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RESEARCH ARTICLE

High-Resolution HDX-MS of Cytochrome c Using Pepsin/Fungal Protease Type XIII Mixed Bed Column

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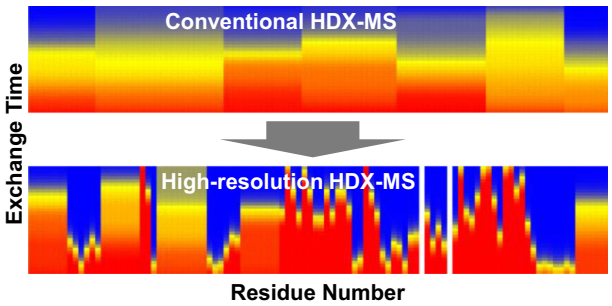

Abstract. A pepsin/FPXIII (protease from *Aspergillus saitoi*, type XIII) mixed bed column significantly improved the resolution of bottom-up hydrogen/deuterium exchange mass spectrometry (HDX-MS) data compared with a pepsin-only column. The HDX-MS method using the mixed bed column determined 65 amide hydrogen exchange rates out of one hundred cytochrome c backbone amide hydrogens. Different cleavage specificities of the two enzymes generated 138 unique high-quality peptic fragments, which allows fine sub-localization of deuterium. The exchange rates determined in this method are consistent within the current study as well as with the previous HDX-NMR study. High-resolution HDX-MS data can determine the exchange rate of each residue not the deuterium buildup curve of a peptic fragment. The exchange rates provide more precise and quantitative measurements of protein dynamics in a more reproducible manner.

Keywords: Cytochrome c, Electrospray ionization, Exchange rate, Fungus protease XIII, Hydrogen/deuterium exchange, Mass spectrometry, Pepsin

Abbreviations ECD, Electron capture dissociation; ETD, Electron transfer dissociation; FPXIII, Protease from *Aspergillus saitoi*, type XIII; GuHCl, Guanidine hydrochloride; HDX, Hydrogen/deuterium exchange; LC, Liquid chromatography; MS, Mass spectrometry; MS/MS, Tandem mass spectrometry; TFA, Trifluoroacetic acid

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Introduction

Determination of backbone amide hydrogens in single-amide resolution is one of the major technical goals in hydrogen/deuterium exchange mass spectrometry (HDX-MS) [1–3]. There are two ways to achieve single-amide resolution in site-specific manner for HDX-MS: the subtraction of the deuterium incorporations in analogous peptides [3–6] and that in analogous daughter ions [7–10].

Relative non-specificity of acid proteases [1–6] and utility of new protease(s) [11–18] can generate pairs of analogous peptides to improve the resolution in bottom-up HDX-MS. For example, the subtraction of the deuteration levels in two analogous peptides, 1–9 and 1–8, can provide the deuteration level at residue 9 (Figure 1b). Although acid proteases, such as pepsin, do not have as strong residue specificity as the proteases active in neutral pH, such as trypsin, they do have preferences [6] and thus the number of analogous peptide pairs generated by a single acid protease is limited [1–3].

Recently, Jørgensen and Rand showed electron capture dissociation (ECD) or electron transfer dissociation (ETD) may generate pairs of analogous daughter ions [7–10]. This strategy can be applicable not only for bottom-up HDX-MS

