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## Epitope Mapping of a 95 kDa Antigen in Complex with Antibody by Solution-Phase Amide Backbone H/D Exchange Monitored by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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### Abstract

The epitopes of a homohexameric food allergen protein, cashew Ana o 2, identified by two monoclonal antibodies, 2B5 and 1F5, were mapped by solution-phase amide backbone H/D exchange (HDX) coupled with FT-ICR MS and the results compared to previous mapping by immunological and mutational analyses. Antibody 2B5 defines a conformational epitope and 1F5 defines a linear epitope. Intact murine IgG antibodies were incubated with recombinant Ana o 2 (rAna o 2) to form antigen:monoclonal antibody (Ag-mAb) complexes. MAb-complexed and uncomplexed (free) rAna o 2 were then subjected to HDX. HDX instrumentation and automation were optimized to achieve high sequence coverage by protease XIII digestion. The regions protected from H/D exchange upon antibody binding overlap and thus confirm the previously identified epitope-bearing segments: the first extension of HDX monitored by mass spectrometry to a full-length antigen-antibody complex in solution.

### Keywords

Hydrogen/Deterium Exchange; Allergen; Cashew; FT-ICR; FTMS

### INTRODUCTION

Characterization of interaction/contact regions between protein antigens (i.e., epitopes) and antibodies is essential for understanding the mechanisms of antibody generation and immune recognition as well as basic aspects of protein-protein interactions. Clinical applications of such information including the rational design of vaccines<sup>1</sup> and efforts to predict the biochemical motifs common to allergenic proteins.<sup>2, 3</sup> The rapidly growing list of therapeutic monoclonal antibodies (mAbs) also stimulates research on epitope characterization.<sup>4–6</sup>

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Mapping the immunoglobulin (Ig)-binding epitopes of a protein antigen (Ag) is a key step in characterization of antigen-antibody complexes. Ig-binding epitopes may be categorized as linear (sequential, continuous) or conformational (discontinuous).<sup>1, 7–10</sup> A linear epitope is typically defined by the binding of an antibody to a single short (8–15 amino acid) peptide sequence.<sup>11–13</sup> A conformational epitope, on the other hand, requires three-dimensional peptide conformation as in a native folded protein. Such an epitope typically (but not necessarily) comprises discontinuous segments brought into close proximity upon protein folding.<sup>11–13</sup> Despite the operational distinction between the two epitope types, even the shortest linear epitopes probably adopt, at least transiently, a defined configuration upon interaction with antibody complementarity-determining regions.<sup>11</sup>

The method used to define a linear epitope normally employs synthetic antigen segments that are then screened in antibody binding assays. That approach usually entails screening complete sets of overlapping peptides encompassing the entire amino acid sequence of the target protein (i.e., peptide scanning).<sup>1, 14, 15</sup> Once identified, the reactive peptides may be mutated and tested to further refine the location of the epitope and identify key residues. In contrast, the techniques employed for identification and characterization of conformational epitopes are considerably more challenging because of the difficulty in maintaining the native conformation of the antigen during preparation and analysis.

Mutagenesis is commonly used to characterize protein-protein interaction, in particular for conformational epitope mapping.<sup>16</sup> Large scale mutagenesis, truncation, and chimeric molecule production can be performed on an antigen to identify the key residues responsible for antigen-antibody complex formation. However, those molecular cloning-based methods are labor intensive, and the altered version may not represent the native conformation of the wild type antigen. In particular, conformational change induced by mutations distant from the actual antigen-antibody contact region, but that alter the epitope structure, can frustrate data interpretation.

Proteolysis monitored by mass spectrometry has also been applied to the mapping of conformational epitopes.<sup>17, 18</sup> Briefly, free antigen and antigen:antibody complexes are subjected to proteolysis and the resulting peptide segments mass-analyzed. The protease functions as a probe to access the antigen surface. Segments covered by antibody binding are protected from digestion. Although straightforward in principle, the practical sequence resolution of this technique is low and depends strongly upon the antigen sequence and the fortuitous locations of enzyme cleavage sites.

When feasible, the most detailed representation of a conformational epitope derives from high resolution X-ray crystallography of an antigen:monoclonal antibody (Ag-mAb) complex. However, crystallization of such complexes is idiosyncratic as well as time- and resource-intensive.<sup>19–21</sup>

Amide backbone hydrogen/deuterium exchange monitored by mass spectrometry (HDX-MS) has become a complementary method for mapping protein surfaces based on solvent accessibility. Both protein dynamics<sup>22–26</sup> and protein-ligand interaction<sup>27–32</sup> can be addressed by HDX-MS. The use of HDX-MS for epitope mapping has recently been reported.<sup>33, 34</sup>

New methodological approaches recently developed for HDX experiments coupled with high resolution FT-ICR MS<sup>35</sup> include: automation,<sup>27, 36</sup> faster chromatographic separation,<sup>37</sup> more efficient digestion,<sup>38</sup> and implementation of enhanced data analysis packages.<sup>36</sup> Extension of HDX to higher molecular weight analytes, combined with fast elution (to minimize D/H back-exchange) requires mass spectral resolution of isotopic distributions of dozens of peptide segments. To that end, the present analyses are

significantly enhanced through the use of a high-resolution (high peak capacity) 14.5 T Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer.<sup>39</sup> Also, our HDX instrumentation has been further optimized to overcome the challenge of high molecular weight Ag-mAb complexes.

Epitope mapping for a small (104 amino acid) model protein (cytochrome c) has been successfully achieved by HDX monitored by NMR,<sup>40</sup> and recently by MS-based HDX<sup>41</sup> for an antibody immobilized on a column during H/D exchange, followed by removal and proteolysis of the antigen. The latter approach effectively reduces spectral complexity by eliminating antibody-derived peptides from the antigen-derived peptides. However, the immobilized antibody approach is subject to antigen carryover and a concomitant reduction in peptide detection; e.g., only 26 peptides were observed for cytochrome c by the MS-based HDX approach. Here we report the mapping of epitopes on a large homotrimeric (monomer mass = 95 kDa) recombinant food allergen in the presence of either of two intact IgG mAbs in solution by HDX-MS. Because of its size and complexity, mapping of the epitopes has not been accessible by NMR or X-ray crystallography.

Food allergies are estimated to affect 6% of children and 3–4% of adults.<sup>42</sup> Food allergy, mediated by IgE, is an adverse immunological (hypersensitivity) reaction to a normally harmless food constituent.<sup>43–45</sup> Here, we apply HDX-MS to the clinically important cashew 11S globulin allergen, Ana o 2 (Ana o 2)<sup>46–50</sup> by comparing backbone H/D exchange of recombinant (r)Ana o 2, free and bound to either of two cognate monoclonal antibodies, 2B5 and 1F5. The 1F5 and 2B5 mAbs have previously been shown to recognize a linear and a conformational epitope, respectively,<sup>47, 50</sup> and thus offer a good test for the application of HDX epitope mapping of a large antigen protein.

## EXPERIMENTAL SECTION

### Expression and purification of MBP-fusion rAna o 2 antigen

Expression and purification of MBP-fusion rAna o 2 was carried out as previously described.<sup>49</sup> rAna o 2 was prepared as ~10 μM monomers (but present as trimers) in phosphate-buffered saline (PBS, EMD Chemicals, Gibbstown, NJ, USA), pH 7.4 for HDX analysis. TCEP (tris(2-carboxyethyl)phosphine), urea, formic acid, acetonitrile, and protease type XIII were obtained from Sigma Aldrich (St. Louis, MO, USA).

