Studying human haptoglobin-hemoglobin structure and interactions using H/D exchange and timsTOF Pro.

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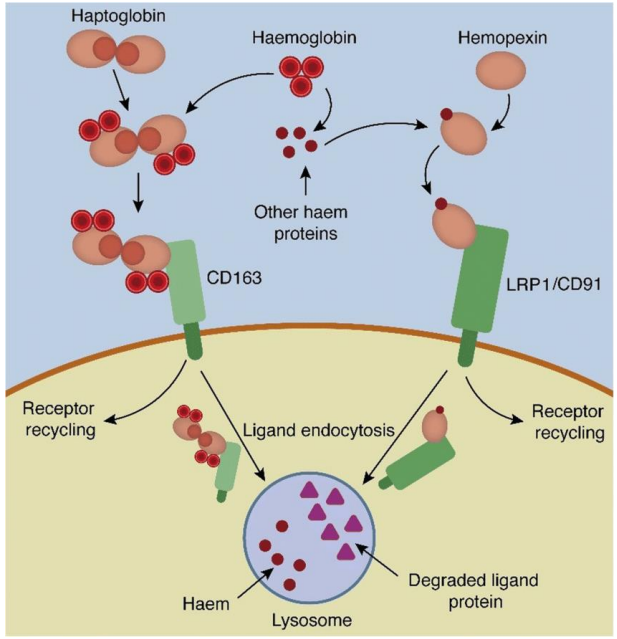
Human Haptoglobin

- one of the most abundant plasma proteins
- antioxidant role through its ability to scavenge haemoglobin (Hb) released from damaged red blood cells
- two allelic variants form three phenotypes - Hp1-1, 2-1, 2-2
- different quaternary structures / organization having different affinity towards the haemoglobin



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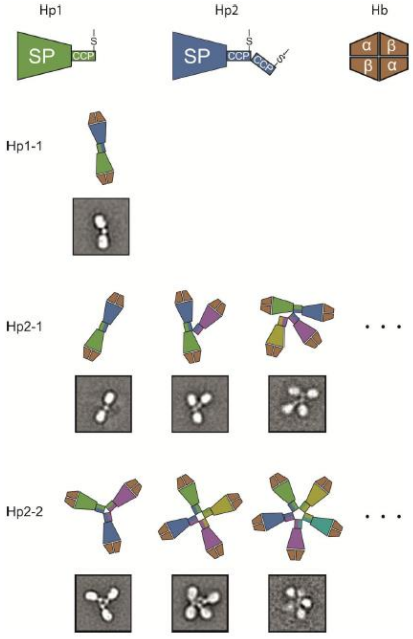

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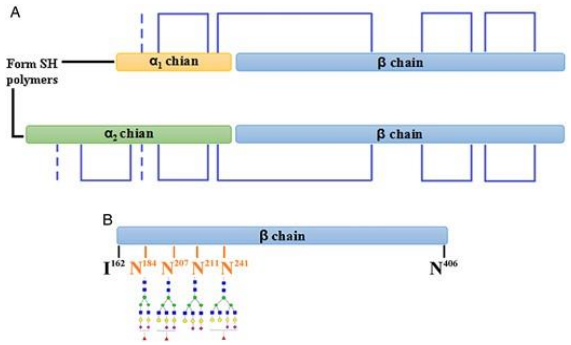
Human Haptoglobin

- structurally composed of alpha and beta subunit. Alpha is mainly involved in oligomerization and exists in two forms – 1 and 2. Alpha 2 contains sequence repetition (CCP - component control protein domain). Beta is involved in Hb binding
- Hp poses an analytical challenge - heavily disulphide bonded (intra and interchain, oligomerization), has 4 N-glycosylation sites (role in binding?) and the above mentioned repetition in alpha2

*MSALGAVIALLLWGQLFA*VDSGNDVTDI**ADDGCPKPPEIAHGYVEHSVRYQCKNYYKLRTEGDGVYTL**NNEKQWINK
AVGDKLPECEAVCGKPKNPANPVQ**R**/ILGGHLDAKGSFPWQAKMVSHHNLTTGATLINEQWLLTTAKNLFLNHSENA
TAKDIAPTLTLYVGKKQLVEIEKVVLHPNYSQVDIGLIKLKQKVSVNERVMPICLPSKDYAEVGRVGYVSGWGRNAN
FKFTDHLKYVMLPVADQDQCIRHYEGSTVPEKKTPKSPVGVQPILNEHTFCAGMSKYQEDTCYGDAGSAFAVHDLEE
DTWYATGILSFDKSCAVAEYGVYVKVTSIQDWVQKTIAEN

