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Differential isotopic enrichment to facilitate characterization of asymmetric multimeric proteins using hydrogen/deuterium exchange mass spectrometry

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Abstract

Hydrogen/deuterium exchange (HDX) coupled to mass spectrometry has emerged as a powerful tool for analyzing the conformational dynamics of protein-ligand and protein-protein interactions. Recent advances in instrumentation and methodology have expanded the utility of HDX for the analysis of large and complex proteins; however, asymmetric dimers with shared amino acid sequence present a unique challenge for HDX because assignment of peptides with identical sequence to their subunit of origin remains ambiguous. Here we report the use of differential isotopic labeling to facilitate HDX analysis of multimers using HIV-1 reverse transcriptase (RT) as a model. RT is an asymmetric heterodimer of 51 kDa (p51) and 66 kDa (p66) subunits. The first 440 residues of p51 and p66 are identical. In this study differentially labeled RT was reconstituted from isotopically enriched (¹⁵N-labeled) p51 and unlabeled p66. In order to enable detection of ¹⁵N-deuterated RT peptides, the software HDX Workbench was modified to follow a 100% ¹⁵N model. Our results demonstrated that ¹⁵N enrichment of p51 did not affect its conformational dynamics compared to unlabeled p51, but ¹⁵N-labeled p51 did show different conformational dynamics than p66 in the RT heterodimer. Differential HDX-MS of isotopically labeled RT in the presence of the nonnucleoside reverse transcriptase inhibitor (NNRTI) efavirenz (EFV) showed subunit-specific perturbation in the rate of HDX consistent with previously published results and the RT-EFV co-crystal structure.

Keywords

Hydrogen deuterium exchange mass spectrometry; isotopic labeling; HIV-1 reverse transcriptase; subunit-selective labeling

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Introduction

Amide hydrogen/deuterium exchange (HDX) coupled to mass spectrometry is a well-established method for probing protein structural dynamics because the amide backbone is highly sensitive to changes in protein conformation.^{1–5} The rate of amide exchange is influenced by the amide hydrogen's local environment.^{6,7} For a protein in its native state, protection from exchange results from hydrogen bonding and reduced solvent accessibility. The exchange rate of an amide hydrogen to deuterium in a folded protein can be greatly increased in the presence of denaturing agents such as urea or guanidinium. In contrast, ligand binding can reduce the rates of amide exchange by strengthening hydrogen-bonding networks and decreasing solvent accessibility.^{8,9}

In an HDX experiment, a target protein is diluted in deuterated (D_2O) buffer for specific time intervals. Back-exchange to hydrogen, which is pH and temperature dependent, is minimized (quenched) by lowering the pH to 2.4 with an acidic buffer at $\sim 0^\circ C$. Denaturants can be added to the quench buffer to aid in digestion by an acid-stable protease—typically pepsin immobilized to a solid support. Peptic peptides are separated by reverse-phase high performance liquid chromatography (RP-HPLC) and analyzed by electrospray ionization mass spectrometry (ESI-MS). The rate of backbone amide hydrogen exchange for each peptide is measured by its mass-to-charge (m/z) shift over time. Protein regions protected from amide exchange are relatively ordered and conformationally stable whereas regions that undergo rapid exchange are more flexible and less ordered. In a differential HDX experiment, on-exchange of the protein of interest is carried out in the presence and absence of a binding partner (protein, DNA, small molecule, etc.). Regions of the protein that reveal perturbations in HDX kinetics due to ligand binding often map to the site of interaction but can also be attributed to allosteric effects.^{10,11}

The requirements of maintaining temperature, pH, and chromatographic separation on a time scale (10–15 min) that avoids unnecessary back exchange with the protonated solvent can pose a potential bottleneck for high throughput HDX of large, complex macromolecular samples.^{12,13} Data processing can also become problematic due to overlapping isotopic peaks resulting from high concentrations of co-eluting peptides. Spectral complexity arising from peak overlap can be addressed by several approaches such as ion mobility,¹⁴ using low D_2O content in exchange solution,¹⁵ isotopic depletion¹⁶ and solid-support immobilization of protein binding partners.^{17,18}