### Monoclonal antibody production for rAna o 2-reactive mAbs (2B5 and 1F5)

Ana o 2-reactive mAbs were generated in the Hybridoma Core Facility at Florida State University according to previously described standard procedures.<sup>48</sup> Twenty monospecific Ana 2-binding mAbs were obtained. MAb 2B5 was identified as recognizing a conformational epitope<sup>47, 50</sup> and chosen for HDX analysis. MAb 1F5, which recognizes a previously defined linear epitope, was also selected as an antibody for HDX analysis.<sup>47</sup> MAbs 2B5 and 1F5 were concentrated to ~20 μM in PBS, pH 7.4. An equivalent molar amount of 2B5 (1:1) or a slight molar excess of 1F5 (1.3:1) was mixed with rAna o 2 and incubated at 25 °C for 30 min to generate the rAna o 2-mAb complexes. Note that each mole of mAb contains two moles of binding sites and the molarity of rAna o 2 was calculated based on the mass of monomeric subunits. Consequently the ratios of mAb binding sites to rAna o 2 epitopes for 2B5 and 1F5 are 2:1 and 2.6:1, respectively.

### Optimization and automation of the hydrogen/deuterium exchange event sequence

An algorithm (HDX integrator<sup>51</sup>) interlaces HDX reactions *in silico*. The HTC Pal autosampler (Eksigent Technologies, Dublin, CA) operating durations for each of three events (initiation of D<sub>2</sub>O labeling, quench/digestion, and injection to LC) for one HDX

reaction are first measured experimentally. The algorithm uses those durations to simulate the entire HDX experiment. Basically, the algorithm interlaces a series of short HDX reactions during the longer reactions, such that no two LC injections overlap. Based on the result, a command list is compiled (by a Python script) for the entire HDX experiment.

A software package, Runmanager, developed in collaboration with Eksigent Technologies, controls the HTC Pal autosampler by use of its command list. The combination of the *in silico* simulation of HDX reactions and the Runmanager software control package permits flexible modification of all HDX parameters: e.g., number of HDX reactions, reaction volume, injection volume, HDX duration, quench duration, digestion duration, and chromatography duration. To evaluate the overall effect of optimization and automation and to compare with the previously reported HDX system,<sup>27, 36</sup> HDX for equine heart myoglobin was performed as described.<sup>38</sup>

### Hydrogen/deuterium exchange

The entire HDX experiment is optimized and automated with the HTC Pal autosampler (Eksigent Technologies, Dublin, CA). A 5 µL stock solution of rAna o 2 (~10 µM monomer concentration) or rAna o 2-mAb complex (~10 µM for rAna o 2 monomer) was mixed with 45 µL of PBS in D<sub>2</sub>O, pH meter reading 7.4, to initiate each H/D exchange period. For the blank control, the initial dilution was made in H<sub>2</sub>O PBS. For the zero-time control, initiation and quenching of HDX reaction are performed simultaneously. The HDX reaction periods were 0.5, 1, 2, 4, 8, 15, 30, 60, 120, 240, and 480 min, each followed by simultaneous quench and proteolysis. Each 50 µL total volume was quenched by rapid mixing with 25 µL of 200 mM TCEP, 8 M urea solution in 1.0% formic acid and 25 µL five-fold dilution of saturated protease type XIII<sup>38</sup> in 1.0% formic acid (final pH ~2.3). The protease digestion was performed for 2 min followed by injection for LC-MS analysis. The column was regenerated for at least 10 min between any two injections. All components for the entire HDX experiment were installed in a chromatography cabinet to maintain the temperature at 1–2°C, as described previously<sup>52</sup>. Each HDX reaction and assay was performed in triplicate.

### On-line LC ESI FTICR MS

After proteolysis, the rAna o 2 (with or without mAb) peptides were separated and desalted with a Jasco HPLC/SFC instrument (Jasco, Easton, MD) interfaced with the HTC Pal autosampler (Eksigent Technologies, Dublin, CA). For LC, 45 µL of the protein digest was injected from a 50 µL loop to a Pro-Zap Expedite MS C<sub>18</sub> column (Grace Davidson, Deerfield, IL), HR 1.5 µm particle size, 500 Å pore size, 2.1 × 10 mm<sup>2</sup>. A rapid gradient from 2% B to 95% B in 1.5 min (A: acetonitrile/H<sub>2</sub>O/formic acid, 5/94.5/0.5; B: acetonitrile/H<sub>2</sub>O/formic acid, 95/4.5/0.5) was performed for eluting peptides at a flow rate of 0.3 mL/min. The LC eluent flow rate is reduced by ~1/1000 by a postcolumn splitter for efficient microelectrospray ionization (micro-ESI).<sup>53</sup>

The ionized LC eluent was directed to a custom-built hybrid LTQ 14.5 T FT-ICR mass spectrometer (ThermoFisher, San Jose, CA).<sup>39</sup> Mass spectra were collected from 380 < m/z < 1300 at high mass resolving power ( $m/\Delta m_{50\%} = 100,000$  at m/z 400, in which  $\Delta m_{50\%}$  is mass spectral peak full width at half-maximum peak height). The total experimental event sequence for each sample was 6.5 min. External ion accumulation<sup>54</sup> was performed in the linear ion trap with a target ion population of three million charges collected for each FT-ICR measurement. LTQ-accumulated ions were transferred (~1 ms transfer period)<sup>55</sup> through three octopole ion guides (2.2 MHz, 250 V<sub>p-p</sub>) to a capacitively coupled<sup>56</sup> closed cylindrical ICR cell (55 mm i.d.) for analysis. The ion accumulation period was typically less than 100 ms during peptide elution and the ICR time-domain signal acquisition period was 767 ms (i.e., an overall duty cycle of ~1 Hz per acquisition). Automatic gain control<sup>57</sup>

and high magnetic field<sup>58</sup> provided excellent external calibration mass accuracy (normally less than 500 ppb rms error).

### HDX data analysis

Data were collected with Xcalibur software (Thermo-Fisher) and analyzed by an in-house analysis package.<sup>36</sup> Time-course deuterium incorporation levels were generated by an MEM fitting method.<sup>59</sup>

## RESULTS AND DISCUSSION

### Optimization of HDX instrumentation

Automation of an HTS Pal robot by a handwritten command list has been shown to increase the efficiency and reproducibility of HDX experiments.<sup>27, 36</sup> The robot utilizes a 50  $\mu$ L syringe to aspirate or dispense sample/solvents for individual HDX reactions in a 96-well plate. One HDX reaction normally includes three events: initiation of D<sub>2</sub>O labeling, quench/digestion and injection to LC. There are typically 39 or more individual reactions (13 different H/D exchange periods, including controls, with 3 replicates for each) for one full HDX experiment. The exchange periods vary from 0.5 to 480 minutes. It is important to program the robot to automatically perform the reactions in a reproducible, interlaced way.

With the help of the *in silico* simulation of HDX reactions and the controlling software package Runmanager, we designed the interlaced HDX experiments and wrote a "command list" to program the robot in seconds. Compared with the "Cycle Composer" based handwritten command list,<sup>36</sup> the new system enables more flexible design and modification of HDX experiments. Moreover, the new system is installed in a chromatography cabinet that provides better cooling capacity for the solvents, HPLC pumps, syringe and column than the previously reported water bath based system.<sup>27, 36, 38</sup>

The overall improvement of the new HDX system is evident from Figure 1. The HDX results for myoglobin performed with the new system exhibit increased deuterium uptake for all segments compared with the previously reported system.<sup>38</sup> The increased deuterium uptake levels indicate reduced back-exchange. For the HDX experiments performed for rAna o 2, several HDX aspects were optimized. For example, the injection volume was adjusted to 45  $\mu$ L and the LC duration was adjusted to 22 min to provide 10 min for column re-equilibration.