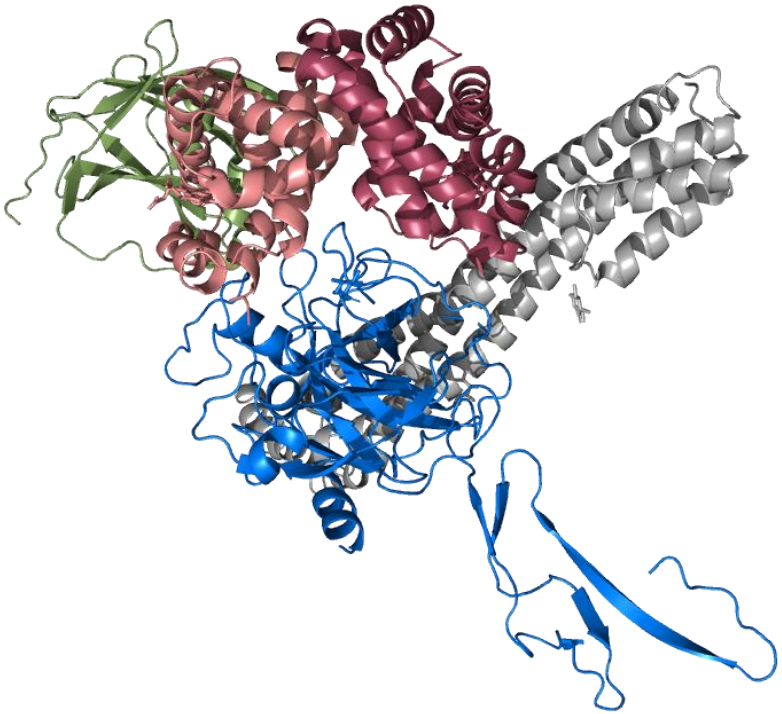
*MSALGAVIALLLWGQLFA*VDSGNDVTDI**ADDGCPKPPEIAHGYVEHSVRYQCKNYYKLRTEGDGVYTL**NDKKQWINK
AVGDKLPECEADDGCPKPPEIAHGYVEHSVRYQCKNYYKLRTEGDGVYTLNNEKQWINKAVGDKLPECEAVCGKPKN
PANPVQ**R**/ILGGHLDAKGSFPWQAKMVSHHNLTTGATLINEQWLLTTAKNLFLNHSENATAKDIAPTLTLYVGKKQL
VEIEKVVLHPNYSQVDIGLIKLKQKVSVNERVMPICLPSKDYAEVGRVGYVSGWGRNANFKFTDHLKYVMLPVADQD
QCIRHYEGSTVPEKKTPKSPVGVQPILNEHTFCAGMSKYQEDTCYGDAGSAFAVHDLEEDTWYATGILSFDKSCAVA
EYGVYVKVTSIQDWVQKTIAEN



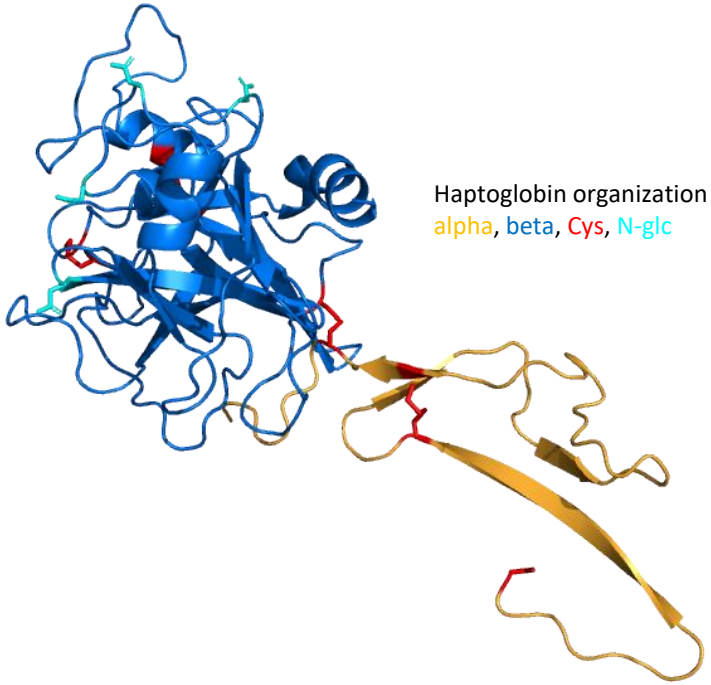
/ alpha / beta subunit and cleavage site (**R**), CCP repetitions and their difference, *signal*, Cys, N-glc,

Human Haptoglobin

- known high-resolution structure (pdb: 4WJG) lacks N-terminal part of the alpha subunit and the protein is stabilized by binding of two other proteins (iron-regulated surface determinant (*S. aureus*) and Hp-Hb receptor (*T. brucei*)



hemoglobin alpha, hemoglobin beta, haptoglobin (92-401)
iron-regulated surface determinant protein (*S. aureus*)
haptoglobin-hemoglobin receptor (*T. brucei*)



Haptoglobin organization
alpha, beta, Cys, N-glc

Goals

Study haptoglobin-hemoglobin interaction and structure in solution by structural mass spectrometry methods (FPOP, chemical cross-linking / labelling, HDX – this presentation) and complement recent native MS+mass photometry findings (*Sem T. et al. PNAS 2020, 117(27): 15554*)

Specifically:

- follow the role of glycosylation

- describe the difference between the variants 1-1, 2-2, 2-1

- extend the in vitro finding to experiments with sera of phenotyped individuals

- adopt the HDX-MS workflow to timsTOF Pro (Bruker Daltonics) with *PASEF*

Methods

Hemoglobin (HbA and HbB), Haptoglobin (form 1-1 and 2-2) were purchased from Sigma

Haptoglobin was natively deglycosylated either by sialidase (neuraminidase) or PNGase F

Proteins were followed either alone (Hb, Hp1-1, Hp1-1neu, Hp1-1png, Hp2-2, Hp2-2neu, Hp2-2png) or in mixture (Hb with the Hp forms) – classical continuous labelling at 25°C

Agilent UPLC + timsTOF Pro with *PASEF. Columns (Phenomenex, Luna Omega Polar C18) and in-house generated protease columns*

Optimization of the HDX-MS workflow - digestion conditions tuning

- quench solution (guanine, urea, urea/thiourea – all with TCEP)
- post-labelling incubation (denaturation and SS bond reduction)
- digestion temperature
- protease columns (pepsin, nepenthesin-1 and 2, rhizopuspepsin, aspergillopepsin, AnPEP, their serial coupling or co-immobilized resins)

Methods

Optimization of the HDX-MS workflow

- setup of LC-MS/MS and LC-MS methods and tuning of the timsTOF Pro with *PASEF* for HDX-MS workflow
    - LC-MS/MS: focuses also on singly charged precursors, number of tims cycles and their duration, number of precursors per tims separation
    - LC-MS: ion transmission and entry set to “low-scrambling” conditions

    (*Weltz Wollenberg DT et al Anal Chem 2020, 92(11):7453*)

- adopting DataAnalysis peak picking and MS export routines for HDX data processing with DeutEx (SW from peterslab)

- developing workflow for assessment of digestion results and comparison of the tested conditions

Detailed analysis of glycosylation pattern and coverage of glycosylation sites in individual studied Hp glycosylation states.
Handling of sequence repetitions.
Optimization of experimental conditions with sera from healthy individuals.