Heterodimeric proteins with shared sequence identity present a challenge for HDX because peptides with identical sequence derived from different subunits cannot be unambiguously assigned. This challenge is well represented by the structure of HIV-1 RT.¹⁹ RT is a heterodimer consisting of 66 kDa (p66) and 51 kDa (p51) subunits. The p66 subunit has polymerase and RNase H enzymatic active sites. The polymerase domain has four subdomains: fingers (residues 1–85 and 118–155), palm (residues 86–117 and 156–236), thumb (residues 237–318), and connection (residues 319–426). The p66 subunit shares its N-terminal 440 amino acid sequences with p51. Despite shared sequence and similar secondary structure, p51 and p66 have different three-dimensional conformations as revealed by many RT crystal structures.^{20,21,22}

The p66 subunit has an open extended conformation whereas p51 is more compact. This presents a further complication for HDX-MS, because peptides of identical sequence can have different solvent environments (Figure 1). Previously this issue was addressed using subunit-selective biotinylation to separate subunits after HDX, but before LC-MS analysis.^{23,24} This process, while effective, required two experiments to obtain a complete data set for the RT heterodimer, and is not suitable for high-throughput automated HDX MS system.

Here, we report a reproducible and novel approach that relies on differential isotopic labeling suitable for automation. We labeled the p51 subunit of RT with ¹⁵N (expressed in isotopically depleted media) and expressed p66 in its isotopically averaged form. After reconstitution and purification of heterolabeled RT, we carried out tandem mass spectrometry to identify labeled and unlabeled peptides. Our data processing software, HDX Workbench,²⁵ was modified to detect and identify differential isotopically labeled samples, allowing peptides to be assigned to their specific subunit based on differences in their m/z values. To validate our approach, we performed differential HDX on RT in the presence and absence of the NNRTI drug, efavirenz (EFV) for comparison with previously reported HDX studies on RT that used biotinylation to separate subunits.²³

Experimental section

Isotopic labeling and expression of p51 and p66

The RT subunit p51 was labeled with the ¹⁵N-isotope using a protocol derived from Xiao et al. 2010.²⁶ Briefly, a single colony of *E.coli* BL-21 DE3 RIL expressing p51 cloned into the plasmid pCDF-2 (EMD Millipore) grown in the presence of 50 µg/mL streptomycin and 34 µg/mL chloramphenicol was inoculated into 0.5 mL of MJ9 minimal media containing 50 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO) and with ¹⁵N-(NH₄)₂SO₄ (Cambridge Isotopes, Cambridge, MA) as the sole nitrogen source and incubated at 37 °C until the culture reached turbidity (~4–6 hrs). 20 µL of the starter culture was diluted into 20 mL of fresh MJ9 media with 50 µg/mL streptomycin and incubated at 37 °C overnight with shaking. 1 mL of overnight culture was diluted into 1 L of MJ9 media with 50 µg/mL streptomycin and incubated at 37 °C with shaking until an OD₆₀₀ of 0.6–0.8 was reached. Cultures were incubated at 4 °C for one hour and p51 was expressed by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) (Gold Biotechnology, St. Louis, MO) to a final concentration of 1 mM. Induced cultures were incubated at 17 °C overnight with vigorous shaking. Cultures were harvested by centrifugation at 6000 × g for 20 minutes at 4 °C. Cell paste was stored at –80 °C until further use. Unlabeled p66 and 51 were expressed separately in *E.coli* BL-21 DE3 RIL cells (Agilent Technologies) from a single colony inoculated into 50 mL Luria-Bertani (LB) broth in the presence of 50 µg/mL streptomycin-sulfate, 34 µg/mL chloramphenicol and incubated overnight at 37 °C with shaking. A 1:20 dilution of the overnight culture into 1 L of fresh LB broth containing 50 µg/mL streptomycin and 0.1% glucose was incubated at 37 °C with shaking until a OD₆₀₀ of 0.6–0.8 was reached. All subsequent steps were carried out as described for ¹⁵N-labeled p51.