### High sequence coverage for free rAna o 2 and rAna o 2-mAb complexes

Native Ana o 2 is biosynthesized as a large proprotein that is posttranslationally cleaved into large (257 aa) and small (171 aa) subunits that remain associated via disulfide bonds.<sup>49, 60</sup> The uncleaved proprotein normally forms a trimer that dimerizes face-to-face to form a hexamer upon maturation to the cleaved form<sup>61–63</sup> Recombinant Ana o 2 is a large 457 aa proprotein linked to a 396 aa maltose-binding protein (MBP) expression tag and adopts a trimeric configuration in solution.<sup>47</sup> The monomer is ~95 kDa and the trimer is ~286 kDa. Although the precise stoichiometry for the Ag:Ab complex is not known, each monomeric subunit (95 kDa) of rAna o 2 likely binds half of an IgG, (one Fab arm of an mAb and associated Fc elements,  $\frac{1}{2}$  mAb mass = 75 kDa) for a total unique sequence mass of 170 kDa.

The recombinant protein is homotrimeric in form, whereas the native protein exists in both trimeric and hexameric forms.<sup>61–63</sup> The hexameric form of the native protein is the result of two trimers interacting by the hydrophobic face (IE face) to form the native hexamer.<sup>61</sup> Although the recombinant is limited to the trimeric form only, those trimers are essential for

further formation of the hexamer. Moreover, each monomer in the trimer/hexamer will display one epitope for Ab binding. Therefore, the presence of the hexamer increases the number of possible Ab binding sites (epitopes) on the molecule but does not affect the overall reactivity or binding affinity of the Ab.

To achieve high sequence coverage for the proteolytic peptides detected after enzymatic digestion and LC/MS, multiple experiments with different digestion conditions were designed and tested. Recently we showed that protease type XIII can efficiently digest an analyte protein in the presence of tris(2-carboxyethyl)phosphinehydrochloride (TCEP•HCl) and urea with increased sequence coverage.<sup>64</sup> The combination of urea and TCEP improved sequence coverage for all rAna o 2 forms (free or in complex) compared with digestion performed without urea and TCEP. Table 1 shows the sequence coverage for free rAna o 2, and rAna o 2 complexed with mAb 2B5 or 1F5 under identical digestion conditions. The binding of mAb slightly reduces the number of observed common segments. Only identical proteolytic peptides from free and complexed rAna o 2 forms are compared for HDX analysis. Figure 2 shows the shared peptides between free antigen and its complexed forms mapped onto the rAna o 2 sequence. The sequence coverage for the analyzed identical segments is ~90% for mAb 2B5 and ~80% for mAb 1F5.

The shared peptides are then analyzed for deuterium incorporation by an in-house HDX data analysis software package.<sup>36</sup> To further increase the sequence coverage and the number of overlapping peptides, a dual-protease digestion approach consisted of 1 min digestion by pepsin followed by 1 min digestion by protease type XIII. As shown in Figure 3, the dual-protease digestion yields a different set of peptides than from single protease digestion. The variety of peptides can be further increased by reversing the order of the two protease digestions (data not shown). High sequence coverage and a high number of overlapping segments are essential for conformational and functional comparisons, especially for epitope mapping of a large antigen.

### Determination of deuterium incorporation

After each H/D exchange period, quenched/digested rAna o 2 segments were separated over a ProZap C<sub>18</sub> column at 0.3 mL/min flow rate. The fast reversed-phase HPLC separation reduces back exchange by ~25% compared to conventional HPLC separations.<sup>37</sup> Data analysis was performed in three steps. The first step is to identify each proteolytic peptide for different rAna o 2 forms unexposed to D<sub>2</sub>O, in the blank controls. The second step is to assign proteolytic peptides following hydrogen/deuterium exchange. The last step is to determine the HDX rate distribution from deuterium uptake versus HDX incubation period profiles. More than 120 segments were analyzed for each rAna o 2 and rAna o 2-mAb sample after each exchange period (in triplicate, plus controls). The software package completed the total analysis in a few hours.

### HDX data analysis confirms rAna o 2 epitope locations for mAb 2B5 and 1F5

MAb 2B5 was initially characterized as reactive against native hexameric Ana o 2 purified from cashew extract as well as the trimeric recombinant cashew proprotein rAna o 2.<sup>47</sup> MAb 2B5 binds to the acidic large subunit of the cashew 11S globulin but only if the large subunit is associated with the small subunit; the isolated cashew large subunits do not react with 2B5. Association of the cashew large subunit with the homologous soybean small subunit restores 2B5 reactivity.<sup>47</sup> Those results suggest a conformational 2B5 epitope. Consistent with this interpretation is the observation that the epitope is highly susceptible to loss of immunoreactivity upon protein denaturation.<sup>47</sup> The 2B5 epitope has been mapped by deletion and point mutations, molecular modeling, and electron microscopy.<sup>50</sup> Key residues

are confined to a 24 amino acid segment near the N-terminus of the large subunit, located between aa 34–57 on the proprotein rAna o 2.<sup>50</sup>

In contrast to 2B5, mAb 1F5 binds to a linear Ana o 2 epitope and, as such, is resistant to denaturation.<sup>48, 49</sup> Screening of a full set of synthetic overlapping peptides shows that the 1F5 epitope is near the C-terminus of the large subunit, e.g., segment 257–271 of the rAna o 2 proprotein.<sup>48, 49</sup>

To assess the ability of HDX to detect these two epitope types (conformational 2B5 and linear 1F5) for this large protein when complexed with antibody, we performed H/D exchange for free rAna o 2, rAna o 2-2B5 complex, and rAna o 2-1F5 complex. 2B5 or 1F5 mAbs were incubated with the antigen in slight antibody excess to generate a soluble Ag:mAb complex. The deuterium uptake level for each proteolytic rAna o 2 segment was determined for each sample. The bar graphs in Figure 4 summarize the HDX results for the 4-hour incubation period for selected assigned proteolytic segments of rAna o 2 complexed with mAb 2B5. The binding of mAb 2B5 to rAna o 2 affords significant protection for aa segments 29–37, 31–41, 32–41, and 41–48 (Figures 5 and 6). The region 31–48 may thus be assigned as the HDX-defined epitope-bearing segment targeted by mAb 2B5, in agreement with the previously determined epitope-bearing segment 34–57.<sup>50</sup>

A similar analysis reveals that segment 265–289 is protected upon the binding of rAna o 2 to mAb 1F5 (Figures 5 and 6). The deuterium uptake profile for segment 265–289 show 36.9% less D uptake upon 1F5 binding (Figure 5, lower left). As for mAb 2B5, the rAna o 2 epitope for binding to mAb 1F5 revealed by the HDX analysis (segment 265–289) is consistent with the previously determined epitope (segment 257–271).<sup>48, 49</sup>

### Epitope segments inferred from H/D exchange

To distinguish protected from unprotected segments the average relative D-uptake difference (ARDD) was calculated for every peptide. Briefly, for each H/D exchange period, the relative difference in D uptake between free and complexed rAna o 2 is divided by the D uptake for free rAna o 2. The relative differences for that segment averaged over all H/D exchange periods define ARDD (see Equation in Figure 5).

