Hydrogen Deuterium eXchange Mass Spectrometry

The joys of clinging onto deuterium…

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Overview

Introduction and background

Why HDX?

Critical elements of bottom-up HDX workflows
Deuterium labelling
Quenching
Digestion
UPLC
MS
Data analysis
Data interpretation

Hydrogen Deuterium eXchange (HDX)

"Probably the best technique in the world…"

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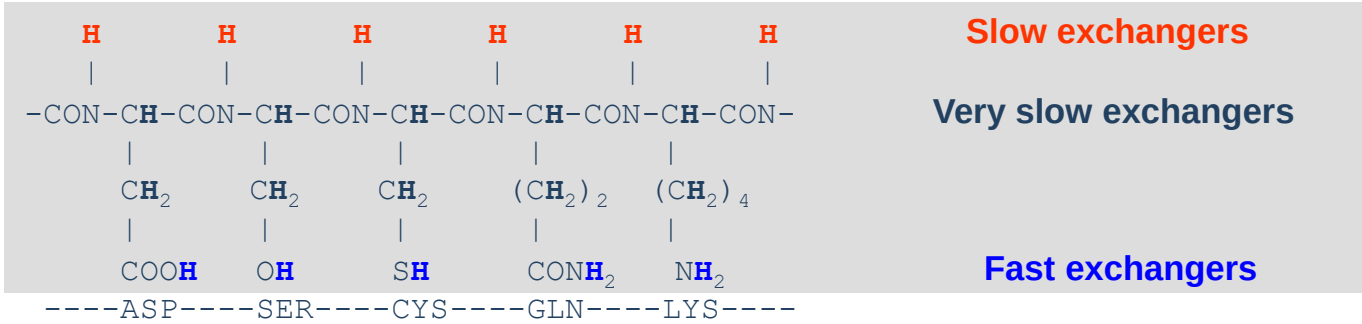
Pioneered in 1954 by Linderstrøm-Lang and co-workers [1]

Labile protons within a protein exchange with deuterium in a solvent increased of ~ 1 amu



Uptake measured using density gradients and since using IR, UV, NMR and MS

1. Hvidt, A., Linderstrøm-Lang, K.Biochim. Biophys. Acta. 14, 574–575 (1954)

Structural effects on amide HDX

The rate of amide exchange is greatly influenced by the protein's structure (by as much as eight orders of magnitude)

Protection from exchange is provided by the protein's secondary, tertiary (and quaternary) structure

Residues coloured blue:

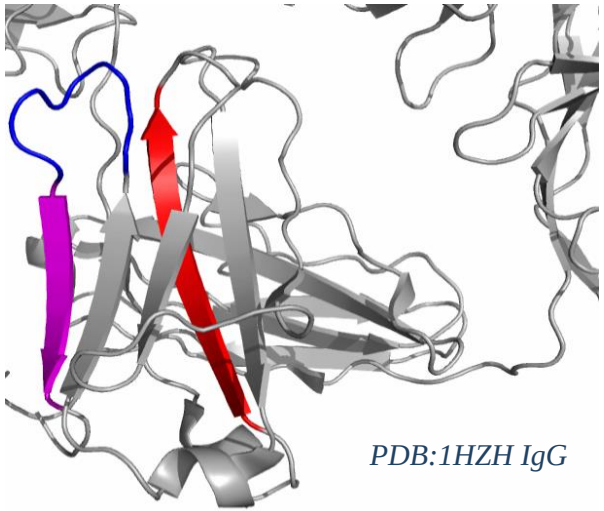
Exposed to solvent and no secondary structure

Residues coloured magenta:

Exposed to solvent, but have secondary structure

Residues coloured red:

Buried in core of protein, shielded from solvent



PDB:1HZH IgG

Intrinsic effects on amide HDX

The rate of exchange is greatly influenced by:

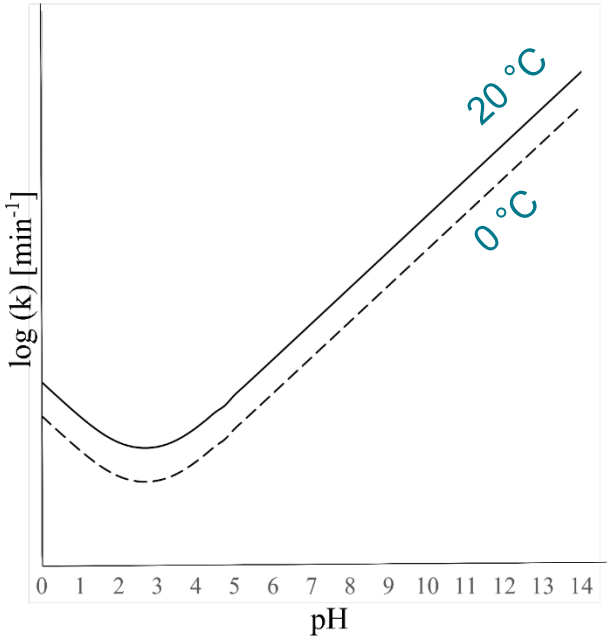
pH
~ 10- fold per pH unit
Temperature
~ 3- fold per 10 °C
Ionic strength
Sequence

Factors must be controlled to study protein structure

HDX is reversible:

Back exchange (10 -30%)

Unstructured poly-DL-ala: [2]
pH 2.5 t1/2 = 25 min
pH 7 t1/2 = 1s



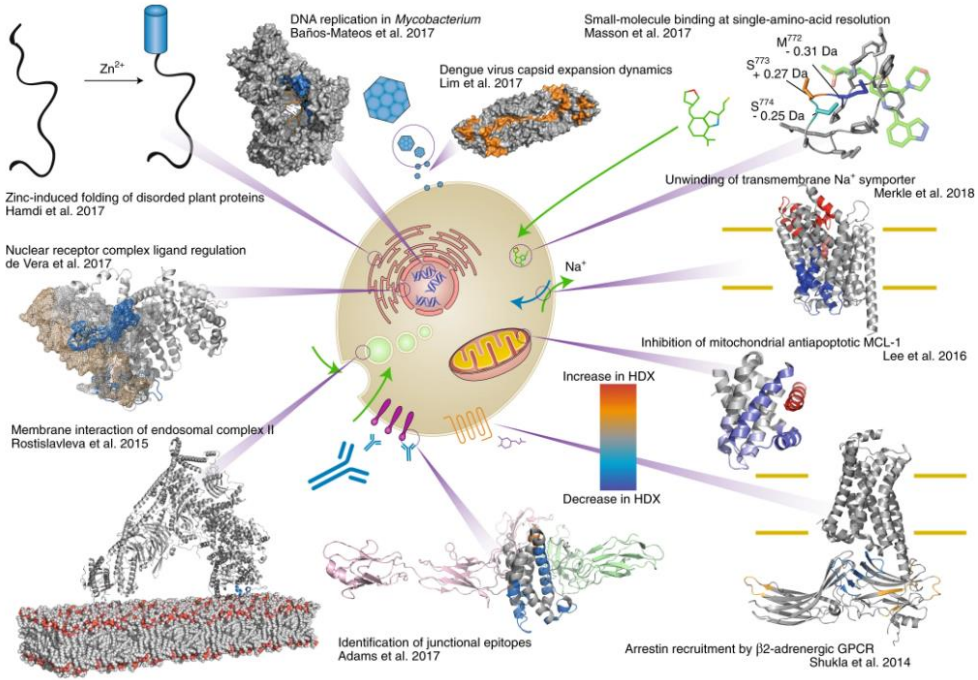
2. Bai, Y., …. Englander, S.W.: Proteins. 17, 75–86 (1993)

Why HDX-MS?

- High resolution technique
- 2°, 3° and 4° structural information
- Low sample requirements (5-50 µM)
- Tolerance to salts and excipients
- Solution phase information
- Unlimited mass range