Results

Digest tuning

Optimal digestion pattern for all proteins (HbA, HbB, Hp1-1, Hp2-2) was obtained by a combination of pepsin and nepenthesin-2. Nep-2 brings many short peptides and pepsin helps to fill gaps and increase redundancy. Co-immobilized column has slightly lower (and more reasonable) redundancy than the serial coupling. *Slightly higher (than usual for other proteins analyzed in the lab) peptide length is caused by the presence of denaturing agents.*

Combination of 3M urea/ 1.5M thiourea / 200mM TCEP provides the best results in terms of protein denaturation and SS bond reduction. Much better denaturation than urea alone (up to 4M) and no effects in LC as in the case of guanidine (complete desalting even at short desalting times)

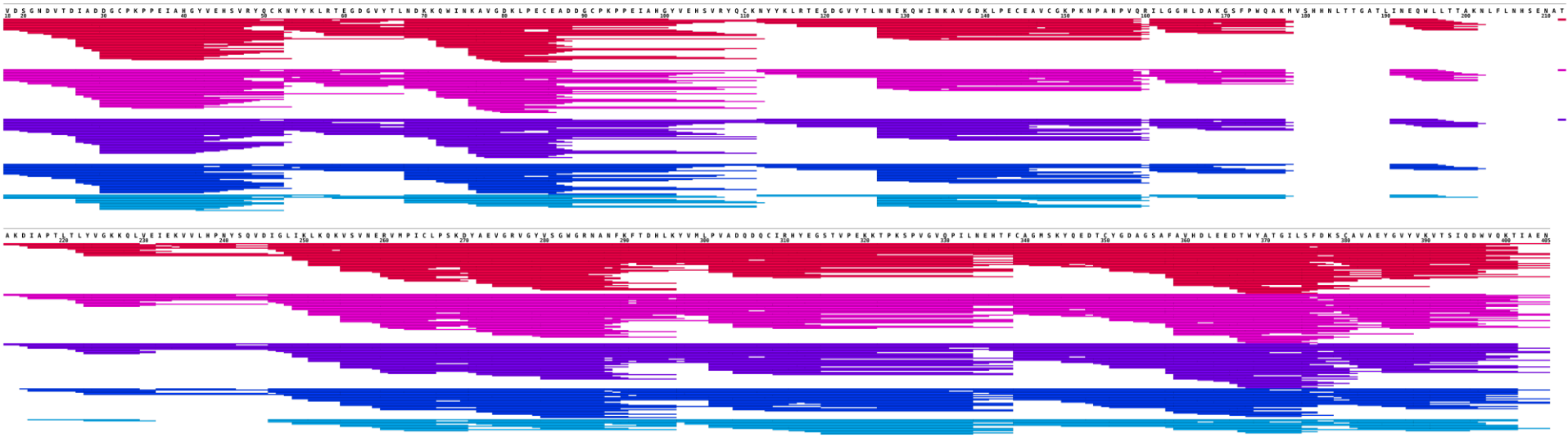
Digestion temperature can be kept at 0°C (lower back-exchange) and post-labelling incubation

Efficiency of the timsTOF employing the PASEF technology is very high – all studied proteins can be analyzed in the LC-MS/MS workflow at once and using the short method (overall method duration 20min) as used for the analysis of deuterated samples. Approx. 250-300 peptides for each hemoglobin and 500 for haptoglobin variants even with quite stringent search results filtering (MASCOT score >20, decoy search + FDR<1%, utilization of crap database containing sequences of HbA, HbB, Hp1 and Hp2). However not all these signals are intense enough to provide good HDX-MS data + comparison of less obvious differences in digestion result is difficult ***-> need for some filtering***

Results

Filtering of peptide IDs

Most efficient and informative ID filtering was obtained through signal intensity thresholding in the generation of mgf (MASCOT generic file).



Example of Hp sequence coverage (redundancy) at different MSMS signal intensities – ordered from top to bottom from no to most stringent filtering. The middle (purple) setting aligns well with the signals providing good HDX data.

Gaps are only around the N-glycosylation sites as this provides raw search result from MASCOT not considering various N-glycans attached.

Plotted with MSTools - http://peterslab.org/MSTools/ (DrawMap)

Results

Filtering of peptide IDs

Most efficient and informative ID filtering was obtained through signal intensity thresholding in the generation of mgf (MASCOT generic file).