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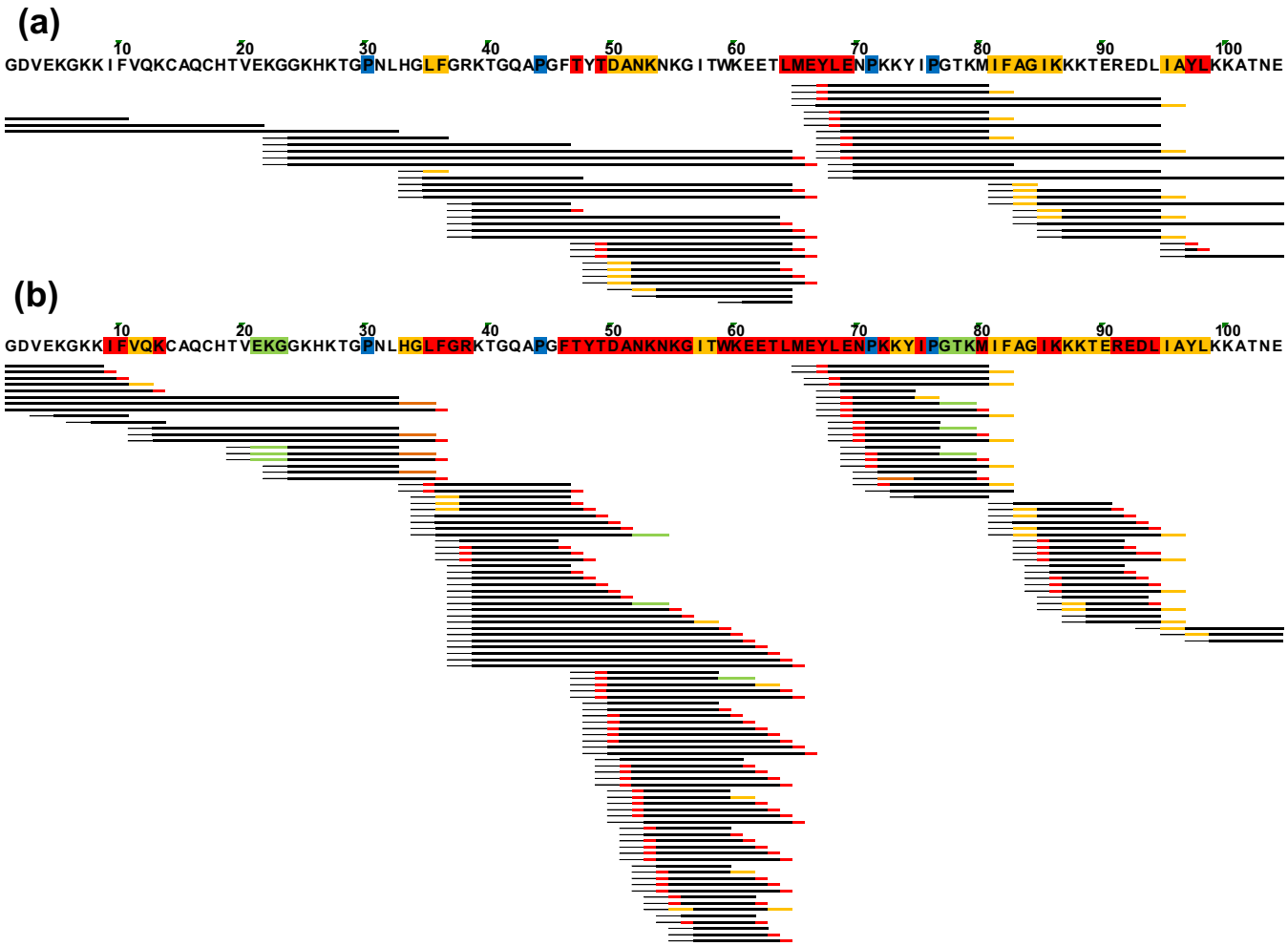

Figure 1. Peptide maps of cytochrome c digested with (**a**) a pepsin only column and (**b**) a pepsin/FPXIII mixed bed column. A line indicates peptide observed. A thin part of each line is the first two residues of each peptide which cannot carry deuterium (except acetylated N-terminal). A thick part of each line is the rest of residues which can carry deuterium. A red part (e.g., the residue 9 in peptide 1–9) indicates the residue which can be sub-localized into single-amide resolution by the comparison with the analogous peptide with one less amino acid (peptide 1–8). An orange part indicates the residues which can be sub-localized into two-residues-long resolution by the comparison with the analogous peptide with two less amino acids. A light green part indicates the residues which can be sub-localized into three-residues-long resolution by the comparison with the analogous peptide with three less amino acids. The sequence of cytochrome c was also color-coded. Red, residues with single-amide resolution; orange, residues with two-residues-long resolution; light green, three-residues-long resolution; and blue, prolines

[19–25] but also for top down HDX-MS [26–30]. One potential issue in this approach is the scrambling of hydrogen and deuterium within a molecule which obscures the location of deuterium [31, 32].