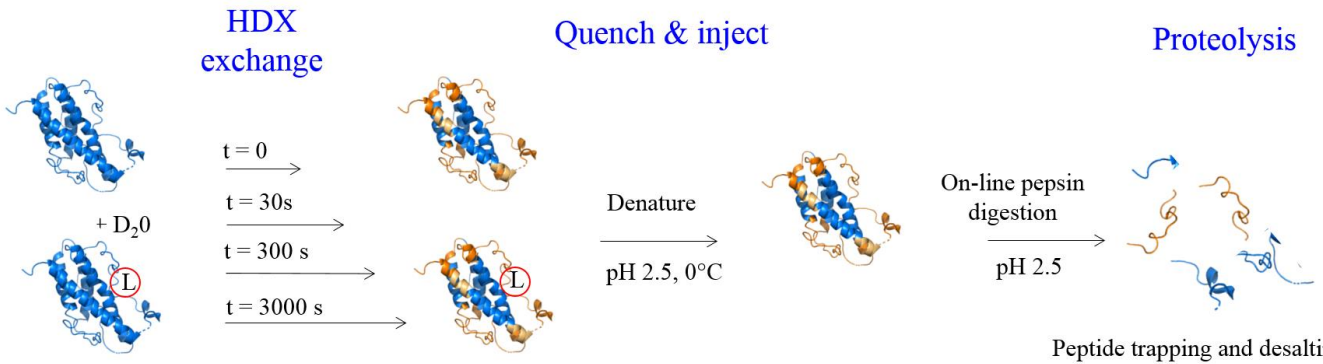
Structural insights into :
- Protein dynamics and function
- Protein-Ligand interactions
- Aggregation
- Protein-Protein interactions
  Epitope/paratope mapping
- Formulation effects

……..



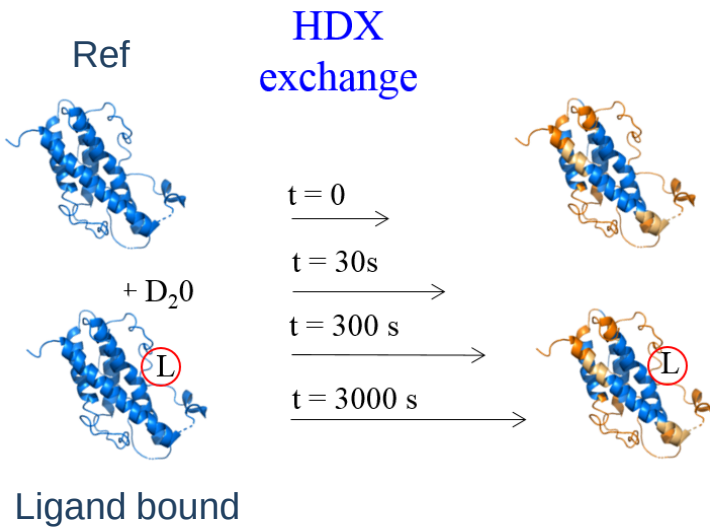
3. Masson, G.R et al. Nature Methods, 16, 595–602 (2019)

HDX-MS: bottom-up workflow



Controlling each stage crucial to producing precise measurements

Deuterium labelling



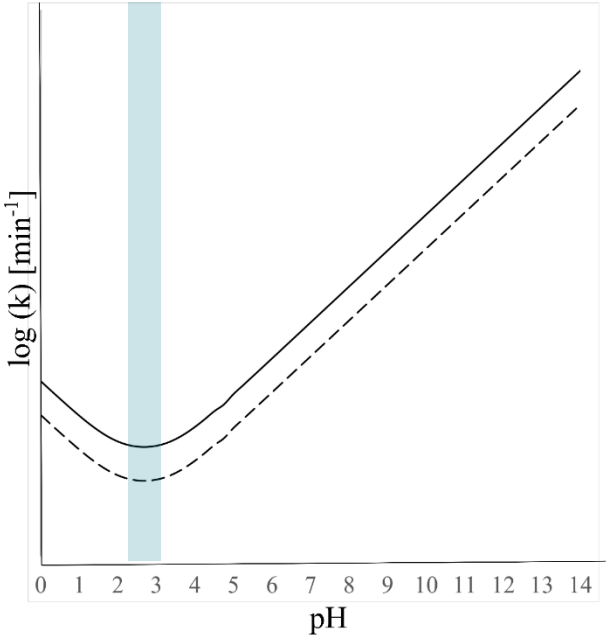
- Typical protein concentration 5 - 50 uM
- Dilution in > 90 % deuterated buffer (10 – 50 fold)
- t = 0, x, 10x, 100x, 1000x…..
- Non- or -automated sample handling
- Differential HDX-MS experiment

Experimental factors to consider:

- pD = pH_{read} + 0.4
- t = x?
- n =3/4
- Salts/excipients
- Temperature
- Internal standards? PPPI peptide [4]

4. Zhang. Z, et al, Anal. Chem. 2012, 84, 4942−4949

Quenching



- Need to stop labelling following a define time
- Portion of the sample is quenched by dilution (~ 2 fold) into a protonated quenching buffer (higher buffer capacity)
- Reduce pH 2.3-2.5, low temperature (1-4 °C)
- Fighting loss of deuterium due to back-exchange
- Introduction of denaturants/reducing agents/detergents
- Flash frozen (-80 °C) or analysed directly

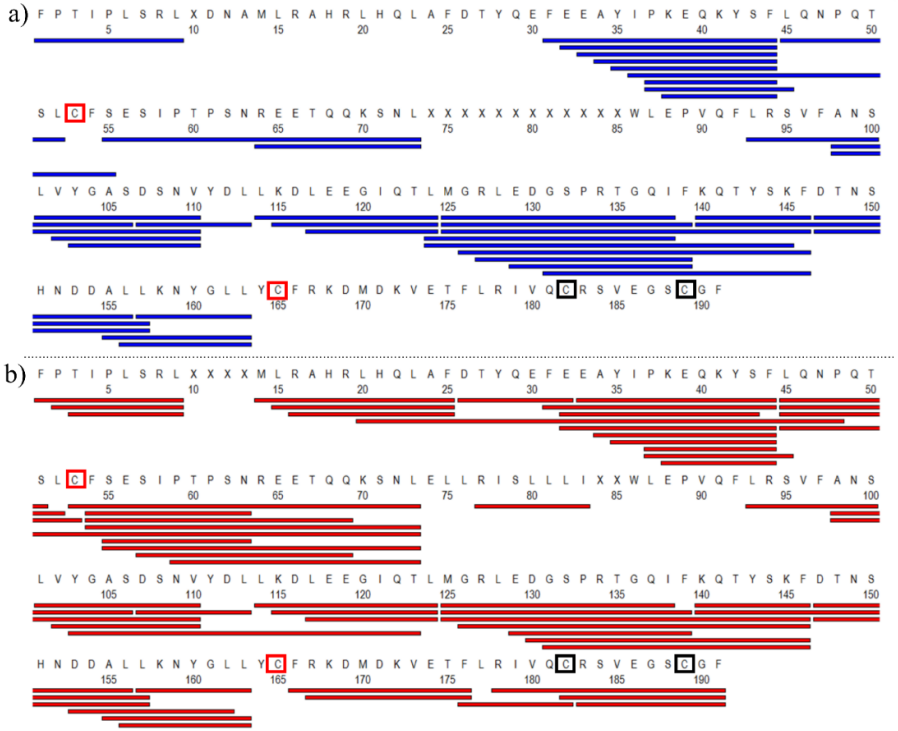
Digestion: bottom-up HDX

- Structural resolution gained by measuring HDX at peptide level
- Can achieve resolution via *non-specific* digestion to produce peptide map of overlapping peptide sequences (t =0)
- Digestion must be:
  fast ~ 1-3min
  acidic ~ pH 2.3-2.5
  low temperature < 25 °C
- Typically performed using Pepsin (Protease Type XIII, XVIII….)
- In-solution or on-line using IMER