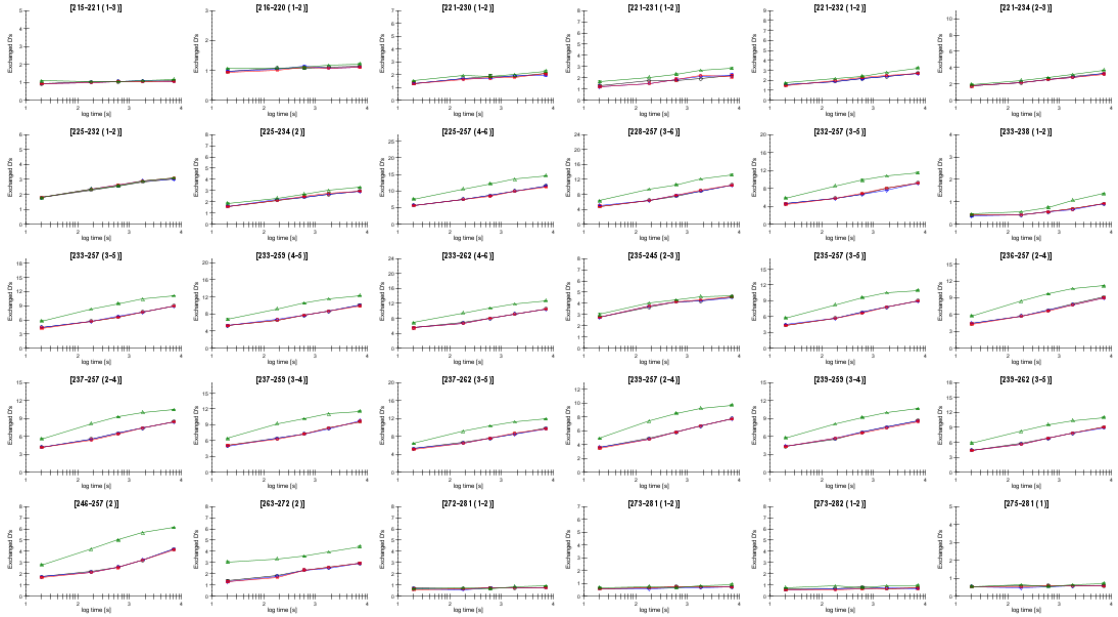
Signal Int	# peptides	Average L	redundancy
0	512	16.1	22.5
10000	500	16.1	21.9
30000	**420**	**16.9**	**19.3**
50000	311	17.2	14.7
100000	161	16.6	7.7

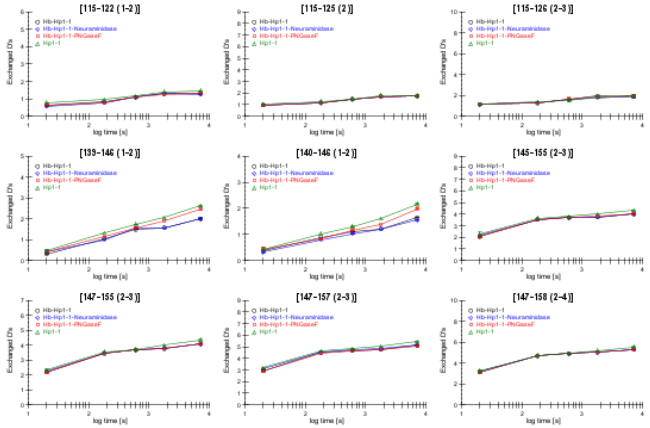
Table parametrizing the previous coverage map

Results

Initial results – set of uptake plots obtained by DeutEx

(Hp1-1 alone, Hp+Hb – glycosylated, desialylated, deglycosylated)





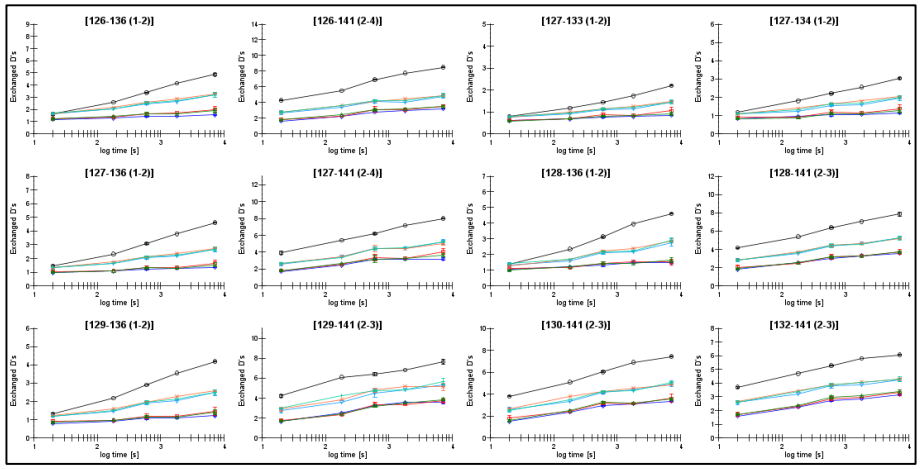
Selected interaction (top) and effect of glycosylation (right) regions.

timsTOF data are having very nice reproducibility.
Control data subset analyzed also on 15T FT-ICR used
in the lab for numerous HDX-MS studies previously and showed good alignment.

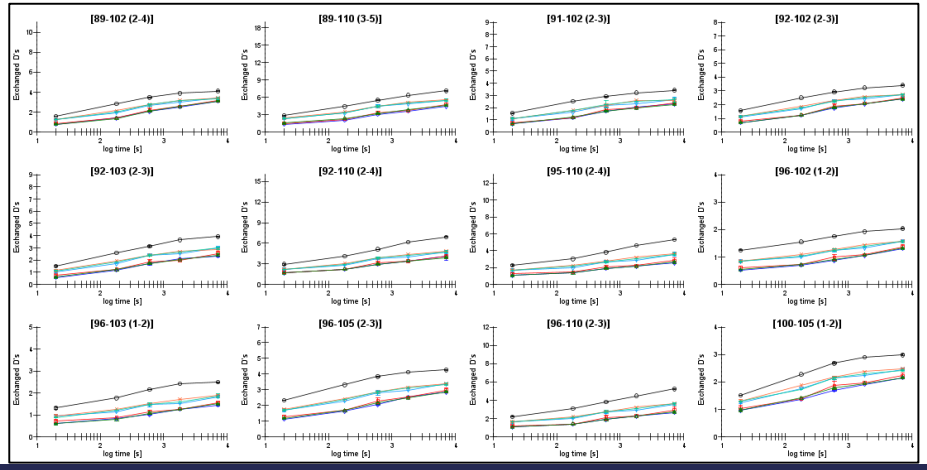
Results

Initial results – set of uptake plots obtained by DeutEx

HbA and HbB – differences in protection by Hp1-1 and Hp2-2 – clustering of curves for 1-1 (more protected) and 2-2 (less protected)



