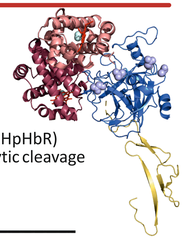
Diving into the complexity of human haptoglobin-hemoglobin interactions.

Pavla Vaňková[1], Petr Man[2], Petr Pompach[1,2], Stuart Pengelley[3], Gary Kruppa[4], Daniel Kavan[2], Petr Novak[2]

1 - BioCeV - Institute of Biotechnology of the CAS, Vestec, Czech Republic **2** - BioCeV - Institute of Microbiology of the CAS, Vestec, Czech Republic **3** - Bruker Daltonik GmbH, Bremen, Germany **4** - Bruker s.r.o., Brno, Czech Republic

INTRODUCTION

Human haptoglobin (Hp)



- One of the most abundant plasma proteins
- Scavenging of haemoglobin (Hb) from damaged red blood cells
- Two subunits - alpha (oligomerization) and beta (Hb binding)
- Two main allelic forms 1 and 2 (seqeunce of alpha 2 has repetition!)
- Phenotypes 1-1, 2-1 and 2-2 - different architecture, affinity to Hb and CD163 (HpHbR)
- Disulfide bonds (intra-/interchain + intermolecular), N-glycosylation, proteolytic cleavage

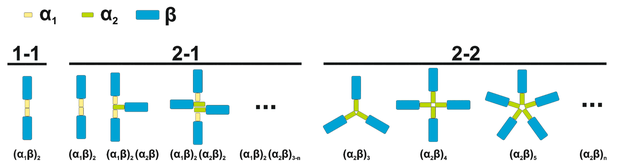


Figure 1: Architecture of haptoglobins and structure of Hp:Hb complex - Hp alpha and beta chain (N-glycosylation sites highlighted), haemoglobin. (PDB: 4WJG)

AIMS / QUESTIONS

- Setup the HDX-MS workflow and data processing on timsTOF Pro with PASEF and high-resolution HDX-MS LC-MS methods on FT-ICR for complex mixture analysis.
- Optimize quenching (guanidine, urea, urea/thiourea), SS bond reduction (TCEP incubation time), proteolysis - temperature and enzyme (pepsin, nepenthesin-1 and 2, rhizopuspepsin, aspergillopepsin, AnPEP, their serial coupling or co-immobilized resins)
- Investigate the role of glycosylation (removal of sialic acid and whole glycans) on Hp:Hb interaction and on Hp structure
- Describe the inter-phenotype differences on isolated proteins and using serum/plasma of healthy donors of three major phenotypes - are there also differences related to individual donors?
- Compare Hp:Hb binding for the ensemble of oligomers and separated/enriched oligomer populations (virtually impossible to go to proteoform level)

METHODS

- Isolated human Hp1-1, 2-1 and 2-2 - native of de-sialylated/-glycosylated
- SEC separation of Hp proteoforms (HPLC 4.6x300mm 1.8um BioZen-2)
- PAGE - Tris-acetate (3-8%)/non-reducing and Bis-Tris (4-12%)/reducing
- Serum of healthy donors - tuning digestion, separation, MS-resolution
- HDX of Hp isoforms, different glycosylation states, serum. All +/- haemoglobin
- UPLC (Agilent 1290 + Luna Omega Polar C18), digestion coi-pepsin/Nep-2
- tims TOF Pro with PASEF and 15T solariX XR (Bruker Daltonics)
- Data processing - MASCOT, DataAnalysis, DeutEx, MSTools (@ peterslab.org)
- Development of timsTOF data acquisition and processing pipeline for HDX-MS

RESULTS

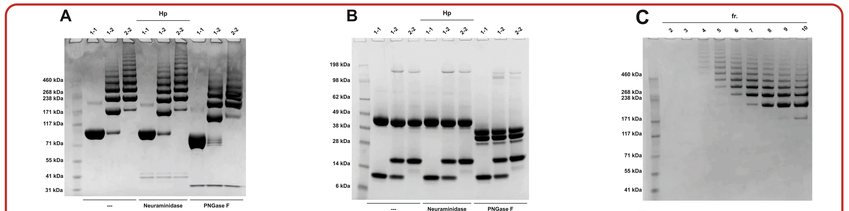


Figure 2: Electrophoretic validation of Hp proteoforms, deglycosylation results and SEC separation. A - non reducing gradient gel of Hp1-1, 2-1 and 2-2 isoforms in the native state, after desialylation and complete deglycosylation. B - Same samples analyzed under reducing conditions which led to disruption of oligomers and separation of alpha and beta subunits. Last three lanes are showing incomplete deglycosylation under native conditions. C+D - SEC separation of Hp2-2 using BioZen SEC column.

