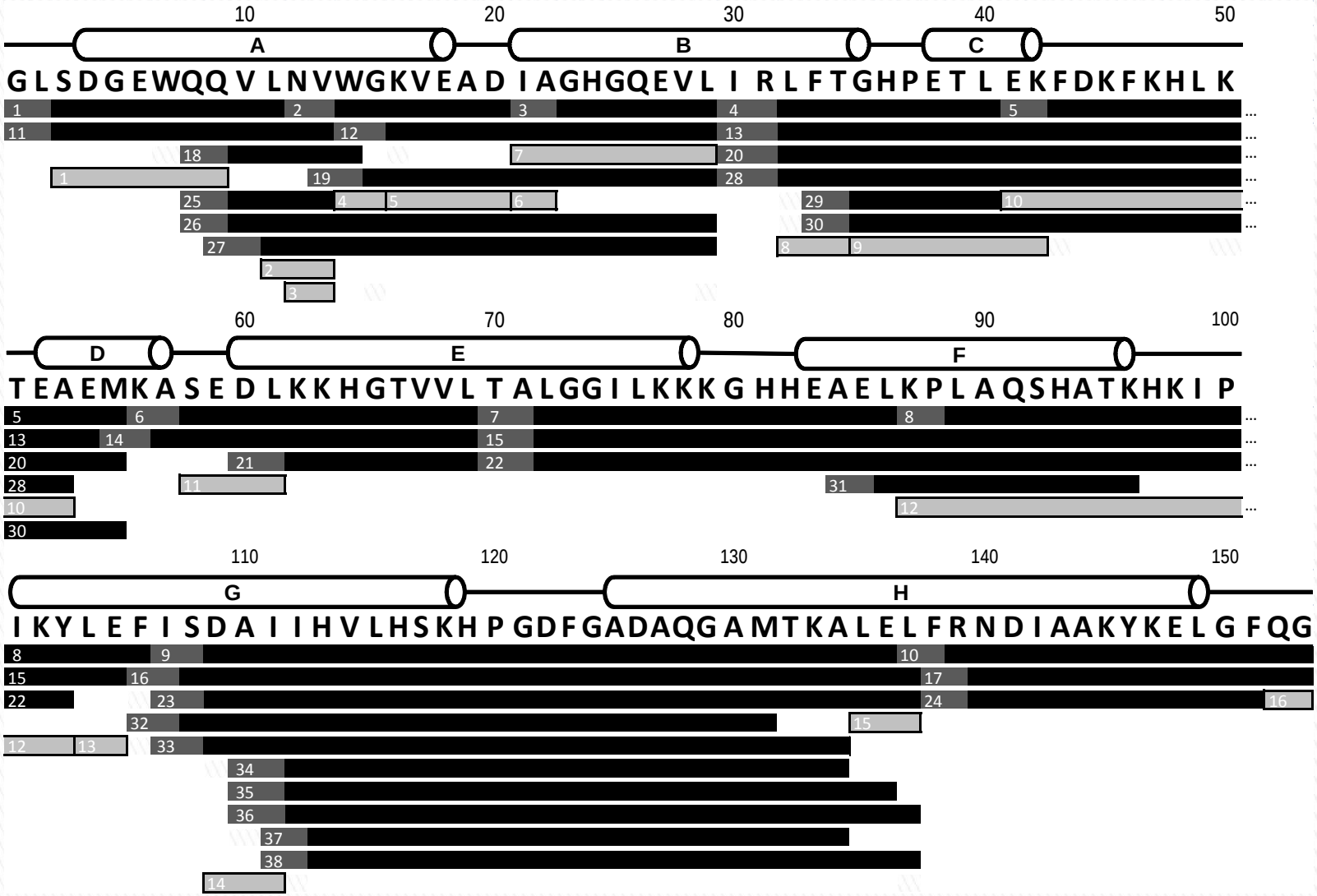
Intact protein: *Myoglobin / sucrose 1:1, 5 °C*



HDX in sucrose is less than mannitol *despite* similar or greater water content → protein structure/dynamics or spatial heterogeneity in water content