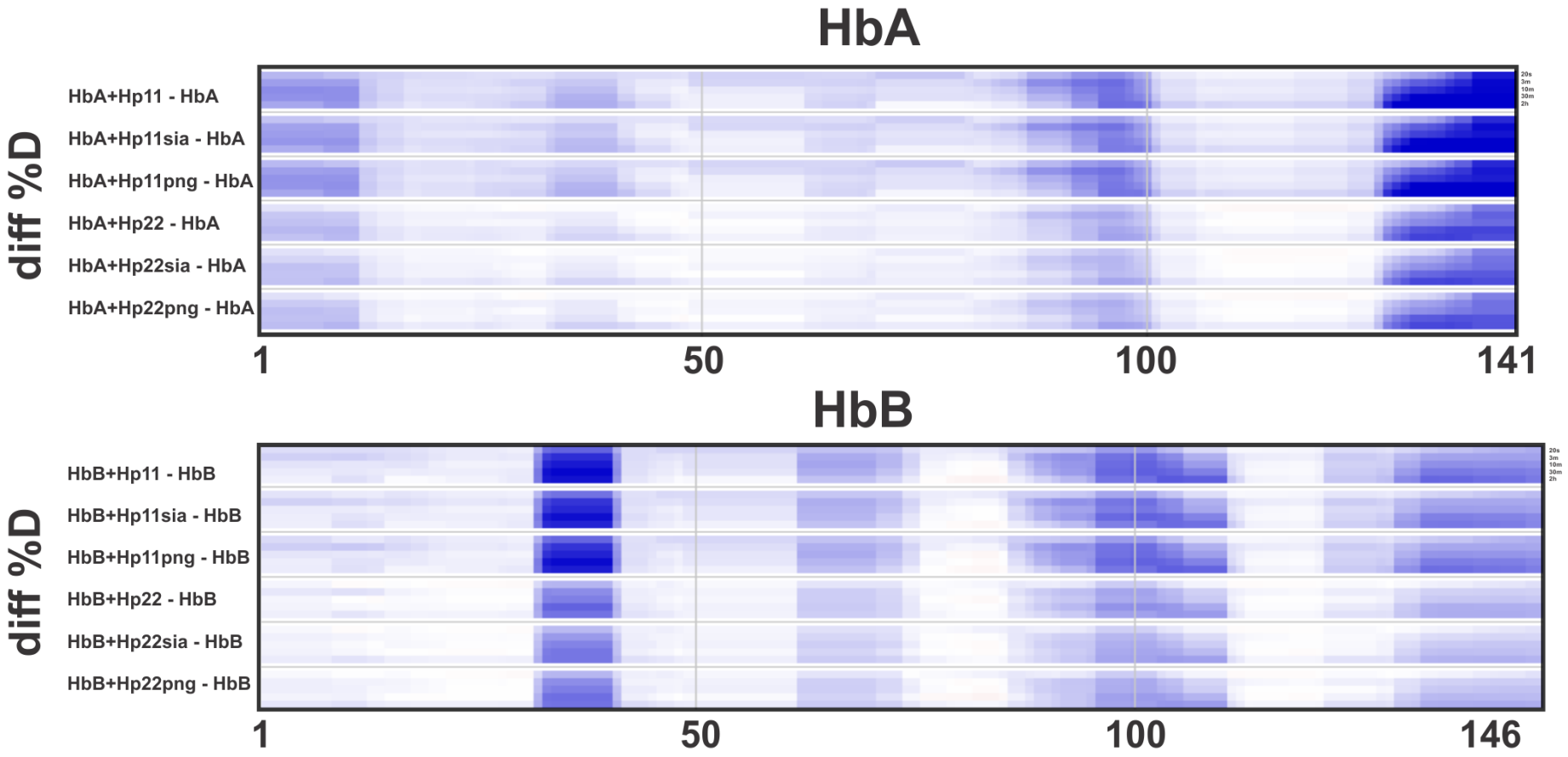
HbA
HbA-Hp1-1
HbA-Hp1-1-sia
HbA-Hp1-1-png
HbA-Hp2-2
HbA-Hp2-2-sia
HbA-Hp2-2-png



HbB
HbB-Hp1-1
HbB-Hp1-1-sia
HbB-Hp1-1-png
HbB-Hp2-2
HbB-Hp2-2-sia
HbB-Hp2-2-png

Results

Differential heatmaps – hemoglobin chains (mature sequence numbering)

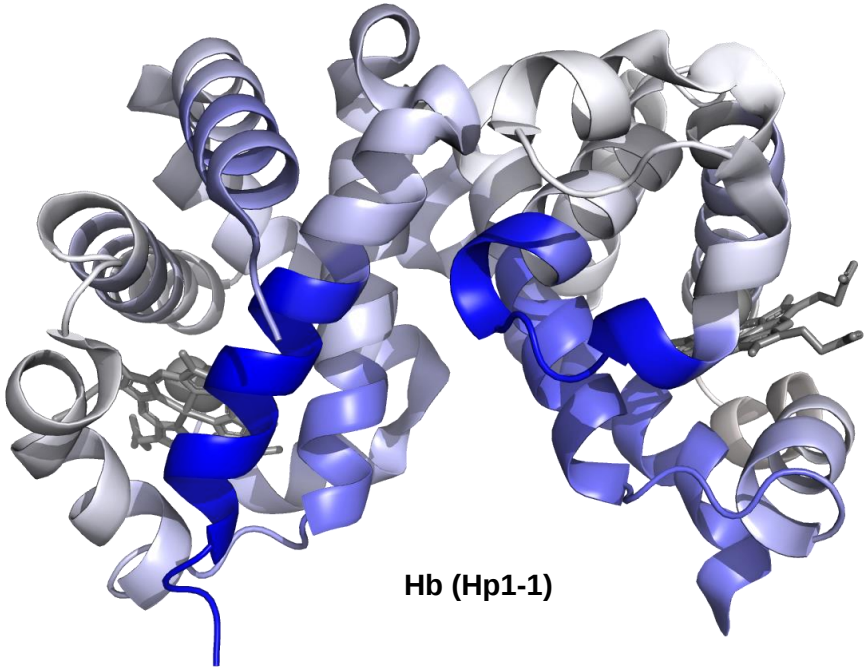


Plotted with MSTools - http://peterslab.org/MSTools/ (Draw Diff H/D Heat Map)

