

Epitope mapping of 7S cashew antigen in complex with antibody by solution-phase H/D exchange monitored by FT-ICR mass spectrometry

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The potential epitope of a recombinant food allergen protein, cashew Ana o 1, reactive to monoclonal antibody, mAb 2G4, has been mapped by solution-phase amide backbone H/D exchange (HDX) monitored by Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS). Purified mAb 2G4 was incubated with recombinant Ana o 1 (rAna o 1) to form antigen:monoclonal antibody (Ag:mAb) complexes. Complexed and uncomplexed (free) rAna o 1 were then subjected to HDX-MS analysis. Five regions protected from H/D exchange upon mAb binding are identified as potential conformational epitope-contributing segments. Copyright © 2015 John Wiley & Sons, Ltd.

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Introduction

Tree nut allergies are one of the most common, severe, and lifelong persistent food allergies among children and adults.^[1,2] Food allergies appear early in life, and the incidence of severe food allergies has increased over the past few decades.^[3–6] Currently, the related costs associated with food allergies in the United States are estimated to be \$25 billion annually.^[7] Reactions to peanut and tree nuts can trigger anaphylaxis in allergic patients. Unlike allergy to milk and egg, peanut and tree nut allergy is often not outgrown and can be observed at a very early age.^[8] Children who are allergic to cashew may face equal or even higher risk of anaphylaxis than those allergic to peanuts.^[9,10]

Cashew nuts are composed of approximately 15–25% protein. Three conserved allergenic seed storage proteins characterized in cashew include the 7S globulin Ana o 1,^[11] the 11S globulin Ana o 2,^[12] and the 2S albumin Ana o 3.^[13] 7S globulins, also known as vicilins, are members of the cupin-like class of proteins. These proteins are characterized by a large β -barrel structural conformation. Vicilins are typically found as homotrimers in their native state, with a molecular weight of 140–180 kDa, comprised of protomers of 40–80 kDa. The cashew 7S globulin, Ana o 1, is a 50-kDa protein in the vicilin family; however, it shares no known common linear epitopes with its homolog, peanut vicilin allergen Ara h 1.^[11] However, cross-reactivity between the closely related cashew and pistachio 7S vicilins has been observed in serum IgE of patients allergic to both.^[14] Likewise, 2G4 has been shown to react with both Ana o 1 and the pistachio 7S, Pis v 1.

The basis for understanding how these molecules are recognized by the immune system is epitope mapping. Epitope mapping identifies the specific sites on the allergen, i.e., the epitopes, responsible for the interaction with antibodies. Epitopes may be categorized as either linear or conformational. A linear epitope is composed of a

short contiguous amino acid segment, whereas a conformational epitope is composed of discontinuous polypeptide segments brought into spatial proximity by the folding of the protein in its native structure.^[15–17] Linear epitopes may be discovered by testing the reactivity of a defined set of peptides with the antibody. A suitable series of peptides can be generated by proteolysis, clonal expression, or liquid or solid phase synthesis.^[18,19] Conformational epitope mapping is more challenging. It is most often determined indirectly by use of binding competition with other antibodies targeting known epitopes or through site-directed mutagenesis. Both methods suffer from the potential influences of undefined allosteric changes leading to misidentification of epitope locations.^[20,21] Moreover, mutations at contact residues do not necessarily result in decreased mAb binding, and mutation of residues that lie outside of the epitope can result in weaker binding. Given recent evidence for the prominence of conformational epitopes recognized by allergic patients' IgE, methods to characterize them at the structural level are gaining impetus.^[22,23]

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Other approaches to epitope mapping include X-ray crystallography,^[23] nuclear magnetic resonance (NMR),^[24] chimeric molecule analysis,^[25] alanine scanning mutagenesis, hydrogen–deuterium exchange,^[26,27] electron microscopy,^[28] and phage display.^[18] The gold standard for epitope characterization is X-ray crystallography of the antigen–antibody complex; however, it is not readily applicable for many antigens and antibodies, and imposes daunting requirements in terms of the amount of material, crystallinity, and data analysis. The major limitation of NMR-based epitope mapping, aside from a high degree of sophistication, technical expertise, and expensive instrumentation, is that it is typically restricted to relatively small proteins (<30 kDa).^[29]

Here we demonstrate an approach based on hydrogen/deuterium exchange (HDX) monitored by high resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) to identify and map epitope-contributing regions of recombinant Ana o 1 protein recognized by mAb 2G4. Recent improvements in HDX analysis monitored by high resolution FT-ICR MS^[30] include automation,^[31] faster chromatographic separation,^[32] more efficient protein digestion,^[33] and an enhanced data analysis package.^[31] The improved HDX-MS methodology has achieved successful epitope mapping in various biological systems.^[26,27,34]

