International Journal of Mass Spectrometry 302 (2011) 53–58

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

MSTools—Web based application for visualization and presentation of HXMS data

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ARTICLE INFO

Article history:
Received 31 May 2010
Received in revised form 16 July 2010
Accepted 29 July 2010
Available online 7 August 2010

Keywords:
Hydrogen/deuterium exchange
Data visualization
Software
Web application

ABSTRACT

Hydrogen/deuterium exchange coupled to mass spectrometry is an excellent technique to study protein structure changes and interactions, especially in cases where traditional high-resolution structural techniques are not applicable. A major drawback of this technique is the absence of universal software tools for data processing and visualization of the results. Here we describe a set of PHP scripts facilitating H/D data visualization and presentation. The application runs on a web server and is freely accessible. It comprises tools helping in preparation of H/D experiments as well as tools for turning simple tables with data on H/D exchange into different ways of representation used by the HXMS community.

1. Introduction

1.1. Hydrogen/deuterium exchange of proteins

Protein hydrogen/deuterium exchange coupled to mass spectrometry (HXMS) is a highly sensitive technique to monitor protein backbone dynamics, solvent accessibility, and protein–protein or protein–ligand interactions [1–6]. In contrast to high-resolution structural techniques it has much smaller requirements in terms of sample amount and concentration. There is virtually no limit with respect to the protein size and its organization, which allows to study large multiprotein complexes or modular proteins [7–9]. Therefore HXMS represents a complementary technique to NMR or protein X-ray crystallography. Its speed and possibility of automation make it a technique of choice in cases where the classical structural techniques meet their limitations [5,10]. The main weakness of HXMS, its relatively low spatial resolution, can be addressed by the use of different proteases, dissociation techniques or site directed mutagenesis [11–20].

1.2. HXMS data processing and visualization

Despite its great potential and capabilities, the use of HXMS is not as widespread as it could be. One of the main reasons is the absence of universal software tools facilitating data processing and visualization. Thanks to the development of protein identification tools, assigning the correct sequence to individual peptides does not represent the problematic stage. The time consuming step is the extraction of deuteration levels represented by the number of deuterium atoms at each peptide and at each time point. The common workflow, frequently used even nowadays, is based on manual extraction of the isotopic envelope of a peptide and calculation of the average mass shift. The latter can be easily done using MagTran programmed by Zhang [21]. However, even for skilled operators this procedure takes days or weeks depending on the size of the protein and the complexity of the problem under investigation. Due to the absence of a universal platform, laboratories working in the HXMS field often design their own software tools for data processing and visualization. The first scripts/programs allowing automated data extraction and processing appeared in 2001 (AUTOHD and DXMS) [22,23]. The DXMS platform, developed in collaboration between Dr. Virgil Woods, jr. and Sierra Analytics (Modesto, CA), is evolving and it represents the only commercial solution. Nowadays it also offers support to various MS data formats and was adopted to high-resolution MS data whose importance in HXMS was demonstrated recently [24,25]. Other tools, either very sophisticated like Dex, TOF2H (MS Excel based) or Hydra or rather simple but not less powerful (HXExpress), followed [26–29]. Recently, the platform called HD Desktop has been introduced with the ambition to become a fully functional freely available tool capable of full data processing, visualization and presentation [30]. Unfortunately, the fact that it operates on a remote server and the data must be uploaded will likely distract some users due to confidentiality issues or simply due to large amount of data to be moved. The individual programs have different options and capabilities.

Abbreviations: AA, amino acid; PDB, protein data bank; HXMS, hydrogen exchange–mass spectrometry; H/D, hydrogen/deuterium.