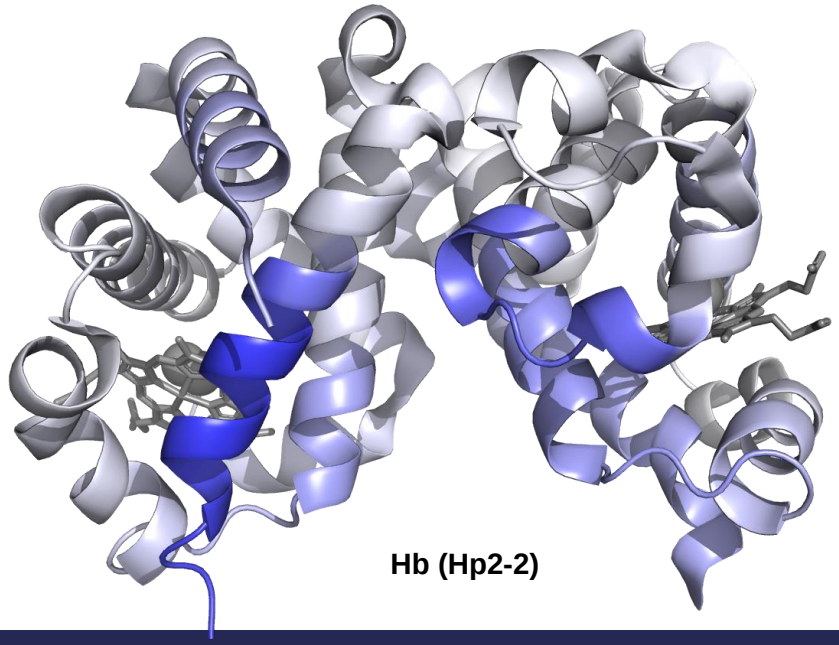
Kavan D and Man P. Int. J. Mass Spectrom. 2011, 302:53. doi:10.1016/j.ijms.2010.07.030

Results

Differences on hemoglobin dimer – bigger protection by Hp1-1 than Hp2-2 binding



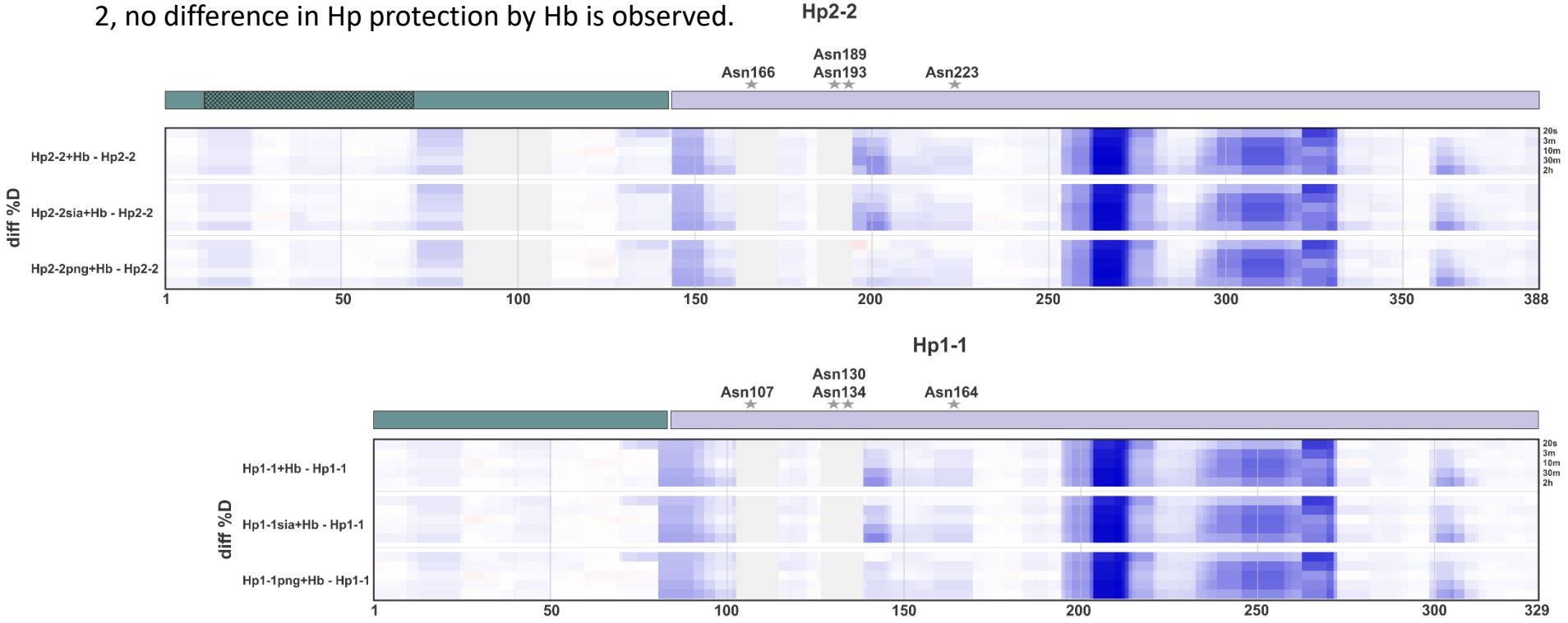
Hb (Hp1-1)



Hb (Hp2-2)

Results

Differential heatmaps – haptoglobin forms - in contrast to difference in Hb protection by Hp1-1 and Hp2-2, no difference in Hp protection by Hb is observed.



