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ANTIGEN–ANTIBODY INTERACTIONS: AN NMR APPROACH

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Abstract—With recent advances in methodology, it now appears that NMR can be used at an unprecedented level of sophistication to obtain new insights into the solution structure and dynamics of the antibody combining site, both free and in its complex with antigen. Most promising in this regard is the Fv fragment (molecular weight ~25 kD) which can be produced by genetic engineering in a form suitable for NMR studies. Isotopic labeling is required to make specific resonance assignments. NMR can also provide information on the conformational preferences of immunogenic peptides and can be used to probe the conformation and dynamics of peptides (appropriately labeled with ^{13}C or ^{15}N) bound to the Fab fragment (molecular weight ~50 kD) of antipeptide antibodies.

The molecular basis by which antibodies recognize protein antigens with such exquisite sensitivity remains a question of considerable importance. X-ray structures of several antibody–protein antigen complexes reported in recent years [1–4] have begun to provide new insights into the intermolecular interactions involved in antigenic specificity. However, it is still not clear to what extent conformational changes in the antibody or the antigen are an integral part of the binding process. Likewise, questions concerning flexibility of the antigen [5, 6] or the antibody combining site remain unresolved. In principle, high resolution NMR spectroscopy should provide answers to many of these questions since it is highly sensitive to local conformational changes and gives direct information on conformational flexibility and dynamics.

In the past, applications of NMR have failed to realize their full potential because of the high molecular weight, ~150 kD, of the antibody molecule. Proteolytic cleavage to the Fab (or Fab′) fragment is frequently possible (Fig. 1), but this still makes detailed NMR studies daunting since the molecular weight is about 50 kD. Nevertheless, many NMR studies of antibody Fab fragments have been reported, the most successful of which have utilized incorporation of deuterated amino acids into the Fab to allow reliable resonance assignments to be made [7–9]. Further proteolytic cleavage into the smaller Fv fragment (~25 kD) which still contains the antigen binding site is only rarely possible and NMR studies have been reported on only relatively few systems [10, 11]. However, with the application of the tools of molecular biology to immunoglobulins, it should now be possible to generate Fv fragments of almost any desired antibody [12–14] such that detailed NMR analysis is feasible. Cloning and expression of Fv fragments also facilitate the incorporation of isotopic labels directly into the protein. Through the combined use of molecular biology and isotope-edited NMR methods [15, 16], there now exists an unprecedented opportunity to investigate the detailed solution structure and dynamics of the antigen-binding domain, both free and in its complexes with protein, peptide or other antigens.

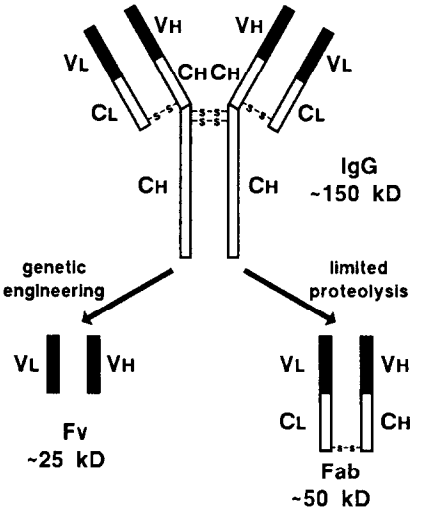

Fig. 1. Schematic diagram showing the derivation of Fab and Fv fragments from the immunoglobulin IgG molecule.

For the purposes of further discussion, we discriminate between the two classes of antibody; antipeptide antibodies induced by peptide fragments of proteins, and antiprotein antibodies induced by the native protein molecule. In other words, in one case the immunogen is a peptide, in the other a protein. The relatively frequent occurrence of antipeptide antibodies which also react with the cognate sequence in the intact protein argues for the importance of conformational preferences in the peptide in the induction of antibodies [17]. The role of mobility of the protein in the cognate region also appears to be significant [18]. The structure of the antigen in the antibody combining site may be unique, neither that observed for the majority of the peptide conformers in solution nor that observed in (for example) the X-ray structure of the protein. Thus,

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Table 1. Immunogenic peptides