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doi:10.1016/j.ijms.2010.07.030

Whereas AUTOHD performs extraction of H/D MS data only, the DXMS provides full platform for peptide sequence assignment and estimation of deuteration levels, but it is not equipped with full data presentation capability. Dex and TOF2H are primarily intended for MALDI-TOF data alone or in conjunction with LC. While Dex can be compared to AUTOHD with respect to its capabilities, the TOF2H provides more sophisticated pipeline covering curation of MS/MS peptide assignments, H/D data extraction and calculation of deuterium uptake plots. Similar capabilities can be found in HYDRA, which is not primarily TOF-based and may serve for H/D + LC–MS or even LC–MS/MS data processing. Finally, HD Desktop is the only platform providing full support for all stages of a typical HXMS experiment even with plotting of the H/D data onto the known 3D model of a protein.

There is no consensus regarding the result visualization and presentation therefore the figures presenting H/D data vary from one publication to another although some common representations can still be found. A first step in HXMS is optimization of the digestion. Thus a peptide map usually appears as a first figure in any HXMS paper. Sequence coverage, number of peptides identified by means of MS/MS and the number of peptides used to extract deuteration rates are usually shown. In pursuit of higher spatial resolution the digestion conditions are varied and proteases with different specificities are employed. This adds more data sets to be included in the peptide map. The next step is the presentation of deuteration rates which are nicely visible from simple graphs plotting number of deuterons or percentage of deuteration versus time. This way of presentation of H/D data, however, becomes less clear if all peptides are shown, especially in case of larger proteins. Such data are better provided as a supplementary material. For clarity of presentation it is more suitable to show figures covering the whole protein sequence and summarizing the deuterium exchange in one picture. This is achieved by so-called heat maps employing color gradients reflecting the increasing deuteration level by the shift from some dark cold color like blue or green into the bright warm tones of red, yellow or orange. Their popularity is due to the fact that they allow representation of all data within one single image. The only disadvantage of this presentation is in its limited sensitivity given by the color change steps which may be, depending on the quality of a print or visual perception of the reader, undistinguishable. A second way of presentation is protection plots, which can easily cover the whole range of deuteration (e.g., from 0 to 100%) without losing the sensitivity. Using the protection plots it is therefore possible to highlight even very delicate and small changes in deuteration on the entire protein sequence. Colorization of known 3D protein models is also a popular way of showing the exchange differences and allows many nice tricks to be employed including animations or highlighting the particular amino acids (AAs) within their natural environment.

Here we describe our web based software suite which evolved from the simple scripts used in our laboratory. These tools are not capable of assignment of deuteration levels or any spectral processing and must be fed with pre-processed data (e.g., tables with deuteration levels for each peptide). However they provide an easy and fast method for HXMS data graphical presentation which can even be used for publication purposes.

2. Materials and methods

MSTools were created as a set of PHP scripts running on a web server with PHP (Hypertext Preprocessor) support. PHP script represents a plain text file containing expressions (commands) in the PHP language executed directly on the server. These scripts can be easily edited and customized in any text editor and are commonly used for production of dynamic web pages. It contains strings/commands in a PHP language which are executed on a web server. A web server application supporting the PHP language (http://www.php.net/) and PHP version 5 with GD library functions (http://php.net/manual/en/book.image.php) are required to run MSTools. The GNU Tar utility (http://www.gnu.org/software/tar/) and the Jmol applet package (http://jmol.sourceforge.net/) are needed for full functionality. Communication with the server is done through any graphical web browser with JavaScript support and the Java runtime environment (http://www.java.com/en/). Browsers not compatible with Scalable Vector Graphics (SVG) format (http://www.w3.org/Graphics/SVG/) require installation of the SVG plugin (http://www.adobe.com/svg/viewer/install/main.html). Detailed installation procedures, an installation CD as well as full support can be obtained from the authors upon request. MSTools are currently running at http://ms.biomed.cas.cz/MSTools/ and a fully functional mirror can be found at http://www.hxms.com/mstools/. The data used here were created *de novo* in order to cover all the aspects shown in the manuscript. The sequence is an N-terminal part of sperm whale apomyoglobin and the model used is 1UFP from protein databank. The web-site contains help sections where some additional data can be downloaded and used for tutorial or testing purposes. The example input files used in this paper can be found on the MSTools web page.