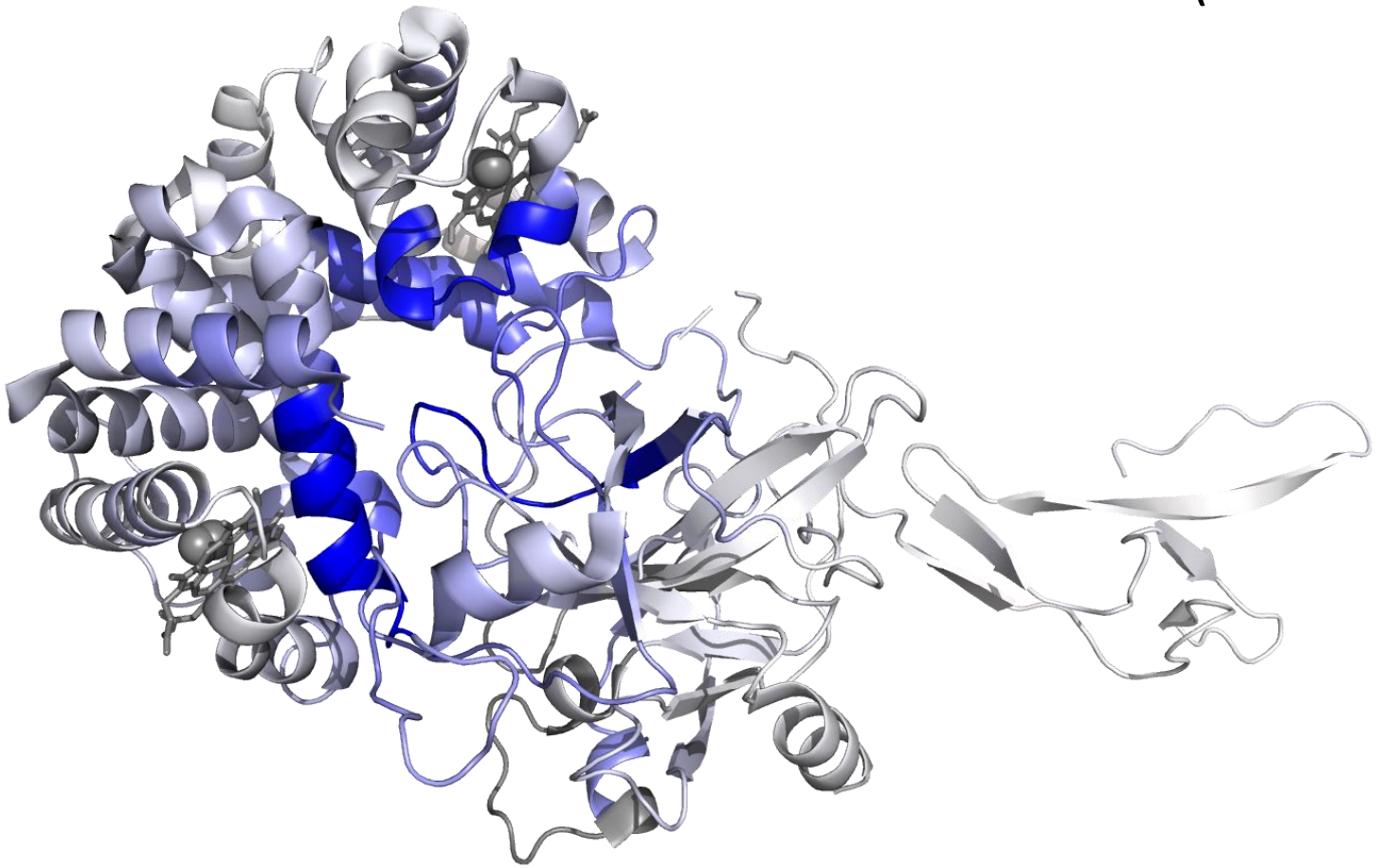
Shaded areas – not covered in this image - shown without glycopeptides and filled repetition – shaded areas (area around 100, which in fact resembles deuteration profile around 50 = same sequence).

Plotted with MSTools - http://peterslab.org/MSTools/ (Draw Diff H/D Heat Map)

Kavan D and Man P. Int. J. Mass Spectrom. 2011, 302:53. doi:10.1016/j.ijms.2010.07.030

Results

Structure of the complex (pdb: 4WJG) colored according to Hp1-1:Hb interaction (2hrs of exchange)



Results

Initial experiments with whole serum (quench and digestion conditions identical to Hp-Hb experiments, however different proteases also tested)