Peptide	Response	Preferred structure in water	Ref.
Influenza virus hemagglutinin (residues 98–106)	B-cell	β-Turn	19
Myohemerythrin (residues 69–87)	B-cell	Nascent helix	20
Sperm whale myoglobin (residues 132–153)	B-cell T-cell	Helix	21
Streptococcus pyogenes 24 M protein (residues 6–34)	B-cell	Helix	22*
Malaria circumsporozoite protein repeat (NPNA)	B-cell	Helical	23†

* Karimi A and Wright PE, unpublished observation.
† Dyson HJ, Satterthwait A and Wright PE, unpublished observations.

the *actual* conformation of an antibody-bound peptide antigen is of considerable interest. In this paper we first review the evidence that immunogenic peptides frequently have a tendency to adopt folded structures in water solution, and then describe the strategies that we are using to investigate both antipeptide and antiprotein antibodies by NMR.

Conformational propensity of immunogenic peptides

NMR methods have been used to investigate the conformational propensities of a number of immunogenic peptides in aqueous solution [19–23]. In all cases examined by us to date, preferential conformations have been found (Table 1). On the basis of these observations, we have proposed that strongly immunogenic peptides which induce antibodies that bind with high affinity to the cognate sequence in the folded protein have such a high propensity to adopt folded conformations that they begin to do so in water solution [17]. This is likely to be particularly important for peptides that induce antibodies which recognize elements of regular secondary structure in the folded protein. Indeed, it is difficult to understand how an immunogenic peptide can induce antibodies that bind to, say, a helix in the native protein, unless the peptide itself has a tendency to adopt helical conformations in water, on the surface of the carrier protein that is used to enhance the immune response or when bound to the B cell receptor.

To investigate any influence of the carrier protein on the conformation of immunogenic peptides, we have developed a methodology for single site coupling of peptides to bovine pancreatic trypsin inhibitor for subsequent NMR studies [24]. Preliminary results indicate that the carrier protein can stabilize folded conformations, presumably by restricting the conformational space available to the peptide.* For some peptides containing a helper T cell epitope, coupling to a carrier protein is unnecessary. Thus, a T cell stimulating peptide corresponding to residues 132–153 of sperm whale myoglobin adopts helical conformations in aqueous solution [21] and is capable

* Ebina S, Dyson HJ and Wright PE, unpublished observations.

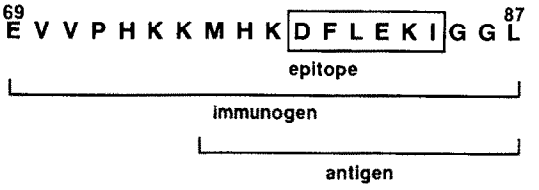

Fig. 2. Amino acid sequence of the peptide immunogen corresponding to the C-helix of *Themiste zostericola* myohemerythrin. The peptide used as an antigen in studies of binding to antipeptide antibody and the residues involved in the epitope are also indicated.

of eliciting a high level of antipeptide antibody response without coupling to a carrier.

In addition to knowledge of the conformational propensity of a given immunogenic peptide free in solution or coupled to a model carrier protein, it is of great importance to determine its conformation when bound to an antipeptide antibody. Clearly, studies of the conformation of a peptide bound to the intact IgG are impossible using current NMR technology. To make this problem tractable, we have begun experiments using the Fab′ fragment of an antibody, with specific isotopic labeling of the peptide to distinguish the resonances of the peptide from those of the antibody.

Conformation of peptides bound to Fab fragments

Experiments to date have focused on the Fab′ fragment of a monoclonal antibody raised [25] against the C-helix peptide (residues 69–87) of myohemerythrin from *Themiste zostericola* [26]. A major epitope was found to consist of the residues shown in Fig. 2 [25]. This region of the peptide was also found to adopt a nascent helical conformation in water solution, a series of turn-like conformations readily stabilized as extended helix in the presence of low concentrations of trifluoroethanol [20]. In the native protein this region is helical [27].