3. Results and discussion

3.1. H/D Exchange Calculator

The *H/D Exchange Calculator* provides information on a number of fast (side chains) and slow (backbone amides) exchangeable hydrogen atoms within a peptide or protein. It calculates accurate monoisotopic and average masses. The calculator takes into account that the peptide bond at the N-terminus of proline does not contain any exchangeable backbone amide hydrogen. There are two ways to upload the input data. The first one requires a protein sequence (in one letter format) and a file which can contain either the peptide limits expressed as numbers of the first/last amino acid (MASCOT users) or a simple list of peptides (Sequest users). The second way uses simple copy-pasting of the list of the peptide sequences directly into the form. In addition to simple H/D statistics, other things are also calculated. This includes multiply charged ions (the upper limit is 10+) or uniqueness which shows how many peptide masses can be fitted to the mass of the individual peptide with a given mass tolerance. The results can be easily transferred to a spreadsheet application and further manipulated there. However, this format is slightly different than the one shown in the browser (data are merged into one table and uniqueness is shown as a number only).

3.2. H/D Experiment Planner

The *H/D Experiment Planner* facilitates the preparation of a feasible and maximally condensed time schedule for any experiment based on the collection of aliquots at different time points. It becomes especially useful when several conditions (e.g., different pH values, various salt concentrations) have to be followed [10]. It also helps in cases where time correction has to be done, e.g., H/D performed at different pH values [5,31]. The result is represented by a simple table showing the times for mixing of the reaction and collection of aliquots. The conditions are differentiated based on the colors defined in the input form.

3.3. Draw Map

The *Draw Map* is used for visualization of protein sequence coverage after enzymatic or chemical digestion. It is also suitable for

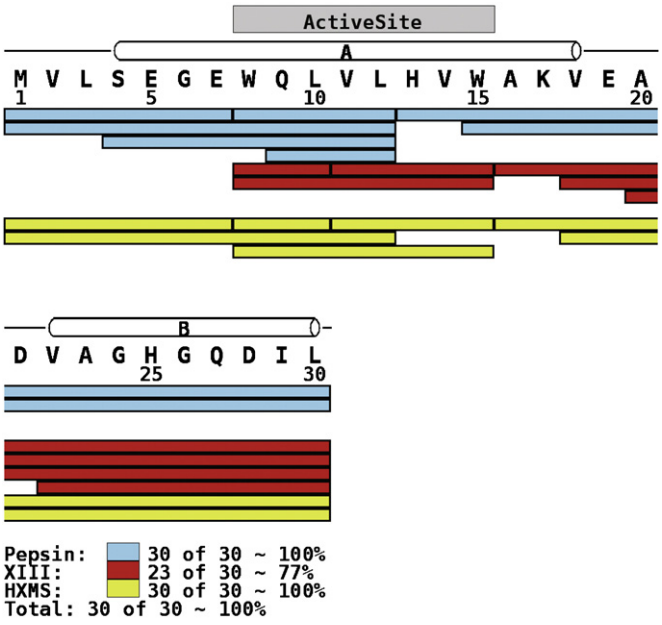

Fig. 1. Output of the *Draw Map* script. Peptide map showing sequence coverage after pepsin (blue bars) and protease type XIII digestion (red bars). Peptides used later for H/D data visualization are shown in green. The figure demonstrates all features that can be drawn with the *Draw Map* script. Besides the bars representing the peptides, structural elements are schematically shown at the top of the sequence (alpha helix = cylinder) together with visualization of the domain (grey bar). The legend at the bottom shows which color was assigned to individual conditions. Sequence coverage in percentage is shown for each digestion condition and for sum of them.