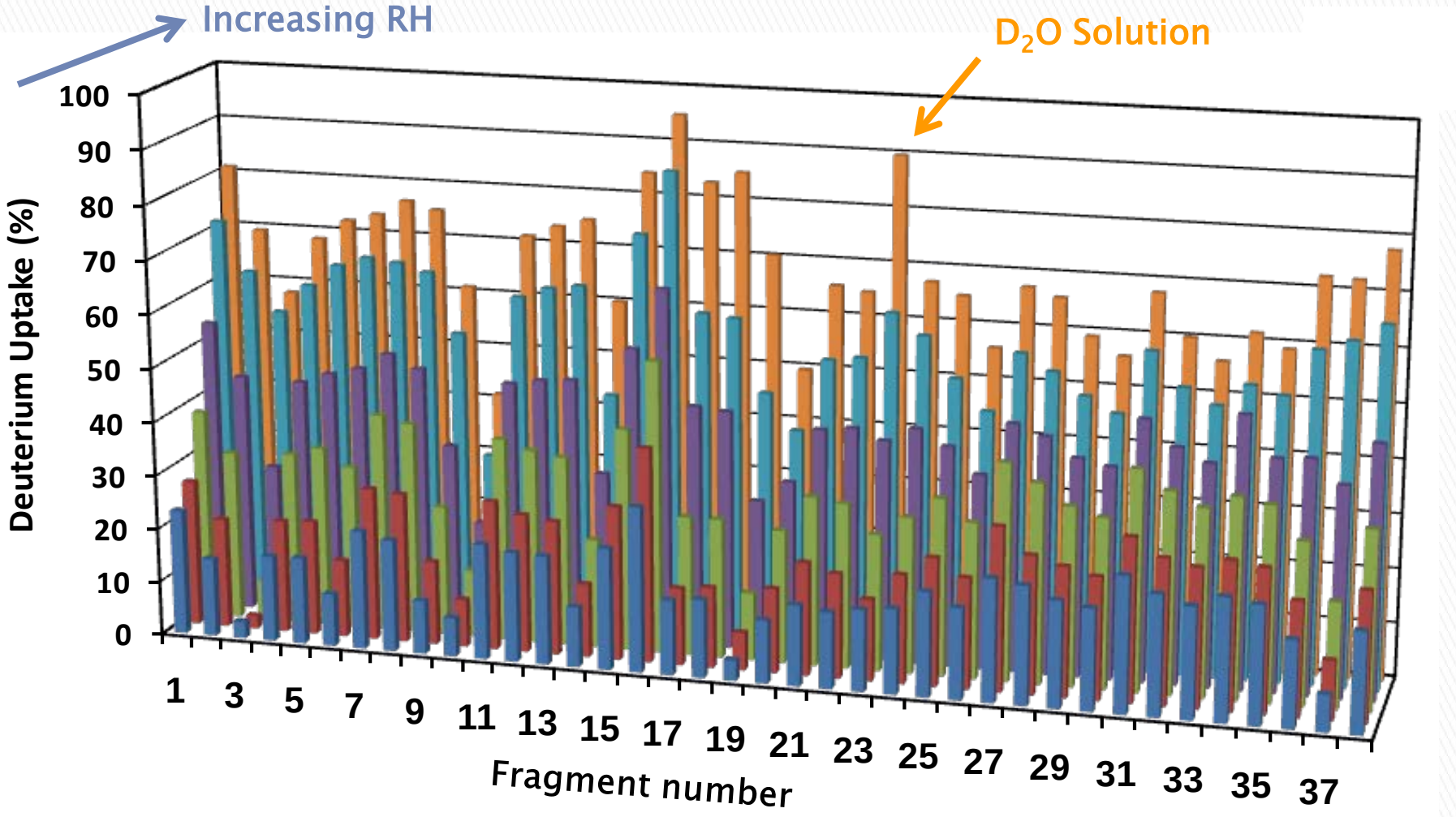
Myoglobin peptide map

Pepsin digestion



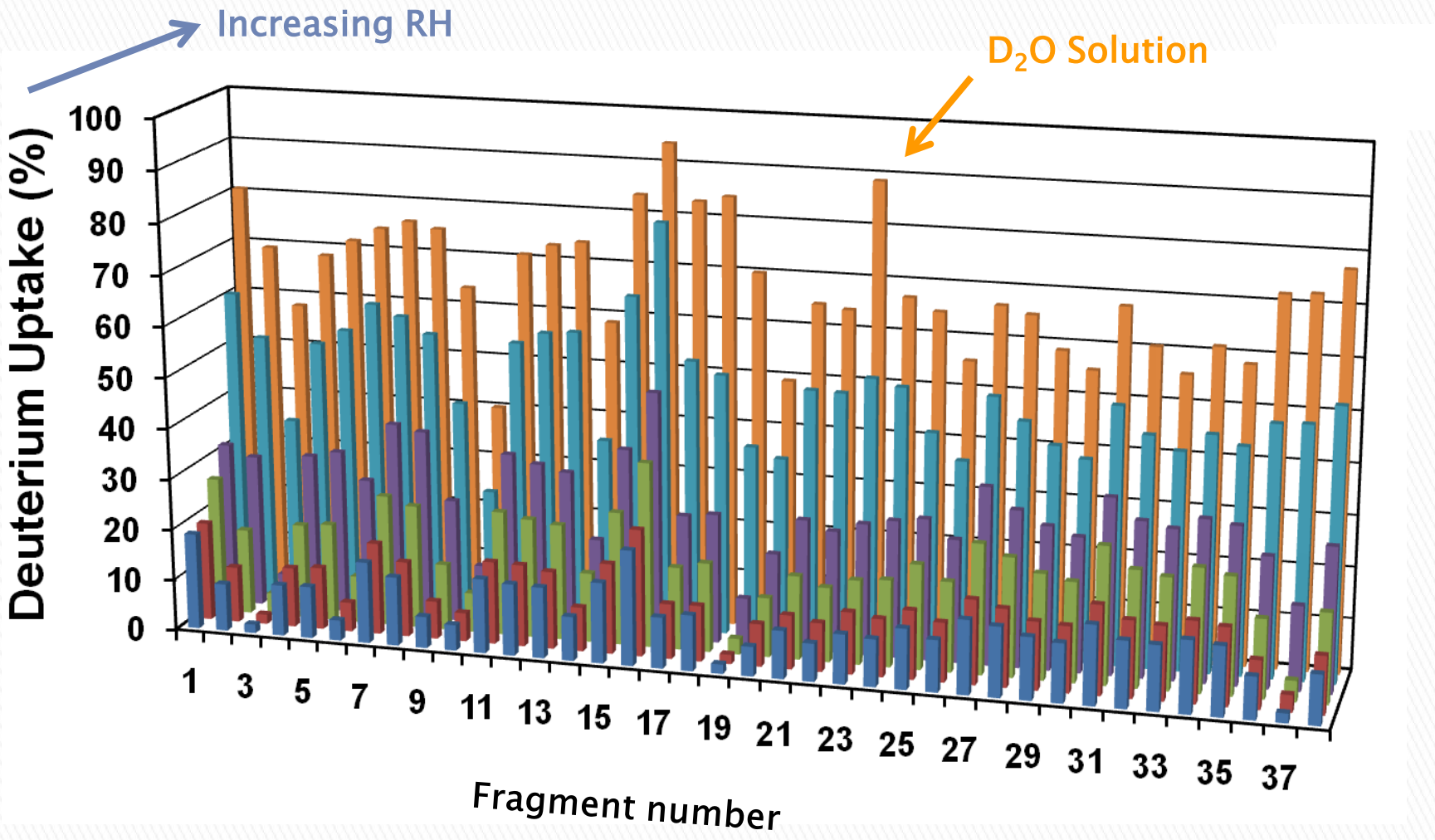
Peptic fragments

Myoglobin / mannitol 1:1, 5 °C



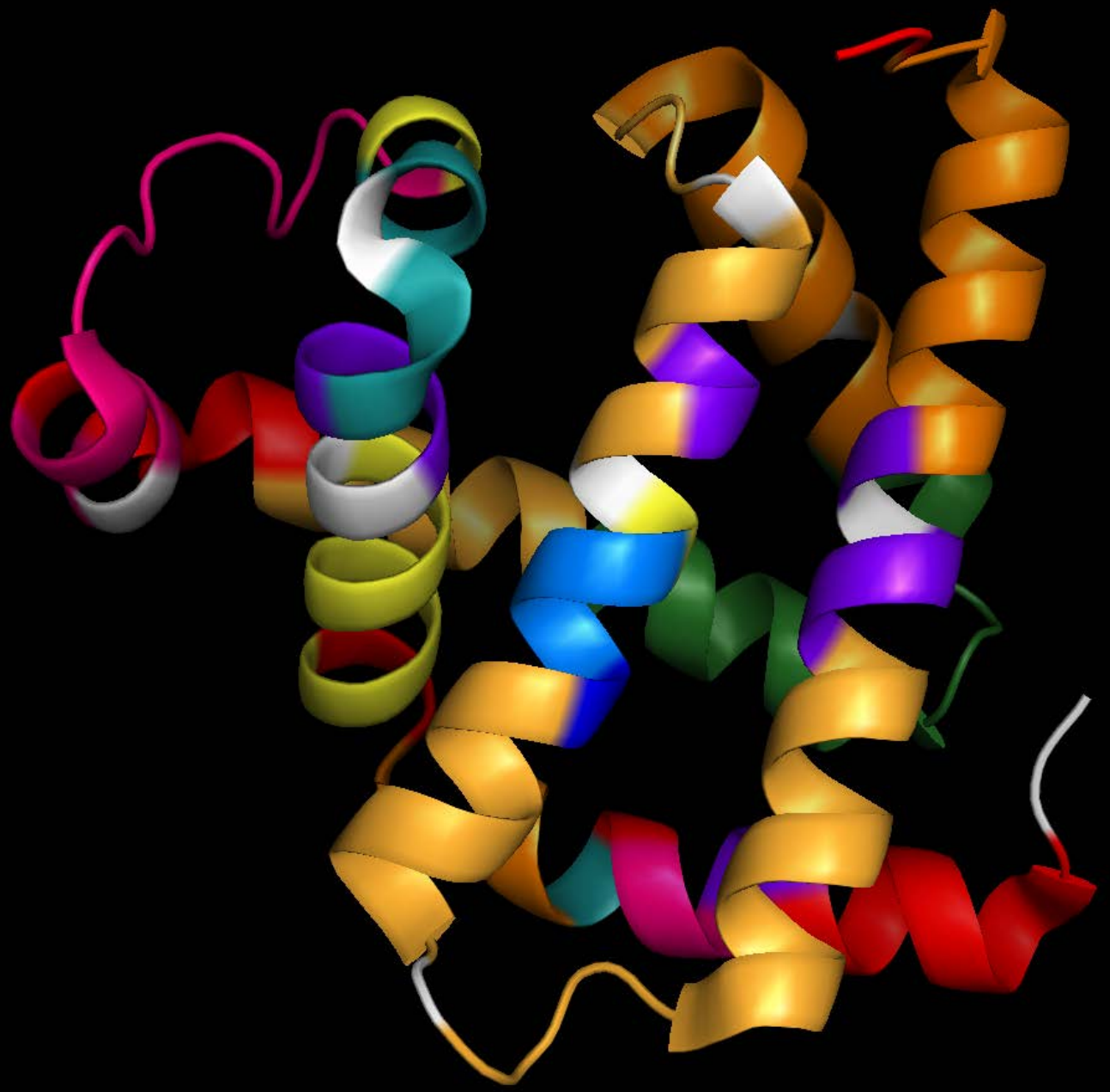
Peptic fragments

Myoglobin / sucrose 1:1, 5 °C



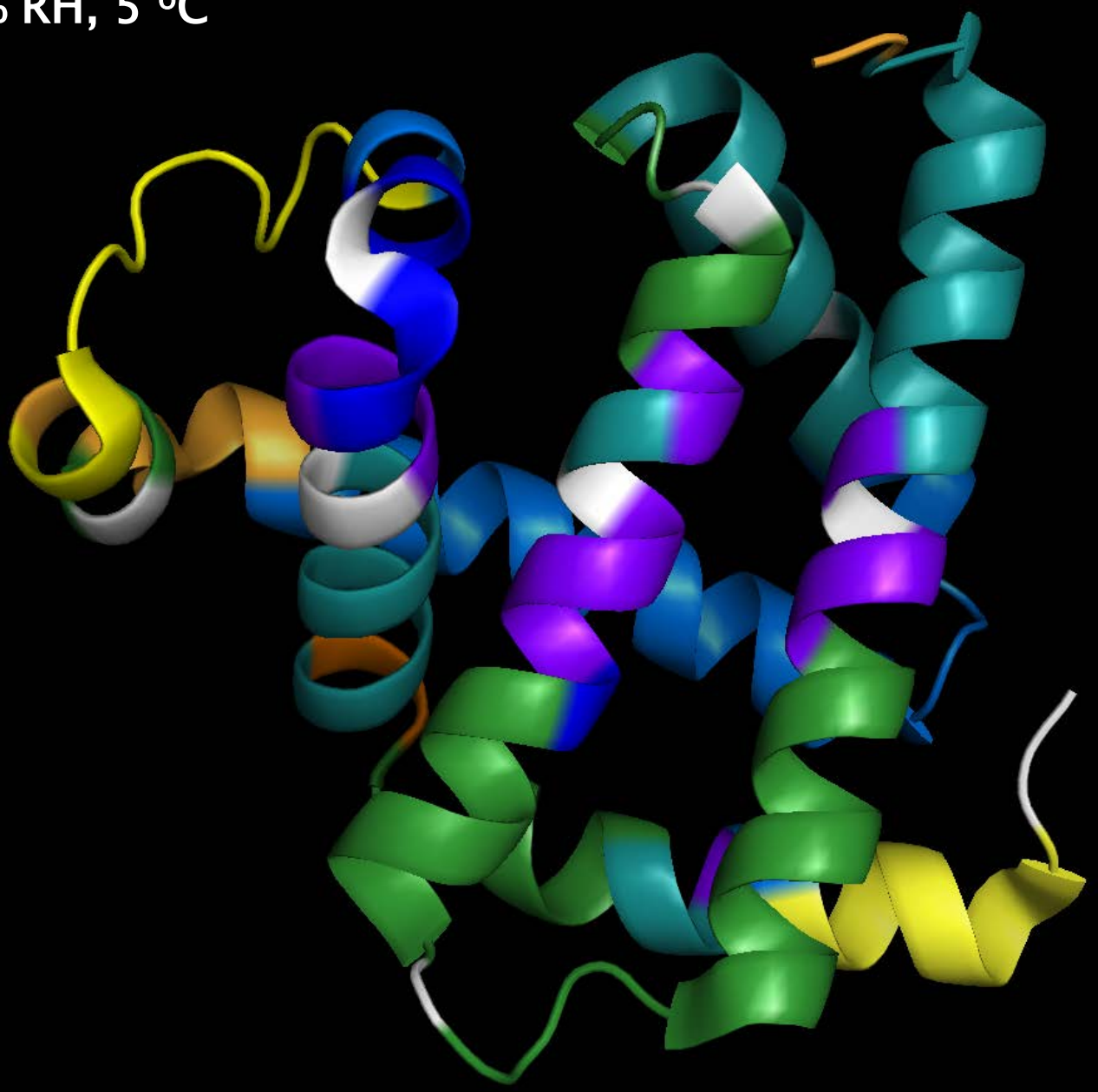
Myoglobin in D_2O solution

5 °C



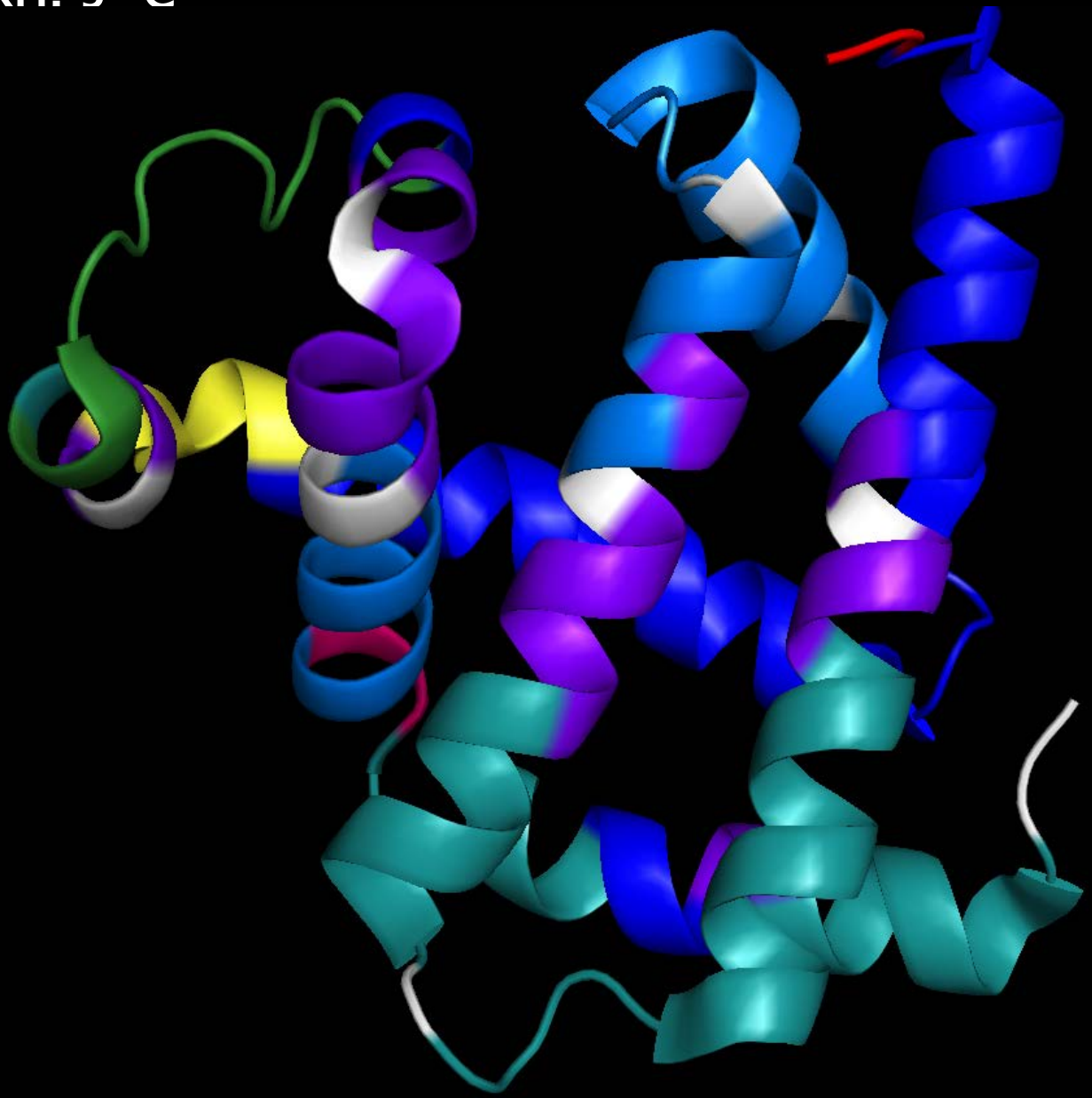
Lyophilized Myglobin: Mannitol

(1:1 w/w), 43% RH, 5 °C



Lyophilized Myglobin: Sucrose

(1:1 w/w), 43% RH. 5 °C



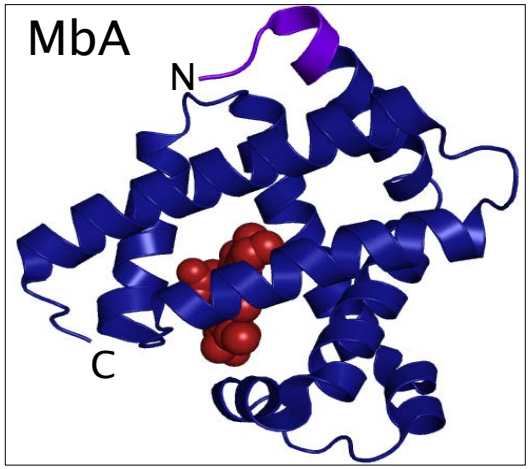
ssHDX and Storage Stability

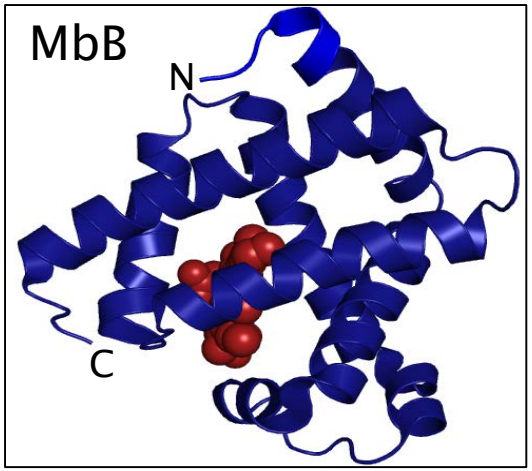
Five Mb formulations, one year storage at 25 and 40C

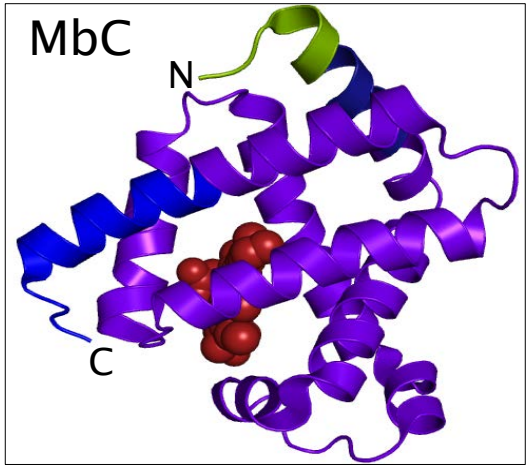
Ingredients	Formulations				
	MbA	**MbB**	**MbC**	**MbD**	**MbE**
Myoglobin (Mb)	1.7 mg/mL, 45% w/w	1.7 mg/mL, 45% w/w	1.7 mg/mL, 45% w/w	3.4 mg/mL, 90% w/w	0.4 mg/mL, 10% w/w
Sucrose	1.7 mg/mL, 45% w/w	–	–	–	3.0 mg/mL, 80% w/w
Mannitol	–	1.7 mg/mL, 45% w/w	–	–	–
NaCl	–	–	1.7 mg/mL, 45% w/w	–	–
Potassium Phosphate, (pH 7.0)	0.4 mg/mL, 10% w/w	0.4 mg/mL, 10% w/w	0.4 mg/mL, 10% w/w	0.4 mg/mL, 10% w/w	0.4 mg/mL, 10% w/w

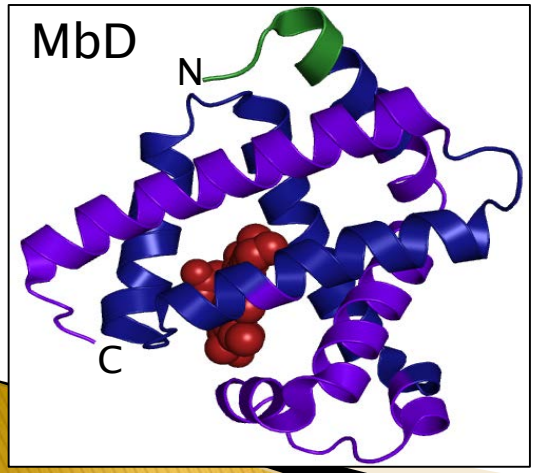
B.S. Moorthy et al., *Mol. Pharm.*, 11/6: 1869–1879, 2014

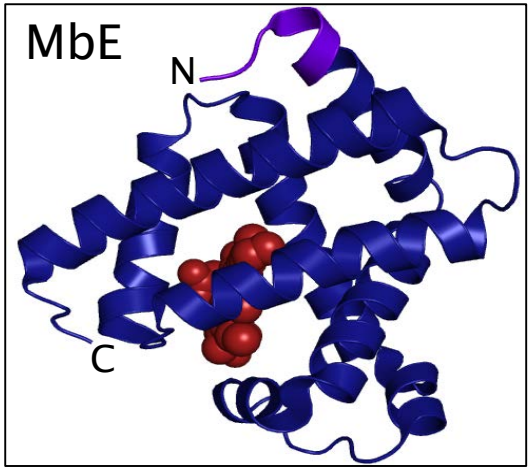
HDX Results Mapped on to the Mb Structure (T=1h)