For binding of the allergen to mAb 2B5, rAna o 2 segments 29–37, 31–41, 32–41 and 41–48 show ARDD greater than 30%. All other segments exhibited either no or small (less than 30%) ARDD upon binding to mAb 2B5. The deuterium incorporation profiles are shown in Figure 5, for both the protected (29–37, 31–41, 32–41, 41–48) and representative unprotected (160–175, 282–293) segments. For binding to mAb 1F5, only segment 265–289 exhibits significant protection upon complex formation (Fig 5, lower left).

Allosteric conformational change can be induced by antibody binding, resulting in a change in D-uptake level for regions remote from the epitope, and can be demonstrated by HDX/MS.<sup>41</sup> In addition to the segments corresponding to the known 2B5 epitope, some other peptide segments generated significant ARDD values. For example, segments 211–217, 212–217, and 373–378 gave ARDD values of 15.4%, 16.0%, and 21.2% (Supplemental Tables S1 and S2). In contrast to the known epitope region, these "remote" segments typically exhibited less than 0.5 D-uptake difference between free and complexed antigen (Supplemental Figure S1). Those smaller differences represent an allosteric effect of antibody binding.

### Molecular modeling for rAna o2 epitopes on binding to mAbs 2B5 and 1F5

A molecular model of trimeric recombinant Ana o 2 was generated by comparison to the X-ray structure of a soybean homologue (glycinin, pdb 1od5). The three identical subunits are

colored gray, black, and blue. The amino acids corresponding to the HDX defined epitope-bearing segments are colored purple (2B5) and red (1F5) (Figure 6, right).

In summary, we have validated the method of solution-phase HDX coupled with high resolution mass analysis as an alternative to traditional biophysical methods for the determination of protein-protein contact regions in molecular complexes involving high molecular weight ligands. In the absence of previously indicated epitope locations, a hierarchy of likely epitope-bearing segments would be evaluated based on the ARDD values. Confirmation of epitope locations would require identification by alternative means such as the more traditional site-directed mutagenesis, peptide screening, or chimeric protein expression. The real advantage of HDX in this context is that epitope searches and fine mapping could be targeted to a specific region, thus requiring considerably less overall effort than starting *de novo* with conventional epitope mapping approaches.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