This paper describes the sub-localization of deuterium from the analogous peptides generated by pepsin/FPXIII (protease from *Aspergillus saitoi*, type XIII) mixed bed column and the determination of backbone amide hydrogen exchange rates in cytochrome c. To obtain the exchange rates, a wide range of exchange time (0.04–120,000 s at 23 °C at pD 7.4) was monitored by varying pH. The approach successfully determined the exchange rates of 65 residues out of 100 backbone amide hydrogens in cytochrome c.

Experimental

Materials

All reagents were obtained from Sigma-Aldrich (St. Louis, MO). Cytochrome c is from equine heart (product number, C7752).

pH

The actual pD (pD_{corr}) value is slightly higher than the readings of a pH meter (pD_{read}) in D_2O ($pD_{corr} = pD_{read} + 0.4$) [33].

On-Exchange Experiment for HDX-MS

On-exchange reaction was initiated by mixing 2 µL of 1 mg/mL cytochrome c in H_2O with 18 µL of deuterated

buffer (20 mM citrate at pD_{corr} 5.4; 20 mM phosphate, pD_{corr} 7.4; 20 mM serine pD_{corr} 9.4). The reaction mixture was incubated for 30, 100, 300, 1000, 3000, or 10,000 s at 3 °C (Figure S1 in Supporting Information). The on-exchanged solution was quenched by the addition of chilled 30 µL of 1.6 M guanidine hydrochloride (GuHCl), 0.8% formic acid and immediately analyzed.

General Procedure for HDX-MS Data Acquisition

HDX-MS analysis was performed with automated HDx3 system (LEAP Technologies, Morrisville, NC) set up analogous to previously described with the syringe chiller on [34, 35]. The columns and pump were as follows: protease, protease type XIII (protease from *Aspergillus saitoi*, type XIII)/pepsin column (*w*/*w*, 1:1; 2.1 × 30 mm) (NovaBioAssays Inc., Woburn, MA); trap, Acclaim PepMap300 C18 (1 × 15 mm) (Thermo Fisher Scientific, Waltham, MA), analytical, Hypersil Gold (1 × 50 mm, 1.9 µm) (Thermo Fisher Scientific); and LC pump, Dionex Ultimate 3000 (Thermo Fisher Scientific). The loading pump (from the protease column to the trap column) was set at 50 µL/min with 0.1% aqueous formic acid. The gradient pump (from the trap column to the analytical column) was set from 8 to 28% acetonitrile in 0.1% aqueous formic acid in 10 min at 45 µL/min.

MS Data Acquisition

Mass spectrometric analyses were carried out using an LTQ™ Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) with the capillary temperature at 220 °C, resolution 60,000, and mass range (*m*/*z*) 300–1300.

HDX-MS Data Extraction

BioPharma Finder 3.0 (Thermo Fisher Scientific) was used for the peptide identification of non-deuterated samples prior to the HDX experiments. HDExaminer version 2.1 (Sierra Analytics, Modesto, CA) was used to extract centroid values from the MS raw data files for the HDX experiments.

HDX-MS Data Analysis

Excel was used to process and present the data.

1. All experimental exchange times were converted into those at pD_{corr} 7.4 and 23 °C (Table S1 and Figure S1 in Supplemental Information) [1, 2, 36].
2. The deuteration levels of two analogous peptides (e.g., 1–9 and 1–8) were subtracted to give the deuteration level at the sub-localized segment (e.g., residue 9) at each time point without using back exchange correction.
3. Steps 1 and 2 yielded the deuterium buildup curve at the sub-localized segment at the standard conditions (pD_{corr} 7.4 and 23 °C; Figure S1 in Supplemental Information).
4. The deuterium buildup curve obtained in step 3 was curve fitted with pseudo first-order kinetic model,

$$D(t) = D_{max}\,[1-\exp(-k\,t)] \quad (1)$$

where t is exchange time, D_{max} is the maximum deuterium retention, and k is the exchange rate. D_{max} and k were optimized to minimize the sum of the squared deviations.