A 12-amino acid segment of the C-helix peptide (Fig. 2), containing the epitope and the region of nascent helix, was used in binding studies. Peptides were synthesized [28] with the backbone amide

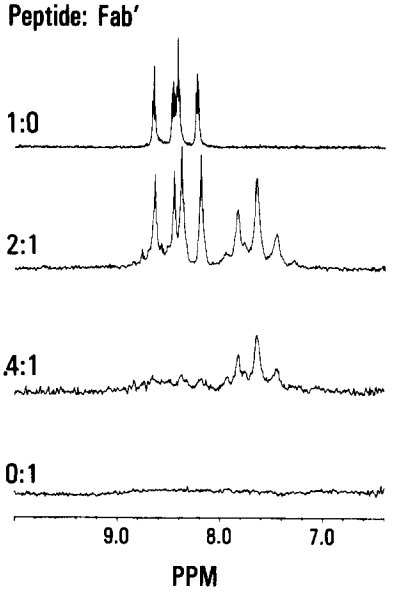

Fig. 3. Reverse-detected spectrum of [^{15}N]glycine-labeled antigenic peptide MHKDFLEKIGGL in the presence of various mole ratios of Fab'. Samples were dissolved in 0.1 M deuteroacetate buffer, pH 5.0, 90% $^{1}H_2O$/10% $^{2}H_2O$. All spectra were recorded at 308° K and at 499.87 MHz.

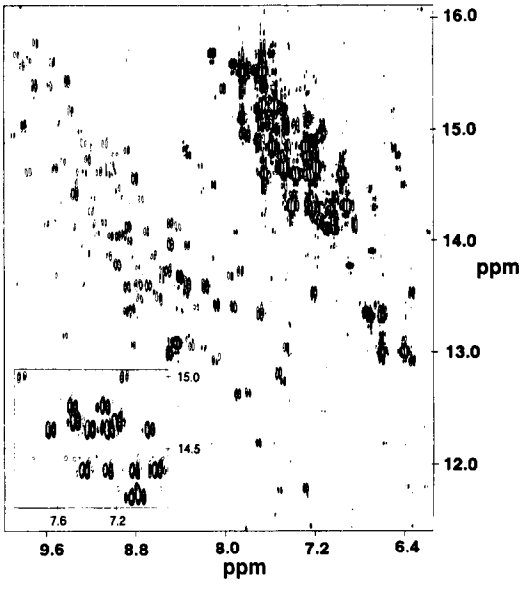

Fig. 4. Portion of 500 MHz phase-sensitive double quantum (2Q) spectrum of Fv fragment in phosphate buffer, pH 7.5, 90% $^{1}H_2O$/10% $^{2}H_2O$. Inset: Aromatic region of an identical spectrum (both chemical shift scales in ppm) recorded for the Fv fragment containing perdeuterated tryptophan and phenylalanine.

labeled with ^{15}N at various positions. These were shown to bind in a 1:1 complex with the Fab' fragment of a monoclonal antibody specific for this epitope, using reverse detection of the ^{15}N-amide protons [26]. Representative spectra are shown in Fig. 3 for peptide doubly labeled with ^{15}N at both glycine residues. Note that the Gly amide proton resonances are significantly shifted and broadened upon complexation, indicating that the environment and correlation times of the amide protons are changed compared to those in the free peptide.

Peptides have been synthesized with ^{15}N labels at Asp 79, Phe 80, Leu 81, Ile 84, Gly 85 and Gly 86 using the residue numbering from myohemerythrin (Fig. 2). In each case, the amide ^{1}H and ^{15}N resonances of Fab' bound peptide can be observed readily and are significantly shifted from those of the free, unbound peptide [29,*]. The ^{1}H resonances have been observed to shift by 0.6 to 1.25 ppm upon binding to the antibody and clearly reflect specific interactions in the bound state. In addition, significant broadening is observed for the peptide NH resonances when bound to the Fab'; the largest linewidths occur for residues within the epitope. The observed linewidth changes are consistent with a model in which residues in the epitope (DFLEKI) are significantly immobilized upon binding to the antibody while residues outside the antibody are much less so [29].

The experiments that have been performed to date [26, 29] demonstrate the feasibility of using isotope-edited NMR experiments to observe the resonances of peptide bound to the Fab fragment of an antipeptide antibody. Clearly, the ultimate goal of such studies is to obtain information on the conformation and dynamics of the bound peptide. More sophisticated NMR experiments are required for this purpose. Our preliminary experiments show that ^{15}N-edited ^{1}H–^{1}H NOE† measurements are feasible and represent the first step towards determination of the structure of the Fab-bound peptide.‡ At a later stage, the dynamic properties of the peptide in its complex with the antibody can be probed through relaxation time measurements of ^{13}C- and ^{15}N-labeled peptide complexes.