Purification of p66 and p51 subunits

Approximately 20 grams of cell paste was resuspended in 50 mL of lysis buffer containing: 50 mM phosphate buffer pH 7.0, 0.6 M NaCl, 0.1% triton X-100, 5 mM imidazole, 1 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonylfluoride (PMSF) (Sigma-Aldrich, St. Louis, MO), added just before use. The resuspended cells were lysed by sonication (Misonix-3000) with a total processing time of 10 minutes and a 30 second on/off pulse at an output of 7.0 (93 watts) in an ice-water bath. The crude lysate was separated into soluble and insoluble fractions by centrifugation at $38,000 \times g$ for 45 minutes at 4 °C in a Sorvall RC 6+ centrifuge with a fixed-angle-rotor. The clarified supernatant was loaded onto a 2 mL nickel-NTA (Qiagen) column equilibrated with lysis buffer w/o PMSF. The column was washed with 20 column volumes of lysis buffer, then with 5 column volumes of lysis buffer containing 1.5 M NaCl, and eluted with 10 mL of 50 mM phosphate buffer pH 7.0, 200 mM NaCl, 1 mM 2-mercaptoethanol (Sigma-Aldrich), and 250 mM imidazole. The p66 subunit, but not p51, was incubated overnight with a 1:20 mass/mass ratio of HRV-14 3C protease, produced in-house, to remove the 6x-histidine purification tag from the N-terminus of p66. 10 mL of eluate was concentrated to 1 mL using a Millipore Amicon Ultra centrifugal concentrator (30 kDa molecular weight cutoff), diluted to 30 mL with 50 mM diethanolamine buffer pH 8.9, and loaded onto a MonoQ 10/100-anion exchange column (GE Healthcare). The column was washed with 100 mL of 50 mM DEA buffer pH 8.9 and eluted with a linear gradient from 0–100% buffer B: 50 mM DEA pH 8.9, 1 M NaCl. Elution was monitored by absorbance at 280 nm and peak fractions analyzed by SDS-PAGE. The p66 and p51 subunits elute at ~120 mM NaCl. Fractions containing the purest sample judged by SDS-PAGE were pooled and buffer exchanged into 10 mM Tris-pH 8.0, 75 mM NaCl using a centrifugal concentrator unit with a 30 kDa molecular weight cutoff. RT subunits were quantitated using an extinction coefficient of $3.1 \text{ (mg/ml)}^{-1} \text{ cm}^{-1}$ for p51 and $3.5 \text{ (mg/ml)}^{-1} \text{ cm}^{-1}$ for p66.

Reconstitution and purification of HIV-1 RT heterodimers

Reconstitution of unlabeled p66 and either unlabeled p51 or ^{15}N -labeled p51 into heterodimeric RT was carried out as described previously.²⁴ For details see supporting information.

HIV-1 RT DNA-dependent DNA polymerase activity

The DNA-dependent DNA polymerase activity of reconstituted ^{15}N -labeled, RT heterodimer was compared to unlabeled RT, where the p66 and p51 subunits were coexpressed in *E. coli* and purified as described previously.²⁷ For details see supporting information.

Intact protein analysis using MALDI-MS

^{15}N incorporation was calculated using MALDI-TOF by subtracting the experimental mass of unlabeled p51 from ^{15}N -labeled p51 and dividing by the total number (600) of nitrogens in p51. ^{15}N incorporation into p51 was calculated to be >95%. For details see supporting information.

Software Analysis with HDX Workbench/MASCOT

The ^{14}N and ^{15}N labeled MS/MS *.raw data files were converted to *.mgf files and then submitted to Mascot (Matrix Science, London, UK) for peptide identification. Peptides with a Mascot score of 20 or greater were included in the peptide sets used for HDX. The MS/MS MASCOT searches were additionally subjected to manual inspection of the MS/MS spectra as well as a decoy database search, and ambiguous identifications were ruled out. This produced two lists of confirmed identifications for both ^{15}N and ^{14}N , which were submitted to HDX Workbench for subsequent analysis. The HDX Workbench software²⁵ was configured to follow a 100% ^{15}N model enabling the software to identify all ^{15}N labeled peptides.