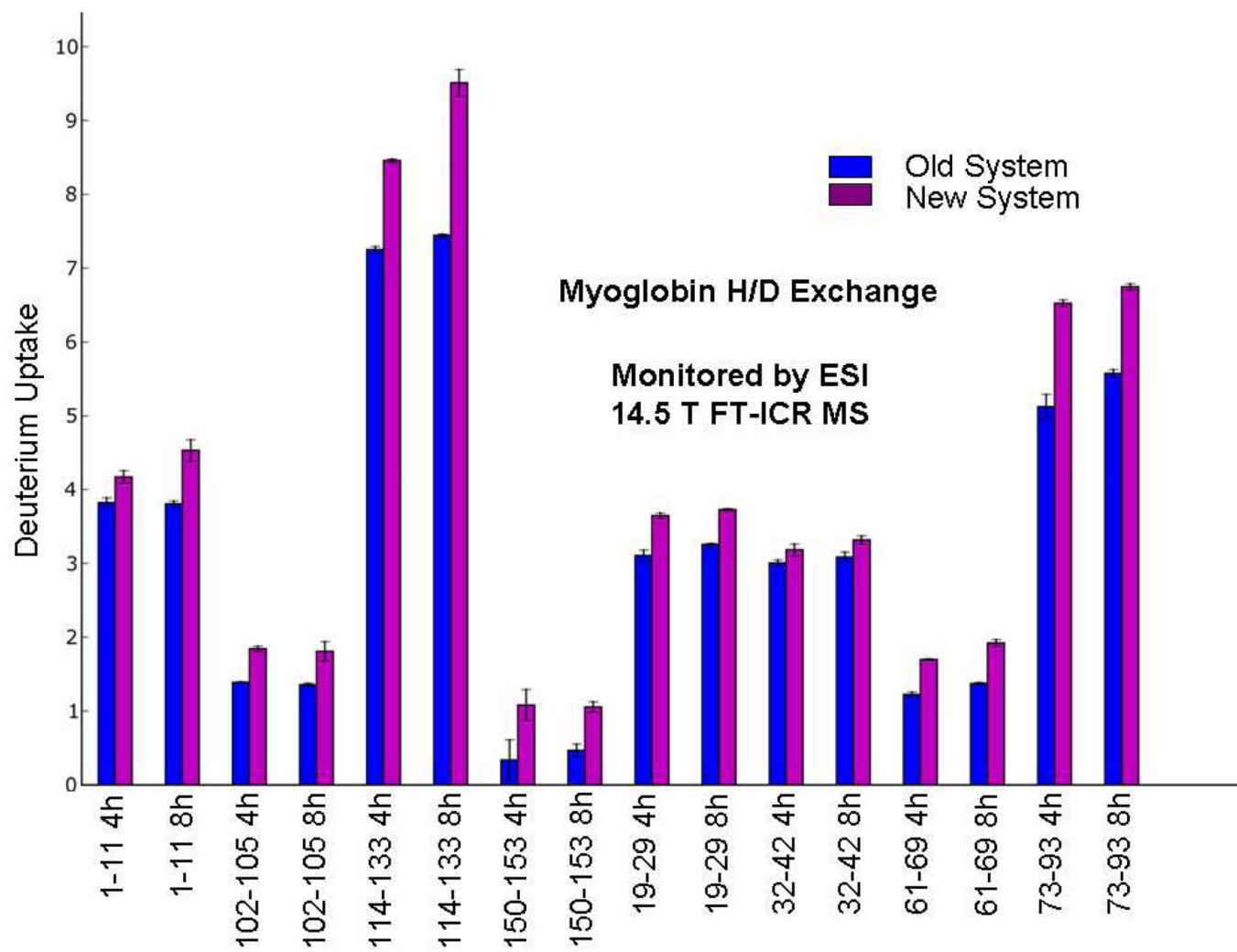
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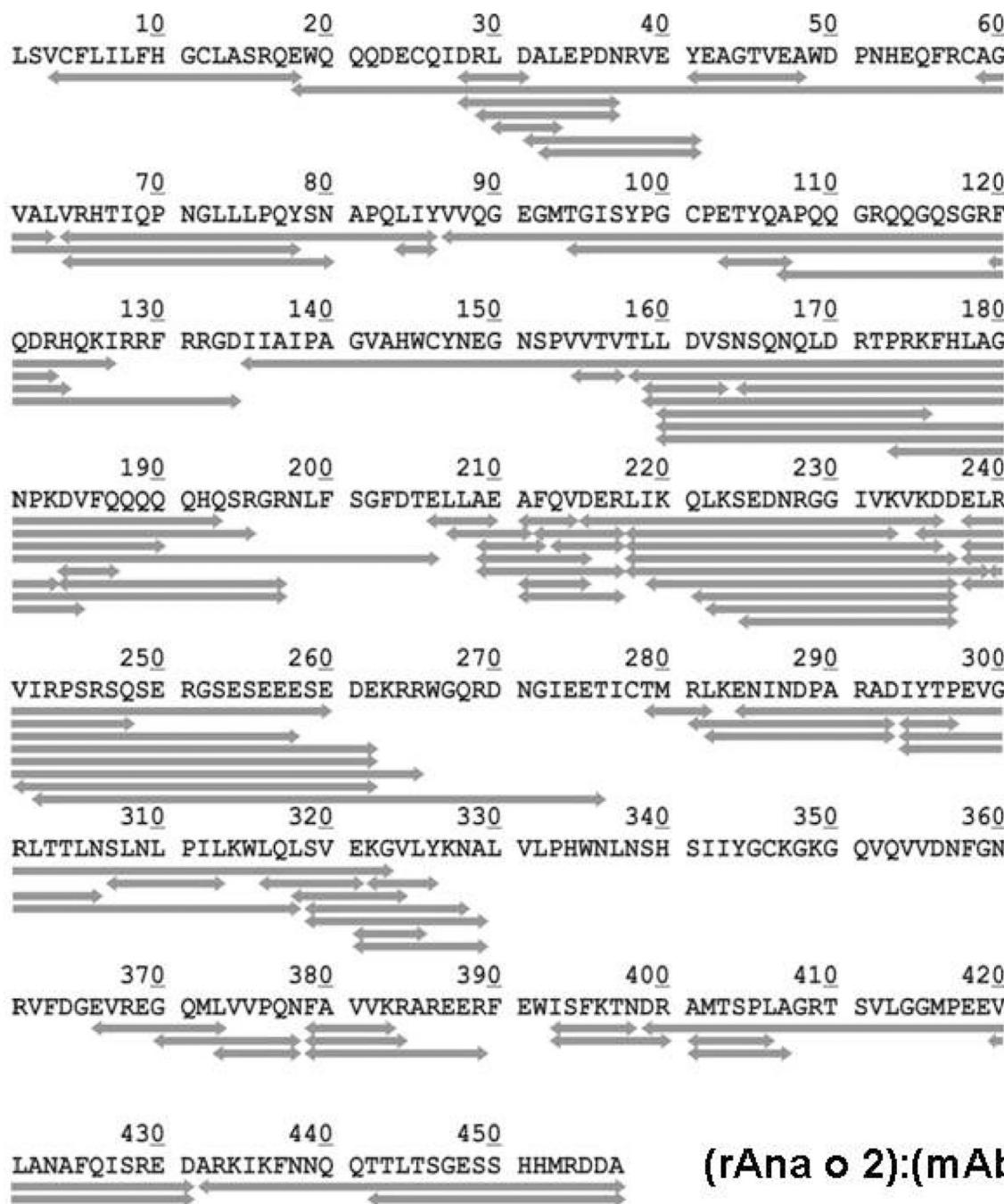
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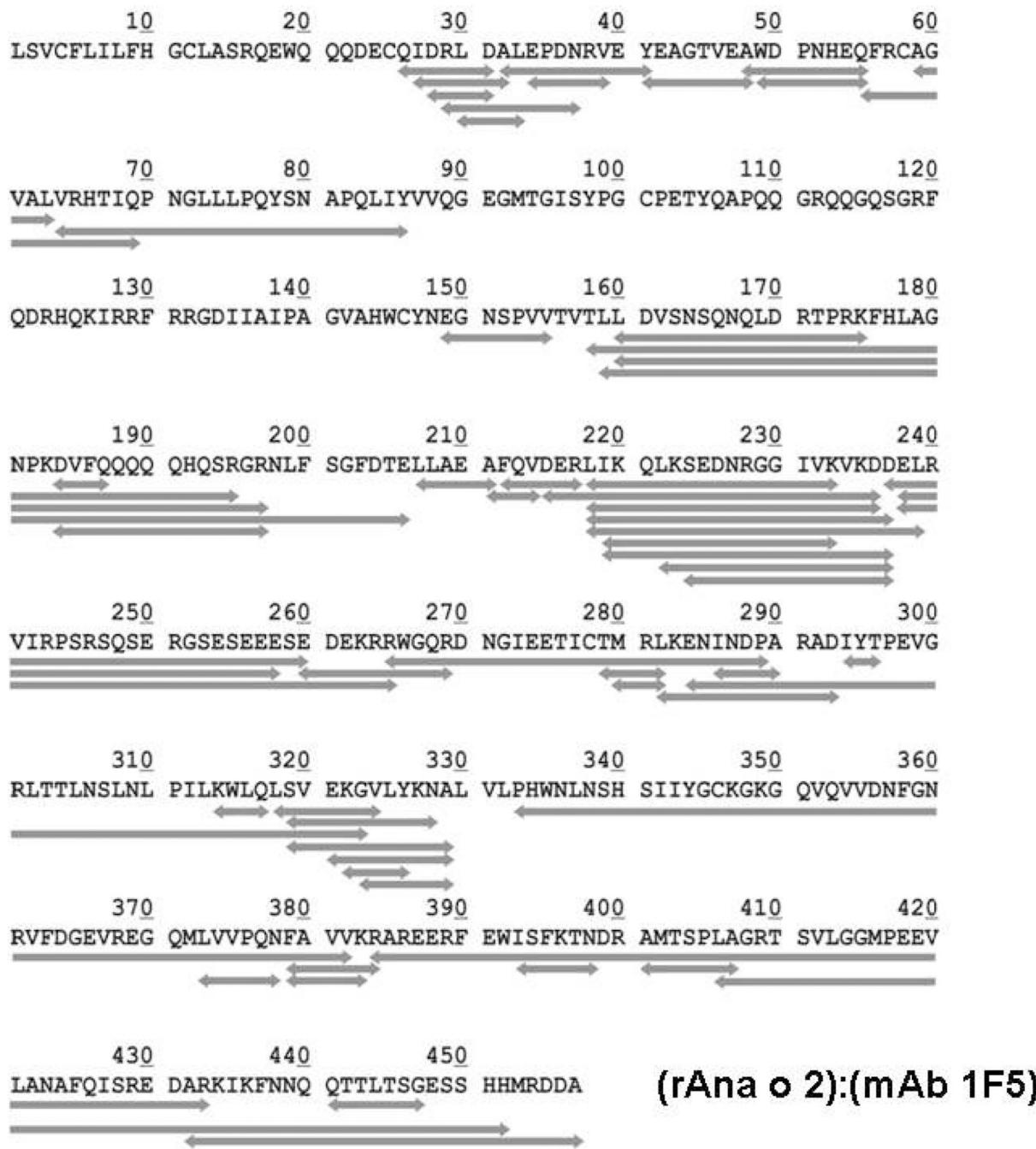
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**Figure 1. Improvement of HDX System (Myoglobin)**