comparison of the digestion with different proteases, under different conditions or for different times (Fig. 1). It requires two simple text files as an input. The first one is a *Sequence file* containing the protein sequence in one letter amino acid code. Here, no check for validity of the characters is done so it is possible to use various symbols (besides the 20 standard amino acids) for specific or modified amino acids. The second input file is a *Digest file* bearing the information about the individual conditions and peptides (numbers specifying the first and last amino acid of each peptide). This file has three columns where the first one is reserved for the description of the conditions and the other ones for numbers stating the first and last amino acid of each peptide. Optionally, a third input file can be used. This is a *Structure file* allowing visualization of structural elements (alpha-helix, beta-sheet), domains, and highlighting of mutations or other interesting features of a protein sequence. Default values in the fields *font size*, *number of AA's per line*, *thickness of bands*, *width of one AA position* influences the quality (size and resolution) of the image. If higher quality (publication ready) is required all the values should be increased/changed proportionally. Compactness of the image is influenced by the aforementioned parameters but also by the way the bars, representing individual peptides, are sorted. There are two representations, *N-term nearest first* and *longest first*. In *N-term nearest first* the set of overlapping peptides is aligned from top to bottom with the peptide having N-terminal end closest to the N-terminus of the protein being on the top. The one closer to the C-terminus will be placed below. In *longest first*, the peptides are sorted according to their length with the longest one being on the top.

Many proteins that are investigated by H/D experiments represent either a part of a larger protein or are expressed as fusion proteins. However, for clarity of presentation it is always advisable to keep the native sequence numbering. For that the values *Added N-term AA's, Start real numbering with*, *Added C-term AA's* are used. If the sequence exactly fits to the native protein or the native sequence numbering is not followed, then the default values (0, 1, 0 respectively) should be used. In other cases the number of amino acids added to the N- (*Added N-term AA's*) or C-terminus (*Added C-term AA's*) of the protein as well as the starting AA (*Start real numbering with*) should be specified. For example, a construct corresponding just to one domain (e.g., 310–435) from a protein with 648 residues could be expressed with a histidine tag (HisTag) at the N-terminus (+12 residues) and three additional amino acids at the C-terminus. Then the values are set as follows, *Added N-term AA's* to 12, *Start real numbering* with to 310 and *Added C-term AA's* to 3. In the resulting figure the residues from HisTag will be numbered as −12 to −1, C-terminal addition as +1 to +3 and the real numbering will start from 310 and end with 435. The resulting image also contains simple legends showing sequence coverage reached under individual conditions and the total sequence coverage summed from the partial ones is also shown.

Besides H/D related mapping this tool can be also used for comparison of tryptic and other enzymatic digestions or in limited proteolysis studies as it shows the structural elements and domains above the sequence and thus allows easy correlation of the cleavage sites with regions of higher accessibility.

3.4. Draw H/D Heat Map

Draw H/D Heat Map is a tool for visualization of HXMS results in the widely used color gradient representation (Fig. 2A). It allows the display of the evolution of deuteration of individual parts of the protein and at the same time it also enables comparison of different conditions. Similarly to the *Draw Map* script it requires the protein sequence, a file with the peptides and optionally, the structure file. Whereas the *sequence* and *structure files* have identical formats to those mentioned above the *digest file* used by this script has a different layout. It contains information about the peptides (starting and ending AA) and their deuteration (expressed in %) at different times and under various conditions. The first line must contain the headers (first three columns) *From*, *To* and *Time*. The next columns are reserved for individual conditions and their description is placed on the first line. The lines bellow header are reserved for the actual data, each line must contain peptide limits, time (converted to seconds) and percentage of deuteration at the respective time point. A specific issue that commonly arises is the case of overlapping peptides. There are two ways to handle the overlaps. One is to make a subtraction of the peptides having one common end (e.g., 1–30 and 1–12), which increases the spatial resolution. The current version of the script does not have this capability yet but it is planned in a future version. The other way of dealing with overlaps is not based on calculations, but requires proper sorting of the input data. That means selecting the shortest peptides covering the entire protein sequence or assembling the overlapping peptides (even those without one common end) in the following manner: The longest peptide is placed first and the shorter ones are overlaid over the first one. During the process of drawing the script will first draw the heat map for the first peptide then place the second peptide over the first one and the third one over the first and second one, etc. For example, the peptides 1–30, 1–12, 6–10 are best placed in the order (from the top of the digest file to the bottom) as written here. If the order is swapped, the 1–30 covers the preceding two and only the entire continuous band (1–30) is drawn in the heat map. It is noteworthy that this strategy does not have any problems with overlapping peptides, which do not have one common end. On the other hand, such a simple overlay finally provides slightly misleading results because the rest of the long peptide, which was not overlaid, still shows deuteration corresponding to the entire peptide. Therefore, the best solution to this problem is to select set of peptides that are continuously covering the entire protein and have no or very small overlaps. The heat map can be drawn either with default colors (rainbow from violet to dark red/brown) or the starting color (0% of deuteration) and the ending one (100% of deuteration) can