The 2G4 epitope of cashew allergen Ana o 1 has not previously been characterized or mapped by any method, and so it remains to be determined whether this epitope is linear or conformational. Here we demonstrate that the cashew 7S globulin allergen, Ana o 1 2G4 epitope is susceptible to denaturation, and we apply HDX-MS to identify likely regions of mAb contact. HDX analysis was accomplished by comparing backbone H/D exchange of free recombinant (r) Ana o 1 to rAna o 1 bound to the mAb 2G4 (rAna o 1:2G4). We show that the 2G4 epitope is conformational, and as revealed by HDX, five discontinuous regions likely contribute to the 2G4 epitope. Two of the regions overlap previously reported linear epitope-contributing regions,^[11] and three are newly identified.

Experimental design

Expression and purification of recombinant Ana o 1 fusion protein

Recombinant Ana o 1 was produced as previously described.^[11] Briefly, *Escherichia coli* BL21 DE3 cells were transformed with an expression plasmid containing the DNA construct for Ana o 1 by the heat shock method. Positive transformants were screened on LB agar plates containing 100 µg/mL ampicillin. Selected colonies were grown in LB medium containing 100 µg/mL ampicillin to an optical density of 0.5 at 600 nm. Expression of the fusion protein was induced with a final concentration of 0.3 mM IPTG.

Cells used for expression of the recombinant fusion protein were resuspended in amylose resin buffer (20 mM Tris, pH 7.4, 200 mM NaCl, 2 mM β-mercaptoethanol, 1 mM EDTA) and lysed by sonication. Particulates and solids were pelleted from the lysate by centrifugation at 10 000 rpm for 30 min. The lysate supernatant was incubated with amylose resin with gentle agitation for 30 min. Amylose resin was pelleted by centrifugation at 2000 rpm for 5 min and washed with 3 column volumes of column buffer 3 times. Recombinant fusion protein was eluted with column buffer containing 10 mM maltose.

Monoclonal Anti-Ana o 1 antibody

The 2G4 mAb was raised and produced in the Hybridoma Facility at Florida State University by use of standard techniques.^[32] The

guidelines for animal care and welfare described in the 'Guide for Care and Use of Laboratory Animals Resources' (National Research Council, National Academy Press revised 1996) were followed. Briefly, mice were immunized with 40 µg of rAna o 1 in RIBI adjuvant at 3-week intervals and were given a final injection of 25 µg of rAna o 1 in saline equally split between intravenous and subcutaneous routes.^[14] The resulting hybridomas were screened and assayed for relative strength and specificity by direct binding ELISA.^[35]

Polyacrylamide gel electrophoresis and protein transfer

Cashew protein extract^[36] (5 µg per 4-mm-wide well) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%). Samples were boiled in SDS loading buffer, both with and without β-mercaptoethanol. After SDS-PAGE, samples were transferred to nitrocellulose membranes (Whatmann GmbH, Dassel, Germany) by wet electroblotting.^[37]

Western blot analysis

Western blotting was performed as previously described.^[38] Briefly, nitrocellulose membrane containing cashew proteins was blocked overnight at 4 °C in phosphate buffered saline (PBS, 10 mM phosphate, 2.7 mM KCl, 140 mM NaCl, pH 7.2, EMD Chemicals, Gibbstown, NJ) with 0.05% Tween 20 (PBS-T) containing 5% non-fat dry milk. The membrane was then probed with hybridoma cell culture media containing the 2G4 antibody, diluted 1:1600 in phosphate buffered saline (PBS-T). The membrane was then washed in PBS-T and developed with a goat anti-mouse-horseradish peroxidase (HRP, Sigma Aldrich, St. Louis, MO) secondary antibody diluted 1:80 000.

Purification of 2G4 mAb

Hybridoma cell culture media supernatant containing the secreted mAb was mixed with equal volumes of purification buffer (0.1 M NaCl, 0.1 M sodium acetate, pH 5.0) and passed over protein G resin (Millipore, Billerica, MA). The resin was washed with 5 column volumes of purification buffer. MAb was eluted from the resin with 0.2 M glycine sulfate pH 2.7. Eluted mAb was neutralized with 1 M Tris base and dialyzed against 1× PBS (10 mM phosphate, 140 mM NaCl, 2.7 mM KCl).