….HYDRGENDETERIMECHANGE…

Digestion: bottom-up HDX

No denaturants



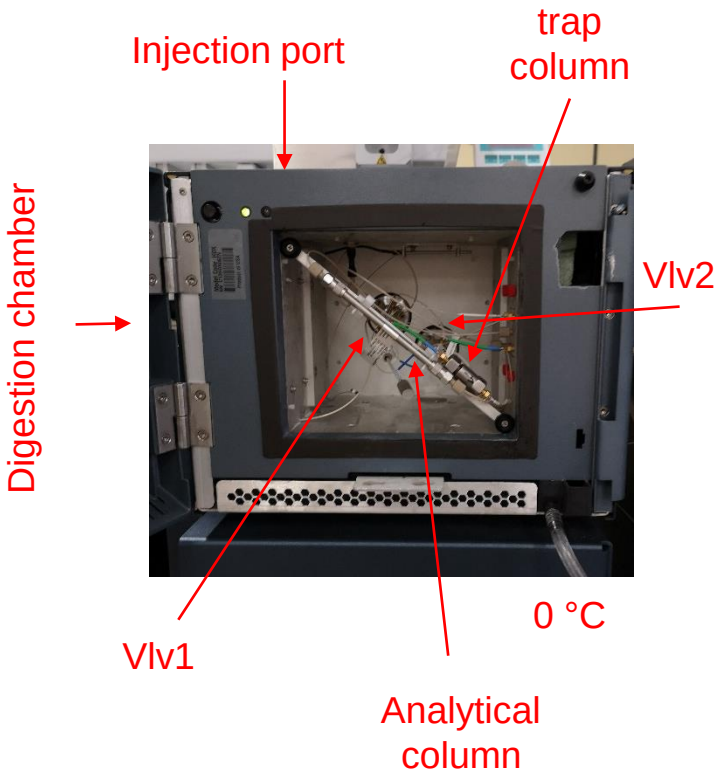
300 mM TCEP, 2 M Gnd.HCl

- ✓ High sequence coverage
- ✓ High overlap (redundancy)
- ✓ High reproducibility

Important to optimise via:

- incubation time/ flow rate
- pressure
- denaturing/reducing agents
- temperature
- proteases

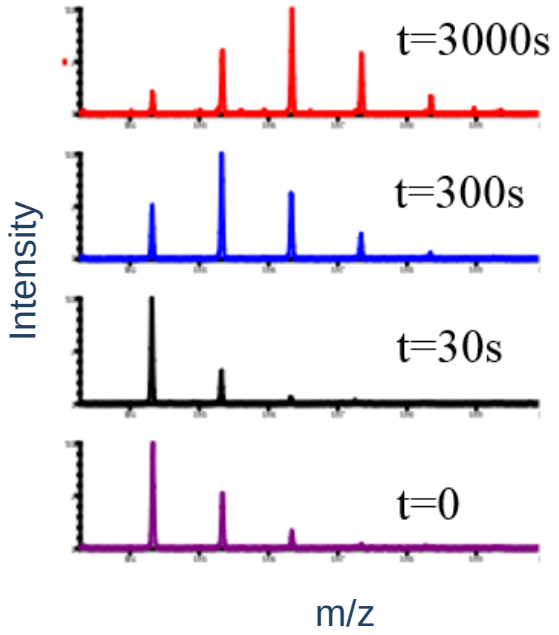
Chromatography



- Fast RP chromatography (gradient 0- 40% ACN in ~ 6-9min) and at 0°C
- Poor conditions for reproducible chromatography
- UPLC system (> 2 µm particle size) pre-cooled and refrigerated
- 10-100 pmol digested protein injected (or flow) onto C18 trap column before switching onto an C18 analytical column
- Often high co-elution of peptides
- IMS-MS can be used to in conjunction to increase peak capacity (especially good for large protein/complexes)

Mass Spectrometry measurement

- Deuterium incorporation = + 1 u/D
- ESI-MS
- Two stages:
  1. Peptide map: Tandem MS for peptide ID, t=0
  2. Deuterated peptide measurement : MS only, t=x
- CID not applicable due to H-scrambling
- ETD/ECD allows for amide D localisation, low efficiency ☹
- Isotopic resolution required (Q-TOF, Orbitraps, FT-ICR)
- Fast scanning speeds to compensate for fast chromatography



Data Analysis : Peptide map generation

- Peptide identification via database search [MASCOT, SEQUEST, PLGS…]
- Non-specific nature of digestion leads to data complexity
- These spectra act as a reference to deuterated spectra, *z*, RT and K (IMS)
- Peptide ID should be reproducible ~ n = 4/5