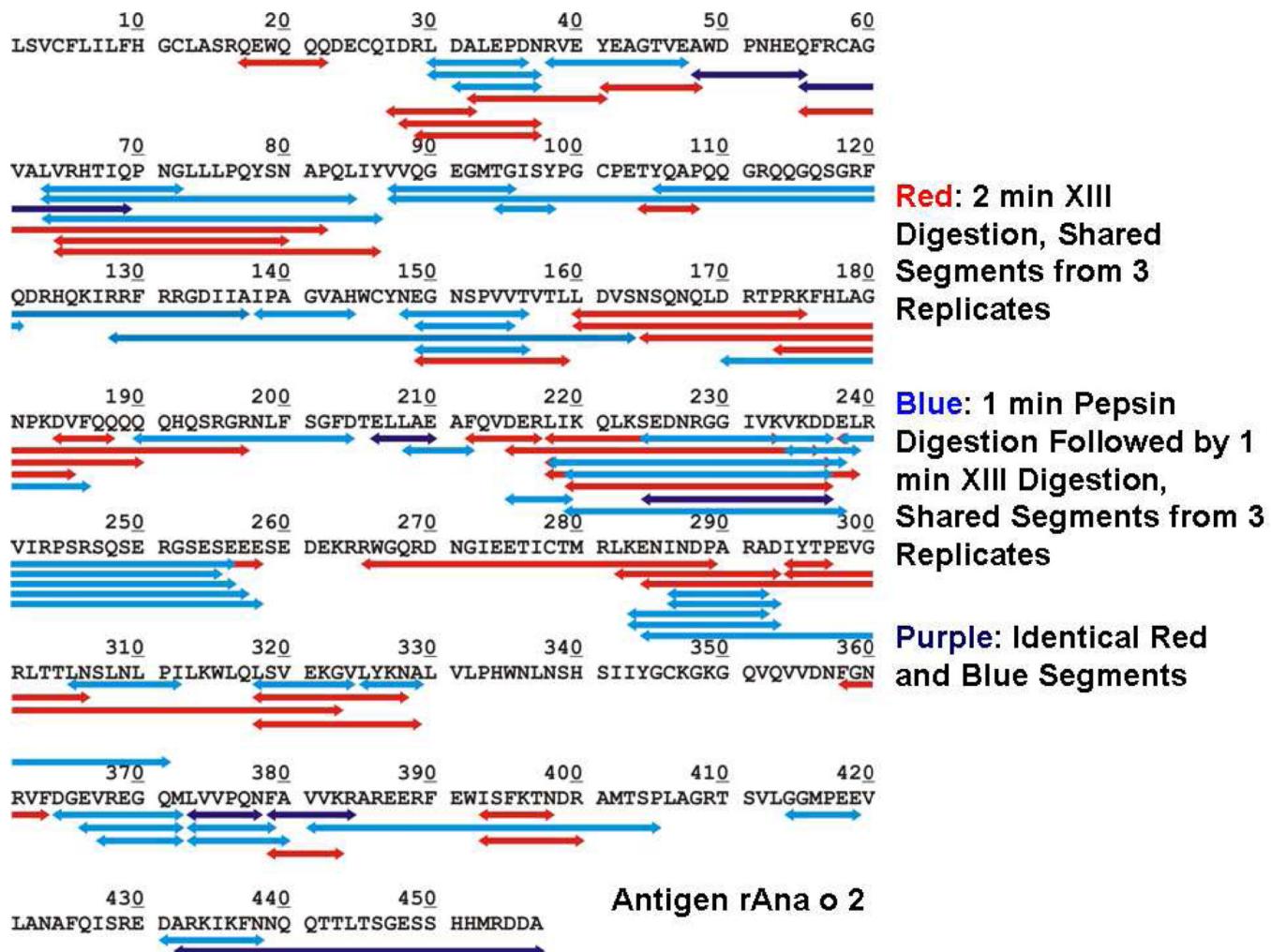
Deuterium uptake for 16 segments of myoglobin after exchange for 4 and 8 hours (old vs new system). The optimization of HDX instrumentation and automation gives increased deuterium uptake for all 16 segments, due to reduced back-exchange.