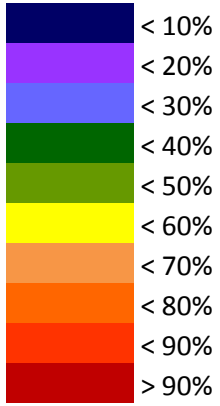




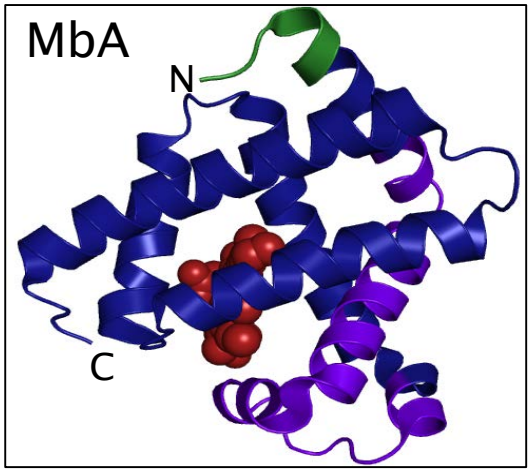


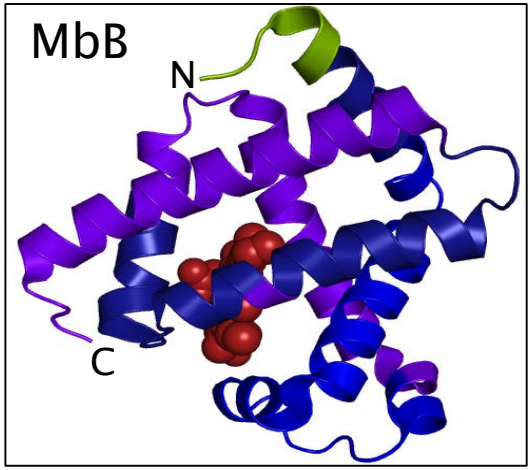


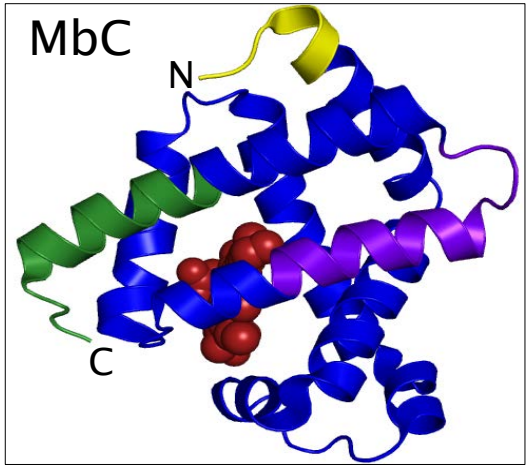
Deuterium Level

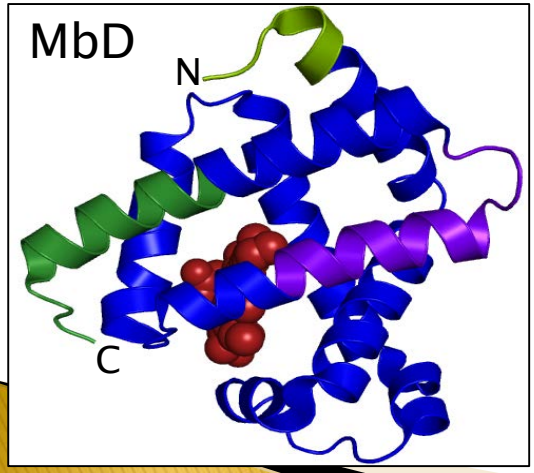


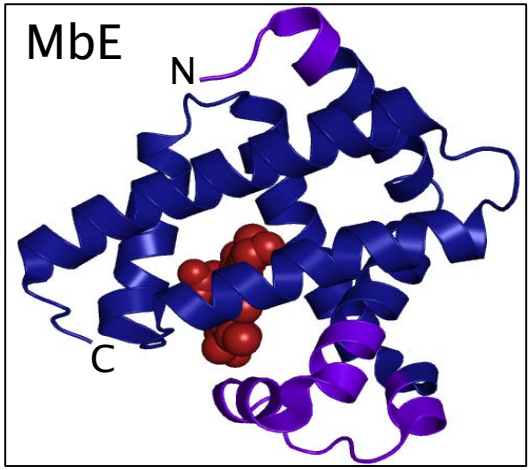
HDX Results Mapped on to the Mb Structure (T=6h)



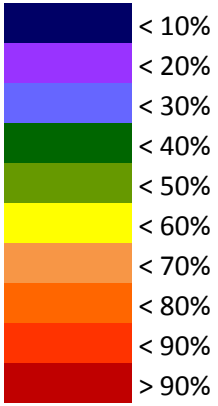




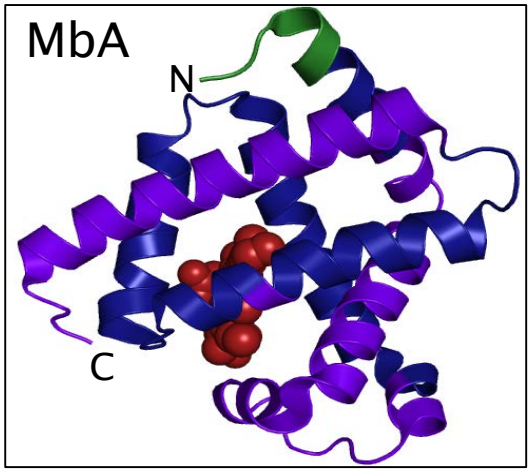


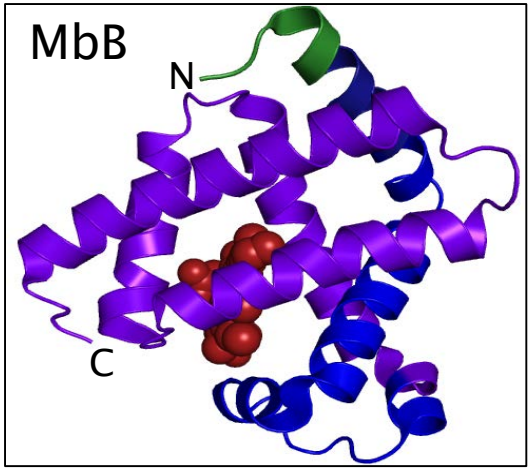


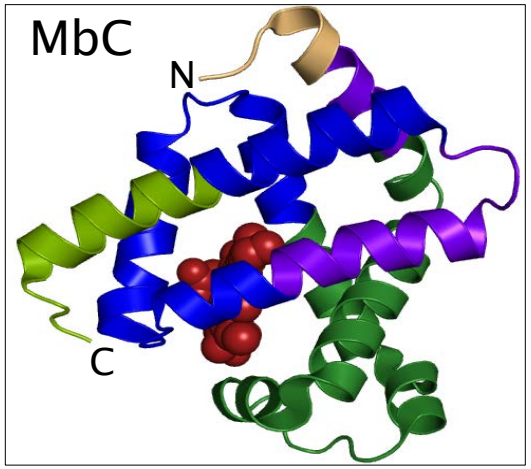
Deuterium Level

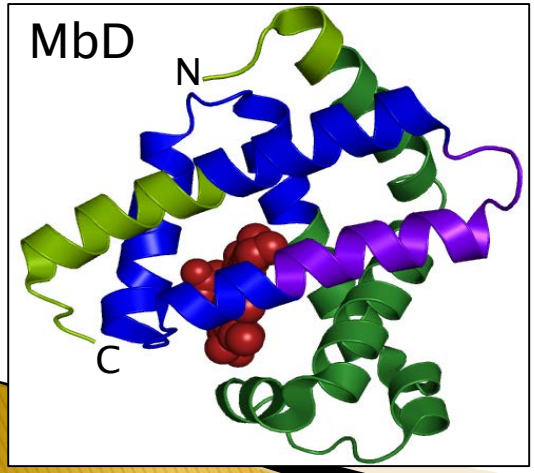


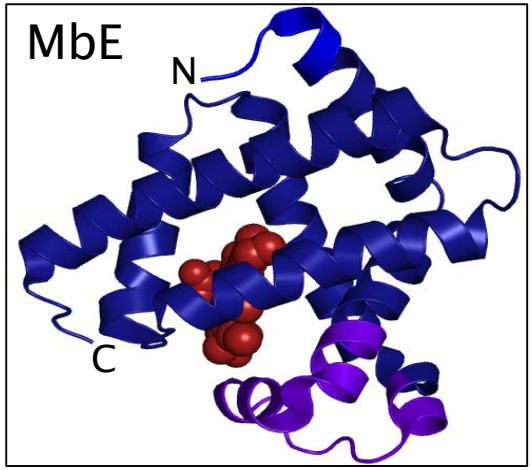
HDX Results Mapped on to the Mb Structure (T=24h)



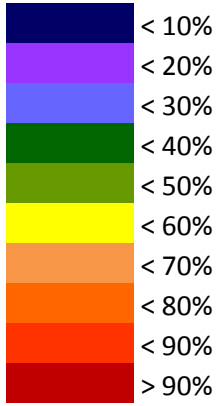




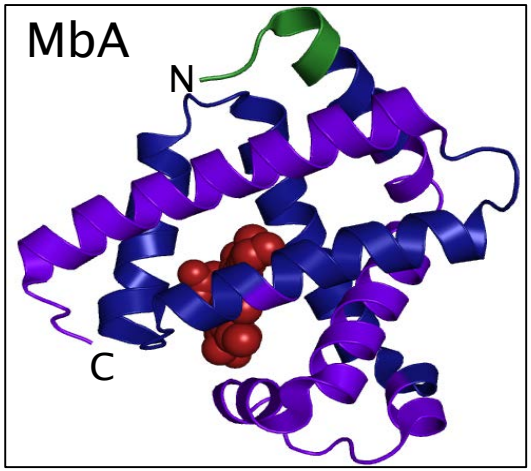


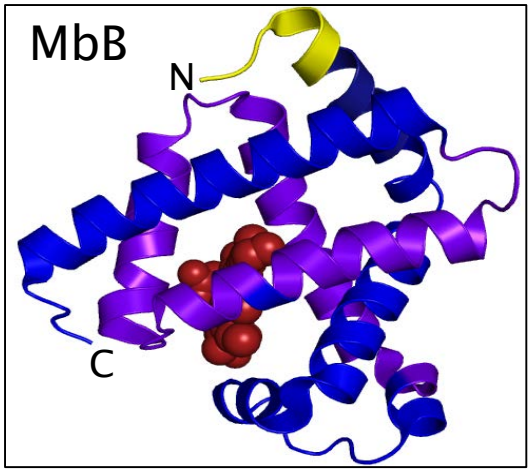


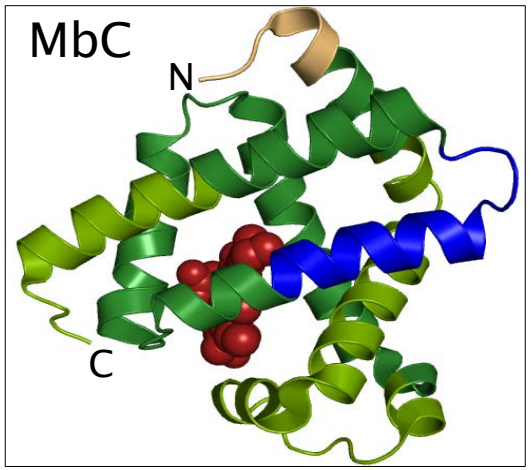
Deuterium Level

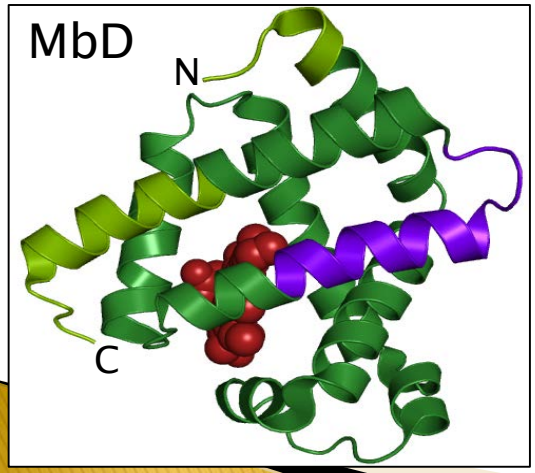


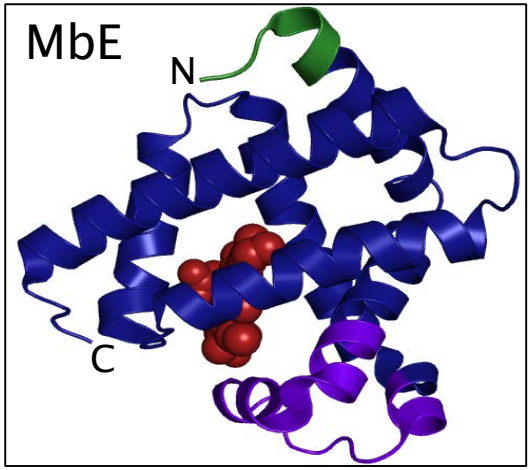
HDX Results Mapped on to the Mb Structure (T=48h)