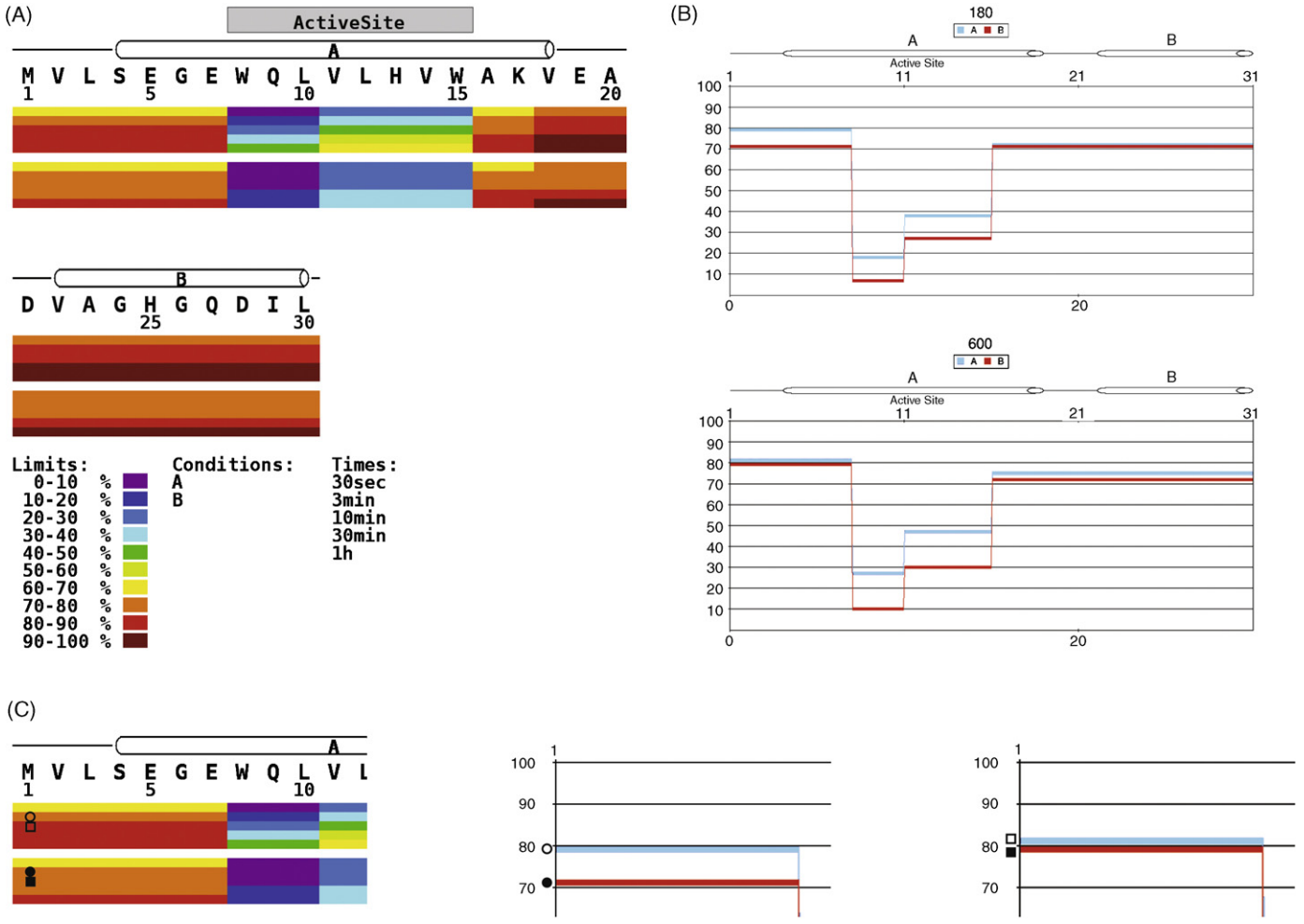

Fig. 2. (A) Result of *H/D Draw Map*. Comparison of H/D kinetics of two hypothetical states **A** (upper thick band) and **B** (lower thick band). Both bands are further divided into thinner bands representing individual time points as indicated in the legend below the heat map. The legend also shows sorting of the conditions (from top to bottom) and assignment of the colors to the levels of deuteration. Similarly to *Draw Map*, structural elements and domain are shown above the sequence. (B) The same data as in (A) were plotted using *Draw H/D Protection Plot* but only two time points (3 min and 10 min are shown). The individual conditions (**A** and **B**) are distinguished by different colors. Their assignments (**A**-blue and **B**-red), together with the times (in seconds) are indicated at the top of each plot. (C) Demonstration of the different sensitivities of *H/D Draw Map* and *Draw H/D Protection Plot*. Circles indicate rather significant difference (9%) in deuteration which was draw by the same color using *Draw H/D Heat Map* but is nicely distinguished by *Draw H/D Protection Plot*. In contrast, a deuteration difference of 2% (squares) nearly overlapping in protection plot was assigned different colors in heat map. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

be specified together with the number (related to the percentage of deuteration) after which the color tone will be changed. Similarly to Draw Map, even here the image resolution is dictated by the fields *font size*, *number of AA's per line*, *thickness of bands*, *width of one AA position* values. The resulting image has the individual conditions split into the individual thick bands, which are further divided into as many parts/lines as the number of time points given in the *digest file*. Conditions are ordered (from top to bottom) in the same way as they were entered in the *digest file* (from left to right). The legend at the bottom of the image provides an overview of the color coding, order of the conditions and the time points displayed.