5. When the deuteration levels of a segment were obtained by multiple peptide pairs (e.g., the deuteration levels of residue 36 can be obtained by the subtraction of 1–36 and 1–35, that of 19–36 and 19–35, or that of 22–36 and 22–35; Figure 1b), all applicable subtracted curves were fitted with a single set of D_{max} and k.
6. When a segment contained more than one residue (e.g., residue 11–12 from peptides 1–12 and 1–10; Figure 1b), multiple sets of D_{max} and k were optimized.

Results and Discussion

Digestion of Cytochrome c with Pepsin/FPXIII Mixed Bed Column

Digestion of cytochrome c with pepsin/FPXIII mixed bed column generated 138 unique high-quality peptides (Figure 1b). Digestion with pepsin-only column generated 58 high-quality peptides (Figure 1a). This improvement is due to the very different cleavage preferences of pepsin and FPXIII [13]. For example, FPXIII can cleave after H, K, P, or R, the site which pepsin does not cleave [6].

The subtraction among the peptides generated by pepsin/FPXIII mixed bed column can yield the deuteration levels of 39 residues at single-amide resolution (red residues in Figure 1b). Similarly, the deuteration levels of 20 residues can be calculated at two-residues-long resolution (orange in Figure 1b) and the deuteration levels of 6 residues can be calculated at three-residues-long resolution (light green in Figure 1b). On the other hand, pepsin digestion can provide the deuteration levels of only 10 residues at single-amide resolution (red residues in Figure 1a) and 14 residues at two-residues-long resolution (orange residues in Figure 1a).

Determination of Backbone Amide Hydrogen Exchange Rates of Cytochrome c

The HDX-MS experiments were carried out at three different pD_{corr}, 5.4, 7.4, and 9.4, to expand the time window [1, 2]. Cytochrome c does not change its dynamic properties within the pD_{corr} range. All exchange times were converted into the exchange times in the standard conditions (pD_{corr} 7.4 and 23 °C) (Table S1 and Figure S1 in Supplemental Information) [36].

The HDX-MS using the mixed bed column determined 65 amide hydrogen exchange rates out of one hundred cytochrome c backbone amides (Table 1). The 65 exchange rates were calculated from 51 unique sub-localized segments: 39

Table 1. Cytochrome c Backbone Amide Hydrogen/Deuterium Exchange Rates (s^{-1}) Determined by Various Methods