Hydrogen/deuterium exchange

Stock solutions of 20 µM rAna o 1 and 20 µM rAna o 1–mAb complex were prepared in 1× PBS. To prepare the complex, we mixed an equivalent molar amount of mAb 2G4 with rAna o 1 (1:1) and incubated at 25 °C for 30 min to generate the rAna o 1–mAb complex. Note that each mole of mAb contains two moles of binding sites, and the molarity of rAna o 1 was calculated based on the mass of monomeric subunits. Therefore, the ratio of mAb binding sites to rAna o 1 epitopes for 2G4 is 2:1. HDX experiments were optimized and automated with an HTC Pal autosampler (Eksigent Technologies, Dublin, CA, USA). Five microliters of free antigen or antigen–mAb complex was mixed with 45 µL of 1× PBS in D₂O to initiate each H/D exchange period. For the blank control, the initial dilution was made in 1× PBS in H₂O buffer. After HDX incubation, each 50-µL sample was quenched by rapid mixing with 25 µL of 200 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), 8 M urea in 1.0% formic acid and 25 µL of a five-fold dilution of saturated protease type XIII (Sigma-Aldrich, St. Louis, MO) in 1.0% formic acid

(final pH ~2.3). Protease digestion was incubated for 3 min at ~1 °C, followed by LC-MS analysis. Each HDX reaction and assay was performed in triplicate. All HDX experiments were performed at ~1 °C, controlled by a Huber power water bath (Peter Huber, Offenburg, Germany).

On-line LC-ESI FT-ICR MS

After proteolysis, the peptides from free and mAb-complexed rAn a o 1 were separated and desalted with a high-performance liquid chromatography/supercritical fluid chromatography (HPLC/SFC) instrument (Jasco, Easton, MD) interfaced with an HTC pal autosampler (Eksigent Technologies, Dublin, CA, USA).^[27] For each LC-MS run, 45 µL of the protein digest was injected from a 50-µL loop to a Pro-Zap Expedite MS C₁₈ column (1.5-µm particle size, 500-Å pore size, 2.1 × 10 mm; Grace Davidson, Deerfield, IL). A rapid gradient from 2% B to 95% B in 2.5 min (A: acetonitrile/H₂O/formic acid, 5/94.5/0.5 vol/vol; B: acetonitrile/H₂O/formic acid, 94.5/5/0.5 vol/vol) was performed for eluting peptides at a flow rate of 0.3 mL/min. The LC flow rate was reduced by ~1/1000 by a post-column splitter for efficient electrospray ionization.

The ionized LC eluent was directed to a custom-built hybrid LTQ 14.5 T FT-ICR mass spectrometer (ThermoFisher, San Jose, CA).^[39] Positive electrospray ionization mass spectra were collected from *m/z* 380–1300 at high mass resolving power ($m/\Delta m_{50\%} = 100\,000$ at *m/z* 400, in which $m/\Delta m_{50\%}$ is mass spectral peak full width at half-maximum peak height).^[40] The total experimental event sequence for each sample was 6.5 min. External ion accumulation was performed in the linear ion trap with a target ion population of three million charges collected for each FT-ICR spectrum. LTQ-accumulated ions were transferred (~1-ms transfer period)^[41] through three octopole ion guides (2.2 MHz, 250 V_{p-p}) to a capacitively coupled^[42] open cylindrical ICR cell (55 mm i.d.) for analysis. The ion accumulation period was typically less than 100 ms during peptide elution, and the FT-ICR time-domain signal acquisition period was 767 ms (i.e., an overall duty cycle of ~1 s per acquisition). Automatic gain control^[43] and high magnetic field^[44] provided excellent external calibration.^[45,46] root mean square (rms) mass error typically less than 500 ppb.

HDX data analysis

Data were collected with Xcalibur software (ThermoFisher) and analyzed by in-house software.^[31,47] Time-course deuterium incorporation levels were generated by a maximum entropy fitting method.^[48]

Results and discussion

The peptide sequences of the cashew allergen protein, rAn a o 1 that are protected by the binding of mAb, 2G4 were mapped by solution-phase amide backbone H/D exchange coupled with FT-ICR MS and the results compared to previous mapping of this same antigen (or antigen-antibody combination) by immunological analyses. As described below, two of the peptide sequences for this mAb, revealed by the HDX analysis (segment 10–21 and 365–379), overlap two previous linear epitopes identified by screening a peptide library with pooled cashew-allergic patients' sera.^[11] Other peptide segments also exhibit decreased D uptake upon binding to the mAb, suggesting that they too contribute to the conformational epitope.

Analysis of the 2G4 epitope by western blotting

Preliminary insight into the nature of the epitope (i.e., conformational or linear) was gained by western blotting. The stability of the epitope was determined by comparing reactivity with 2G4 under reducing and non-reducing conditions. As seen in Fig. 1, the 7S protein is recognized by 2G4 under non-reducing conditions only: i.e., reducing conditions denature the target protein to the point that mAb 2G4 no longer binds, thus demonstrating the conformational nature of the epitope. However, boiling in the presence of SDS, a treatment that can also denature many proteins, while leading to the dissociation of the trimer into its constituent subunits, did not destroy the epitope. Therefore, it can be safely assumed that the 2G4 epitope does not depend on the quaternary structure of the protein but rather is disrupted by the loss of the disulfide bonds coupled with the protein unfolding properties of the heat/SDS treatment, thereby classifying it as a conformational epitope.