3.5. Draw H/D Protection Plot

Draw H/D Protection Plot represents an alternative way of H/D data visualization over the protein sequence (Fig. 2B). The color gradient (heat map) representation has a major drawback: a limited sensitivity to smaller differences. This is especially true when a wide range of deuteration levels (e.g., 0–100%) is used but the differences fall just into the color change step (e.g., 41 and 49). This can be solved by setting smaller color change step but still users/printers may have problems to distinguish delicate differences in the tone of the same color (Fig. 2C). This tool provides higher sensitivity to small differences in deuteration, in comparison with the *Draw H/D Heat Map* tool. It uses the same input files but the results are graphs, not color gradients. The graphs are plots with the peptide backbone on the *x*-axis and the percentage of deuteration (number of acquired deuterium atoms can be used as well) on the *y*-axis. The script allows two methods of grouping of the data. The first method groups the data with the same conditions into one image. This is helpful when the time evolution of the H/D kinetics is followed. The second method, which is based on grouping of the same times, allows facile comparison of different conditions. It is also possible not to group the data and have a separate graph for each time point of each condition. Numbering of the protein backbone is again similar to the one described for the previous two tools. The difference is in the way the image size is calculated. In contrast to *Draw Map* and *Draw H/D Heat Map* it is not done through setting of the image parameters but the image size in pixels is specified in the input form. It must be pointed out that this tool is not really suitable for presentation of overlapping peptides. The only problem is in the increased complexity of the image, especially if more conditions appear (are grouped) in one figure. However if overlapping peptides are used, the option *draw connecting lines* should be turned off.