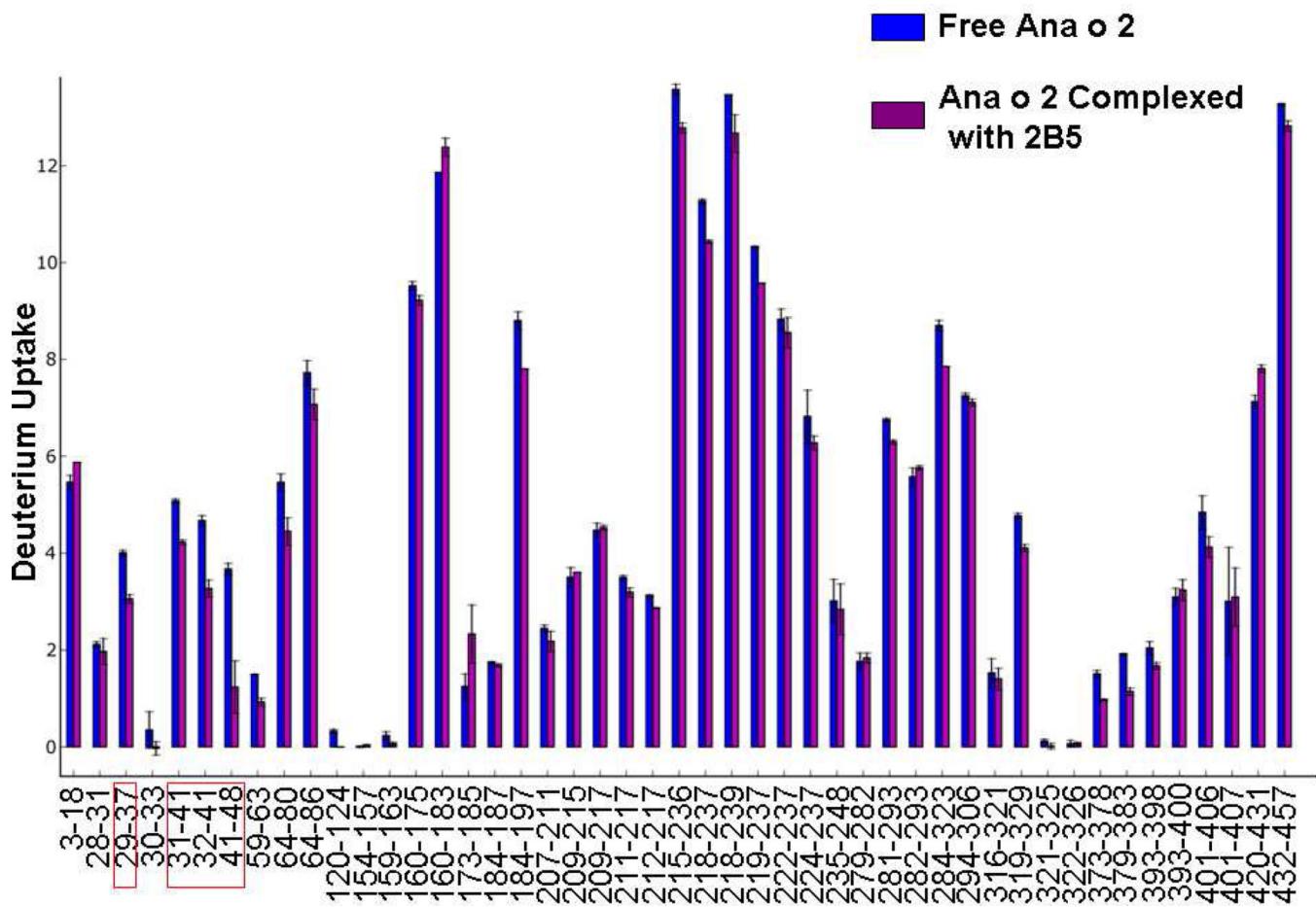


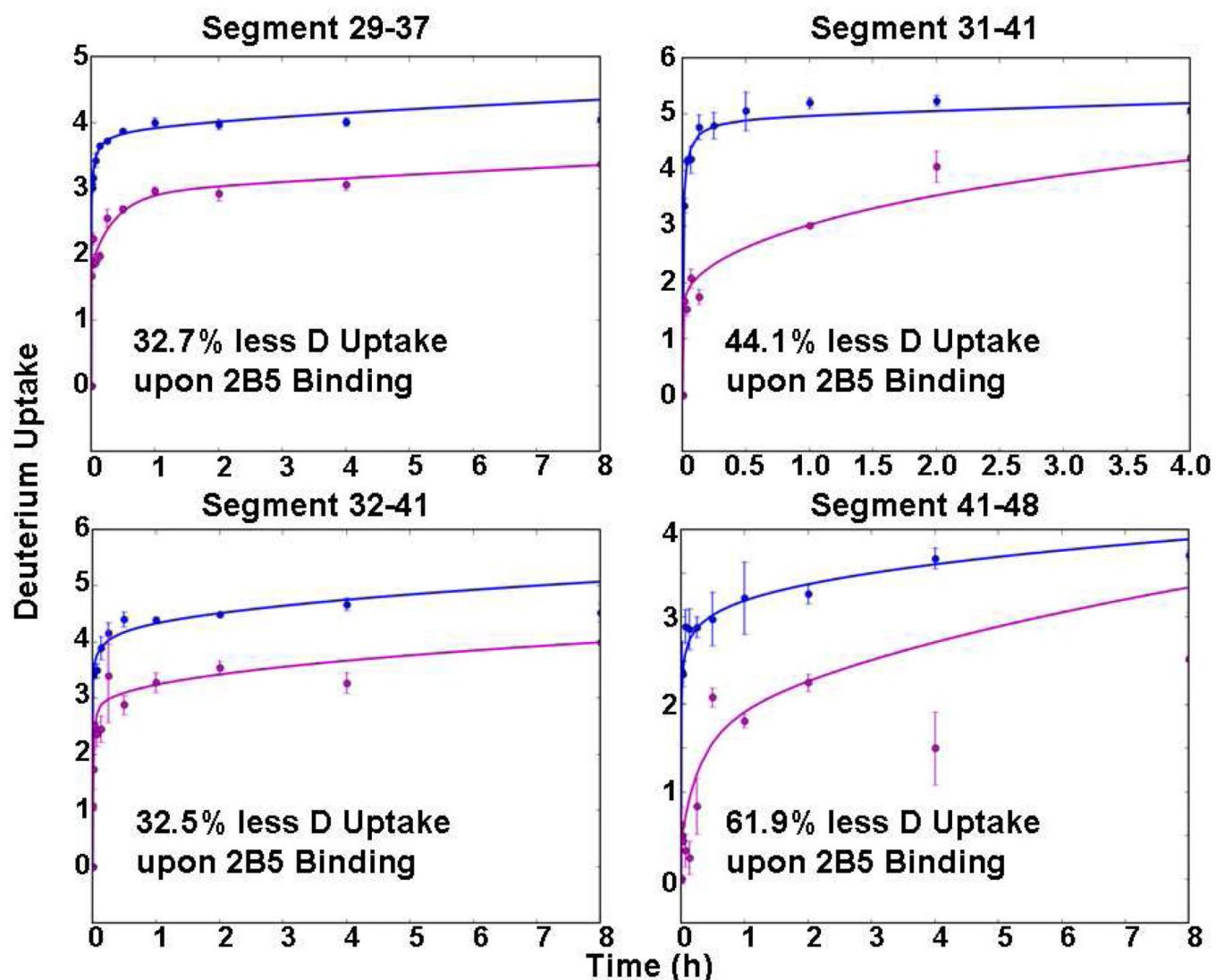
**Figure 2. Sequence Coverage for Common Segments for Both Antibodies**

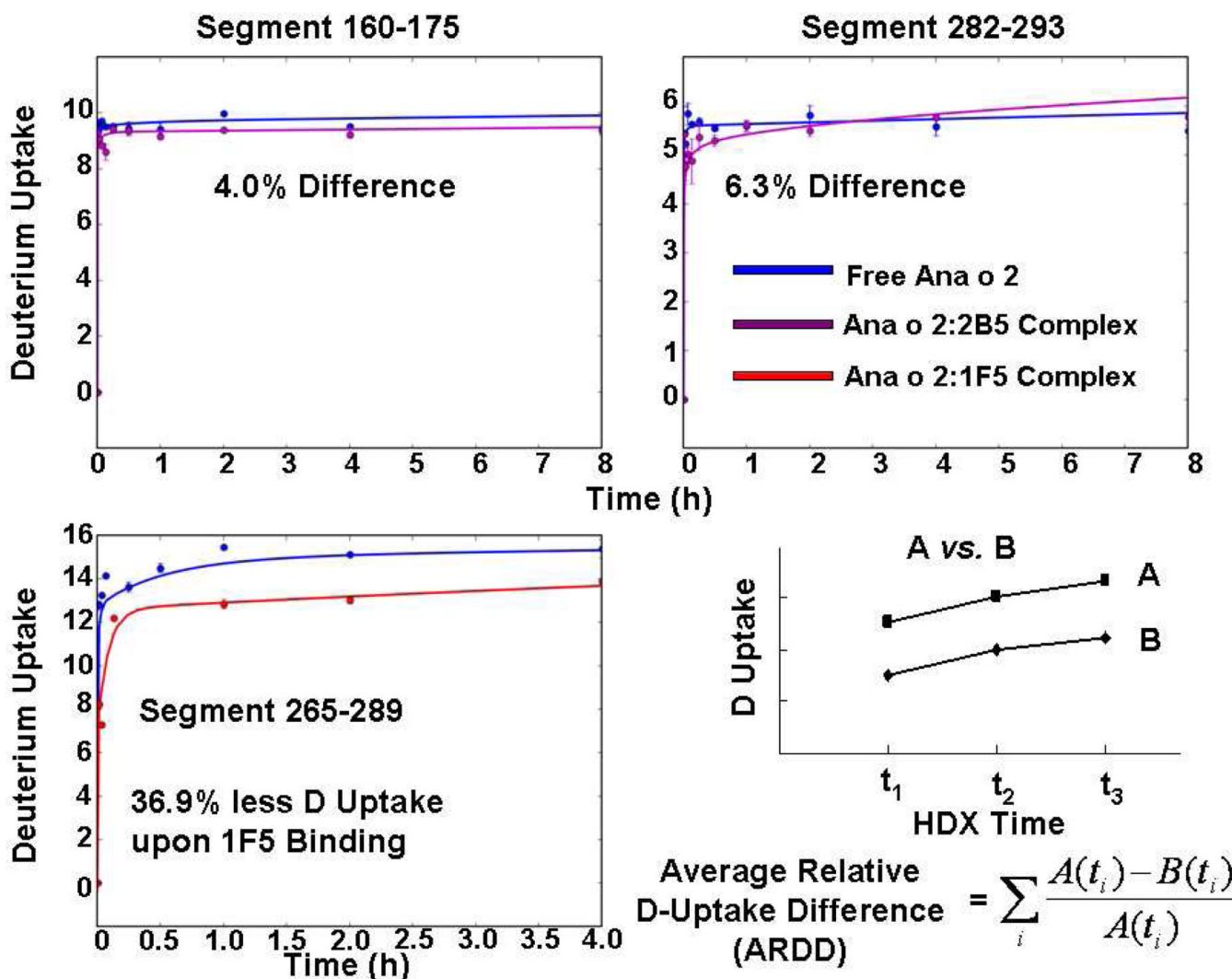
Sequence coverage for analyzed identical segments of different rAna o 2 forms. Sequence coverage for identical proteolytic segments of free rAna o 2 and rAna o 2 complexed with mAb 2B5 (top) or mAb 1F5 (bottom). The segments cover 90% (mAb 2B5) and 80% (mAb 1F5) of the sequence based on 126 (mAb 2B5) and 77 (mAb 1F5) identical segments. All segments for both complexes were identified in at least 2 of the 3 replicates for every H/D exchange period.