HDX 'heat' map for free rAn a o 1 allergen

Recombinant rAn a o 1 is a protein with 512 amino acids. Epitopes for rAn a o 1 have been previously studied by immunoblotting of a solid phase overlapping peptide array.^[11] Eleven 15-amino-acid-long peptides were dot-blotted against the sera of cashew-allergic patients and 11 linear epitopes identified. To identify the peptide segments that likely contribute to the conformational 2G4 epitope and compare them to the previously identified linear epitopes, we used HDX to compare the deuterium uptake of rAn a o 1 in the absence and presence of mAb 2G4.

Initially, aliquots of free rAn a o 1 were incubated in deuterium buffer for 0.5, 1, 2, 4, 8, 15, 30, 60, 120, and 240 min, with each incubation period performed in triplicate (Fig. 2). HDX-MS analysis revealed some regions of rAn a o 1 that are relatively resistant to HDX and other regions that exchange more rapidly. Among the detected peptides, residues 10–21, 81–109, and 134–162 at the N-terminal region present higher deuterium levels, showing that the N-terminus is relatively more solvent-accessible. The mid-region (residues 163–203) is relatively less solvent-accessible, suggesting that it is buried and/or more structured. Segment 365–379 is reported to contain a linear epitope for rAn a o 1

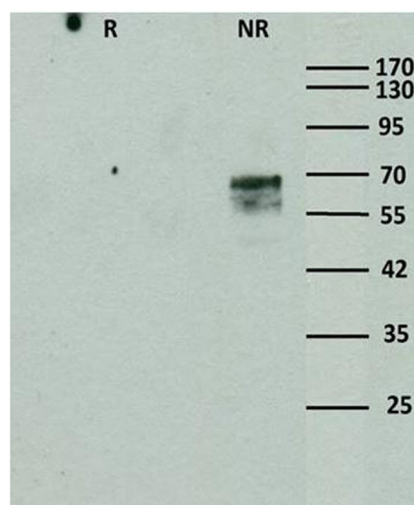


Figure 1. Western blot of cashew protein extract. Non-reduced (NR) and reduced (R) cashew aqueous extracts were electrophoresed, transferred to nitrocellulose and probed with mAb 2G4.