Accession	Protein	#Peptides	SC [%]	MW [kDa]	Scores
ALBU_HUMAN	Serum albumin OS=Homo sapiens OX=9606 GN=ALB PE=1 SV=2	868	96.1	69.3	44268
APOA1_HUMAN	Apolipoprotein A-I OS=Homo sapiens OX=9606 GN=APOA1 PE=1 SV=1	303	93.3	30.8	14661.1
A2MG_HUMAN	Alpha-2-macroglobulin OS=Homo sapiens OX=9606 GN=A2M PE=1 SV=3	261	65.4	163.2	11175
IGG1_HUMAN	Immunoglobulin gamma-1 heavy chain OS=Homo sapiens OX=9606 PE=1 SV=2	227	71.7	49.3	10841.5
CO3_HUMAN	Complement C3 OS=Homo sapiens OX=9606 GN=C3 PE=1 SV=2	219	66.2	187.0	9306.6
HBB_HUMAN	Hemoglobin subunit beta OS=Homo sapiens OX=9606 GN=HBB PE=1 SV=2	152	99.3	16.0	9264.2
HBA_HUMAN	Hemoglobin subunit alpha OS=Homo sapiens OX=9606 GN=HBA1 PE=1 SV=2	146	99.3	15.2	8719
IGHG2_HUMAN	Immunoglobulin heavy constant gamma 2 OS=Homo sapiens OX=9606 GN=IGHG2 PE=1 SV=2	175	72.4	35.9	8171.9
TRFE_HUMAN	Serotransferrin OS=Homo sapiens OX=9606 GN=TF PE=1 SV=3	191	41.4	77.0	8006.6
HPT_HUMAN	Haptoglobin OS=Homo sapiens OX=9606 GN=HP PE=1 SV=1	152	79.8	45.2	7552.9
IGHG3_HUMAN	Immunoglobulin heavy constant gamma 3 OS=Homo sapiens OX=9606 GN=IGHG3 PE=1 SV=2	155	66.8	41.3	7240.1
IGHG4_HUMAN	Immunoglobulin heavy constant gamma 4 OS=Homo sapiens OX=9606 GN=IGHG4 PE=1 SV=1	151	70.6	35.9	7181.1
A1AT_HUMAN	Alpha-1-antitrypsin OS=Homo sapiens OX=9606 GN=SERPINA1 PE=1 SV=3	116	80.1	46.7	5873.5
CERU_HUMAN	Ceruloplasmin OS=Homo sapiens OX=9606 GN=CP PE=1 SV=1	136	73.1	122.1	5519.4
IGK_HUMAN	Immunoglobulin kappa light chain OS=Homo sapiens OX=9606 PE=1 SV=1	109	75.7	23.4	5336.2
IGKC_HUMAN	Immunoglobulin kappa constant OS=Homo sapiens OX=9606 GN=IGKC PE=1 SV=2	96	100.0	11.8	4895.3
HEMO_HUMAN	Hemopexin OS=Homo sapiens OX=9606 GN=HPX PE=1 SV=2	108	68.6	51.6	4734.8
CO4A_HUMAN	Complement C4-A OS=Homo sapiens OX=9606 GN=C4A PE=1 SV=2	106	50.7	192.7	4653.5
CO4B_HUMAN	Complement C4-B OS=Homo sapiens OX=9606 GN=C4B PE=1 SV=2	105	50.6	192.6	4563.5
TTHY_HUMAN	Transthyretin OS=Homo sapiens OX=9606 GN=TTR PE=1 SV=1	75	86.4	15.9	3712.9

Table 1: List of top 20 proteins identified in the whole serum+spiked Hb using HDX-MS digestion/LC-MS setup. Samples processed using "HDX-like" protocol - quenching by urea, thiourea and TCEP with 3min incubation on ice and subsequent online digestion on pepsin+Nep-2 column. Incomplete coverage of Hp is due to the repetitive sequence (search result "bug") and missed glycosylation sites. The number of peptides usable for HDX is approx half with no major drop in coverage.