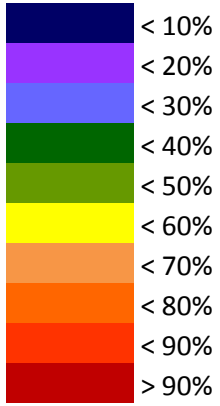




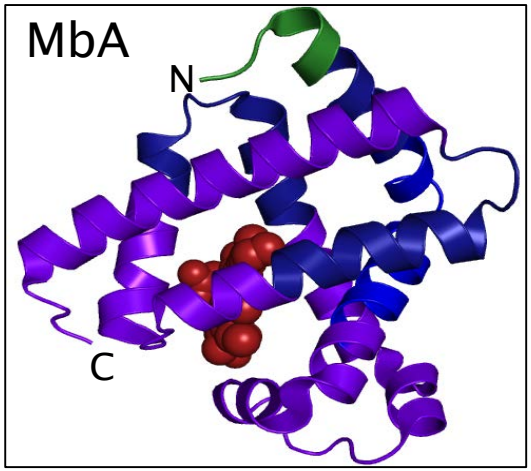


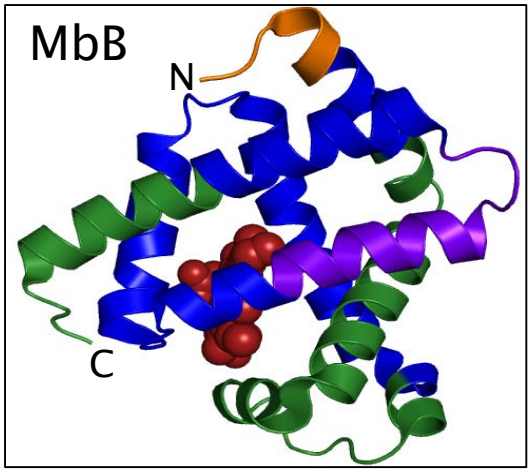


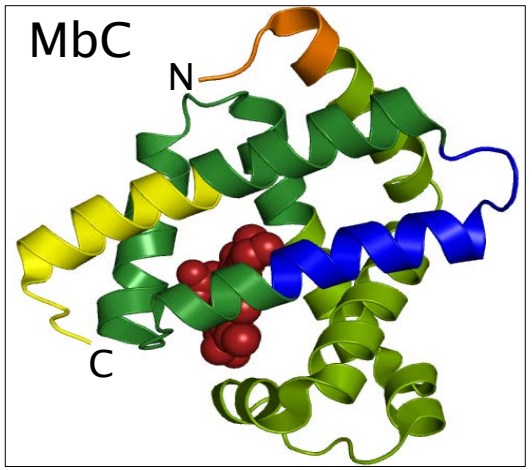
Deuterium Level

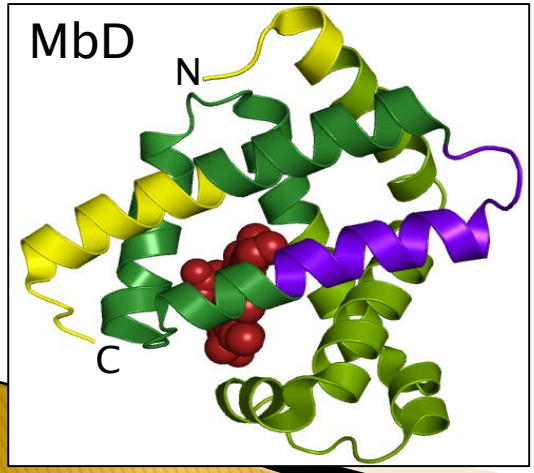


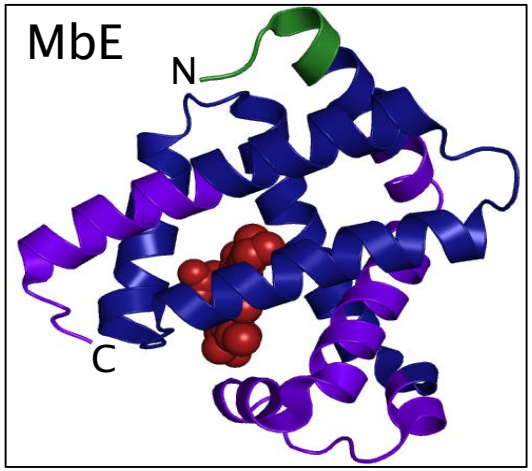
HDX Results Mapped on to the Mb Structure (T=120h)



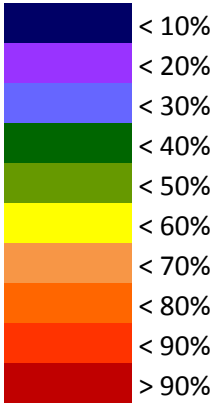




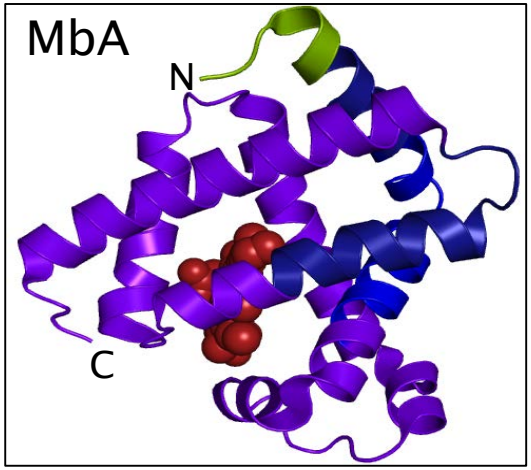


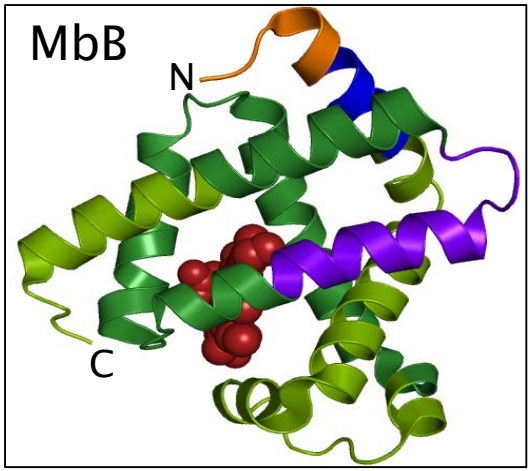


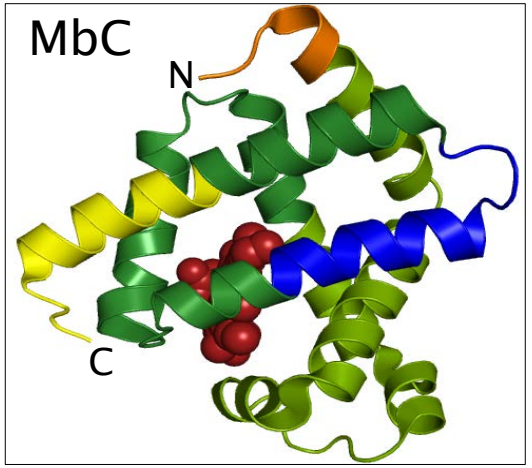
Deuterium Level

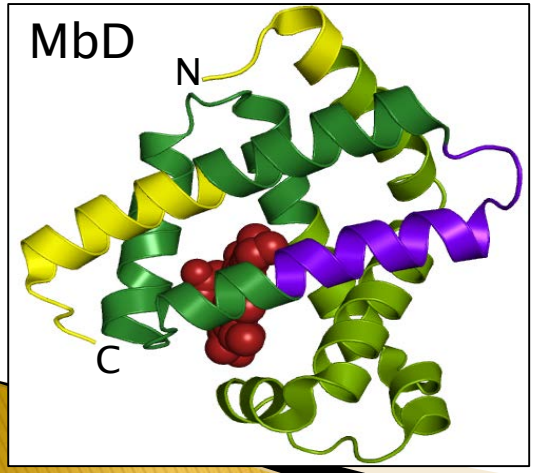


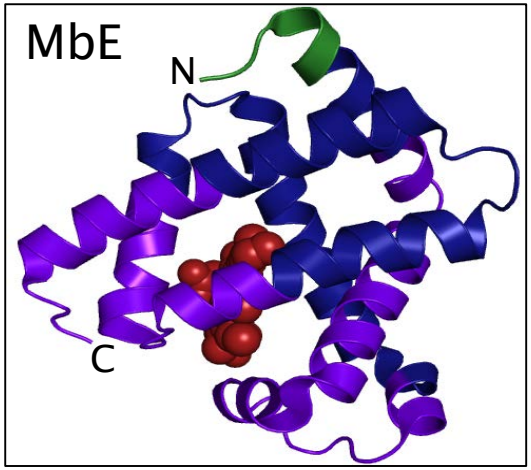
HDX Results Mapped on to the Mb Structure (T=240h)



