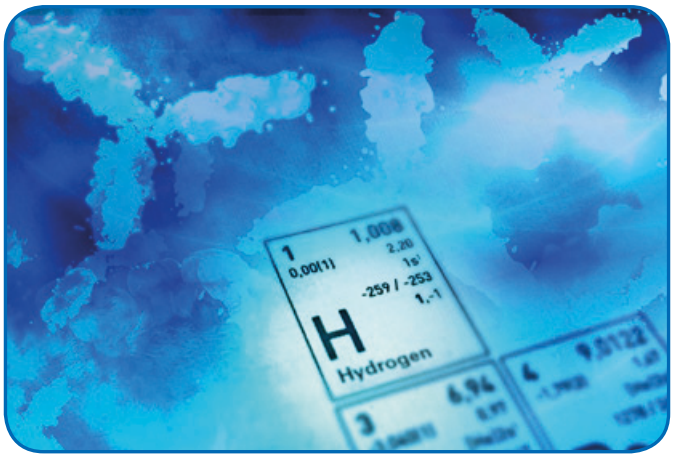

Poster Note PN-28

A Fully Automated Robotic Set-Up based on Ultrahigh-Resolution QTOF Mass Spectrometry for Hydrogen Exchange Experiments

Introduction

The activity of most therapeutic antibodies is directed by their ability to link to a target antigen. Localizing the amino acids (AAs) responsible for this interaction is mandatory from a mechanistic point of view, but also for intellectual property protection. Epitope mapping is performed with an array of techniques, including hydrogen exchange mass spectrometry (HX-MS). With HX-MS solvent accessibility of the polypeptide backbone is measured and related to the proteins secondary structureas well as monitoring conformational changes occurring during antigen binding. While HX-MS experiments provide valuable information which is difficult to obtain by alternative techniques, they are also demanding in several aspects. On one hand they require exact timing and reproducibility in sample preparation. On the other hand full sensitivity at high mass resolution and isotopic fidelity is necessary for data interpretation. Here we established a system to meet these requirements **(Fig. 1)** and studied its performance using a biologically relevant model system.

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Sophisticated liquid handling robot specialized for HX-MS experiments

UHR-QTOF

Fig. 1 Key components of the robotic setup for automatic HX-MS experiments

Methods

- Instrumental Setup: Liquid handling robot (HDX PAL3, LEAP Technologies), UHPLC-system (1290 Infinity II, Agilent) and a ultra-high resolution (UHR) QTOF mass spectrometer (maXis, Bruker).
- Benchmark system used as model for detecting conformational changes caused by binding: E. coli Hsp90 (HtpG), a 140 kDa dimer, which undergoes significant conformational changes in response to ATP binding [Graf et al. EMBO J. 28, 602-613 (2009)].
- HX-MS Experiment: HtpG and HtpG+ATP samples were prepared at 5 different labeling time points as detailed in **Fig. 2**. Each time point was run in triplicate resulting in 30 LC-MS runs. In addition 0% and 100% control ("Full-D") samples were included (**Fig. 2**).

	1 Pre-Incubation	2 Labeling	3 Quenching	4 Digest	5 Separation
HtpG + ATP	20 min with ATP Sample (7 µL) + ATP (7 µL)	10 / 30 / 100 / 300 / 1000 s Sample (5 µL) + D2O (95 µL)	2% FA pH 2.35 0.5 °C Sample (20 µL) + Quench (70 µL)	10 pmol 180 s Digest @ 8 °C Trap @ 0.5 °C	10 min gradient 5 - 55 %B (0.5 °C) A: 0.3% FA in H2O B: 0.3% FA in ACN
HtpG	20 min without ATP Sample (7 µL) + H2O (7 µL)	10 / 30 / 100 / 300 / 1000 s Sample (5 µL) + D2O (95 µL)			
HtpG unx	20 min without ATP Sample (7 µL) + H2O (7 µL)	10 s Sample (5 µL) + H2O (95 µL)			
HtpG 100%		10 s Sample (5 µL) + D2O (95 µL)			

Fig. 2 Flowchart representing the tasks automatically performed by the robotic setup for a complete HX-MS experiment on HtpG/HtpG+ATP.

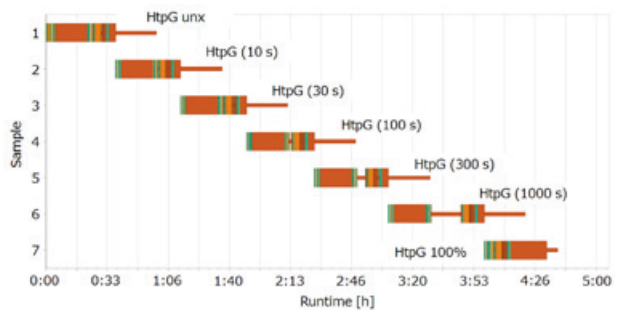

Fig. 3 Sample Table as calculated by Chronos. Idle times - e.g. D2O labeling, LC runtime - are utilized to prepare the next samples, thereby saving 135 min compared to a dataset with sequential measurements.

Methods (cont.)