	NMR[a]	Pepsin[b]	ETD[c]	FPXIII/pepsin[d]		NMR[a]	Pepsin[b]	ETD[c]	FPXIII/pepsin[d]
V3	–	–	1.6E−01	–	W59	–	–	2.0E-04	< 1.0E−06
E4	–	–	4.9E−03	–	K60	–	–	9.0E-05	2.4E−06
K5	–	–	2.3E−03	–	E61	–	–	1.2E−01	4.9E+02
K7	2.6E−04	–	–	–	E62	–	–	3.1E+01	2.2E−01
K8	7.9E−04	–	–	6.2E−05	T63	–	–	4.8E−02	7.6E−03
I9	3.2E−05	–	–	1.4E−05	L64	1.5E−05	3.6E−05	1.0E−03	6.2E−06
F10	5.7E−08	–	–	< 1.0E−06	M65	2.6E−06	1.7E−05	–	< 1.0E−06
V11	1.6E−05	–	–	1.3E−05	E66	5.8E−04	1.6E−04	–	4.0E−05
Q12	2.1E−04	–	–	8.4E−05	Y67	3.3E−05	2.3E−05	–	2.4E−05
K13	4.9E−05	–	–	1.5E−05	L68	1.2E−08	3.5E−06	–	< 1.0E−06
C14	7.6E−05	–	–	–	E69	9.4E−06	–	–	1.3E−04
A15	4.7E−04	–	–	–	N70	7.4E−04	–	1.8E−02	1.7E−04
H18	4.3E−04	–	–	–	K72	–	–	–	7.5E−03
T19	3.6E−04	–	–	–	K73	7.1E−03	–	–	4.9E−05
E21	–	–	–	2.9E+02	Y74	2.1E−04	–	1.7E−03	7.0E−04
K22	–	–	–	4.0E−01	I75	1.0E−04	–	7.3E−04	3.6E−05
G23	–	–	–	< 1.0E−06	G77	–	–	2.2E−04	5.5E−04
G29	1.1E−04	–	–	–	T78	–	–	6.1E−04	3.5E+01
N31	2.2E−02	–	–	–	K79	2.1E−02	–	2.3E−02	1.1E−02
L32	4.0E−06	–	–	–	M80	3.1E−02	–	1.2E−02	3.0E−03
H33	2.0E−05	–	–	2.6E−06	I81	–	2.2E−01	6.8E−02	1.6E−01
G34	–	–	–	< 1.0E−06	F82	–	3.1E+01	2.0E+01	2.4E+01
L35	3.9E−04	–	–	< 1.0E−06	A83	–	9.4E+02	1.1E+03	6.1E+01
F36	8.4E−05	–	–	1.5E−05	G84	–	2.2E−01	3.7E−01	9.1E−02
G37	1.0E−03	–	–	1.4E−04	I85	1.1E−03	–	1.4E−03	1.9E−04
R38	1.5E−03	–	–	9.4E−05	K86	–	–	6.6E+01	3.8E+01
T40	3.9E−03	–	–	–	K87	–	–	–	9.1E+01
G41	–	–	5.8E−03	–	K88	–	–	–	5.3E−01
Q42	5.5E−03	–	–	–	T89	–	–	–	4.4E+00
A43	1.4E−02	–	–	–	E90	–	–	–	1.3E−03
G45	–	–	1.2E−02	–	R91	1.2E−05	–	–	< 1.0E−06
F46	–	–	4.1E+02	1.1E−02	E92	6.6E−06	–	–	< 1.0E−06
T47	–	1.5E+02	–	5.3E+01	D93	1.4E−05	–	–	< 1.0E−06
Y48	–	–	–	5.0E−01	L94	3.8E−08	–	–	< 1.0E−06
T49	2.6E−02	8.8E−03	–	6.1E−03	I95	3.8E−09	9.4E−09	< 7.1E−07	< 1.0E−06
D50	–	–	–	1.5E+01	A96	3.7E−08	5.3E−08	< 1.4E−06	< 1.0E−06
A51	–	–	–	4.0E−01	Y97	2.5E−08	1.1E−07		< 1.0E−06
N52	9.1E−03	–	–	3.7E−03	L98	3.8E−10	9.5E−08	< 5.3E−08	< 1.0E−06
K53	1.3E−02	–	–	3.6E−03	K99	1.2E−07	–	–	–
N54	2.1E−02	–	–	4.0E−01	K100	2.5E−04	–	–	–
K55	–	–	–	8.6E−03	T102	–	–	4.2E−04	–
G56	–	–	–	4.0E−01	N103	–	–	3.1E−03	–
I57	–	–	–	3.8E−01	E104	–	–	5.9E−02	–
T58	–	–	–	2.0E−02					

All exchange rates were converted to those at pH_{read} 7.0 and 23 °C. The minimum exchange rate for FPXIII/pepsin method is 1.0E−06, due to the exchange time employed. [a]Reference [37]; [b]reference [2]; [c]reference [32]; and [d]the current study

single residues, 10 with two residues long, and 2 with three residues long (Figure S1 in Supplemental Information).

The sub-localization by the subtraction can be done with two analogous peptides with the common N-terminal (Figure 2a, b, e) or those with the common C-terminal (Figure 2c, d, f). The sub-localization at C-terminal (with the common N-terminal) is simple. The extra deuterium in the longer peptide can be assigned to the extra backbone amide hydrogen at the C-terminal. On the other hand, the sub-localization at N-terminal (with the common C-terminal) is not as straightforward, because the first two residues of each peptide cannot retain deuterium [36]. In this case, the extra deuterium in the longer peptide is assigned to the third residue of the longer peptide. For example, the subtraction between the deuterium incorporations in peptides 36–46 and 37–46 yields the deuterium incorporation at residue 38 (Figure 2a).

