Antigen mobility in the combining site of an anti-peptide antibody

(antigen recognition/NMR/crossreactivity/synthetic vaccines)

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ABSTRACT The interaction between a high-affinity antibody, raised against a peptide incorporating the loop region of hen egg lysozyme (residues 57–84), and a peptide antigen corresponding to this sequence, has been probed by proton NMR. The two-dimensional correlated spectroscopy spectrum of the antibody–antigen complex shows sharp, well-resolved resonances from at least half of the bound peptide residues, indicating that the peptide retains considerable mobility when bound to the antibody. The strongly immobilized residues (which include Arg-61, Trp-62, Trp-63, and Be-78) do not correspond to a contiguous region in the sequence of the peptide. Examination of the crystal structure of the protein shows that these residues, although remote in sequence, are grouped together in the protein structure, forming a hydrophobic projection on the surface of the molecule. The antibody binds hen egg lysozyme with only a 10-fold lower affinity than the peptide antigen. We propose that the peptide could bind to the antibody in a conformation that brings these groups together in a manner related to that found in the native protein, accounting for the high crossreactivity.

One of the most intriguing aspects of antibody recognition is the ability of some antibodies raised against peptide fragments of proteins to crossreact with intact native proteins (1–3). This phenomenon not only has considerable significance from the point of view of protein structure and folding but also has generated much interest in the possibility of using small peptides in a pharmacological role, particularly as synthetic vaccines (4, 5). Little is known, however, about the underlying principles that determine whether a particular peptide is able to act as a good mimic of a protein epitope, primarily because of the shortage of structural information pertaining to antibodies complexed with their peptide antigens. To our knowledge there has, for example, been only one report of the crystallographic structure of an anti-peptide antibody–peptide complex (6). In contrast a number of structures of anti-protein antibodies complexed with their protein antigens have been determined (7–11). As part of a long-term study in our laboratories of the structural origins of antibody recognition and protein–peptide crossreactivity, a panel of antibodies (Gloop1–Gloop5) specific for the loop determinant of hen egg lysozyme was raised against a peptide immunogen that incorporated residues 57–84 of the protein sequence (12)—known as the “loop” peptide. This sequence corresponds to a large surface loop in the native protein structure and contains a disulfide bridge between residues 64 and 80. The NMR studies have focused on one of the antibodies, Gloop1. The antibody binds two forms of the loop peptide, where the disulfide is either reduced or oxidized, with equal affinity (Kd ≈ 10⁻⁸ M). The antibody is also strongly crossreactive with the native protein; it binds to hen lysozyme itself with only a 10-fold drop in affinity (12, 13). Crystallographic studies have yielded a structure for the Gloop1 Fab (R.E.G. and A.R.R., unpublished results), but to date all attempts to cocrystallize a complex of the antibody with either its peptide or protein antigen have failed. Some limited information relating to the binding of lysozyme by Gloop1 has been obtained from serological studies, but the interactions of only 3 of the 28 amino acid residues in the loop sequence could be characterized by this method.

Proton (1H) NMR studies of peptide antibody complexes have been shown to yield valuable information about the nature of antibody–antigen interactions in cases where the binding affinity is low enough for rapid exchange to occur between the bound and free ligand (14, 15). NMR methods employing isotopically labeled peptides have been demonstrated to be useful in cases where tighter binding exists (16). Here we report the direct observation of two-dimensional (2D) 1H NMR spectra of the complex between the high-affinity anti-peptide antibody Gloop1 and the reduced form of the loop peptide. The spectra show well-resolved crosspeaks from at least half of the peptide residues, indicating that much of the peptide retains a substantial degree of mobility when bound in the combining site. Moreover, the residues that are most strongly immobilized in the bound complex do not correspond to a contiguous length of the peptide sequence, but form a discontinuous epitope. Examination of the NMR results in the light of the conformation of the analogous sequence in the crystal structure of hen egg lysozyme provides some insight into the possible origins of the high crossreactivity of the Gloop1 antibody.

MATERIALS AND METHODS

Materials. Peptide synthesis reagents were obtained from Applied Biosystems. The sizing column used to purify the antibody Fab was obtained from Pharmacia; iodoacetamide and all other reagents and buffers were purchased from Sigma.