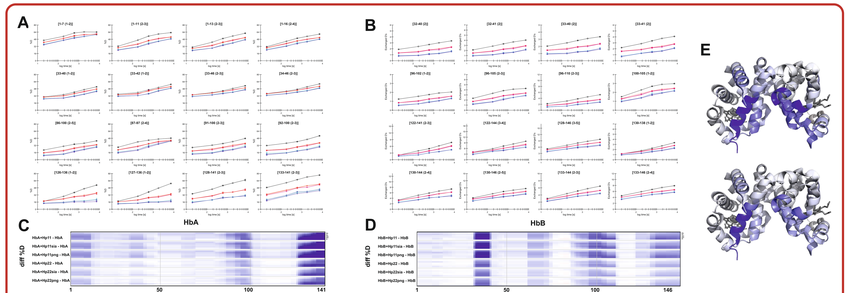


Figure 3: HDX analysis of haemoglobin alpha and beta after interaction with native and deglycosylated forms of Hp1-1 and Hp2-2. A and B - selected uptake plots showing differences induced by Hp:Hb interaction. Clustering to Hp1-1 (blue tones) and Hp2-2 (red tones) groups points to different strength of binding for Hp1-1 and Hp2-2. C and D - all data are summarized and displayed in a form of differential heat maps (plotted with MSTools) where free Hb (A-alpha or B-beta) state was subtracted from the state with Hp forms. No apparent effect of glycosylation states on the changes induced to Hb. E - projection of these data onto Hb dimers - top - Hp1-1, bottom - Hp2-2 interaction. Within each structure, the HbA is on the left and HbB on the right.

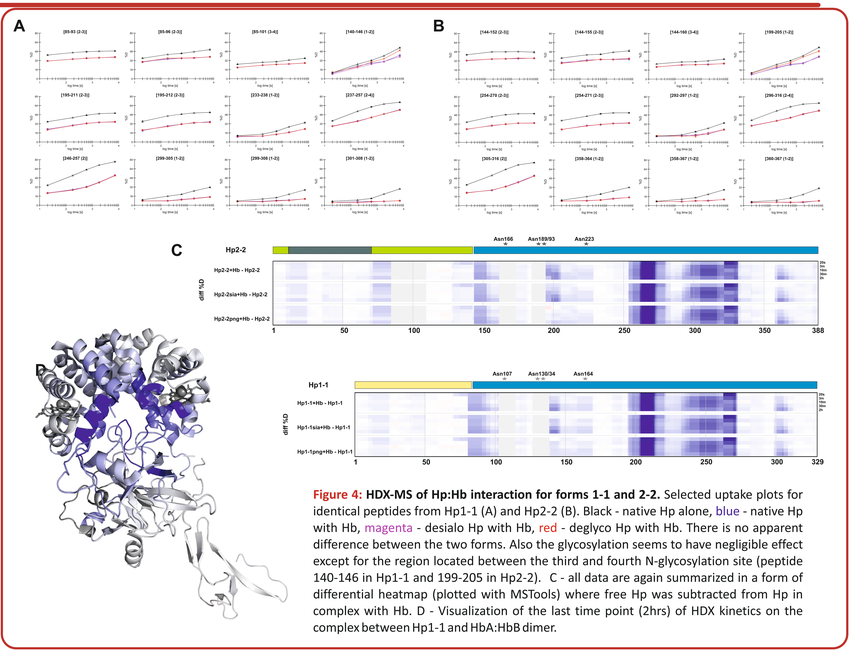


Figure 4: HDX-MS of Hp:Hb interaction for forms 1-1 and 2-2. Selected uptake plots for identical peptides from Hp1-1 (A) and Hp2-2 (B). Black - native Hp alone, blue - native Hp with Hb, magenta - desialo Hp with Hb, red - deglyco Hp with Hb. There is no apparent difference between the two forms. Also the glycosylation seems to have negligible effect except for the region located between the third and fourth N-glycosylation site (peptide 140-146 in Hp1-1 and 199-205 in Hp2-2). C - all data are again summarized in a form of differential heatmap (plotted with MSTools) where free Hp was subtracted from Hp in complex with Hb. D - Visualization of the last time point (2hrs) of HDX kinetics on the complex between Hp1-1 and HbA:HbB dimer.

CONCLUSIONS AND DIRECTIONS

Interaction between two Hp isoforms and haemoglobin was probed by HDX-MS (Hp2-1 remains to be done). The results align well with the known crystal structure but here the interaction was studied in solution and without additional two stabilizing proteins that were used in the X-ray experiment. Sialic acid on the N-glycans has likely no role in the interaction, but complete removal of glycan affects one particular region close to the Hp:Hb interaction interface. However, the deglycosylation was not complete (Fig. 2B) and thus the effects might be even stronger if full glycan removal is achieved. This will be further addressed. There was also clear difference in Hb protection caused by Hp1-1 or Hp2-2 that fits well to the known (but so far unexplained) difference in Hp:Hb affinity. These effects will be studied in more detail with fractionated Hp oligomers. Initial experiments were also done on Hp:Hb interaction using full serum/plasma showing potential of such studies in complex matrices.

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