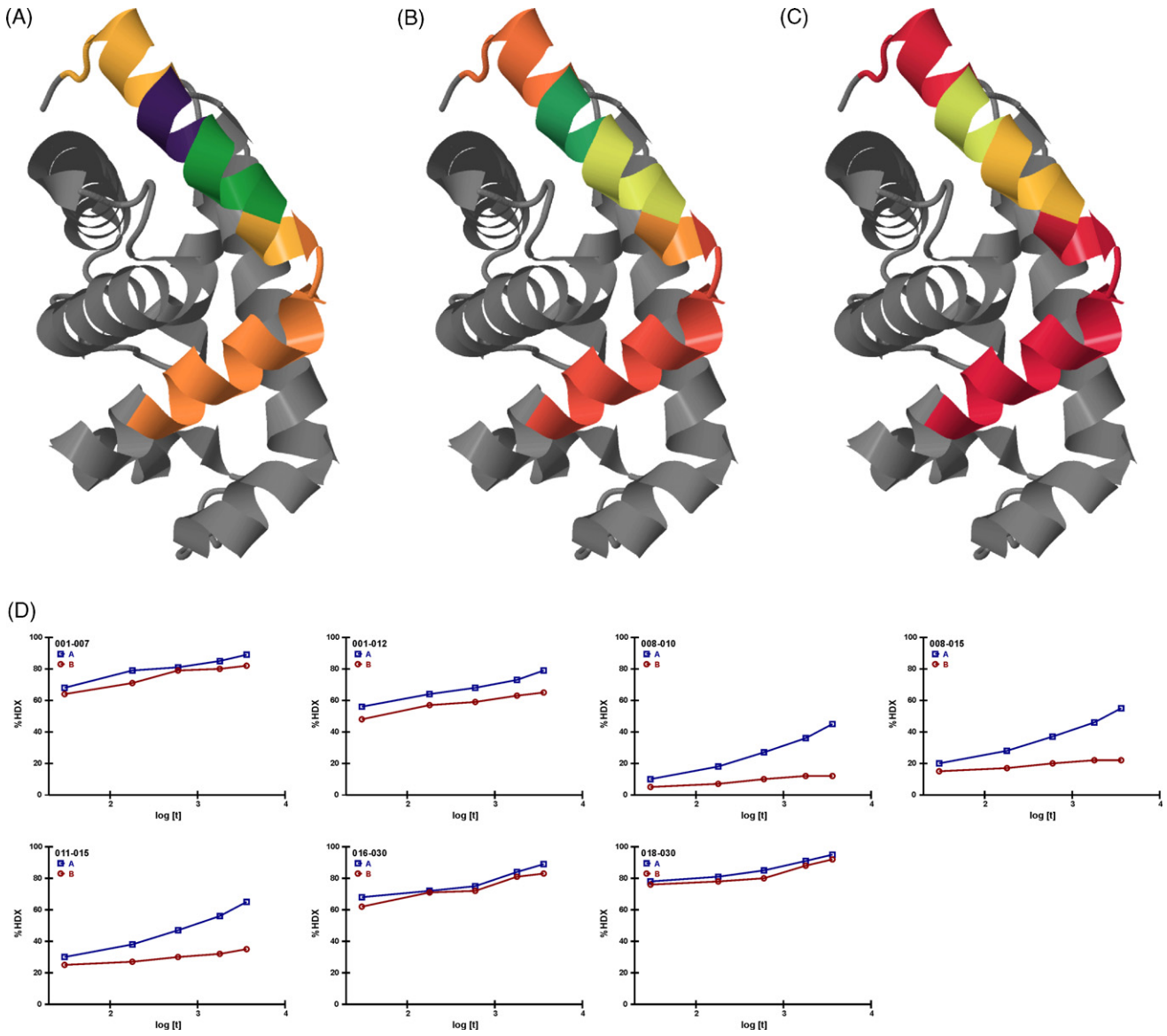

Fig. 3. Result of *Jmol Scripter*. Deuteration of the N-terminal part of apomyoglobin, hypothetical state **A**, rendered onto the know structure (1UFP) at three time points (A—30 s; B—3 min; C—1 h). The color gradient is the default one of *Jmol Scripter* and runs from purple to red via shades of turquoise, green, yellow, orange and pink. Section D shows corresponding H/D exchange plots as created by the script *Draw H/D Exchange Plots*. The time points corresponding to the models in A–C are the first, middle and last one.

3.6. Jmol Scripter

Jmol Scripter uses the *digest file* (same format as for heat map and protection plot) to color the known 3D model of a protein (e.g., PDB file) according to the H/D data (Fig. 3). Deuteration levels of each peptide are converted to colors (the default rainbow scheme or user defined scheme) and rendered on the model. A single image is created for each time point and each condition. The script performing this action can be downloaded and used offline in Jmol. In addition to the static images, an animation (with speed adjustable by the parameter *frame delay*) showing evolution of the deuteration is prepared and the corresponding script can be also downloaded. Alternatively, the models can be manipulated in the web browser (requires Java and JavaScript). Besides the color scheme, there are other parameters to be set. Very important is the PDB offset fitting the digest file to the protein sequence in PDB file. Here the users are advised to proceed carefully and check the exact sequence (and its numbering) used to obtain the model. The option *visualize* is used to show just the protein or also other molecules included in the PDB file (cofactors, lipids or other co-crystallizing compounds). Taken from the graphical part, the users can also set the way the structure will be visualized (spacefill, cartoon, wireframe, etc.), background color or a color corresponding to the parts of the protein for which no H/D data were obtained (grey by default). If any overlapping peptides occur, they should be handled as in the case of *Draw H/D Heat Map*. That means that the longest peptide is placed first and the shorter ones are overlaid over the first one. The legend showing the assignment of the colors to the deuteration percentages is printed below the Jmol frame.