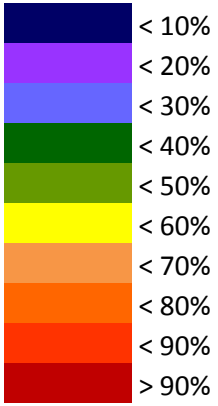






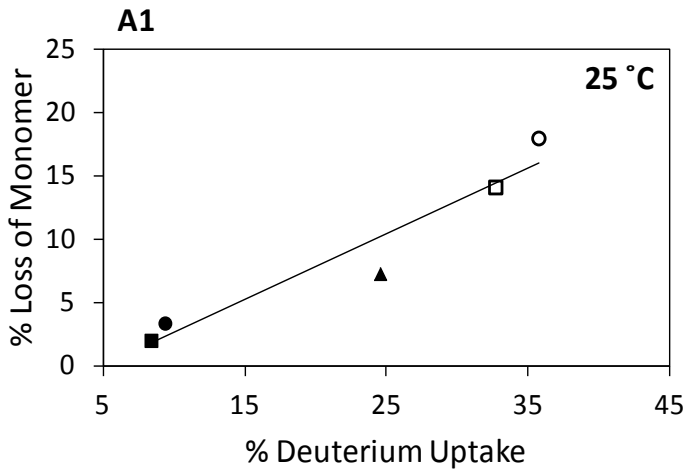


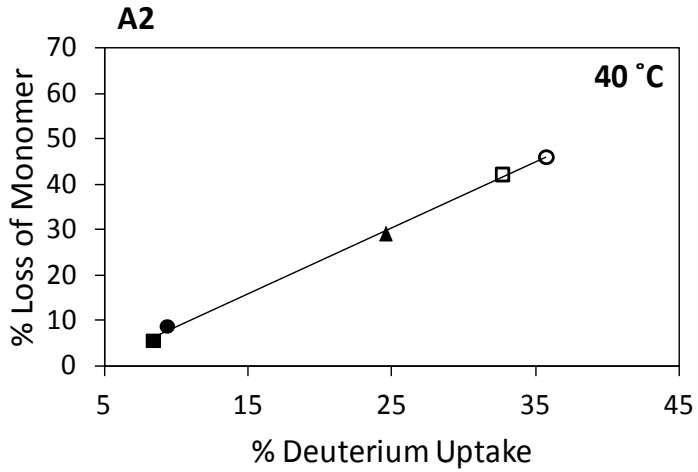
Deuterium Level

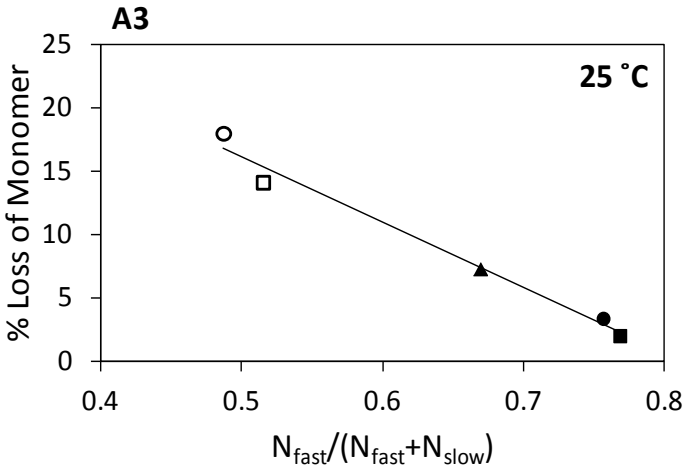


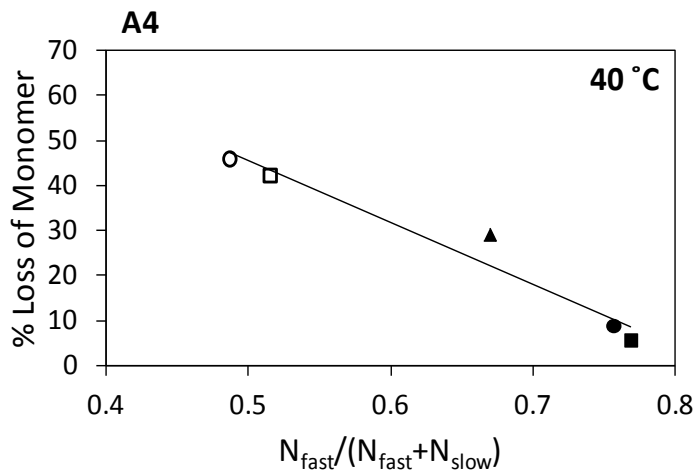
ssHDX and storage stability

Storage for 360 days, 25 and 40 °C; stability by SEC; 48 h ssHDX



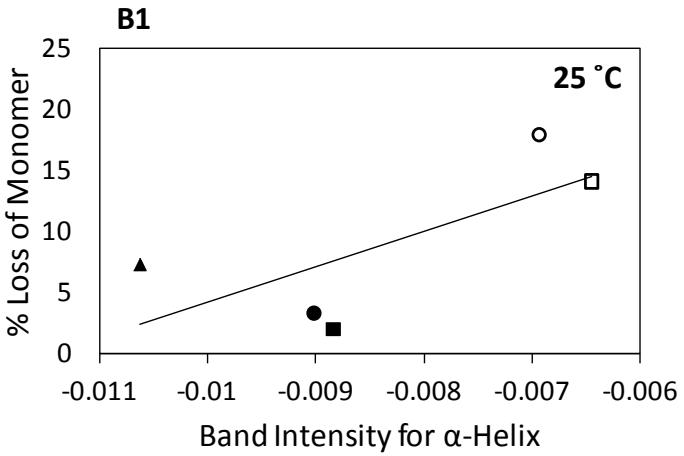


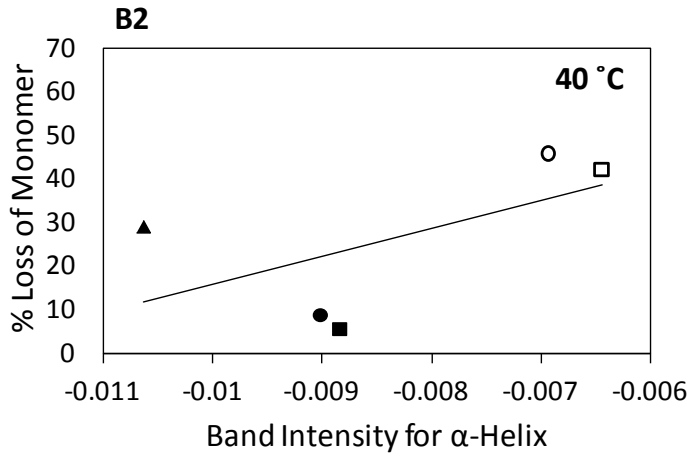


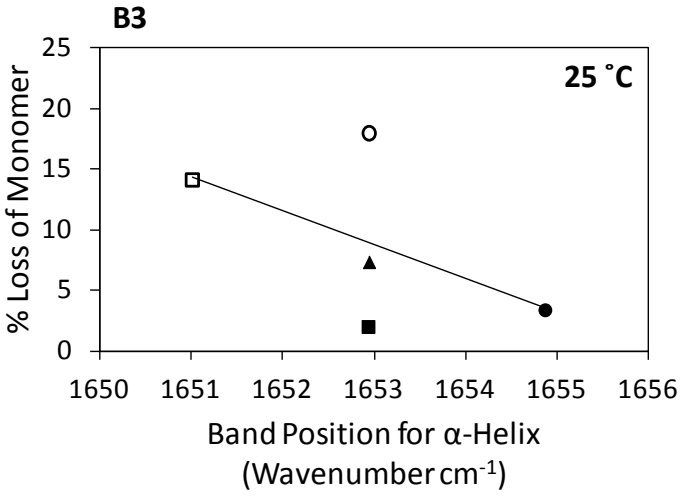


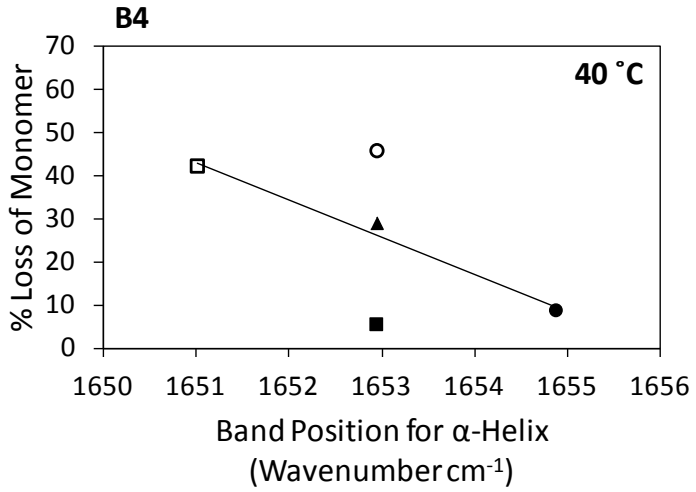
FTIR and storage stability

Storage for 360 days, 25 and 40 °C; stability by SEC









Summary

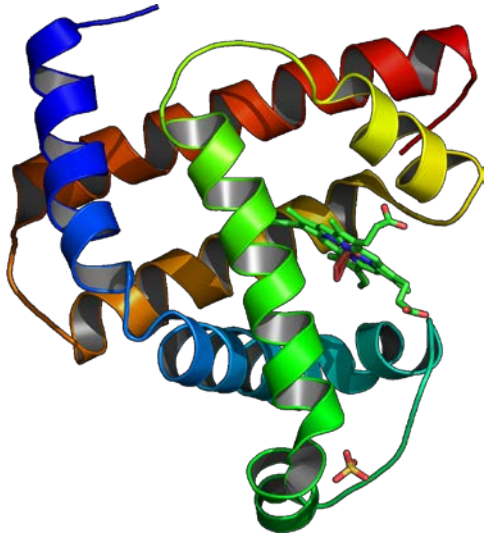
- ssHDX can be performed in powders using $D_2O(g)$ as a deuterium source.
- ssHDX rates are much slower than vapor sorption.
- Rate and extent of ssHDX are affected by RH, excipient type and amount, temperature.
- For Mb in lyo powders, ssHDX parameters are highly correlated with extent of aggregation on 1-yr storage.

Unknown

Relative contributions of protein conformation, protein dynamics and excipient interactions to ssHDX results.

Solution HDX

- Solvent exposure
- Conformation
- Dynamics



Solid-state HDX

- D_2O exposure
- Conformation
- Dynamics; local?
- Matrix / excipient interactions?

http://emedicine.medscape.com/article/1007814-overview

Solid-state photolytic labeling (ssPLL)

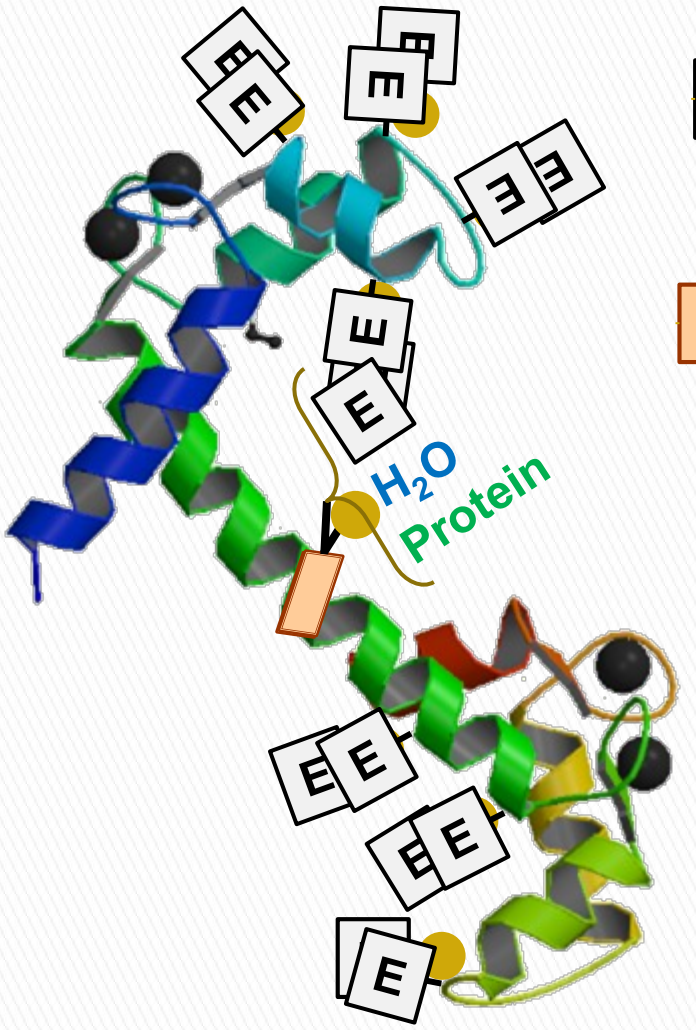
- Mapping protein-protein interactions and the side-chain environment of proteins in amorphous solids

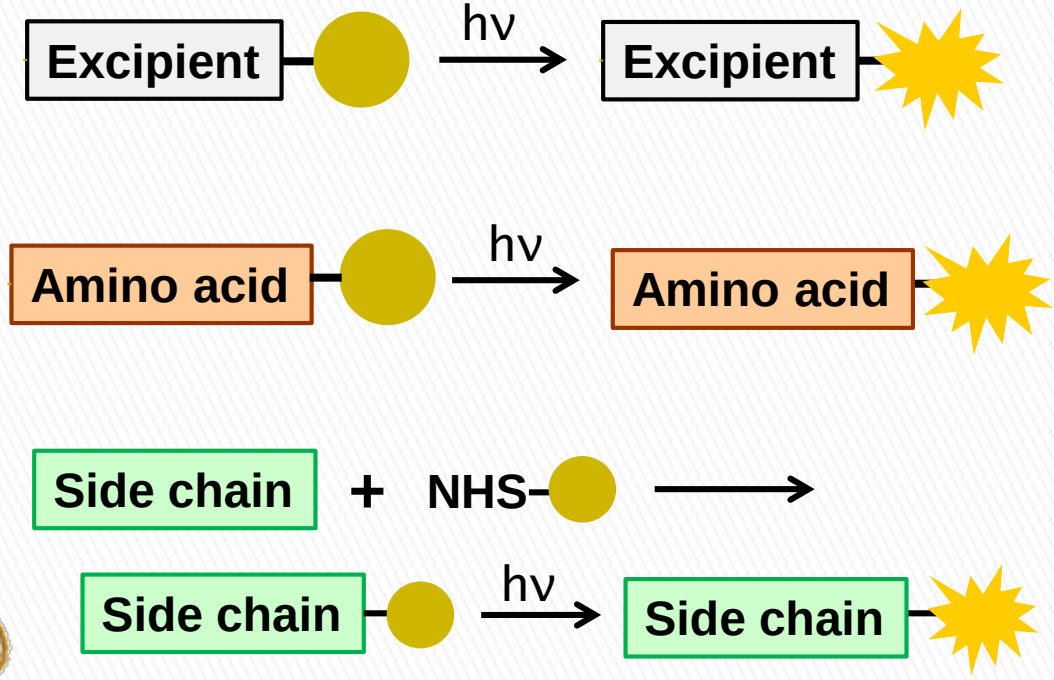
L. Iyer et al., *Mol. Pharm.*, 10: 4629-4639, 2013
B.S. Moorthy et al., *JoVE*, in press
L. Iyer et al., *Mol. Pharm.*, in preparation

Covalent labeling with MS analysis

- Developed as an alternative and complement to HDX.
- Eliminates back exchange.
- Used to study protein-protein interactions (PPI) in cells.
- A variety of chemical crosslinking strategies, with triggers initiating reaction.
- Here, we evaluate photo-activatable crosslinkers in amorphous lyophilized solids.

