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Mapping invisible epitopes by NMR spectroscopy

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Defining discontinuous antigenic epitopes remains a substantial challenge, as exemplified by the case of lipid transfer polyproteins, which are common pollen allergens. Hydrogen/deuterium exchange monitored by NMR can be used to map epitopes onto folded protein surfaces, but only if the complex rapidly dissociates. Modifying the standard NMR-exchange measurement to detect substoichiometric complexes overcomes this time scale limitation and provides new insights into recognition of lipid transfer polyprotein by antibodies. In the future, this new and exciting development should see broad application to a range of tight macromolecular interactions.

Like other types of antibodies, IgE is made in response to the presentation of various antigens. Once produced, IgE binds to mast cells, where it stands ready to trigger the future release of antihistamines, cytokines, and other defensive factors if the antigen is encountered again. Typically, IgE is a low-abundance isotype of immunoglobulin, but it can be sometimes found at higher circulating abundance; in this case, presentation of the antigen can lead to extreme responses associated with allergic disease and asthma (1). Identification and eventual prediction of allergen epitopes could yield crucial insights into allergic disease to manage IgE activity and prevent these extreme and potentially fatal events, but progress has been slow. This bottleneck has been especially difficult to overcome for antigens such as lipid transfer proteins (LTPs) that adopt a folded state during allergic sensitization. In these cases, the epitope recognized by the antibody depends on three-dimensional structure, not on a linear peptide motif that can be readily identified using routine screening methods or MS. An exciting new NMR-based methodology for epitope mapping (2) has the potential to accelerate allergen epitope identification, while also having clear applicability to a broad range of tight-binding macromolecular interactions.

To demonstrate their technique, Di Muzio and colleagues studied Art v 3, an LTP from mugwort pollen. LTPs are found in a variety of foods and pollens and are similar enough that an antibody generated in response to one allergen can often cross-react with the allergens from other, eliciting potentially life-threatening clinical manifestations (3). To explore this system, the authors first raised and purified a set of three murine mAbs against Art v 3. The authors confirmed that the selected mAbs recognize physiologically relevant epitopes by testing their ability to block the interaction between Art v 3 and immunological sera from 21 patients allergic to mugwort pollen. Then Di Muzio *et al.* set out to determine the binding epitope associated with each mAb.

When antibodies are raised against folded proteins, leading to structural epitopes, the best strategy to directly reveal the binding epitope is often determination of a co-crystal structure. However, successful attempts to crystallize complexes of lipid transfer proteins and mAbs remain extremely rare. Arguably, the gold-standard method for mapping protein-ligand interactions in solution is NMR chemical shift perturbation (4). However, high-resolution solution NMR is constrained by well-documented size limits, and antibody-antigen complexes are far too large for easy and rapid direct observation. Indeed, when Di Muzio attempted to record a representative NMR spectrum of the Art v 3–mAb complex, no chemical shift perturbations were observed. Instead, due to the slow tumbling of the large complex in solution, resonance intensity decreased uniformly toward a total loss of spectral peaks at saturating mAb concentration. One popular NMR approach to study the dynamics of protein complexes that are not directly detectable is chemical exchange saturation transfer spectroscopy (5). However, chemical exchange techniques depend on the complex displaying a lifetime of seconds or less; surface acoustic wave measurements of the Art v 3 interaction with the various mAbs investigated showed kinetic dissociation rates on the time scale of hours, precluding the use of standard techniques.

To overcome the problems imposed by slow complex dissociation, Di Muzio *et al.* turned to hydrogen/deuterium exchange of labile amides in the protein backbone. Motivated by the classic work of Englander and Kallenbach (6), this technique has seen decades of application to monitor protein structure and dynamics. Further, these techniques are readily applied to monitoring protein-ligand interactions (7). Unfortunately, traditional hydrogen exchange experiments, like chemical exchange saturation transfer, depend on faster dissociation of the complex than observed for Art v 3. In a clever modification of the traditional protocol, the authors added substoichiometric quantities of the mAb to Art v 3 samples prior to the onset of hydrogen/deuterium exchange. In a continuous NMR assay collected over 5–9 days, which the authors name hydrogen/deuterium exchange memory spectroscopy (HDXMEM; Fig. 1), the rate of hydrogen/deuterium exchange is recorded through serial acquisition of NMR spectra for unbound Art v 3 in equilibrium with the mAb-bound complex. Exchange rates for mAb-bound Art v 3 are compared with those of Art v 3 alone, allowing the differences in exchange time between the two data sets to identify regions of Art v 3 that undergo significant changes in chemical environment upon complex formation. In this way, the authors demonstrated that the three mAbs studied each bind structural