3.7. Draw H/D Exchange Plots

The last tool is a script for drawing H/D exchange graphs. It plots percentage of deuteration (or number of deuterons) versus time for each peptide provided in the *digest file*. It can handle up to eight different experimental conditions in one graph. It is possible to use logarithmic or linear scale on the x-axis and it supports user defined limits on the y-axis. The result files are in SVG format and thus can be edited in any vector graphic editor that can handle the format.

4. Conclusions

Here we described our web based suite that can aid in preparation of H/D experiments as well as in visualization of HXMS data. It speeds up the whole process of graph, figure or plot creation and thus can be used anytime during data processing for quick verification of the results. Moreover, it can also provide publication ready images. Besides the features described above, the applications (*Draw H/D HeatMap*, *Draw H/D Protection Plot* and *Jmol Scripter*) can be also used to show difference between two states. The application is currently running on two independent servers and the installation is available upon request. Even though it is described as a web based application, the features of PHP allow, after a small change of the scripts, running it locally from a command line. Our future work will be aimed at fine tuning of the existing parts, addition of more tools and re-writing of the scripts into a standalone application which can easily allow users to set and influence many parameters.

Acknowledgements

This work is dedicated to the memories of Petr Sedmera, good friend and former head of our laboratory. The authors thank all collaborators and users of MSTools for their valuable comments and suggestions. We also thank J.R. Engen for his encouragement, comments and invitation to contribute to this special issue. Financial support from Czech Science Foundation (P207/10/1040), grant agency of the ASCR (KJB500200612), Ministry of Education Youth and Sports (LC545, LC07017) and Institutional Research Concept AVOZ50200510 is gratefully acknowledged.

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