Sequence	Start	End	MHP	RT
⊟ 27-217				
FPTIPLSR	1	8	930.5407	6.52
PTIPLSRL	2	9	896.5564	7.47
FDNA	10	13	466.1932	4.33
FDNAM	10	14	597.2337	5.70
MLRAHRLHQLAF	14	25	1492.8318	5.61
LRAHRLHQLAF	15	25	1361.7913	5.42
RAHRLHQLAF	16	25	1248.7072	5.26
FDTYQE	25	30	802.3254	5.49
DTYQE	26	30	655.2570	4.28
DTYQEF	26	31	802.3254	6.56
TYQE	27	30	540.2300	4.06
TYQEF	27	31	687.2984	6.40
FEEA	31	34	495.2086	4.87
FEEAYIPKEQ	31	40	1253.6048	5.71
FEEAYIPKEQK	31	41	1381.6998	5.25
EEAYIPKEQ	32	40	1106.5364	5.00
EEAYIPKEQK	32	41	1234.6314	4.54
EAYIPKEQK	33	41	1105.5888	4.64
AYIPKEQK	34	41	976.5462	4.15
YIPKEQK	35	41	905.5091	3.92
PKEQ	37	40	501.2667	4.58
PKEQK	37	41	629.3617	4.15
KEQK	38	41	532.3089	4.05
LQNPQTS	45	51	787.3945	4.01
LQNPQTSL	45	52	900.4785	5.71
LQNPQTSLC	45	53	1003.4877	5.87
FSESIPTPSNREE	54	66	1492.6914	5.53

Data analysis : deuterium uptake

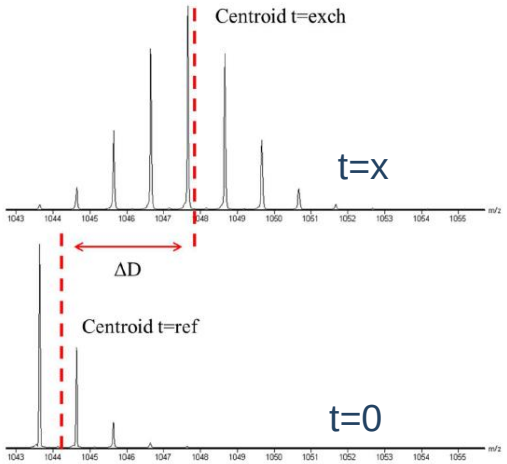
- Measurement of the centroid of isotopic distribution

$$centroid\ mass = \frac{\sum m_i I_i}{\sum I_i}$$

m/z

Intensity of spectral peak

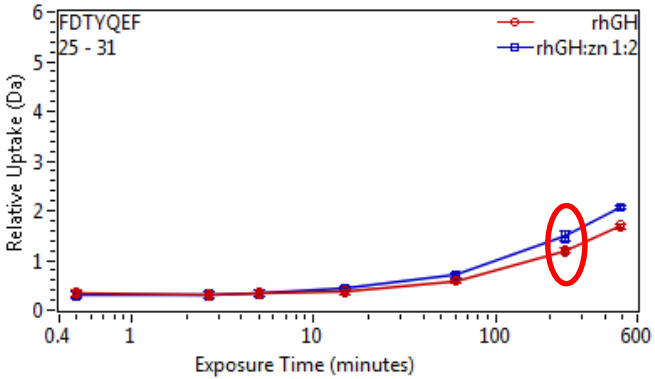
Total intensity of isotopes



- Subtraction of centroid of undeuterated peptide to give deuterium incorporation
- Deuterium uptake expressed in (Da) or as relative deuterium uptake (%)
- Automated software [HDX Workbench, MS Tools, DynamX and HDExaminer….]