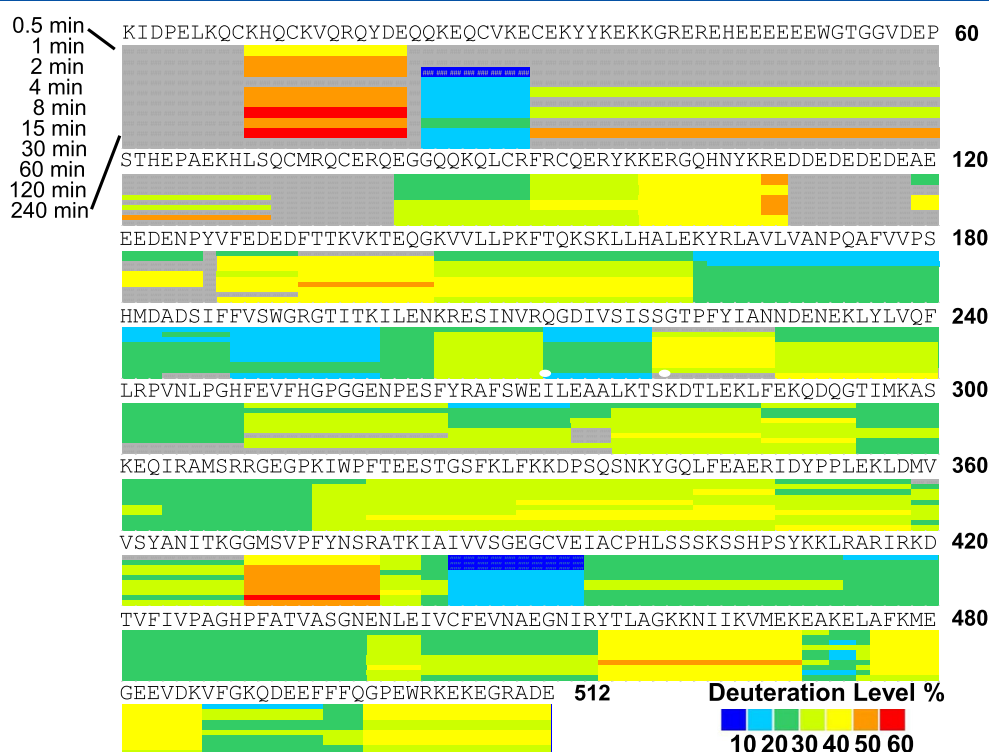


Figure 2. HDX 'heat' map for free rAna o 1 allergen. The deuterium level (in percentage) is calculated by dividing the observed deuterium uptake by the maximum possible deuterium uptake for each peptide (for example, a peptide consisting of n amino acids can take up a maximum of $n - 1$ observable deuteriums if no prolines are present). For each peptide, the calculated deuterium level for each of the experimental HDX incubation periods (top left, proceeding from top to bottom, shown for the first 60 AA segments: incubation periods of 0.5, 1, 2, 4, 8, 15, 30, 60, 120, and 240 min) is mapped onto the sequence. Residues are colored grey if no data are available for that residue for that incubation period. (This figure is available in colour online at wileyonlinelibrary.com/journal/jms.)

recognized by antibodies in sera from cashew-allergic patients.^[11] The high HDX rate for that segment in the free rAna o 1 HDX-MS data is consistent with location of that segment on the surface of the antigen and available for mAb binding: an important validation of the previous non-structurally constrained peptide-level finding.

HDX peptide recovery for free and complexed rAna o 1

After tenfold dilution in D₂O to initiate H/D exchange, the final concentrations of the antigen and mAb are relatively low (1 μ M) and in the presence of a high concentration of non-volatile salt. A special gradient was designed to compensate for the low sample concentration, followed by a divert valve to desalt each digest by directing the first-half-minute of eluent, mainly salt, to waste. We performed two identical sets of HDX experiments for the free antigen and the antigen–mAb complex. The HDX incubation periods were 0.5, 1, 2, 4, 8, 15, 30, 60, 120, and 240 min, each performed in triplicate. Only proteolytic peptides common to free and complexed rAna o 1 forms were compared for HDX analysis. For quality control, we also performed a parallel digestion experiment for the free and complexed antigen with no D₂O exposure. Figure 3 shows the common peptides between free antigen and its complexed forms mapped onto the rAna o 1 primary amino acid sequence. Without any exposure to D₂O, free antigen or its complexed form was denatured, digested by Protease XIII, and subjected to LC-MS analysis. That experiment serves as a blank control to ensure good sequence coverage by peptides common to free and complexed antigen, and good sequence resolution for deuterium uptake level. The sequence coverage for the common segments for mAb 2G4 is 76%,

with many overlapping peptides, providing spatial resolution at the 'peptide level'.

Determination of deuterium incorporation

After each H/D exchange period, the reaction was quenched and digested for 3 min. The resulting rAna o 1 segments were separated by passage through a ProZap (Grace Davidson, Deerfield, IL) C₁₈ column at 0.3 mL/min, and analyzed by positive ESI FT-ICR MS. The data analysis is a three step process. First, blanks (with no D₂O exposure) were analyzed to identify the proteolytic peptides for free and complexed rAna o 1. Second, the common segments were chosen for targeted assignment of proteolytic peptides following hydrogen/deuterium exchange. In the last step, a natural abundance isotopic distribution was generated, and multiple isotopic envelopes of all possible deuteration states were calculated. The isotopic distributions for deuterated peptides were calculated to search through the experimental data and identify the actual isotopic peaks for each HDX incubation period. The identified peak lists were then sorted according to signal-to-noise ratio (with a peak-picking threshold magnitude of at least 6 σ of baseline noise) and retention time. More than 140 segments were analyzed for each free and complexed rAna o 1 sample for each exchange period (in triplicate, plus controls). The total analysis was completed in a few hours with previously described software.^[30]

Potential epitope regions from HDX results

The HDX-MS experiment was repeated for the complexed rAna o 1 and the deuterium uptake for the free and complexed rAna o 1 was



Figure 3. Sequence coverage for proteolytic segments common to free rAn a 1 and rAn a 1 complexed with mAb 2G4. Without exposure to D₂O, free or complexed antigen was unfolded by urea and TCEP, digested by Protease XIII, and subjected to LC-MS analysis. The segments span 76% of the sequence based on 75 common segments. All segments for both complexes were identified in at least two of the three replicates for each H/D exchange period.