Consistency of Backbone Amide Hydrogen Exchange Rates

The deuterium buildup curves generated by the subtractions of two analogous peptides were consistent within the current dataset (Figure 2). There are many sub-localized segments whose deuterium buildup curves can be obtained from multiple pairs of analogous peptides. For example, the deuteration levels of Glu62 can be obtained by the subtraction of the deuteration levels in peptides 48–62 and 48–61 or that in peptides 51–62 and 51–61 (Figure 2c).

The backbone amide hydrogen exchange rates determined by the subtraction of two analogous peptides and ions generally agreed well with those determined by HDX-NMR (Table 1 and Figure 3) [37]. Most of the exchange rates determined were within tenfold of those determined by the HDX-NMR method. One discrepancy in the current study and the NMR study is the

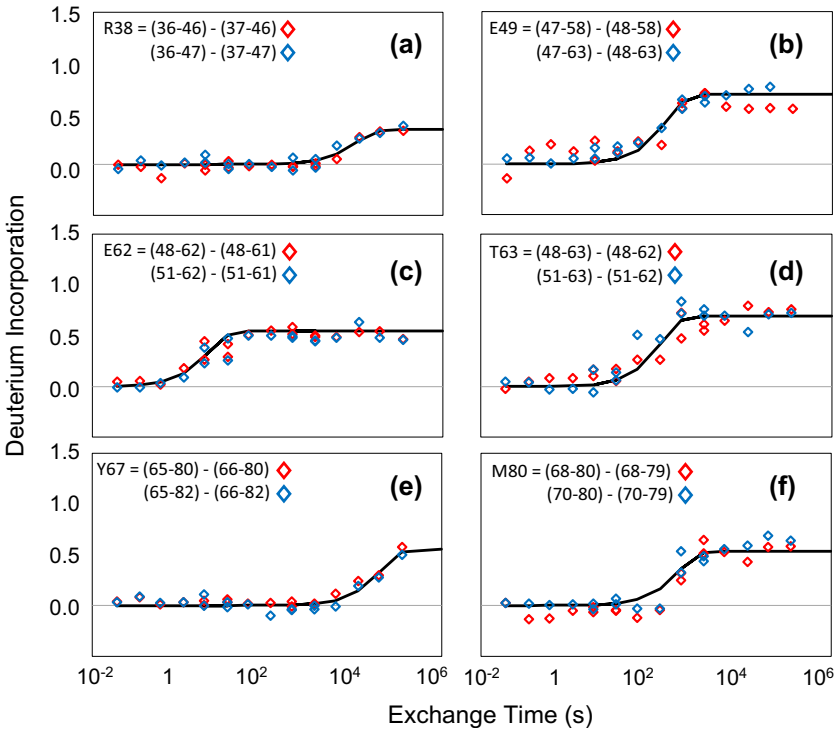

Figure 2. Deuterium buildup curves of selected sub-localized segments. (**a**) Arg38, (**b**) Glu49, (**c**) Glu62, (**d**) Thr63, (**e**) Tyr67, and (**f**) Met80. Diamonds are experimental deuterium incorporations at the residue by the subtraction of two analogous peptides. A black line is the curve fitted to the model described in the Experimental section

time window. In the current study, the shortest time point was 0.037 s and the longest time point was 120,000 s at pH 7.4, 23 °C (Table S1 in Supplemental Information). In the NMR study, the shortest time point was about 200 s and the longest time point was about 2,000,000 s after the exchange times were converted to the same condition [37]. As a result, the NMR study could not determine the fast exchange rates due to the lack of short time points and the current study could not determine the slow exchange rate due to the lack of long time points (Table 1).