Peptide Synthesis. A synthetic form of the loop peptide (QINSRWWCNDGRTGSPRLANIPCSALL, residues 1–28) was synthesized on an Applied Biosystems model 430A peptide synthesizer using standard fluoren-9-ylmethoxycarbonyl (Fmoc) reaction cycles. The peptide (residues 1–28) corresponds to residues 57–84 in the natural hen egg lysozyme sequence, with the single substitution of a Ala residue for Cys at position 76 in the synthetic sequence. This substitution causes no detectable change in binding affinity. The free thiol was protected with iodoacetamide, and the blocked peptide was separated from the reaction mixture and purified by reverse-phase HPLC.

Abbreviations: 2D, two dimensional; COSY, correlated spectroscopy; DQF, double quantum filtered.

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2Residues within the peptide are numbered throughout the text according to their corresponding positions in the protein sequence.
Preparation of Antibodies and Fab. IgG material was produced by cell culture in a hollow fiber bioreactor in collaboration with the Wolfson Cytotechnology Laboratory (University of Surrey) from stock cells produced in ascites as described in ref. 12. Fab was prepared from the IgG by papain digestion and purified on a Sepharcll S-200 (Pharmacia) sizing column. Further purification of the Fab, to remove contaminating small molecules, was then carried out by mixing it with a molar equivalent of its protein antigen, hen egg lysozyme, and then separating the Fab from the complex by using a TSK 3000 gel-filtration column (Bio-Rad), at pH 5.5 in phosphate buffer.

\[ ^3 \text{H} \text{NMR Assignments in the Reduced Loop Peptide.} \] Full sequential assignments were obtained for the peptide in a straightforward manner, using conventional methods (17) at 27°C and pH 4.0 and at pH 7.1 (the pH of binding to antibody). \( d \alpha N \) (i, j + 1) nuclear Overhauser effect connectivities were obtained by using the rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY) experiment (18), with a mixing time of 500 msec. Assignments for the peptide are given in Table 1.

Titration of Fab with Peptide Antigen. A sample of the antibody was titrated with peptide ligand such that the ratio of peptide to Fab varied from 0.22:1 at the start of the titration, through the fully bound complex (1:1), to an end point where the ligand was present in excess (1.33:1). The Fab sample (1.2 mM in \( ^2 \text{H}_2\text{O} \)) was placed in the NMR tube, and peptide was added in 5-μl aliquots via a Hamilton syringe. For each point in the titration, a double quantum filtered (DQF) J-correlated spectroscopy (COSY) experiment was performed.

NMR Data Collection. 2D DQF COSY spectra were recorded for the unbound peptide, the uncomplexed Fab, and the bound peptide. All spectra were obtained under identical conditions (see legend to Fig. 1). The molar ratio of antibody to antigen in the sample used to record the bound peptide spectrum was 1.0:0.9. pH readings were made with a glass electrode and are not corrected for any isotope effect. Spectra were recorded on a Bruker AM-500 spectrometer using 1024 \( T_2 \) increments with 32 scans per increment. Phase-sensitive data were collected by using the method of time proportional phase incrementation (21), with a sweep width of 12.5 ppm. The data matrix was multiplied by a phase-shifted sine bell (6°) in both dimensions and zero filled once in \( T_2 \) and twice in \( T_1 \) before transforming, to give a digital resolution of 3 Hz per point in the final spectrum. All data processing was carried out by using FTNMR (Hare Research, Woodinville, WA). The changes in chemical shifts and in the crosspeak fine structure of the bound resonances were small enough that the bound spectrum could be assigned by reference to the spectrum of the free reduced peptide.

Simulated Line Broadening of COSY Crosspeaks. The NMR data from the free peptide (i.e., no antibody present in the sample) were processed with a number of simulated line broadenings of between 1 and 15 Hz. The resulting spectra were then contoured at identical levels and compared with the spectrum of the bound peptide. In this way it was possible, for each residue in the peptide, to give a lower limit and in some cases a finite range to the estimated increase in the linewidth of its resonances on binding to the antibody, on the basis of the observed reduction in the intensity of the corresponding COSY crosspeaks.