As an alternative to isotopic labeling, the transferred NOE may, in suitable cases, be used to investigate the conformation of a peptide bound to an antipeptide antibody. This approach has been utilized to study the interactions between a peptide of cholera toxin and an antipeptide antibody [30]. This method is less general than the isotope labeling strategy in that it is only applicable if the dissociation rate of the bound antigen is fast relative to the T_1 relaxation time of antigen and antibody protons. In addition, the transferred NOE method is only reliable when it can be demonstrated that there is a single binding site on the antibody and that antigen binds in a unique conformation. In this regard, we note that ^{15}N-edited spectra of antibody-bound MHKDFLEKIGGL, with the peptide labeled at Asp 79 and Phe 80 (myohemerythrin numbering, Fig. 2), show evidence of a lower affinity secondary binding

* Tsang P and Wright PE, unpublished observations.

† Abbreviations: NOE, nuclear Overhauser effect; TOCSY, total correlation spectroscopy; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; and COSY, two-dimensional correlated spectroscopy.

‡ Tsang P, Rance M and Wright PE, unpublished observations.

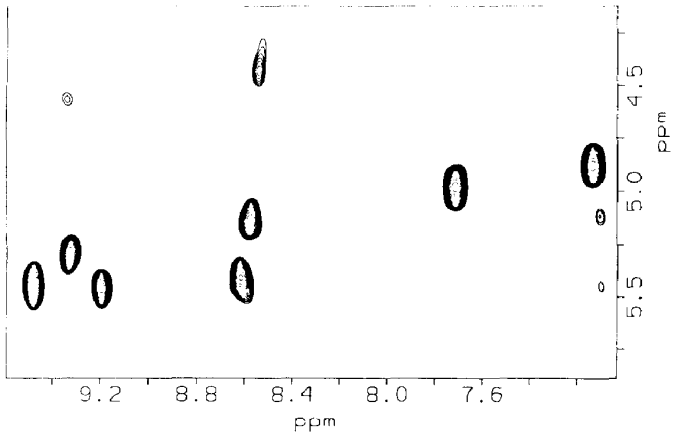

Fig. 5. Fingerprint region of a reverse-detected ^{15}N-filtered TOCSY spectrum of Fv fragment containing ^{15}N-labeled tyrosine. Sample conditions were as given for Fig. 4. The spectrum was recorded at 600 MHz with ^{15}N decoupling and 30 msec isotropic mixing.

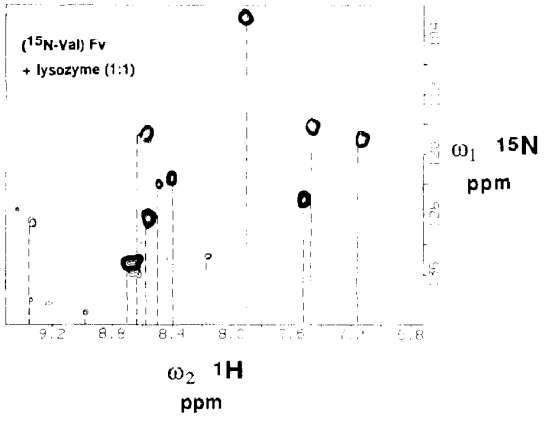

Fig. 6. Portion of a 600 MHz ^{15}N-^{1}H correlated spectrum of a 1:1 Fv–lysozyme complex. The Fv is labeled with [^{15}N]-valine. The broken vertical lines indicate the positions of the amide proton resonances in the uncomplexed Fv fragment.

site in addition to the primary, high-affinity antigen binding site [29].

NMR approaches to antibody structure and dynamics

It is generally recognized that structural studies by high resolution ^{1}H NMR on systems with molecular weight in excess of 20 kD are extremely difficult due to broadness and overlap of the ^{1}H resonances. Although many NMR studies of antibody Fab fragments have been reported [7–9 and references therein], the smaller Fv fragment (molecular weight ~25 kD) is far more attractive for detailed NMR investigations. Such an approach is now feasible, using the powerful methods of molecular biology to clone and express Fv fragments together with isotope labeling strategies to provide assignments of the NMR spectra. These advances open the way to high resolution structural studies of antibody–antigen interactions.