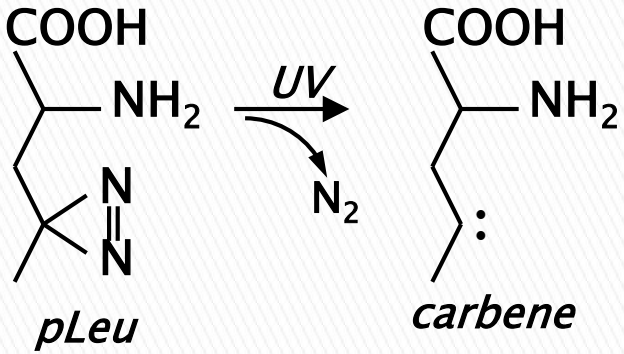
Photolytic labeling



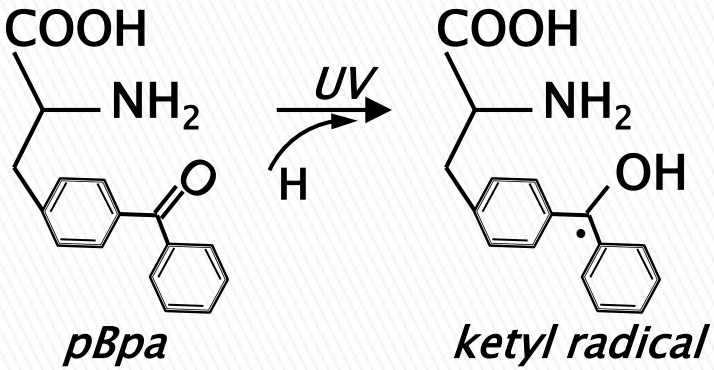


Photoamino acids

Photoleucine *(pLeu)*

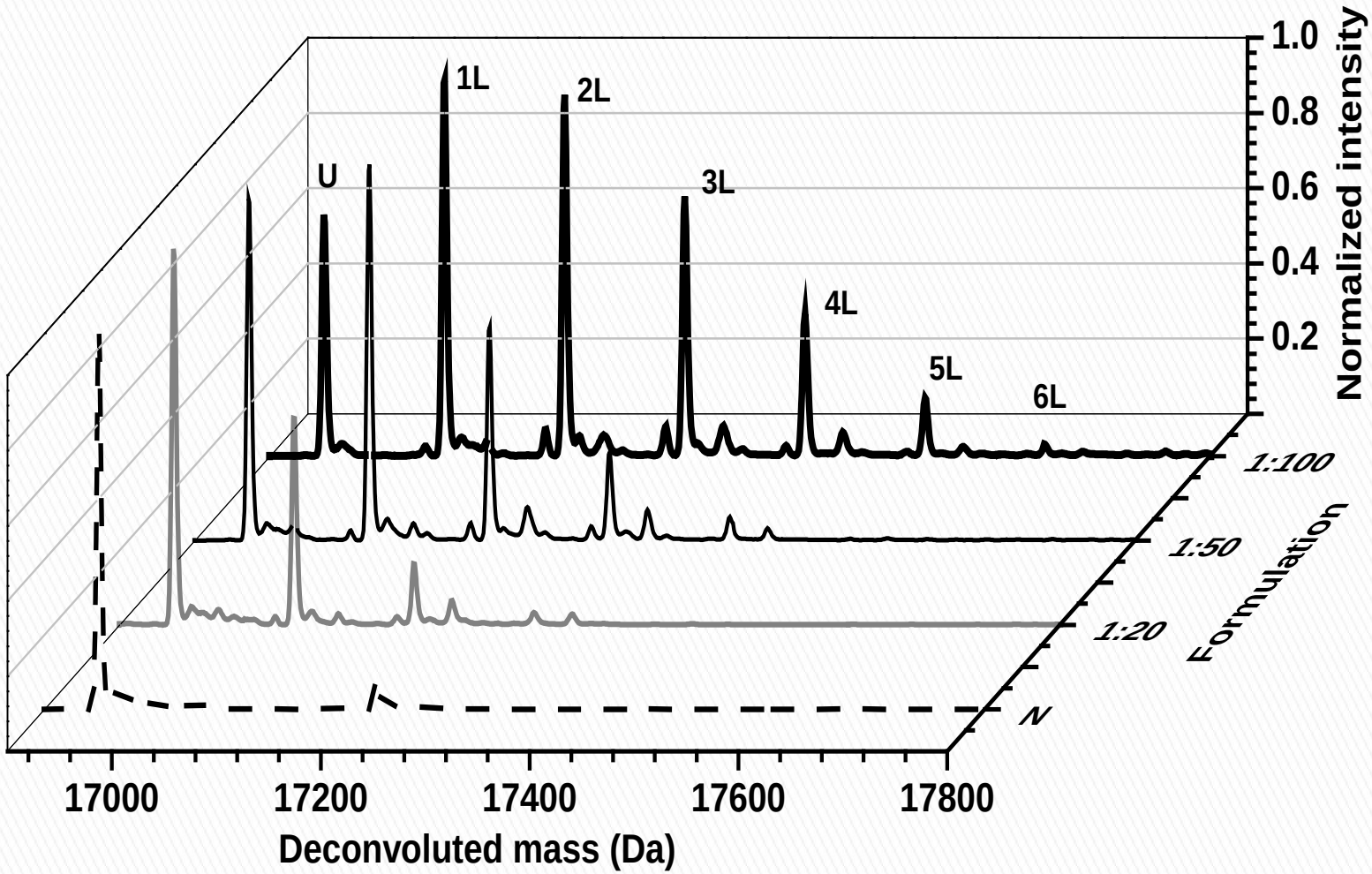


p-benzoyl phenylalanine *(pBpa)*



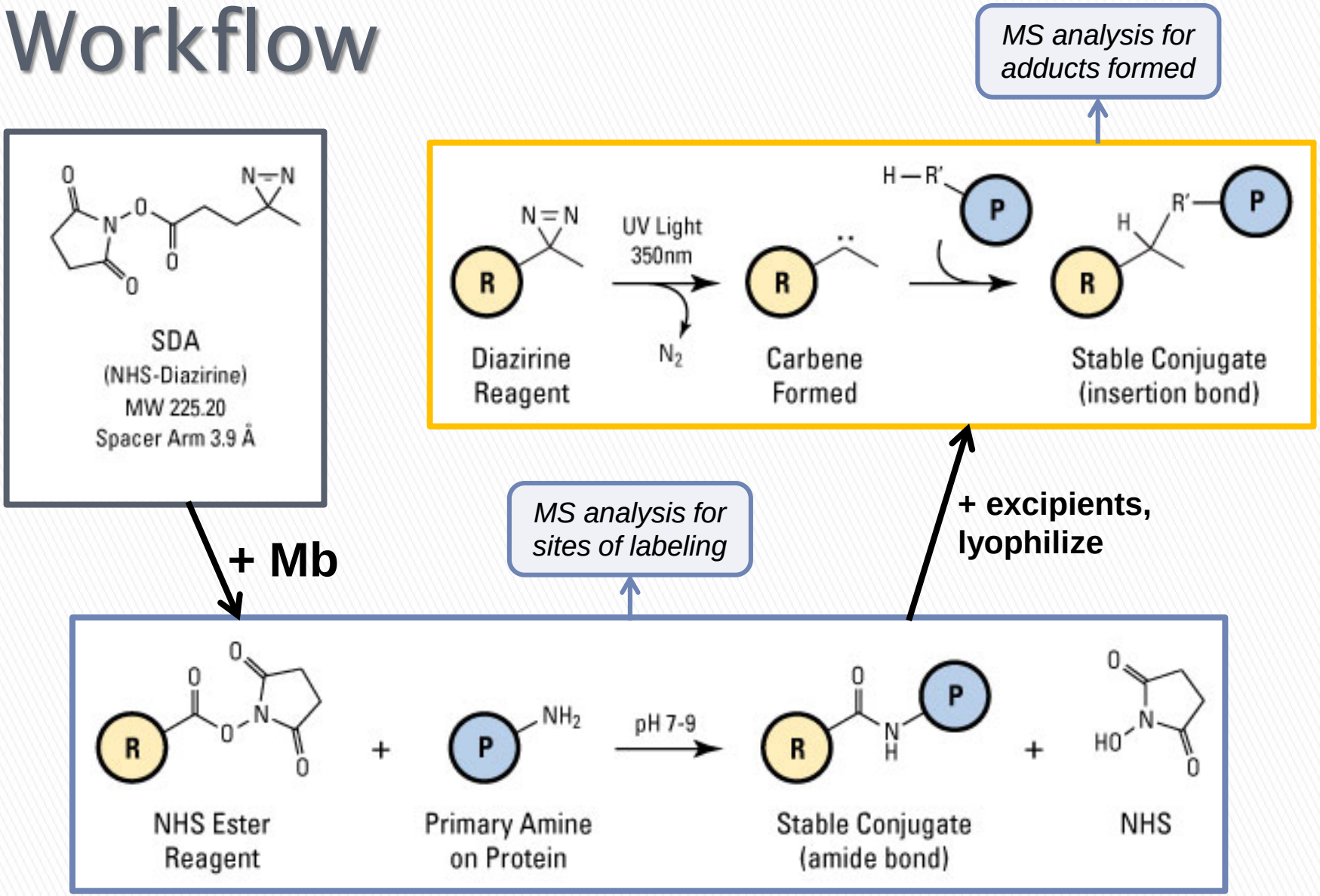
Diazirine reacts in solids

Mb + pLeu + *sucrose (1:2 w/w), 365 nm*



L. Iyer et al., *Mol. Pharm.*, 10: 4629–4639, 2013

Workflow



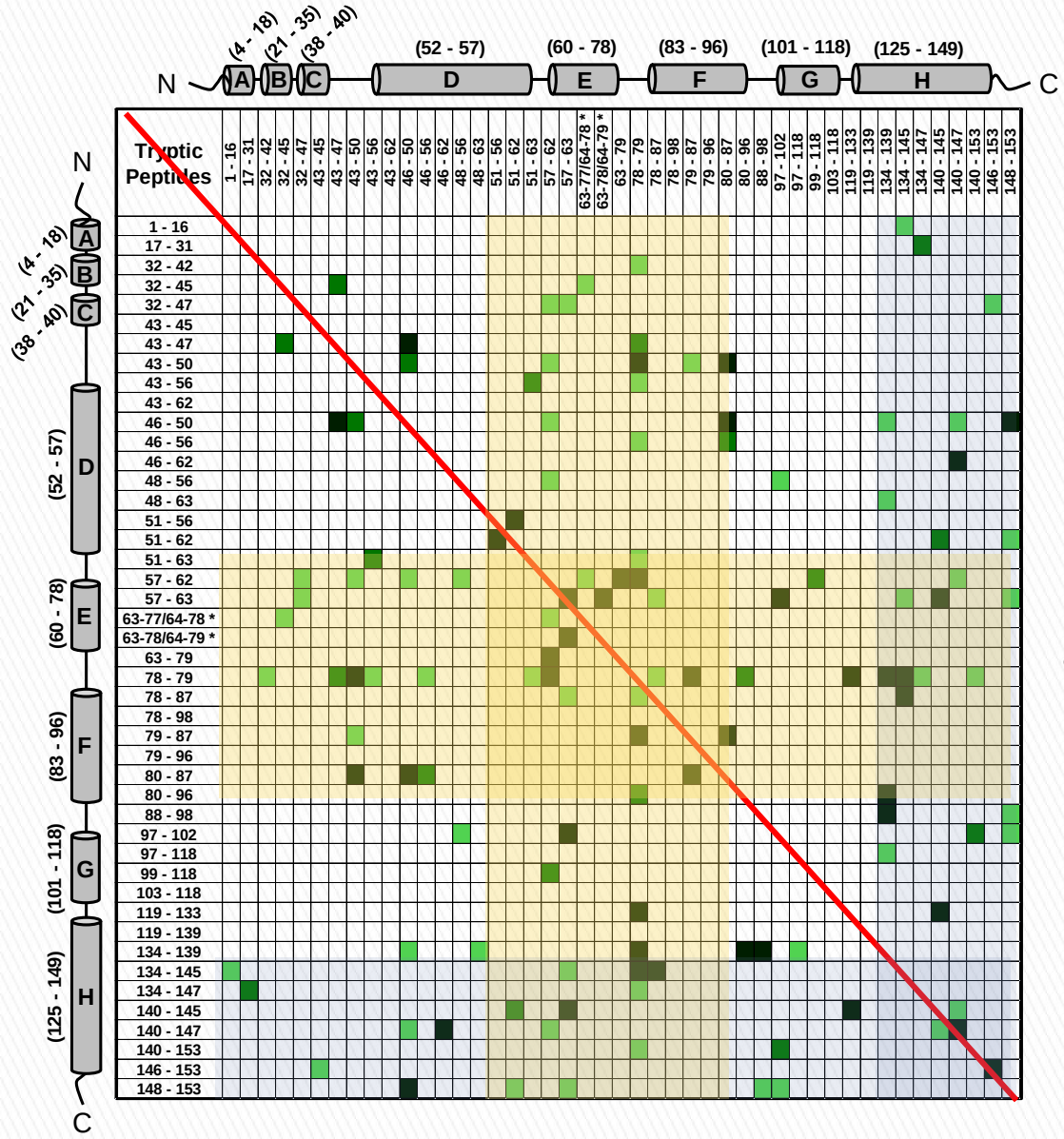
www.piercenet.com

MS analysis

- Types of adducts
  - Protein–protein adducts
  - Protein–water adducts
  - Protein–excipient adducts
- ESI LC–MS and MS/MS, trypsin digestion
- “Detection” (mass list)
  - Up to four missed cleavages (trypsin)
  - Dead-end modifications (N_2 loss without adduct)
  - Multiple SDA (0–4 labels per peptide)
  - Adducts of two peptides

Protein–protein interactions

Lyo Mb, no excipient



- Map shows peptide-peptide adducts detected in 1, 2 or 3 injections
- Symmetric about the diagonal
- Many interactions involving the E and F helices and C-terminus detected

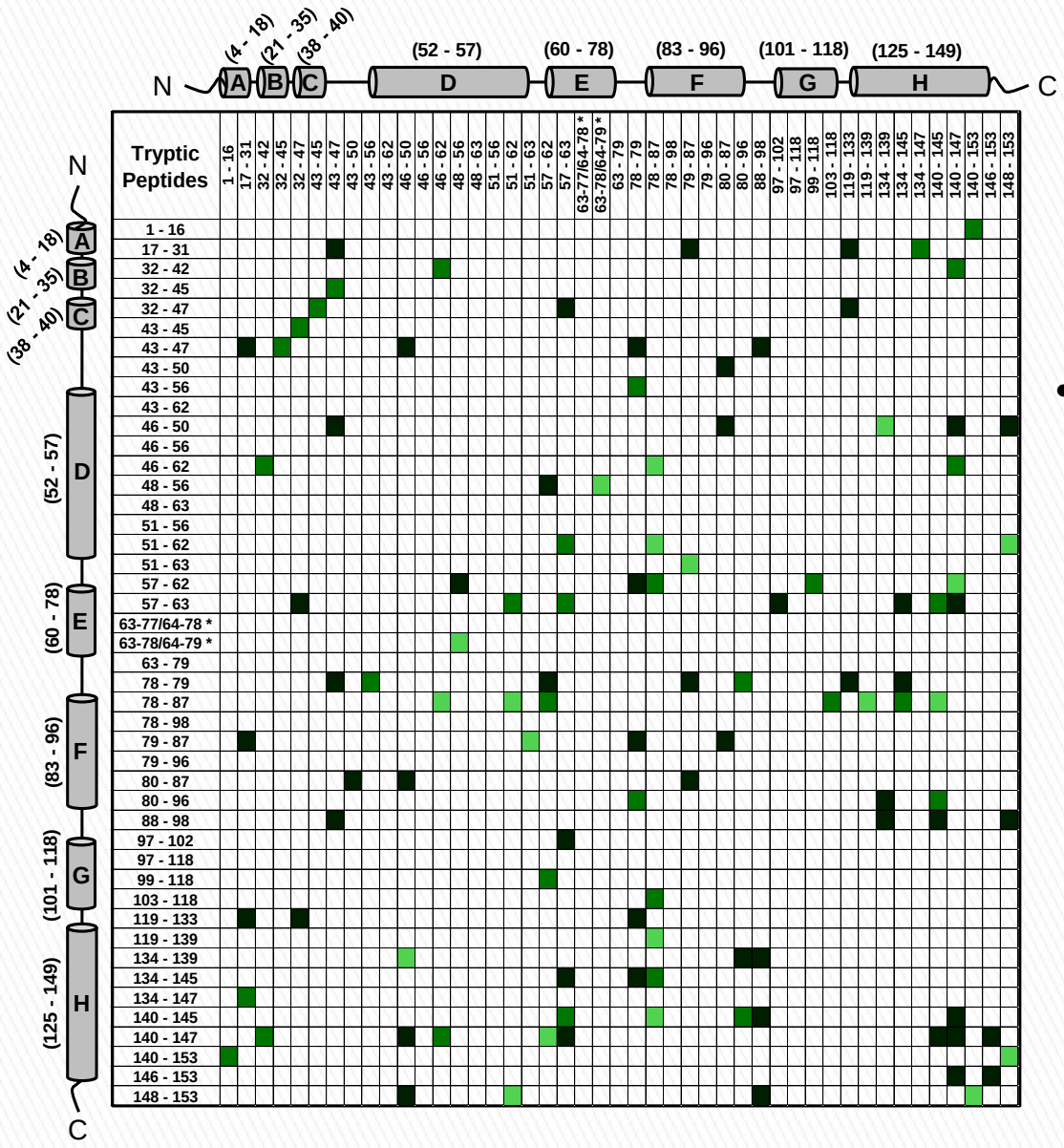
Detected in 1/3 injections

Detected in 2/3 injections

Detected in 3/3 injections

Protein–protein interactions

Lyo Mb, + raffinose



- Interactions somewhat more distributed than in excipient-free formulation.