**Figure 3. Effect of Dual Enzyme Digestion**

Dual enzyme digestion of the rAna o 2 antigen. The red segments are generated by 2 minute protease XIII digestion (with 4 M urea to unfold the proteins and TCEP to break disulfide bonds) and the blue segments are generated by 1 min pepsin digestion followed by 1 min protease XIII digestion. Only those segments identified in all 3 replicates are pictured. The dual enzyme digestion approach produces more segments than obtained by protease XIII digestion alone and provides better sequence resolution, especially for large proteins.







**Figure 5. D Uptake Profils for Selected Segments**

Deuterium incorporation vs. H/D exchange period for selected segments of free and complexed rAna o 2. Segments 29–37, 31–41, 32–41 and 41–48 exhibit less deuterium uptake for rAna o 2 complexed with mAb 2B5, thereby defining the 2B5 epitope. Deuterium uptake profiles are similar for segments 160–175 and 282–293. For the binding of mAb 1F5, only one segment (265–289) shows significant protection on complexation, thereby defining its epitope.

— TSSKFNECQLNNLNALEPDHRVESEGGLIETWNSQHPELQCAG 43  
 LASRQEWWQQQQECQIDRLDALEPDNRVEYEAGTVEAWDPNHEQFRCAG 60  
 . : \*\*\*:., \*:\*\*\*\*\*:\*\*\* \*, \* :\*:\*, . \* :\*:\*\*\*

LHLPSYSPYPQMIIIVVQGKGAIGFAFPGPCPETFEKPQQQSSRRGSRSQ 103  
 LLLPQYSNAPQLIYVVQGEGMTGISYPGCPETYQAPQQ—GRQQGQSG 118  
 \* \*\*.\*\* \* \*\*: \* \*\*\*\*: \* \*;:\*\*\*\*\*: \*\*\* .\*: .:\*

FNEGDVLVIPPGPVWYNTGDEPVVAISLLDTSNFNNQLDNPRVFY 163  
 FRRGDIIIAIPAGVAHWCYNEGNSPVVTLLDVSNSQNQLDRTPRKFH 177  
 \* , \*\*:., \*\*.\*\*. \* \* \*\*:., \*\*\*:\*\*\*, \*\* :\*\*\*\*:.\*:\*

MQQQQQQQKSHGGRKQQHQQQEEEGSVLSGFSKHFLAQSFNTNEDTA 223  
 FQQQQQQHQSR—————GRNLFSGFDTELLAEAFQVDERLI 219  
 :\*\*\*\*\*:;\*: \* .:\*\*\*. .:\*\*\*;\*: .:\*

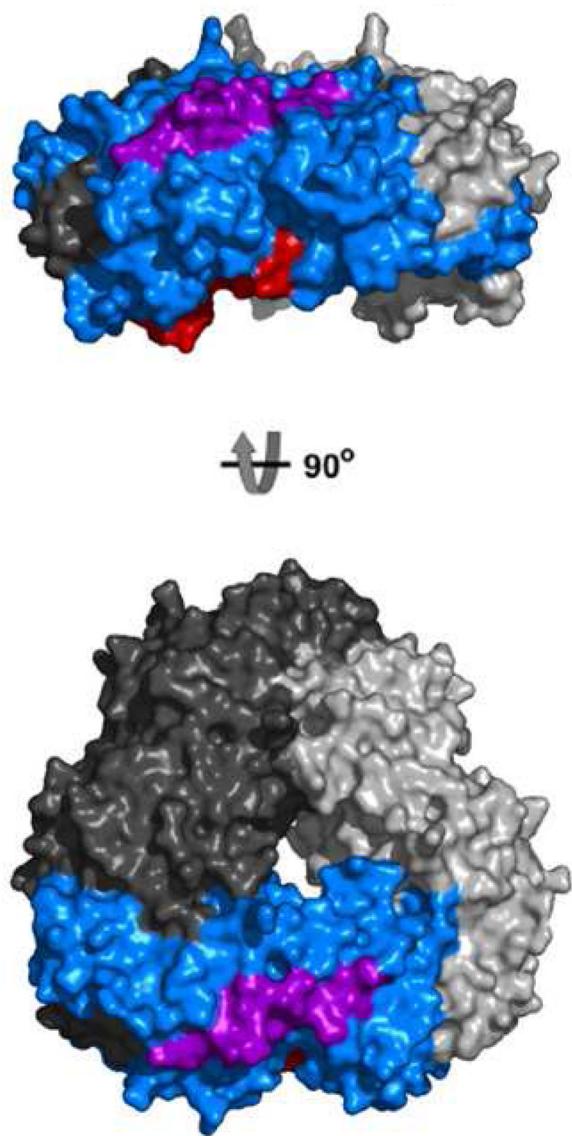
IVTVEGG—LSVISPKWQEDEDEDEDEEYEQTSPYPPRRPSHGKHD 282  
 IVVKVDDDELRVIRPSRSQSE————RG 252  
 \*\*.\*\*.: \* \*\* \*, .:.\* ..

PDHPPQRPSRPEQQEPGRGCQTRNGVEENICTMLHENIARPSRADF 342  
 —————RWGQRDNGIEETICTMRLKENINDPARADI 294  
 \* \* \* :\*\*\*.\*\*\*\*:\*\*\*:\*\*\* \* :\*\*\*:

SLTPALRQFGLSAQYVVLRYRNGIYSPHWNLNANSVIYVTRGKGRVRV 402  
 SLNLPILKWLQLSVEKGVLKYKNALVLPHWNLNHSIIYGCKGKGQVQV 354  
 \*\*.\*\* \*: : \*\*. : \*\*\*:\*. : \*\*\*\*\*:\*\*\*:\*\*\* :\*\*\*:\*\*\*

LRRGQLLVVPQNFFVAEQGGEQGLEYVVFKTHHNNAVSSYIK—DVFR 459  
 VREGQMLVVPQNFAVVKRAREERFEWISFKTNDRAMTSPLAGRSTVLG 414  
 :\*.\*:\*\*\*\*\*:\*, .:.\*: :\*\*\*:\*\*\*:.\*:.\*: .:.\*:

LGQSQVRQLKYQGNGPLVNP————— 492  
 ISREDARKIKFNNQQTTLTSGESSHHMRDDA 457  
 .:..:.\*:.\*:..:..:.\*..



**Figure 6. HDX Epitope Mapped onto X-ray Structure**

Structural models for trimeric Ana o 2. Left: The sequences of Ana o 2 and its glycinin homologue are aligned with the HDX-defined 2B5 and 1F5 epitopes boxed in purple and red, respectively. Asterisk (\*) denotes aa identity, colon (:) indicates aa similarity. Right: The epitopes are colored similarly for the trimeric X-ray model (pdb ID 1od5).

**Table 1**

Proteolysis results for different rAna o 2 forms. Shared segments are shown in Figure 2.

	Sequence Coverage	Number of Segments
Free rAna o 2	97%	211
rAna o 2 in complex with 2B5	91%	157
rAna o 2 in complex with 1F5	89%	152
Shared segments between		
rAna o 2 and Ana o 2-2B5 complex	90%	126
Shared segments between		
Ana o 2 and Ana o 2-1F5 complex	80%	77