Accession	Protein	#Peptides	SC [%]	Scores
ALBU_HUMAN	Serum albumin OS=Homo sapiens OX=9606 GN=ALB PE=1 SV=2	813	97.0	44829.2 (M.score:44829.2)
CO3_HUMAN	Complement C3 OS=Homo sapiens OX=9606 GN=C3 PE=1 SV=2	261	77.2	11807.3 (M.score:11807.3)
A2MG_HUMAN	Alpha-2-macroglobulin OS=Homo sapiens OX=9606 GN=A2M PE=1 SV=3	219	70.0	9885.6 (M.score:9885.6)
APOA1_HUMAN	Apolipoprotein A-I OS=Homo sapiens OX=9606 GN=APOA1 PE=1 SV=1	176	91.0	9079.8 (M.score:9079.8)
A1AT_HUMAN	Alpha-1-antitrypsin OS=Homo sapiens OX=9606 GN=SERPINA1 PE=1 SV=3	148	82.1	8786.1 (M.score:8786.1)
TRFE_HUMAN	Serotransferrin OS=Homo sapiens OX=9606 GN=TF PE=1 SV=3	190	84.0	8623.8 (M.score:8623.8)
IGG1_HUMAN	Immunoglobulin gamma-1 heavy chain OS=Homo sapiens OX=9606 PE=1 SV=2	167	71.0	8088.8 (M.score:8088.8)
IGHG4_HUMAN	Immunoglobulin heavy constant gamma 4 OS=Homo sapiens OX=9606 GN=IGHG4 PE=1 SV=1	152	82.3	7129.5 (M.score:7129.5)
IGHG2_HUMAN	Immunoglobulin heavy constant gamma 2 OS=Homo sapiens OX=9606 GN=IGHG2 PE=1 SV=2	137	76.1	6164.8 (M.score:6164.8)
IGK_HUMAN	Immunoglobulin kappa light chain OS=Homo sapiens OX=9606 PE=1 SV=1	93	72.4	4797.0 (M.score:4797.0)
HPT_HUMAN	Haptoglobin OS=Homo sapiens OX=9606 GN=HP PE=1 SV=1	95	78.1	4728.9 (M.score:4728.9)
IGHG3_HUMAN	Immunoglobulin heavy constant gamma 3 OS=Homo sapiens OX=9606 GN=IGHG3 PE=1 SV=2	102	62.6	4599.1 (M.score:4599.1)
TTHY_HUMAN	Transthyretin OS=Homo sapiens OX=9606 GN=TTR PE=1 SV=1	85	86.4	4537.7 (M.score:4537.7)
IGKC_HUMAN	Immunoglobulin kappa constant OS=Homo sapiens OX=9606 GN=IGKC PE=1 SV=2	85	100.0	4386.4 (M.score:4386.4)
CO4B_HUMAN	Complement C4-B OS=Homo sapiens OX=9606 GN=C4B PE=1 SV=2	89	49.6	4308.4 (M.score:4308.4)
CO4A_HUMAN	Complement C4-A OS=Homo sapiens OX=9606 GN=C4A PE=1 SV=2	86	49.1	4213.7 (M.score:4213.7)
CERU_HUMAN	Ceruloplasmin OS=Homo sapiens OX=9606 GN=CP PE=1 SV=1	68	57.1	2972.8 (M.score:2972.8)
APOB_HUMAN	Apolipoprotein B-100 OS=Homo sapiens OX=9606 GN=APOB PE=1 SV=2	74	16.5	2954.6 (M.score:2954.6)
HEMO_HUMAN	Hemopexin OS=Homo sapiens OX=9606 GN=HPX PE=1 SV=2	58	65.4	2938.1 (M.score:2938.1)
IGLC2_HUMAN	Immunoglobulin lambda constant 2 OS=Homo sapiens OX=9606 GN=IGLC2 PE=1 SV=1	38	94.3	2206.8 (M.score:2206.8)
HPTR_HUMAN	Haptoglobin-related protein OS=Homo sapiens OX=9606 GN=HPR PE=2 SV=2	41	60.3	1978.7 (M.score:1978.7)
IGL1_HUMAN	Immunoglobulin lambda-1 light chain OS=Homo sapiens OX=9606 PE=1 SV=1	35	50.9	1968.3 (M.score:1968.3)
APOA2_HUMAN	Apolipoprotein A-II OS=Homo sapiens OX=9606 GN=APOA2 PE=1 SV=1	35	77.0	1940.3 (M.score:1940.3)
AACT_HUMAN	Alpha-1-antichymotrypsin OS=Homo sapiens OX=9606 GN=SERPINA3 PE=1 SV=2	44	61.9	1829.9 (M.score:1829.9)
IGLC3_HUMAN	Immunoglobulin lambda constant 3 OS=Homo sapiens OX=9606 GN=IGLC3 PE=1 SV=1	31	94.3	1724.8 (M.score:1724.8)
ITIH2_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H2 OS=Homo sapiens OX=9606 GN=ITIH2 PE=1 SV=2	41	36.0	1723.7 (M.score:1723.7)
FETUA_HUMAN	Alpha-2-HS-glycoprotein OS=Homo sapiens OX=9606 GN=AHSG PE=1 SV=2	28	52.0	1566.8 (M.score:1566.8)
APOC1_HUMAN	Apolipoprotein C-I OS=Homo sapiens OX=9606 GN=APOC1 PE=1 SV=1	28	68.7	1526.6 (M.score:1526.6)
ITIH1_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H1 OS=Homo sapiens OX=9606 GN=ITIH1 PE=1 SV=3	31	39.3	1353.7 (M.score:1353.7)
VTDB_HUMAN	Vitamin D-binding protein OS=Homo sapiens OX=9606 GN=GC PE=1 SV=2	27	39.5	1238.2 (M.score:1238.2)

Hp coverage and number of peptides can be increased if longer gradient is used

Conclusions

HDX-MS workflow was successfully adopted to timsTOF Pro with PASEF and provides very significant improvement in terms of identified peptides = higher resolution in HDX-MS

So far, Hp1-1 and Hp2-2 were compared in their binding to hemoglobin and showed different extent of protection on the Hp-Hb interaction interface which aligns well with their know different affinities.

While desialylation of the N-glycans had no or very small effect on the structure/interaction, complete removal of the glycan likely alters the Hp-Hb contact and Hp structure. This fits to a recent independent study published in PNAS last year (doi: 10.1073/pnas.2002483117). Further investigation is now being carried out.

Interaction of Hp-Hb in the whole serum was partially optimized. Now sera phenotyping is carried out to select samples matching the individual forms and will be subjected to the HDX workflow done with individual proteins.

All results will be then integrated with the data from other structural MS techniques being gather by the members of peterslab.

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http://peterslab.org/