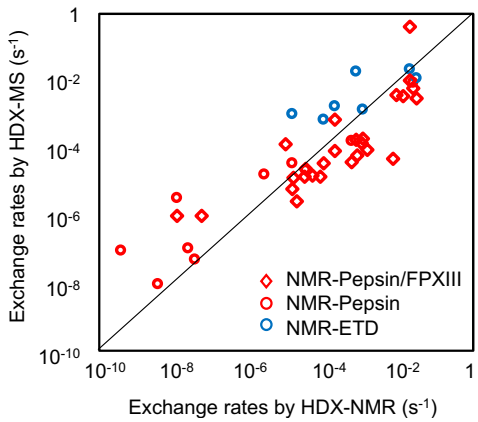

Figure 3. Comparison of backbone NH exchange rates determined by HDX-NMR and HDX-MS. Red diamond, comparison of the value determined by HDX-NMR versus that determined by the subtraction of two analogous peptides with pepsin/FPXIII digestion; red circle, comparison of the value determined by HDX-NMR [4] versus that determined by the subtraction of two analogous peptides with pepsin digestion [2]; and blue circle, comparison of the value determined by HDX-NMR [4] versus that determined by the subtraction of two analogous ions generated by ETD [32]. A linear regression R^2 value for HDX-NMR versus HDX-MS was 0.78

Keys to Determine Backbone Amide Hydrogen Exchange Rates

There are three elements to determine backbone amide hydrogen exchange rates by HDX-MS: (i) generation of many analogous peptides by proteases or analogous ions by gas-phase fragmentation, (ii) subtraction of deuterium incorporation in a pair of analogous peptides or ions, and (iii) usage of a wider time window.

A combination of multiple proteases in a mixed bed column or tandem columns format may generate many analogous peptides [3]. Although pepsin may be considered to have a weak preference, it does have preferred cleavage sites [6]. Other acid proteases also have some preferences which are different from pepsin [3, 11–18]. Therefore, the usage of multiple proteases can yield more cleavage sites and lead to high-resolution HDX-MS.

Electron capture dissociation (ECD) or electron transfer dissociation (ETD) may generated many analogous daughter ions [7–10, 19–32]. The biggest issue of this approach is randomization of exchangeable hydrogen and deuterium atoms (scrambling) in gas phase. Although there are several examples

of deuterium sub-localization [19–30], there are very few examples of exchange rate determination by this approach [32].

Presenting the deuterium incorporation in a sub-localized segment by subtracting the deuterium incorporations in a pair of analogous peptides or ions is more challenging than presenting the deuterium incorporation in each peptide or ion. In a sub-localized segment, the error in the deuterium incorporation is larger than a single peptide or ion, because the subtraction adds the errors from the two analogous peptides or ions. On the other hand, the deuterium incorporation in a sub-localized segment is smaller than the original two analogous peptides or ions. Therefore, a sub-localized segment has a smaller signal (deuterium incorporation) with a larger uncertainty (error) than the original peptides or ions.

High-resolution HDX-MS approach should be combined with a wider time window. The real benefit of high-resolution HDX-MS is the determination of an exchange rate instead of just following a deuterium buildup curve. To determine an exchange rate, the transition from near zero deuterium incorporation to near 100% deuterium incorporation must be observed (Figures 2 and 4b). A wider time window increases the chance of observing the transition (the yellow part of a single-amide resolution residue in Figure 4b), especially knowing many proteins have wide ranges of backbone amide hydrogen exchange rates [2, 37].

Benefits of Determining Backbone Amide Hydrogen Exchange Rates

There are two major benefits of obtaining exchange rates through high-resolution HDX-MS instead of deuterium buildup curves. One is generating more reproducible and archivable data and the other is enabling quantitative arguments.

Exchange rates of backbone amide hydrogens are likely to be more reproducible among different laboratories and better suited for archiving than deuterium buildup curves. Inter-laboratory comparison of HDX-MS data may be challenging [38], primarily due to the variations among laboratories in the post exchange procedures, such as digestion and separation. The deuterium buildup curve of a single amide hydrogen, $D(t)$, is described in Eq. (1). In this equation, the exchange rate, k, is independent of the post exchange conditions and should be constant among the laboratories. On the other hand, the maximum deuterium retention, D_{max}, depends on the post exchange conditions and may vary among the laboratories. As a result, the deuterium buildup curve of a single amide hydrogen, $D(t)$, is also variable depending on the laboratory settings. A peptide deuterium buildup curve, which is the sum of multiple deuterium buildup curves, also depends on the laboratory settings. In the end, the exchange rate, k, should be more consistent among different laboratories than the peptide deuterium buildup curve.