HDX analysis

Solution-phase amide HDX was carried out on our automated system as described previously.²⁸ For on-exchange experiments, the intensity weighted average m/z value (centroid) of each peptide's isotopic envelope was calculated using HDX workbench software with modified features. For details see supporting information.

Results and Discussion

^{15}N labeling, purification and enzymatic activity

We addressed the problem of identifying peptides originating from either subunit in RT by isotopically labeling the p51 subunit with ^{15}N during protein expression (see Experimental Section for details). The p51 and p66 subunits were expressed and purified separately prior to forming hetero-labeled RT *in vitro*. We chose to label p51 to avoid contaminating RT with unlabeled p51 that can spontaneously break down from p66.²⁹ We also used a two-stage purification procedure that took advantage of the individually 6x-histidine-tagged subunits. The ^{15}N -labeled p51 and unlabeled p66 were expressed and purified as described in experimental section. Prior to forming RT heterodimers, the 6x-histidine tag of p66 was removed using HRV-14 3C protease. After forming RT a second Ni-NTA affinity purification was performed to remove untagged p66 and p66 homodimers. A second treatment with protease was carried out to remove the 6x-histidine tag from p51. A final ion-exchange purification step (MonoQ) was carried out to remove protease and contaminating homodimers. Protein purity was estimated at 95% by SDS-PAGE. Incorporation of ^{15}N for p51 in the reconstituted RT was determined to be 95% by MALDI-TOF mass spectrometry (detailed in Experimental Section and Supplementary Figure S1). We tested the DNA-dependent DNA polymerase activity of assembled RT using a heteropolymeric DNA template-primer in a filter-binding assay. RT polymerase activity was measured by uptake of ^3H -TTP into acid precipitable material. The ^{15}N -labeled RT showed comparable activity to unlabeled RT for which p66 and p51 were coexpressed in *E. coli* (Figure 2).

Detection of isotopically labeled peptides: Configuration of HDX Workbench software

We carried out peptide identification by MS/MS under HDX conditions and used a modified version of the program Mascot to identify ^{15}N -labeled peptides originating from p51 and unlabeled peptides originating from p66. We made two separate peptide lists: one consisting of only ^{15}N labeled peptides (p51) and the other having naturally abundant ^{14}N (p66)

peptides. We used these peptide sets with the software package HDX Workbench to calculate the rates of deuterium exchange for each peptide.

A feature of HDX Workbench is its automated detection of peptides in MS1 space. Determination of a peptide's elemental composition and theoretical isotopic distribution (factoring in charge state) is compared to the theoretical distribution in the experimentally observed spectra. For flexibility, the software produces text-based files allowing end users to configure and define modifications and chemical elements as needed. Files were configured to follow a 100% ^{15}N model enabling the software to detect all ^{15}N -labeled peptides. In both samples, the mass spectral peaks corresponding to all peptides were visually inspected to confirm the identifications. The automated detection of a representative +2 peptide “NVLPQGWKGSPAIF” (elemental composition = $\text{C}_{72}\text{H}_{109}\text{N}_{18}\text{O}_{18}$) from RT is demonstrated in Figure 3. The naturally abundant ^{14}N (unlabeled) species is shown with a monoisotopic m/z of 757.41 and a centroid m/z of 757.88. The labeled (^{15}N) species is shown with a monoisotopic m/z of 766.38, and a centroid m/z of 766.81. The light grey vertical bars represent regions in which correct peptide peaks are expected to reside. The first bar corresponds to the monoisotopic peak. While the peptide has the same elemental composition and charge state in both panels of Figure 3, the software follows a 100% ^{15}N model in the bottom panel in order to identify the peptide. With this configuration HDX of differentially labeled peptides can be carried out under the same experimental scheme. The software is freely available and the configuration files are available to, and can be modified by, the end users.