We have carried out preliminary NMR studies of an engineering antibody Fv fragment expressed in myeloma cells that clearly demonstrate the feasibility of this approach. The antibody contains the antigen binding site from the mouse anti-lysozyme antibody D1.3 [1] combined with framework regions from human antibodies [12]. Using ^{15}N- and ^{2}H-labeled protein and a combination of heteronuclear and homonuclear (^{1}H) two-dimensional NMR experiments, a general strategy for unambiguous amino acid spin system identification has been developed.* This approach should have general applicability to proteins of molecular weight greater than 20 kD.

In general terms, our spin system assignment strategy is as follows:

(i) ^{1}H Homonuclear double quantum spectroscopy is used to establish, as completely as possible, scalar coupling networks for side chain protons. Assignments can be confirmed, and spectra can be simplified, using Fv labeled with one or more perdeuterated amino acids.

(ii) The side chain proton resonances are connected to the appropriate amide proton resonances using ^{15}N ω_2-filtered TOCSY and NOESY experiments performed on Fv specifically labeled with ^{15}N amino acids.

Once spin system assignments have been made, sequence specific assignments can be obtained by observation of $C^{\alpha}H_i$-$^{15}NH_{i+1}$ NOE connectivities (using a separate Fv sample containing perdeuterated amino acid at position i to confirm the connectivity, if necessary). In regions of the protein where sequential NH_i–NH_{i+1} NOEs predominant, connectivities may be followed through selective ^{15}N double labeling, and observation of $^{15}NH_i$–$^{15}NH_{i+1}$ NOEs.

Homonuclear double quantum spectroscopy plays an important role in the spin system assignment strategy. Our practical experience both with the antibody Fv fragment (molecular weight ~25 kD) and

* Riechmann L and Wright PE, manuscript in preparation.

myoglobin (molecular weight 18 kD) [31] is that double quantum spectroscopy often provides superior sensitivity and resolution compared to the more conventional double quantum filtered COSY experiment. This is because most peaks in multiple quantum spectra have predominantly in-phase multiplet structure in the ω_1 dimension, thus avoiding the loss of intensity due to self-cancellation of antiphase multiplets which occurs in COSY spectra as the protein becomes larger and the linewidth increases [32]. A representative double quantum spectrum of the Fv fragment is shown in Fig. 4. The inset to this figure demonstrates the dramatic spectral simplification that can be obtained by perdeuteration of specific amino acid residues, allowing, in the example shown, unequivocal identification of the tyrosine ring spin systems. An example of a ^{15}N-filtered TOCSY spectrum obtained from ^{15}N-Tyr-labeled Fv is given in Fig. 5. The enormous degree of spectral simplification achievable by use of ^{15}N labeling is obvious: only NH_i–$C^{\alpha}H_i$ cross peaks of the [^{15}N]tyrosine residues appear in the spectrum.

While the representative spectra of Figs. 4 and 5 make it clear that high quality data can be obtained for the antibody Fv fragment such that detailed information on the structure and dynamics of the free antibody fragment should be forthcoming, can useful NMR data be acquired for the antibody–antigen complex? In the present case, the combined molecular weight of the Fv–lysozyme complex is ~40 kD. A ^{15}N–^{1}H correlated spectrum of ^{15}N-valine-labeled Fv in its 1:1 complex with lysozyme is shown in Fig. 6. This spectrum clearly demonstrates the feasibility of NMR studies of the Fv–protein antigen complex. Although the ^{15}N and ^{1}H resonances of the valine residues of the Fv are only slightly shifted upon antigen binding, spectra of ^{15}N-tyrosine-labeled Fv (not shown) exhibit significant chemical shift changes upon binding of antigen. It is of considerable interest, therefore, that the X-ray structure of the Fab D1.3–lysozyme complex reveals five tyrosine residues (but no valines) of the antibody in contact with lysozyme [1]. Thus it appears that NMR can be used as an exquisitely sensitive probe of local interactions within the antibody combining site in Fv–antigen complexes.

With recent advances in methodology, both in NMR spectroscopy and antibody engineering, it now appears that NMR can be used at an unprecedented level of sophistication to obtain new insights into the solution structure and dynamics of the antibody combining site, both free and in its complex with antigen. In particular, it should be possible to address directly questions concerning conformational changes, in both the antibody combining site and the antigen, that accompany antigen binding. The availability of isotope-labeled Fv and antigen (protein or peptide) should facilitate studies of the dynamics and flexibility of antibody and antigen in both the free and bound states.

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