Supplementary data

MS/MS-confirmed peptide library building by nanoLC-MALDI

The size of the refMPL began to saturate early (**SuppFig. 1a**), with nearly 250 members after just the initial MS/MS experiment, yet only 382 members after the subsequent eight experiments irrespective of various changes of experimental condition (**SuppFig. 1a**). These changes in experimental condition (switch from automated to manual nanoLC, instrument overhaul and tuning, change from immobilized to solution-phase pepsin and switch to an electrospray mass spectrometer (LTQ)) led to no more than transient “up-kicks” in library complexity, which plateaued quickly (**SuppFig. 1a**). The final library complexity of 382 peptides provided 98.6% sequence coverage of VP55 with just two short contig breaks (3 and 4 aa). In separate experiments, 94.7% sequence coverage of VP39 was achieved, over 142 peptides, with just 3 short contig breaks. The 382 member VP55 library represented ~4% of the 9893 theoretical, nonspecifically-terminated fragments of VP55 with masses in the 600 – 3000 Da range. Experimental coverage of theoretical fragment space of just 4% may be attributed in large measure to the non-random cleavage specificity of pepsin protease [1] but perhaps also to secondary or tertiary structural considerations (even at low pH), and to mass spectrometry considerations such as the efficiency of peptic peptide ionization and/or positive ion CID fragmentation. In contrast to the library-building MSMS experiments, HDX-MS experiments benefited markedly by switching from immobilized to in-solution pepsin, with this change leading to a 3-fold enrichment in the number of experimental peptide masses matching the above refMPL (**SuppFig. 1b**). This improvement was observed at the MS level albeit there was only a negligable corresponding increase the numbers of peptides amenable to high-quality MS/MS (**SuppFig. 1a**, experiment #7) - even in the context of the LTQ mass spectrometer (**SuppFig. 1a**, experiment #8) whose wide dynamic range might be expected to facilitate the detection of low abundance peptides.

The increased complexity is attributed to the presumed better steric properties of the solution pepsin. Among the total set of non-redundant masses detected in a typical HDX experiment, typically ~50 – 75% matched the theoretical refMPL containing the 9893 theoretical fragments of VP55 (described above), indicating the apparent richness of HDX datasets in ionizable fragments of VP55, and that the key factor limiting experimental MS/MS library complexity (only 382-members, above) may therefore have been the efficiency with which peptic peptides were fragmented by CID in either the MALDI-TOF/TOF or the LTQ mass spectrometer to generate MSMS spectra able to yield strong MS/MS ions scores.

Thorough consideration of potential ambiguities in peptide assignment in MS experiments

The potential for ambiguity was apparent at several levels all of which are addressed here: Ambiguity at three levels related to mass-matching between HDX and MS/MS library-building experiments (above), namely (**a**) an HDX query peptide matching two VP55 library members of extremely similar mass (maybe less than 1 ppm difference) whose respective MS/MS fragmentation patterns had indicated, with high confidence, that they represented distinct peptic peptides; (**b**) two experimental masses which differed by more than 40 ppm and were therefore regarded as distinct members of the HDX dataset, both matching a single MS/MS-confirmed library member whose mass bisected them and scored as a match to either; (**c**) an HDX query peptide, in experiments containing VP55 + VP39, matching both VP55 and VP39 library members. In 30 – 50% of cases, peptides flagged as ambiguous according to one of the above three criteria could be disambiguated by use of a tool [2] that enabled the chromatographic outlier of the pair to be identified for peptides in HDX and MS/MS experiments, by alignment of z-number (solvent elution concentration). According to this tool, 26 of the 119 peptides in **Table S1** were either chromatographic outliers or peptides that could not be

disambiguated via the above analyses, and are indicated as such in the table. Elimination of the 26 chromatographic outliers (**Table S1**) left 94 peptides in total (82 of them in the VP55-alone dataset) whose maximum deuteration (as a % of theoretical value) was quantifiable (**Table S1**). These 82 peptides covered 74% of VP55's sequence.

HDX by LC-MALDI back-exchange: Direct measurements

Attempt were made to estimate back exchange rates directly [3], from control experiments in which F2H peptic digests were treated as described above but, prior to sample drying and data acquisition, sample spots were allowed to remain on the chilled MALDI target plate for various periods. These incubations led to estimates of back exchange during the forward exchange experiments in the range of just 4.9 – 8.4% (**Table 1**), indicating nonlinearity of BE during the experiment with respect to time. Attempts to estimate back-exchange based upon pre-chromatographic incubations also led to low values (data not shown). Rates of BE in the gas phase (in the spectrometer) were calculated, via data data re-acquisition, to be ~0.013 Da of centroid mass back-shift per minute (data not shown). Since experimental BE at atmospheric pressure was in the range ~0.3 Da/min (calculated from a 12 min experiment time, an average of 8.14 exchangeable amides per peptide and the experimental percentages of theoretical maximum deuteration indicated above), in-instrument BE was not considered to be a major factor for HDX by nanoLC-MALDI experiments, even over a 30 min acquisition time. By process of elimination, the bulk of BE presumably occurred during LC.