^{15}N Labeling doesn't interfere with inherent conformational dynamics of the protein

In order to test if heavy isotopic labeling interferes with the inherent dynamics of p51 we performed HDX to compare the backbone dynamics of ^{15}N -labeled and unlabeled (isotopically averaged) p51. The software HDX Workbench was modified to calculate the average percentage of deuterium uptake for each peptide over six time points (10, 30, 60, 300, 900 and 3600 sec). Figure 4 presents a comparison of the average deuterium uptake for peptides from labeled and unlabeled p51. The conformational dynamics of both proteins are very similar. The residues of the thumb subdomain (230–250 and 285–300) and the C-terminus (420–440) showed a high degree of flexibility. In contrast, the p51 N-terminal residues 30–60, residues of the palm (185–210), and connection regions (370–395) showed more ordered, less dynamic conformations. The deuterium build-up curves of three select peptides from labeled and unlabeled p51 are shown: one from the N terminus (102–106), one from the C terminus (381–295), and an internal peptide (302–306). Significant differences in deuterium build-up between samples were not observed.

In heterolabeled RT p51 and p66 subunits assume different conformations

After confirming that isotopic labeling does not affect the intrinsic conformational dynamics of p51, we measured the conformational dynamics of RT with ^{15}N -labeled p51 and unlabeled p66. Tandem mass spectrometry of heterolabeled RT under HDX compatible conditions gives 75% and 68% sequence coverage for p51 and p66, respectively (Supplementary Figure S2). After on-exchange, pepsin digestion and LC separation were carried out at 4°C within 15 minutes to minimize back exchange to H. Figure 5 describes

the average percentage of deuterium incorporation for selected peptides across the length of p51 and p66. Different conformational dynamics for p66 and p51 subunits were observed. For example, peptides covering region 230–245 in p51 showed 36–45% of average D₂O uptake over one hour, while the identical peptides in p66 showed D₂O uptake of 72%–74% (Supplementary Figure S3 a,b). This suggests that although they share identical amino acid sequences, the N-terminal residues of p66 and p51 possess different conformational dynamics in agreement with observations from RT crystal structures.^{21,22,20} This observation also confirms that we were able to detect identical peptides from a single pool originating from different subunits based on their isotopic labeling and could determine their respective levels of D₂O incorporation and conformational states. We also compared the HDX pattern of ¹⁵N-labeled p51 alone and in reconstituted RT (Supplementary Figure S4). In the context of the heterodimer p51 subunit showed an overall stabilized conformation compared to p51 alone. It has been reported that p51 has a 700-fold greater affinity for p66 than for p51³⁰ suggesting that the predominant species is p66/p51.

HDX analysis of labeled RT binding to efavirenz (EFV)

To validate our approach, we carried out a differential HDX experiment with heterolabeled RT in the presence and absence of the antiretroviral NNRTI drug, efavirenz (EFV). EFV is an allosteric inhibitor of RT that blocks viral replication. HDX of RT in the presence of EFV was reported in an earlier study by Wintrod and colleagues.³¹

We carried out differential HDX on RT and RT-EFV using a previously described method.³² Previous results showed that EFV makes contact with 14 residues in p66 and none in p51.^{33,34} Peptides in regions 100–105, 165–182, 187–192, 232–246 and 301–328 contain most of the residues involved in EFV binding. Peptides in those regions along with allosteric regions showed significant stabilization of various magnitude, (Figure 6 and Supplementary Figure S5 a,b). The observed changes in deuterium uptake at direct binding and allosteric sites are in agreement with previous work.²³ Figure 5 shows the average percentage of deuterium exchange corrected for back exchange (see experimental section) over 1 hr mapped onto the crystal structure of RT (PDB ID: 1IKW). Deuterium build-up curves for three representative peptides with identical sequence, 62–77, 231–246 and 283–300 are presented. Despite having identical sequences, peptides originating from p51 (¹⁵N labeled) or p66 (unlabeled) showed different deuterium uptake patterns. Moreover, we were able to detect significant changes in deuterium uptake between apo and EFV-bound RT. Residues 66–77 in p66, but not p51, exhibited significantly increased HDX behavior, and were found to be more dynamic, in the presence than in the absence of EFV. This observation is consistent with the RT-EFV crystal structure, for which this region is disordered.

In this report we used RT as a model protein to validate the differential isotopic enrichment approach. Based on the results presented it is clear that this method has general applicability and can be used to analyze other heterodimeric proteins that contain subunits of identical sequence. To this end Supplementary Figure S6 illustrates a general workflow for implementing this strategy to other proteins.