then compared. The calculated average relative increase in antigen D-uptake on binding to antibody (ARDD) for each common peptide (with a peptide length of 5–30 aa) reveals whether that segment is protected or unaffected by mAb binding. As shown in Fig. 3, there are few common peptides between the free and complexed rAn a 1 in the region 22–80, due mostly to abundant glutamic acid residues that hinder protease digestion.^[33] Indeed, we observe multiple exceptionally long peptides within that region. Those long acidic peptides are also more difficult to detect and thus are inconsistently observed. To circumvent this problem, peptides of any length, digested from the free or complexed protein, spanning the segment of interest but not necessarily of the same length, were used to calculate the ARDD for segments in that region. The HDX heat map for region 22–80 in both free and complexed rAn a 1, and the corresponding ARDD are shown in Fig. 4. For each H/D exchange period, the relative increase in D uptake on binding of rAn a 1 to the antibody is divided by the D uptake for free rAn a 1. The relative changes for that segment averaged over all H/D exchange periods define ARDD as described previously.^[27] For the present analysis, the potential epitope-contributing antigen segments are defined as the regions with overlapping peptides with ARDD values more negative than –10% (i.e., 10% reduction in H/D exchange on binding).

As shown in Fig. 5, five segments exhibit significant protection on antibody binding. The deuterium uptake profile for segment 10–21 shows more than 40% lower D uptake upon 2G4 binding. The decrease in D uptake for segment 352–362 is ~30% and for segment 365–379 is ~20%. The binding of 2G4 to rAn a 1 affords protection for aa segments 61–70, 204–211 and 277–287. Those regions may thus be assigned as the HDX-defined epitope-contributing segments targeted by mAb 2G4. Notably, the regions 10–21 and 365–379 are in agreement with previously determined epitope-

bearing segments 13–27 and 365–379.^[11] In addition, regions such as 23–46, 229–239, and 286–308 exhibit more than 10% increase in deuterium uptake upon antibody binding. The deuterium incorporation profiles are shown in Fig. 6, for the representative protected segments (10–21, 204–211, 277–287, and 365–379).

The homolog model of peanut Ara h 1 and the crystal structure of canavalin from jack bean were used to map the potential epitopes (Fig. 7). Two regions, segment 10–21 and 61–70, are not mapped because neither model/crystal structure contains the N-terminal region, which indicates that the current model does not fully explain the Ag:Ab interaction. Segments 277–287 and 352–379 are spatially close to each other and could function together in antibody binding. Segment 204–211 is located on a loop, distant from the other two mapped segments; therefore, its decrease in deuteration level more likely results from conformational change upon antibody binding. Canavalin is the closest homologous protein to allergen rAn a 1, with most of its sequence crystallized and resolved by X-ray crystallography. However, although the large N-terminal extension is present in the jack bean 2CAV crystal,^[49] it is not resolved in the crystallographic analysis, presumably because it exhibits more than one conformation. That hypothesis is supported by our results showing that the N-terminal region of allergen rAn a 1 has higher deuteration level, suggesting that it is most likely on the surface and/or unstructured. This finding demonstrates a distinct advantage of the HDX experiment over X-ray crystallography: namely that HDX enables the discovery of potential epitopes for dynamically structured regions.

Of the five potential epitope-contributing regions revealed by HDX, two closely overlap with those previously mapped by peptide-antibody dot blot. The observation of significantly decreased deuteration level in the region 10–21 and 365–379 upon antibody binding agrees with Wang et al.'s identification of 13–27

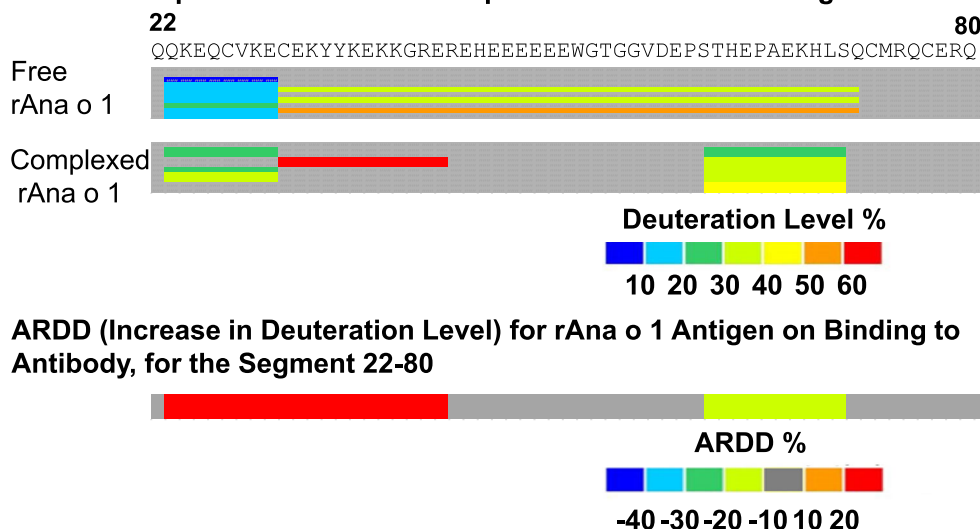
Deuterium Uptake for Free and Complexed rAna o1 for the Segment 22-80

Figure 4. Top: Heat map for the rAna o 1 antigen region 22–80, based on increase in H/D exchange for rAna o 1 antigen on binding to antibody. In this region, there are few common peptides between the free and complexed antigen, but several peptides of different length are found within the same segment. Bottom: Increase in deuterium uptake for the antigen region 22–80 on binding to antibody. Because of the lack of common peptides throughout most of this region, the ARDD of a segment is calculated based on the peptides, digested from the free or complexed protein, covering the segment of interest but not necessarily of the same length.