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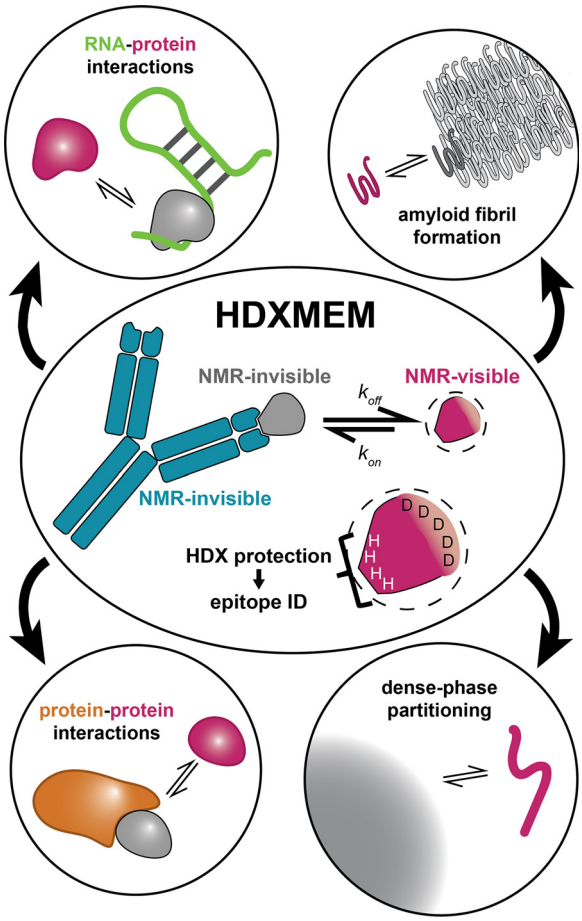


Figure 1. The HDXMEM technique and its potential applications to slowly dissociating macromolecular complexes. When a protein of interest (POI) (*pink*) associates very tightly with a large binding partner or assembly, it is unfeasible to detect the bound-state POI (*gray*) by traditional NMR methods. HDXMEM relies on protection of the binding surface in the NMR-invisible state under subsaturating conditions, where the unbound POI can be detected with a "memory" of the bound state. HDXMEM may advance detection and description of high-affinity, slowly dissociating complexes, such as protein-protein and protein-RNA interactions, the formation of amyloid fibrils, and dense-dilute phase equilibria.

epitopes spanning multiple units of secondary structure and/or loops. Further binding assays using other LTPs helped to refine the predicted binding epitopes, whereas assays using Art v 3 point mutants validated the predictions. Interestingly, there was only partial overlap between the determined epitopes for the three mAbs. Finally, the authors tested the point mutants against the sera from allergic patients. The mutations clearly disrupted binding in general, but the authors also observed a highly individual profile for each set of antibodies, suggesting we still have much to learn about the cross-reactivity that is frequently associated with LTP allergens in the clinic.

Whereas the results of this study are significant with respect to IgE function and lipid transfer protein allergy, perhaps its greatest impact rests in the broad utility of the new HDXMEM spectroscopy (Fig. 1). For example, RNA-protein interactions are an area where extremely tight binding can be expected (8), often driven by extremely slow dissociation of the complexes, and where determining structures is not generally easy. Hydrogen exchange monitored by MS has been applied to monitor amyloid fibril formation (9), as have NMR-based "dark state exchange" methods, but as mentioned before, this technique relies on dissociation being fast relative to the time scale of the NMR measurement. Where exchange between the soluble phase and the fibril is slow, this new method may help to conclusively investigate these interactions. Finally, this method could be extended to interrogate the mechanisms of liquid-liquid phase separation and, potentially, to monitor the transition from condensed liquid phases into gel states (10), which would lead to significant advances in understanding nonmembranous organelle formation in cells. Despite the rich history of hydrogen/deuterium exchange in the NMR field, it is clear that the innovative extension to very slowly dissociating macromolecular complexes will yield rich new biochemical insights in studies to come.

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Abbreviations—The abbreviations used are: LTP, lipid transfer protein; HDXMEM, hydrogen/deuterium exchange memory spectroscopy; POI, protein of interest.

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