Conclusions

In this report we have described a new strategy for characterizing asymmetric heterodimers with shared sequence by HDX-MS. We applied subunit-selective labeling of RT with ^{15}N in order to detect peptides with identical sequences originating from either subunit based on their mass and determined the deuterium uptake level for on-exchange HDX reactions. We demonstrated the applicability of our strategy using heterodimeric HIV-1 RT and that isotopic labeling does not affect the intrinsic protein dynamics. We showed both subunits (^{15}N -labeled p51 and unlabeled p66) assume different conformational states in the heterodimer which is consistent with X-ray structures of RT. Finally, we validated our approach by carrying out differential HDX experiments with RT and EFV and showed comparable results to those published previously.²³ The software HDX Workbench, was modified for analyzing differential isotopically labeled samples and is freely available (http://hdx.florida.scripps.edu/hdx_workbench/Home.html).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Subdomains and conformations of HIV-1 RT p66 and p51 subunits. (a) Subdomains of the p66 subunit of HIV-1 RT shown by color: fingers (blue), palm (red), thumb (green), connection (yellow), RNase H (orange). The p51 subunit is shown in cyan. (b) The overall conformations of p66 and p51 subunits. Subdomains in both p66 and p51 are colored as in (A).

Figure 2.

DNA-dependent DNA polymerase activity of ^{15}N -labeled assembled (open circles) and unlabeled, coexpressed (black circles) HIV-1 RT. Activity was carried out on an oligomeric 90-nucleotide template annealed to a 15-nucleotide primer at 37 °C for 5, 10, and 20 minutes. Uptake of ^3H -TTP was converted to pmoles of dNTP incorporated into acid-precipitable material as described in the Experimental Section. Activity at each time point is the average of three independent measurements.

Figure 3.

Automated detection of the peptide “NVLPQGWKGSPAIF” (+2 charge state) using HDX Workbench. (Top panel) The unlabeled (^{14}N) species is shown with a monoisotopic m/z of 757.41, and a centroid m/z of 757.88. (Bottom panel) The labeled (^{15}N) species is shown with a monoisotopic m/z of 766.38, and a centroid m/z of 766.81. Extracted ion chromatograms highlighting the retention time for both the labeled and unlabeled peptide (eluting at 4.7 – 4.9 mins within the eight minute gradient) are presented as insets. The light grey bars indicate regions in which correct peptide peaks are expected to reside, the first bar indicates the monoisotopic peak. Both the labeled and unlabeled peptides share identical elemental compositions and charge states. The software follows a 100% ^{15}N model in the bottom panel to identify the ^{15}N -labeled peptide.

Figure 4.

Heavy isotopic (¹⁵N) labeling doesn't interfere with the intrinsic conformational dynamics of the protein. On-exchange reaction of ¹⁵N-labeled and unlabeled p51 was carried out in parallel, and the average percentage of deuterium uptake over a 1 hr time period for all peptides was plotted using GraphPad Prism software. For both the labeled (red) and unlabeled (blue) sample, average deuterium uptake is very similar. Deuterium build-up curves of representative peptides are shown in the inset.

Figure 5.

¹⁵N-labeled p51 and unlabeled p66 assume different conformational states in RT. Assembled ¹⁵N labeled RT heterodimers were subjected to on-exchange. The average percentage of deuterium uptake over 1 hr time period for all peptides was plotted using the software Graphpad prism. The N-terminus of p51 (red) is more dynamic compared to that of p66 (blue) despite having identical sequence in that region.

Figure 6.

Differential HDX analysis of RT binding to efavirenz. (a) The difference in HDX of the RT heterodimer in the presence and absence of the NNRTI drug efavirenz is mapped onto the atomic coordinates of the RT-EFV co-crystal structure (PDB ID: 1IKW). Regions of the structure shown in grey indicate peptides for which no net HDX behavior was observed in the presence of RT-EFV compared to apo-RT. Regions where net HDX behavior was observed are shown in color according to the legend. Encircled and boxed areas show the same peptides in p66 and p51 subunits. Build up curves of representative peptides, (b) 283–300, (c) 231–246 and (d) 62–77 are also shown.