Note 1: In Fig. 3a, four of the six peptides shown had not quite reached saturation at 15 min exchange time. However, in the dataset as a whole, 50% of peptides had either completely saturated,

or were within 1% of saturation based on the difference between 5 min and 15 min deuteration levels. 75% were within 5% of saturation, and all were within 11% of saturation based on the same criterion.

Fig. S1. Peptic peptide identification under evolving experimental conditions. (**a**) Cumulative growth in numbers of MS/MS-confirmed peptides in VP55's non-redundant peptide library over eight MS/MS experiments. Changes in experimental condition during library building are indicated. (**b**) Numbers of VP55 MS hits to the library developed in (a), in the F1H timepoint of ten separate VP55-alone HDX experiments. Notable changes in conditions from one HDX experiment to the next are indicated. Experiments 9 and 10 were not full HDX experiments, but comprised F1H timepoints only, and therefore did not contribute further to this study.

Fig. S2. Deuterium uptake plots for peptides in the absence and presence of VP39. See text for details.

Table S1. Peptides analyzed in HDX/LC-MALDI experiments of VP55 alone and VP55+VP39. In each section of the table, "*Max mean %Deu*" represents the maximum peptide deuteration within the experimental deuteration timecourse as a percentage of the theoretical maximum (non-BE-corrected data). The timepoint corresponding to the maximum typically was the longest (900 Sec). The analysis was applied to the mean deuterium uptake plot between replicate experiments. "*Mean SASA value*" is the mean solvent accessible surface area for the exchangeable amide protons of the peptide, calculated by rolling a solvent-size ball over the solvent-excluding surface of the protein (Materials & Methods), and "*% SASA residues*" represents the percentage of exchangeable residues in the peptide that have any solvent exposure (SASA value) at all. "*Propn Exchangeable Hbonded*" represents the proportion of exchangeable mainchain amide protons that are predicted to be hydrogen bonded within the structural model, and "*Tot Hbonds as Propn of Exchangeable*" represents the total number of

hydrogen bonds as a proportion of exchangeable amide protons (since some amide protons are doubly hydrogen bonded). "*Max mean %Deu/mean SASA*" shows the ratio of column 2 ("*Max mean %Deu*") divided by column 3 (/"*Mean SASA value"*), and "*Max mean %Deu/mean propn free*" is the ratio of "*Max mean %Deu*" to (1 – "*Propn Exchangeable Hbonded*") as a measure of deuteration as a proportion of numbers of non-hydrogen bonded exchangeable amides. "*%Deu plotarea VP55 / VP55VP39*" values were calculated for peptides in **Table S1** yielding data with both VP55 alone and the VP55-VP39 heterodimer. %Deu values at all common timepoints were summed individually for VP55 and VP55/VP39, and a ratio was then taken. The column "*Shared peptide*" indicates peptic peptides with entries in both VP55 alone and VP55+VP39 sections of the table (contributing data for both groups of experiments). Blue colored entries represent peptides yielding the all-timepoint rates curves (with mostly replicate data for all timepoints and short error bars). "*#rep*" = number of repeats of deuterium uptake expt and data processing for this peptide. "*NA*" = values not available (residues preceding ile24 in the recombinant protein were invisible in crystal structure). Potentially ambiguous peptide ID based on chromatographic properties (left side of table): "*Solvent outlier*": % solvent elution in HDX experiment does not correspond to that during MS/MS identification of same mass peptide (see text). "*Q->2LVP55":* Query mass (HDX) matched two VP55 library masses in TOF2H-Processor search output, unresolved which is likely to be correct. "*Q-> L-VP55 & L-VP39*": Query mass (HDX) matched a VP55 library mass and a VP39 library mass in TOF2H-Processor search output. "*2Q ->L-VP55*": – Two query masses could match the library mass in TOF2H-Processor search output. "*XIC & potential mass-shift overlap*": Overlap in plots of timepoint vs. normalized XIC peak fraction. Later development of the search loop in TOF2H (unpublished) led to ~20% increases in numbers of hits identified, which would have provided a total of ~150 hits for the experiment series in **Table S1**.

References

1. Hamuro, Y., et al., *Specificity of immobilized porcine pepsin in H/D exchange compatible conditions.* Rapid Commun. Mass Spectrom, 2008. **22**: p. 1041-1046.
2. Cauich, E. and P.D. Gershon, *Two new tools for applying chromatographic retention data to the mass-identification of HDX peptides during HD-exchange experiments by nanoLC-MALDI*, in *57th ASMS conference on Mass Spectrometry & Allied Topics, Philadelphia, PA (May 31 – June 4, 2009). Wed. Poster #420.* 2009.
3. Nikamanon, P., et al., *"TOF2H": A precision toolbox for rapid, high density/high coverage hydrogen-deuterium exchange mass spectrometry via an LC-MALDI approach, covering the data pipeline from spectral acquisition to HDX rate analysis.* BMC Bioinformatics,, 2008. **9**: p. 387.