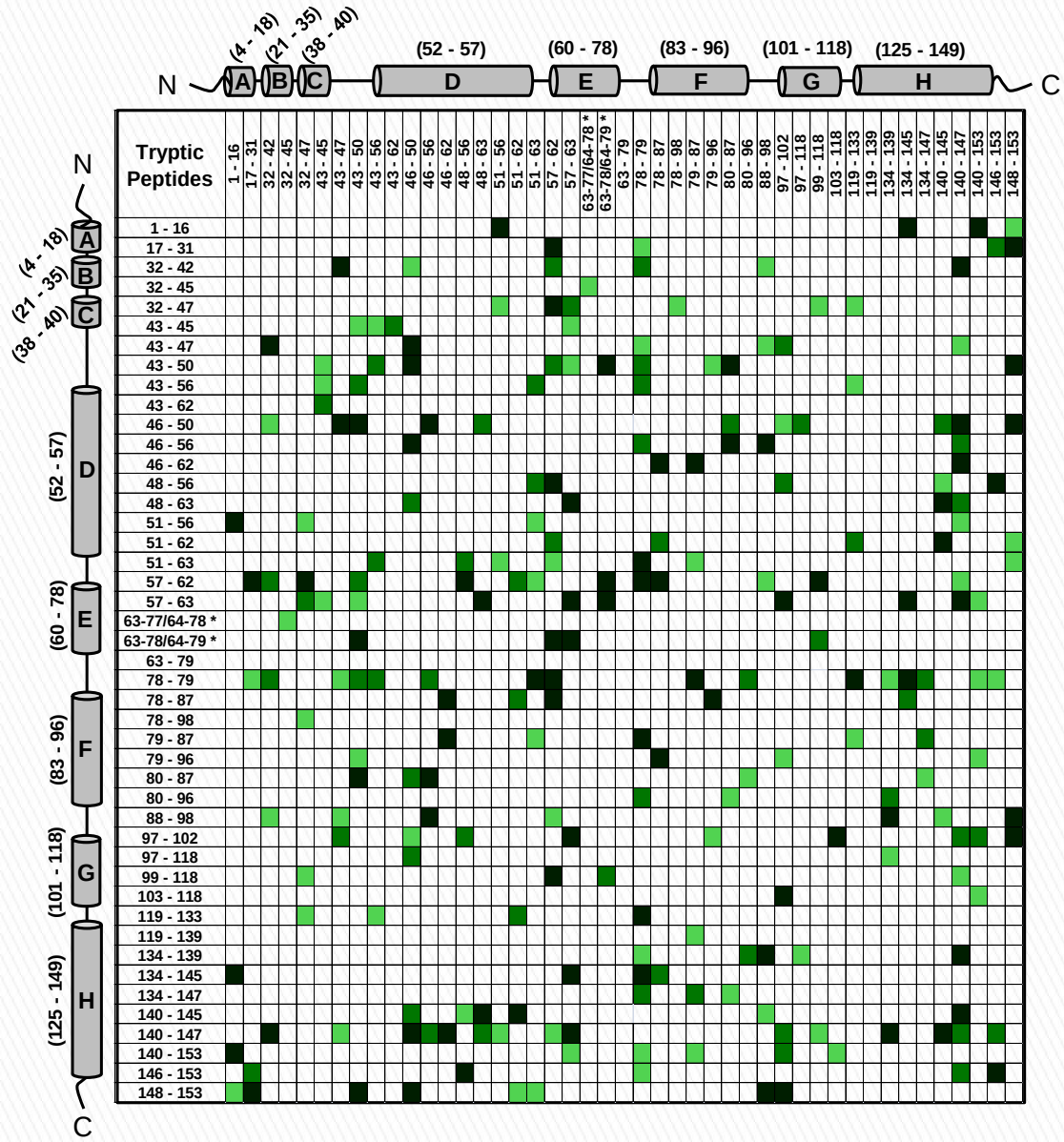
Detected in 1/3 injections

Detected in 2/3 injections

Detected in 3/3 injections

Protein–protein interactions

Lyo Mb, + Gdn HCl



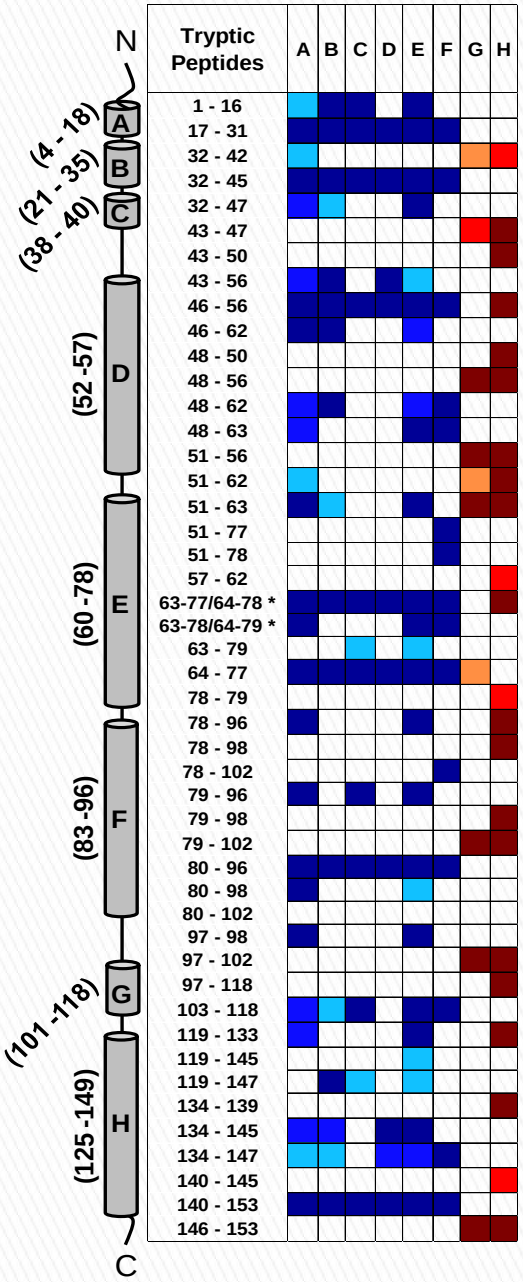
- Many more interactions detected.

Detected in 1/3 injections

Detected in 2/3 injections

Detected in 3/3 injections

Water and excipient interactions



KEY

Peptide/water adducts

A – Mb alone, lyo

B – Mb alone, soln

C – Mb + raffinose, lyo

D – Mb + raffinose, soln

E – Mb + Gdn HCl, lyo

F – Mb + Gdn HCl, soln

Peptide/raffinose adducts

G – Mb + raffinose, lyo

H – Mb + raffinose, soln

Detected in 1/3 injections

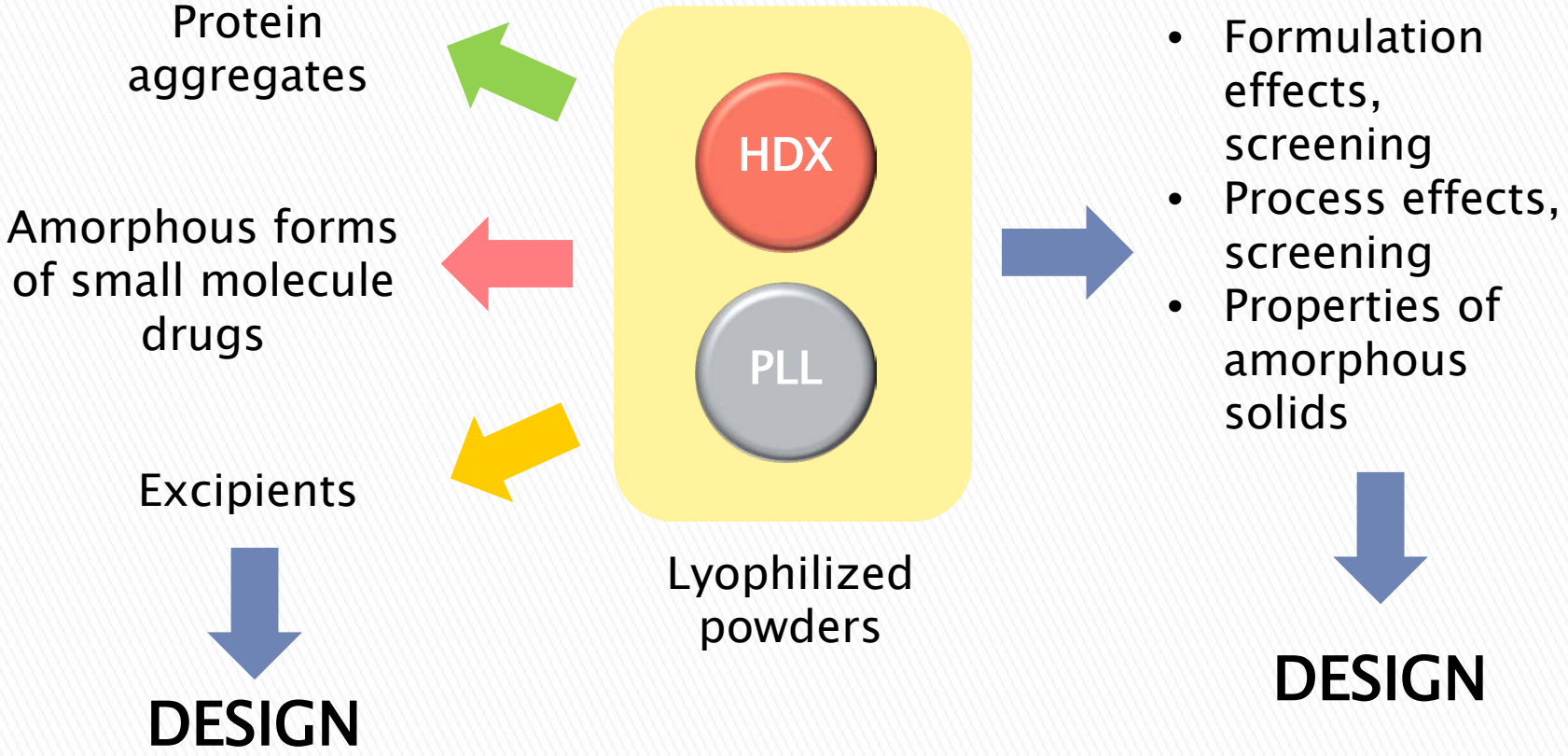
Detected in 2/3 injections

Detected in 3/3 injections

Summary

- Photolytic labeling can be performed in solid powders using diazirine chemistry.
- SDA-labeled Mb forms adducts with protein, water and excipient (raffinose) in lyo solids.
- Formulation affects the adducts formed.
- Method allows water replacement hypothesis to be tested.

For discussion



"Can we have all the answers yesterday?" Hong-Ren Wang, Vertex

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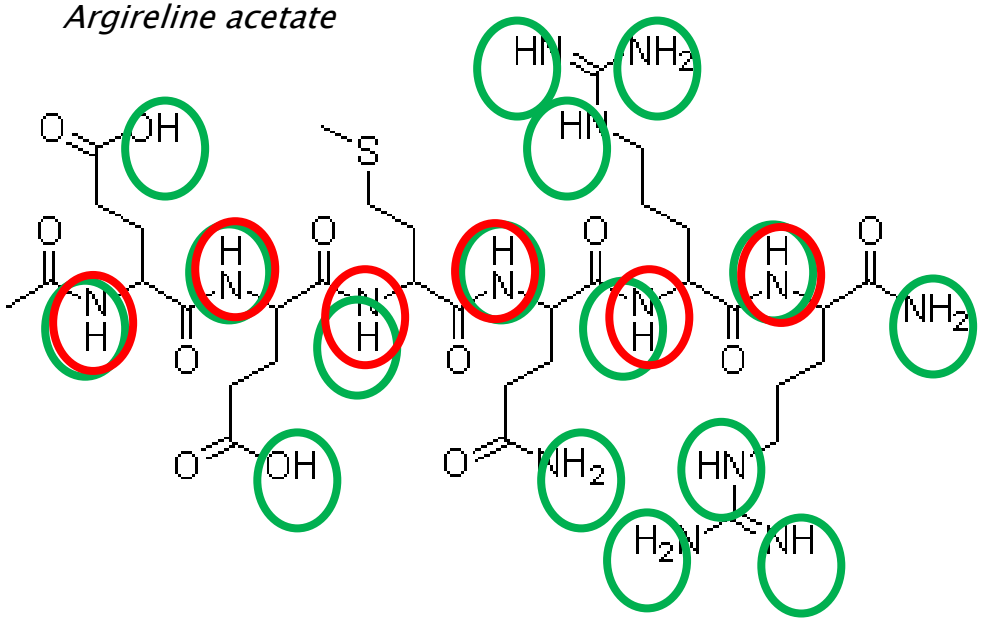
Topp Lab, May 23, 2015

Supplemental Slides

Back exchange

and the information content of HDX in solution

Argireline acetate

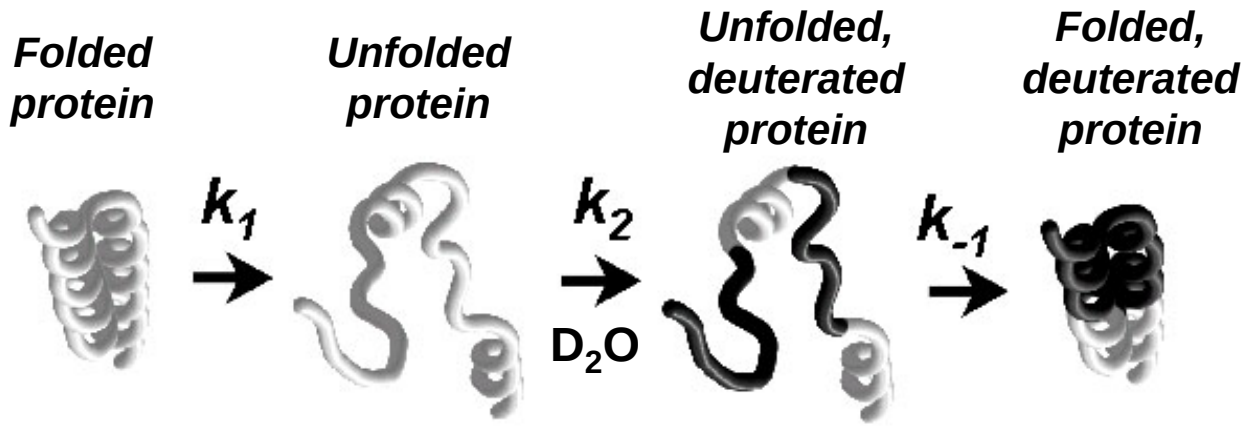


- HDX can occur at any labile hydrogen.
- Back exchange is rapid for side chains; only HDX of peptide bonds is detected.
- In larger proteins, HDX occurs slowly for H-atoms involved in secondary structure or buried in the core.
- HDX reports the “exposure” of the backbone to exchange.