Quantitative biophysical arguments are possible with backbone amide hydrogen exchange rates, while deuterium buildup curves only enable qualitative arguments. An exchange rate can be converted into the protection factor upon protein folding using Bai's parameters [36] and then the protection factor can be converted into the free energy at each site in the case of EX2 mechanism [39]. The exchange rates determined by HDX-MS enable to discuss the effects of perturbation on an analyte

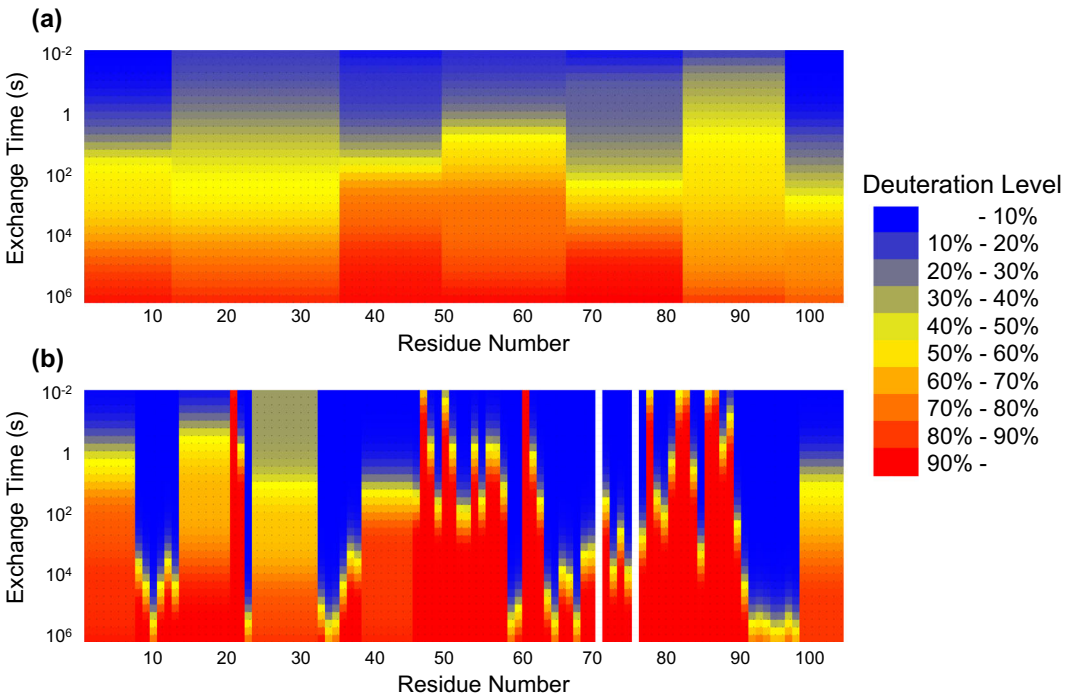

Figure 4. HDX-MS data, (**a**) without sub-localization and (**b**) with sub-localization. This figure was reconstructed from the exchange rates determined in a previous study [2] and this study. (**a**) was generated using the deuterium buildup curves of seven peptides which cover the entire cytochrome c sequence without sub-localization. (**b**) was generated using all sub-localization described in this paper. White columns in (**b**) are Pro71 and Pro76. Pro30 and Pro44 were averaged in segments 24–32 and 39–46, respectively

protein, such as ligand binding, formulation change, and mutation, quantitatively in terms of the energy change (kcal/mol) at each residue. On the other hand, the discussion with HDX-MS deuterium buildup curve data remains qualitative.

High-resolution HDX-MS data determined backbone amide hydrogen exchange rates which provide quantitative measurements of protein dynamics in a more reproducible manner. Traditionally, HDX-NMR has described the dynamics of relatively small proteins quantitatively by determining the backbone amide hydrogen exchange rates [37], while HDX-MS has described the dynamics of larger proteins qualitatively at a lower resolution. This manuscript shows the analysis of a small protein in a quantitative manner by high-resolution HDX-MS using a pepsin/FPXIII mixed bed protease column. The strategy can be applicable to the quantitative analysis of more challenging proteins and protein complexes which cannot be analyzed by NMR.

Acknowledgments

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