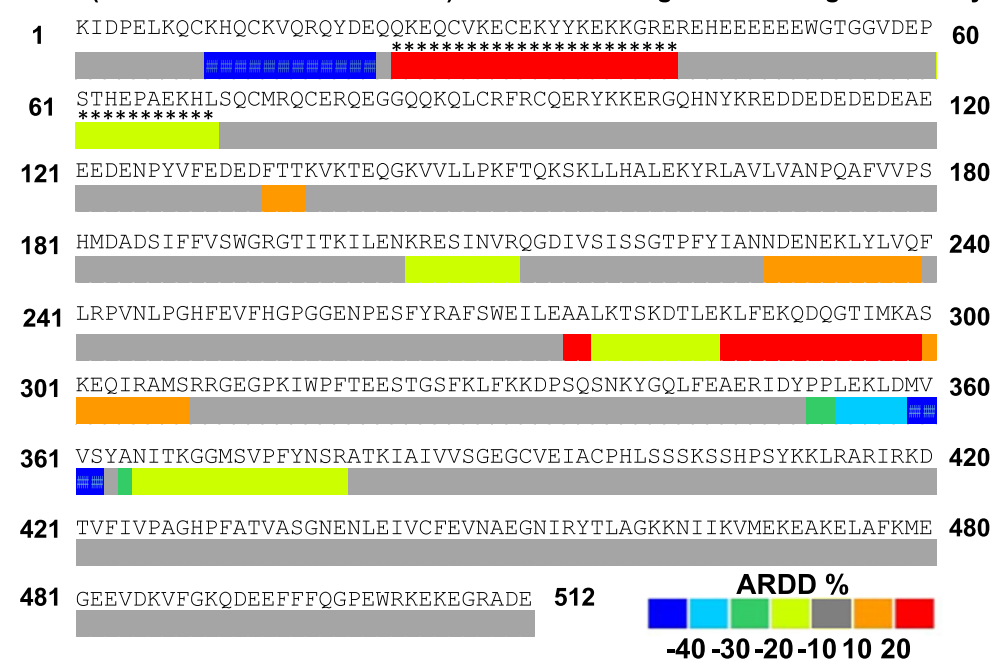
ARDD (Increase in Deuteration Level) for rAna o 1 Antigen on Binding to Antibody

Figure 5. H/D exchange results for free rAna o 1 and rAna o 1 complexed with mAb 2G4. For each of the proteolytic peptides common to free rAna o 1 and rAna o 1 complexed with mAb 2G4, the relative D-uptake increase for the antigen on binding to antibody (ARDD) is calculated as described previously.^[26] Rather than calculation based on common peptides, the ARDD calculated based on peptides, covering the segment of interest but not necessarily of the same length, are annotated by asterisks. Color coding for the ARDD value is shown at bottom right.

and 365–379 segments as linear IgE epitopes recognized by a pool of sera from 12 patients who were allergic to cashew.^[11] We propose that the D uptake decreased regions 10–21 and 352–379 are spatially proximal, at least upon antibody binding, and contribute to the conformational epitope. Because the mAb 2G4 binds to rAna o 1, the structure may open and expose the adjacent segments to

the solvent. Therefore, as the mAb 2G4 binds to potential epitope-contributing segments (e.g., segments 10–21 and 352–379, and possibly segment 61–70, 204–211, and 277–287 (labeled in blue or green) and alters the structure, the deuterium uptake by segments 23–46, 229–239, and 286–308 (labeled in orange and red) increases. Segments 10–21 and 352–379 appear to be the