http://www.lavemc.com/cosmetic-peptide/238-argireline-acetate-acetyl-hexapeptide-3.html

HDX kinetics

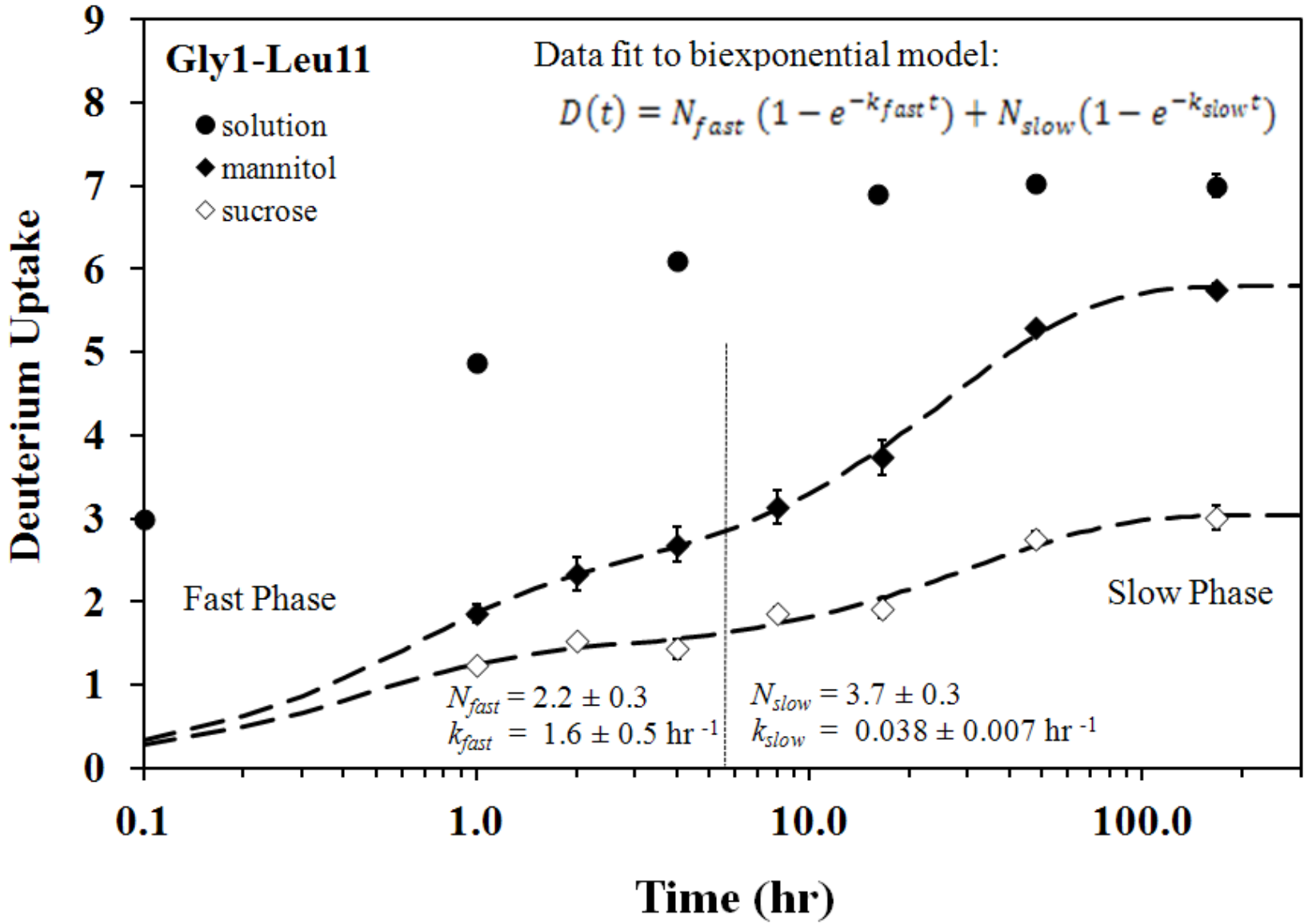
Protein folding and deuterium exchange



- Two kinetic processes: (i) protein unfolding and refolding, (ii) deuterium exchange.
- Two kinetic extremes:
  - Exchange much faster than refolding (EX1: $k_2 >> k_{-1}$)
  - Refolding much faster than exchange (EX2: $k_{-1} >> k_2$)

http://www.umass.edu/karbon13/research.htm

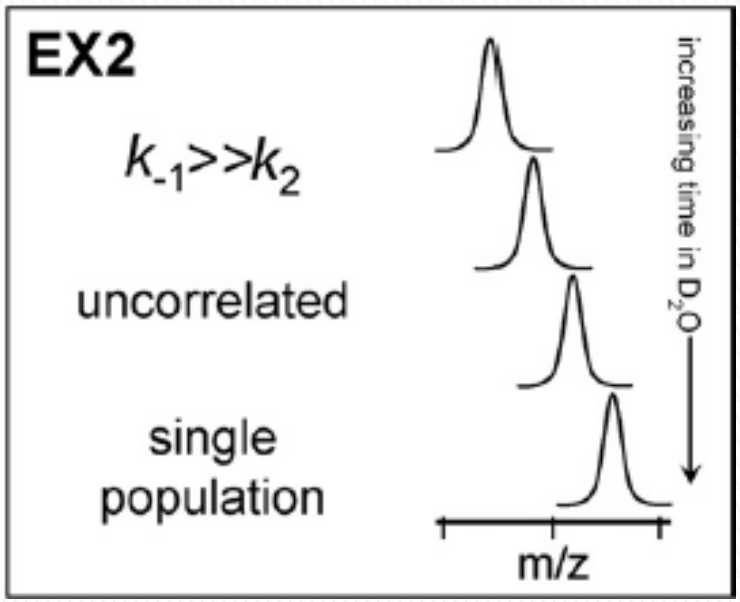
Myoglobin HDX kinetics

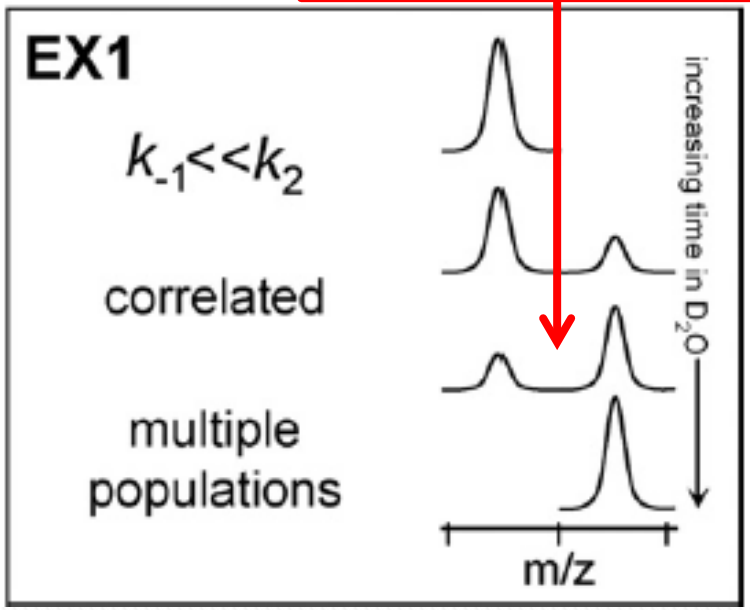


HDX theory – EX2 and EX1 limits

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

Broadening of peak width may be observed instead if populations not well resolved.





most situations

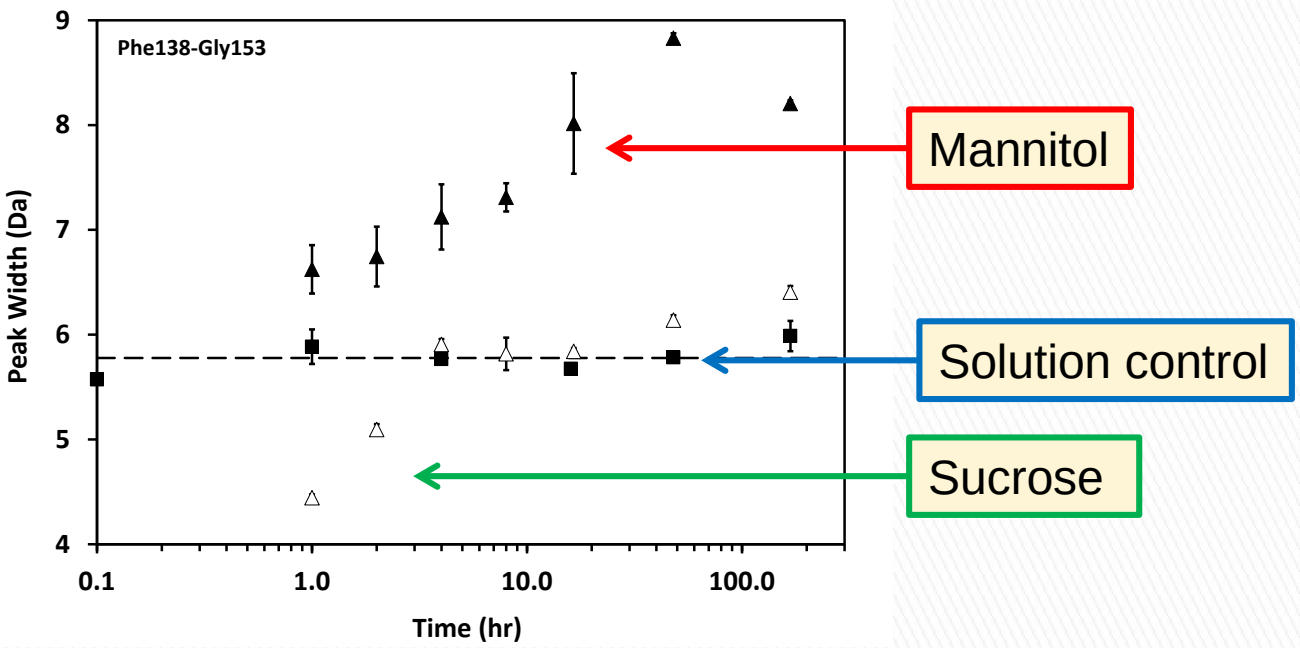
$k_{obs} = k_1 * k_2 / k_{-1} = K_{op} * k_2$

less common

$k_{obs} = k_1$

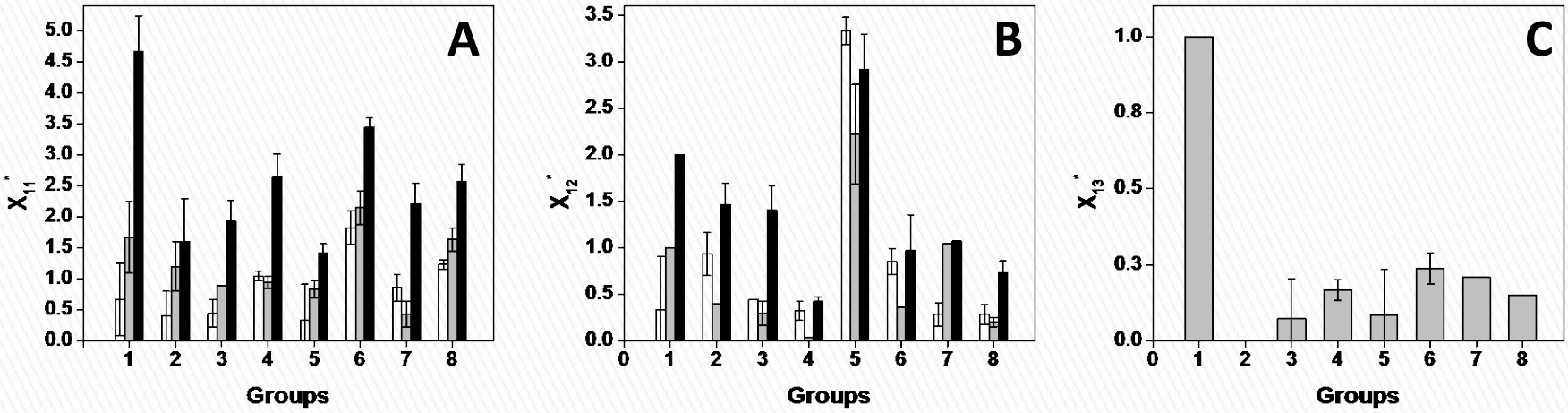
EX2/EX1-like peak width broadening

Example: Phe138-Gly153 in Mb



- Greater peak widths in mannitol formulations suggest mixed EX1/EX2 behavior, structural perturbation.
- Consistent with greater deuterium uptake overall.

Data analysis – ssPLL



Bar graph of (A) peptide-peptide adducts, (B) peptide-water adducts and (C) peptide-raffinose adducts detected by LC-MS. White bars represent Mb-SDA lyophilized in the absence of excipients (blank), grey bars represent Mb-SDA lyophilized with raffinose and black bars represent Mb-SDA lyophilized with Gdn HCl. X_{1n} values were counted for peptides assigned to 8 groups. Bars represent mean normalized X_{1n} values (X_{1n}^{*}) ± SD (n=3). Note that in the abscissa for panel (C), Group (6) spanning residues Lys^{78}-Lys^{98} was expanded to Lys^{78}-Lys^{102} to accommodate peptide Lys^{79}-Lys^{102} that was found to form raffinose adducts.