- Software: Sample preparation, including preincubation, labeling, quenching and digest as well as LC-MS data acquisition was controlled by Chronos (Axel Semrau) which also provides an optimization routine for scheduling all LC-MS runs of an HX-MS experiment (**Fig. 3**). Data analysis of the recorded HX-MS data was performed using HDExaminer (Sierra Analytics) resulting in deuterium uptake plots and heat maps. The heat map data were exported from HDExaminer to PyMol (Schrödinger) which allows the visualization of structural changes using 3D crystal structures of the protein

Results

- MS/MS experiments of non-deuterated HtpG after pepsin digestion yielded 179 identified peptides with 97% sequence coverage. After manual evaluation and curation of the data, the list was reduced to 47 peptides representing 95% sequence coverage. These 47 peptides were used for the subsequent data analysis (**Fig. 4**).

- ATP binding induced pronounced conformational changes, resulting in a slow exchanging tensed state (**Fig. 4** and **Fig. 5**), with the highest degree of HX protection in the N-terminal ATP binding domain. The high reproducibility provided by the robotic setup is reflected in the small error bars of the deuterium uptake plots shown in **Fig. 5**.

- EX1 exchange mechanism could be observed (**Fig. 6**), indicating the coexistence of two populations of proteins. This is due to the slow hydrolysis of ATP and subsequent dissociation of ADP and transition through the more flexible nucleotide-free state.

Conclusion

- A fully automated robotic setup based on UHR-QTOF MS was established providing the following advantages for HX-MS:
    - Exact timing of sample preparation
    - Full sensitivity at high mass resolution combined with isotopic fidelity result in accurate deuterium uptake kinetics determination even in difficult cases (coeluting peptides, EX1 kinetics)
- These advantages were demonstrated using a model system. The obtained data are fully comparable with previously published data from a manual HX-MS system but are of higher overall quality and reproducibility.

Fig. 4 Heat map generated in HDExaminer showing nucleotide-dependent changes in HtpG after 30 s in D2O. Green lines above the sequence indicate the identified peptides. High solvent accessibility is represented by red colors. Upon ATP addition a protection (represented in blue) can be observed.

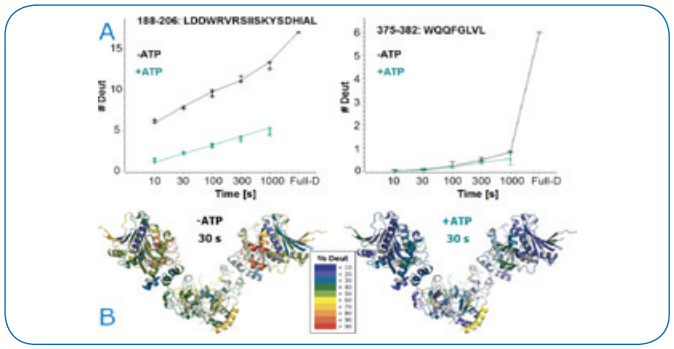

Fig. 5 A: D2O uptake plots of HtpG reveal shifts in solvent accessibility upon ATP addition (left), indicating conformational changes. The middle domain mostly stays protected (right).
B: Secondary structure representation of the crystal structure of full-length E. coli HtpG colored according to exchange at 30 s.

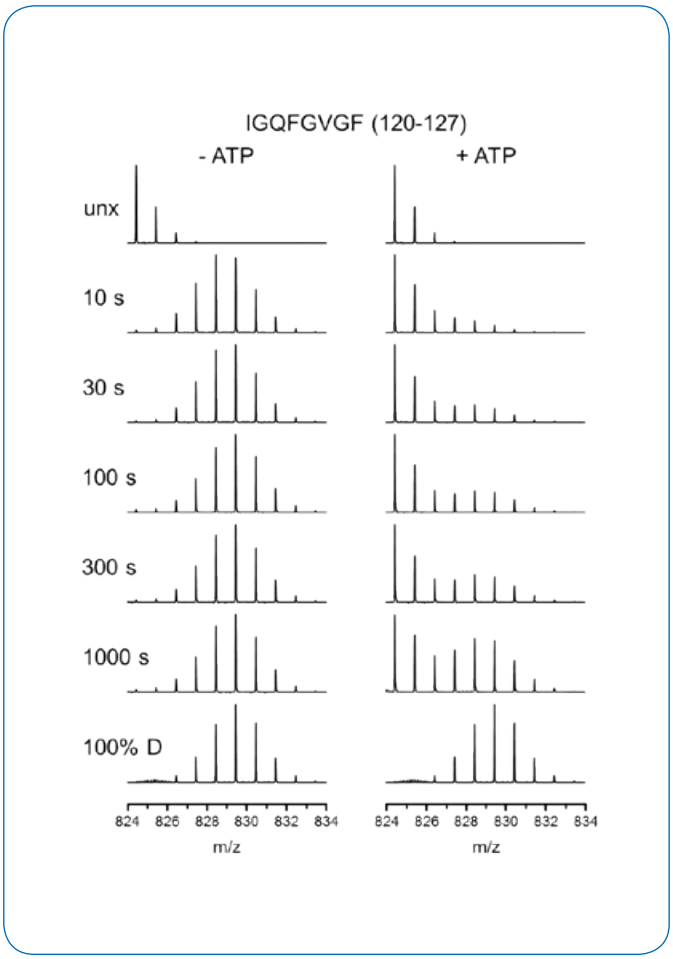

Fig. 6 Bimodal distribution in the presence of ATP suggesting an EX1 amide hydrogen exchange mechanism. EX1 indicates the coexistence of two populations, due to the low rate of ATP hydrolysis.

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