Max uptake = N - P - 1

Data analysis : displaying results



Uptake plots

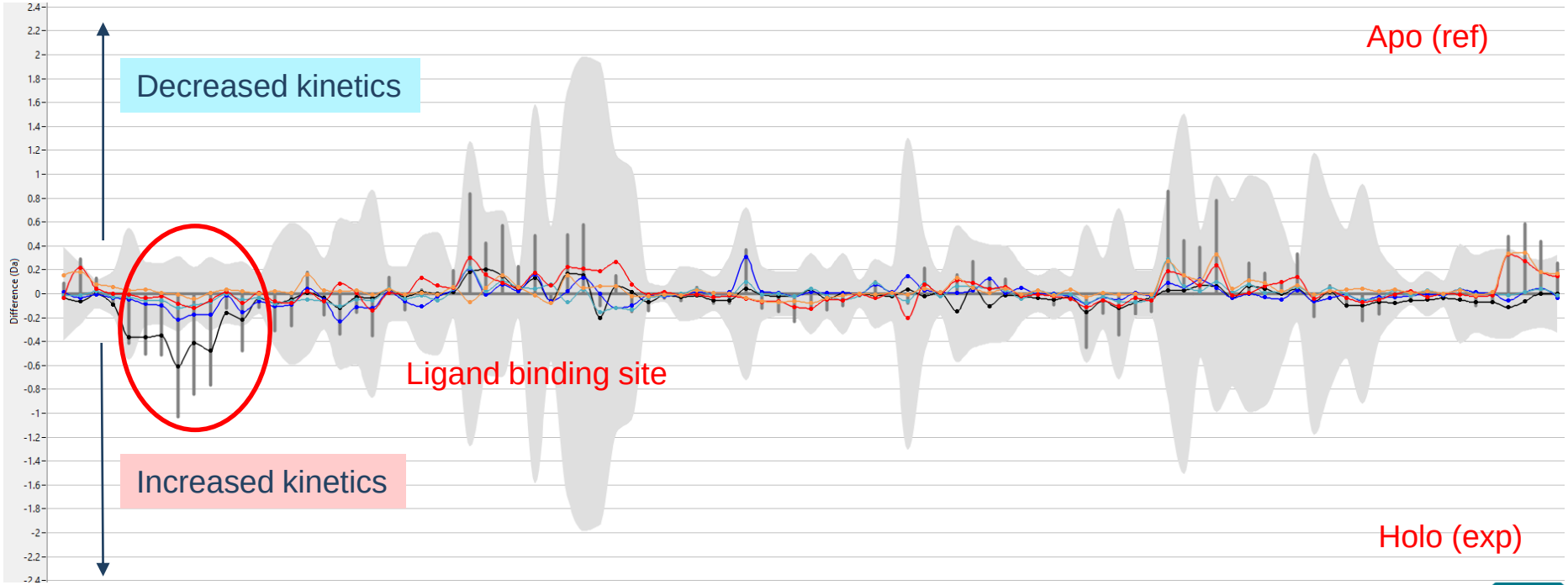
Reproducibility of measurements

Variability is peptide specific!

Data analysis: displaying results

Differential butterfly plots

$Uptake_{ref} – Uptake_{exp}$



Data analysis: displaying results

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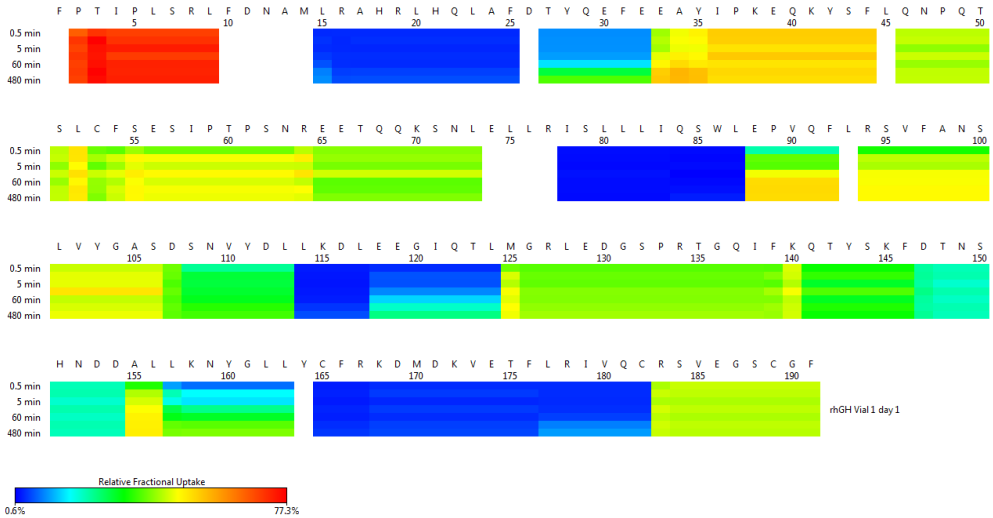
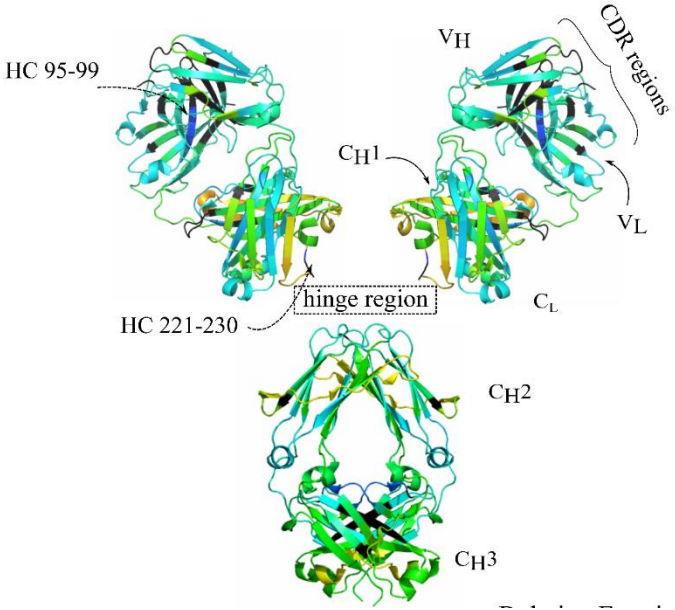
Heat map