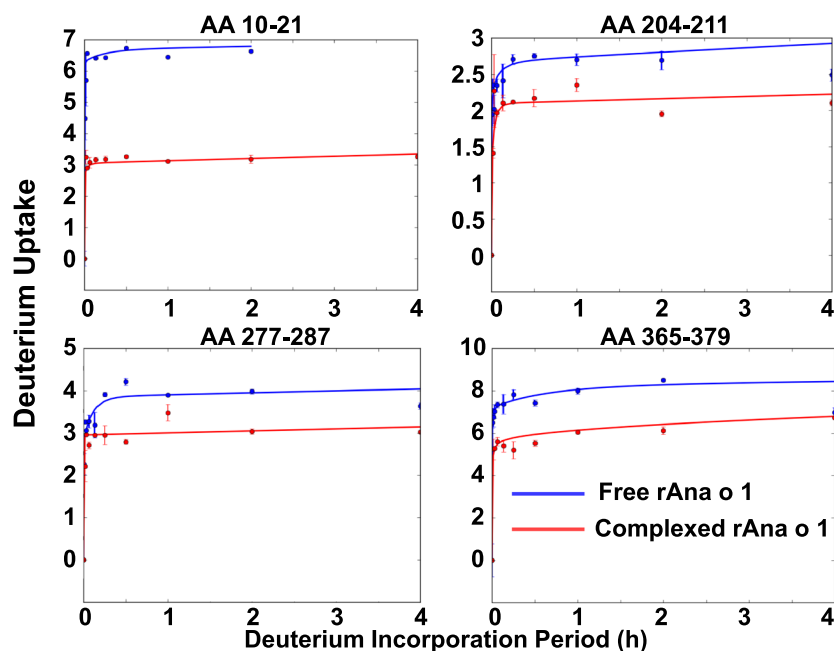


Figure 6. Deuterium incorporation versus H/D exchange period for selected segments of free and complexed rAna o 1. Segments 10–21, 204–211, 277–287, and 365–379 exhibit less deuterium uptake for rAna o 1 complexed with mAb 2G4, thereby defining the 2G4 epitope. Two of those peptide segments (10–21 and 365–379) are consistent with previously determined linear epitopes.^[11] (This figure is available in colour online at wileyonlinelibrary.com/journal/jms.)

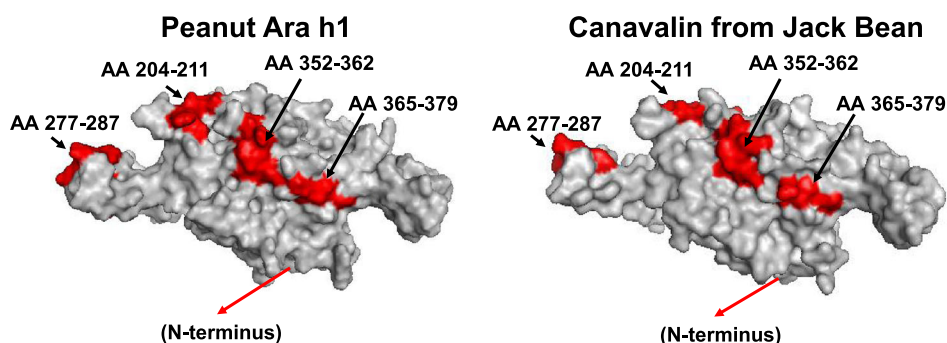


Figure 7. Potential epitope-contributing regions of Ana o 1 mapped onto the homolog model of Ana o 1. Left: Ara h 1 from peanut (P43237, SwissProt), Right: Canavalin from jack bean (pdb 2CAV). The N-terminus, about 120 amino acid long, is not structured, and is therefore not mapped onto either model. (This figure is available in colour online at wileyonlinelibrary.com/journal/jms.)

primary contributors to antibody binding, based on their large decrease in ARDD, and the decreases in D uptake at 61–70, 204–211, and 277–287 may be because of either direct interactions with the mAb or secondary allosteric conformational effects. We propose that elements of these segments may function both as independent linear epitopes when complexed with some antibodies and together may contribute to one or more conformational epitopes.

Because of the significant deuterium uptake decrease in the segment 10–21 upon antibody binding and its relatively high unbound HDX rate, we propose that the N-terminus of rAna o 1 may be on the surface of the molecule and at least partially unstructured. Conversely, the center of the protein sequence is more highly structured, due either to buried residues or hydrogen-bonding. The other epitope-contributing region 365–379 is close to the diproline segments 'PP', which is mostly associated with a helix turn^[50] and therefore may be located on the surface near the segment 10–21. The segment 365–379 is shown to be on the surface of the structure in Fig. 7. Therefore, the segments 10–21 and 365–379 may function together as a conformational epitope, and the binding

event may bring those segments closer together, resulting in an opening of the structure and increased deuterium level of other distal segments.

In summary, we have used solution-phase HDX monitored by high-resolution mass spectrometry as an alternative to traditional immunological/biophysical methods for the determination of regions contributing to a mAb reactive epitope. The present HDX experiments demonstrate that two of the previously reported linear epitope-contributing peptide segments also contribute to a conformational epitope and reveal three new peptide segments that may play a role in epitope expression. The precise degree to which the identified segments make direct contact with the mAb or have altered exposure to the solute as an indirect effect of antibody binding will require additional study.

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