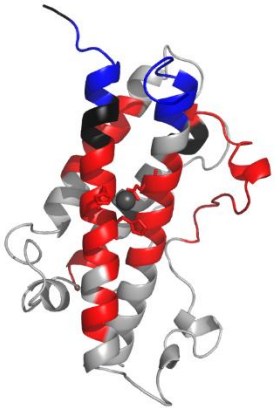
XRC



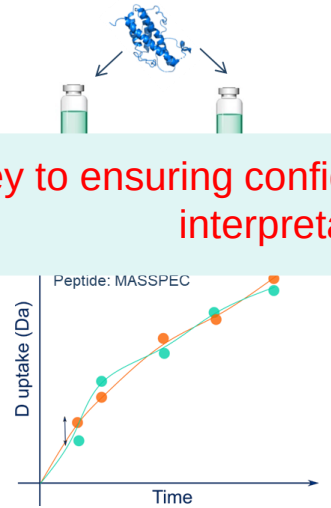
Data interpretation: establishing significance

Multiple approaches considering:

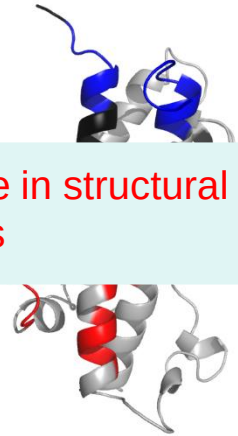
Intra-day reproducibility :3 σ, statistical significance (inc. Tukey multiple comparison, t-tests) [5,6]
Inter-day reproducibility: vial-to-vial thresholds, SEM [6] or peptide specific [7]



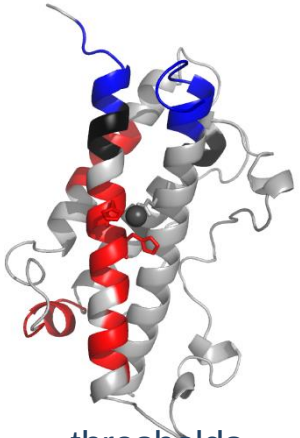
3 σ



Key to ensuring confidence in structural interpretations



Statistical significance



thresholds

5. Chalmers, M.J, et al. Int J Mass Spectrom. 2011 April 30; 302(1-3): 59–68.
6. Hourdel, V. et al, Bioinforma. Oxf. Engl. 32, 3413–3419 (2016)
7. Houde, D. et al J. Pharm. Sci. 100, 2071–2086 (2011).
8. Groves, K. et al, J.Am. Soc. Mass Spectrom. 31, 3, 553-564 (2020)

Further reading…

Hydrogen Exchange Mass Spectrometry of Proteins: Fundamentals, Methods, and Applications, David D, Weis (Editor), John Wiley & Sons (2016)

Konermann, L., Pan, J. & Liu, Y.-H. Hydrogen exchange mass spectrometry for studying protein structure and dynamics. Chem. Soc. Rev. 40, 1224–1234 (2011).

Engen, J. R. & Wales, T. E. Analytical aspects of hydrogen exchange mass spectrometry. Annu. Rev. Anal. Chem. 8, 127–148 (2015)

Recommendations for performing, interpreting and reporting hydrogen deuterium exchange mass spectrometry (HDX-MS) experiments, Masson, G.R et al. Nature Methods, 16, 595–602 (2019)