RESULTS

In Fig. 1 we present a region of the 2D DQF COSY spectra of the uncomplexed reduced peptide, the antibody Fab, and the 1:1 Fab–peptide complex. The spectrum of the reduced peptide (Fig. 1A) contains a large number of intense

### Table 1. Sequence-specific assignments in the reduced loop peptide and changes in chemical shift and linewidth on antibody binding

<table>
<thead>
<tr>
<th>Residue</th>
<th>Chemical shifts,* ppm</th>
<th>( \Delta LW, \text{Hz} )</th>
<th>RA, Å²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-57</td>
<td>H&lt;sup&gt;+&lt;/sup&gt; 4.02 (0.01) H&lt;sup&gt;+&lt;/sup&gt; 2.08 (0.01) H&lt;sup&gt;+&lt;/sup&gt; 2.34 (0.03)</td>
<td>1–3</td>
<td>1–3</td>
</tr>
<tr>
<td>Ile-88</td>
<td>4.14 (0.02) 1.81 (0.00)</td>
<td>&gt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>Asn-59</td>
<td>4.51 2.74, 2.66</td>
<td>&gt;6</td>
<td>9–10</td>
</tr>
<tr>
<td>Ser-60</td>
<td>4.30 3.82 (0.00), 3.73 (0.00)</td>
<td>&gt;3</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Arg-61</td>
<td>4.02 1.46</td>
<td>&gt;3</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Trp-62</td>
<td>4.55 3.22</td>
<td>&gt;3</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Thr-69</td>
<td>4.36 3.10, 2.82</td>
<td>&gt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>Asp-66</td>
<td>4.63 2.82, 2.69</td>
<td>&gt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>Arg-68</td>
<td>4.37 1.77</td>
<td>&gt;3</td>
<td>&gt;3</td>
</tr>
<tr>
<td>Thr-69</td>
<td>4.56 4.16 (0.03)</td>
<td>&gt;5</td>
<td>6–8</td>
</tr>
<tr>
<td>Pro-70</td>
<td>4.34 2.25, 1.92</td>
<td>&gt;5</td>
<td>5–8</td>
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<tr>
<td>Ser-72</td>
<td>4.40 (0.05) 3.89 (0.08), 3.82 (0.04)</td>
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<td>5–6</td>
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<tr>
<td>Arg-73</td>
<td>4.30 1.82, 1.70</td>
<td>&gt;3</td>
<td>1–3</td>
</tr>
<tr>
<td>Asn-74</td>
<td>4.65 2.69, 2.83</td>
<td>&gt;6</td>
<td>&gt;6</td>
</tr>
<tr>
<td>Leu-75</td>
<td>4.28 1.60</td>
<td>&gt;6</td>
<td>6–8</td>
</tr>
<tr>
<td>Ala-76</td>
<td>4.25 (0.07) 1.35 (0.04)</td>
<td>6–8</td>
<td>6–8</td>
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<tr>
<td>Asn-77</td>
<td>4.65 2.83, 2.69</td>
<td>&gt;6</td>
<td>&gt;6</td>
</tr>
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<td>Ile-78</td>
<td>4.42 1.84</td>
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<td>&gt;10</td>
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<tr>
<td>Phe-79</td>
<td>4.40 2.25 (0.00), 1.91 (0.00)</td>
<td>&gt;5</td>
<td>6–8</td>
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<tr>
<td>Cys-80</td>
<td>4.49 (0.02) 3.03 (0.03), 2.98 (0.03)</td>
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<td>6–8</td>
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<tr>
<td>Ser-81</td>
<td>4.37 (0.01) 3.89 (0.01), 3.82 (0.01)</td>
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<td>1–3</td>
</tr>
<tr>
<td>Ala-82</td>
<td>4.33 (0.00) 1.37 (0.00)</td>
<td>1–3</td>
<td>1–3</td>
</tr>
<tr>
<td>Leu-83</td>
<td>4.33 (0.00) 1.60 (0.01)</td>
<td>1–3</td>
<td>1–3</td>
</tr>
<tr>
<td>Leu-84</td>
<td>4.18 (0.01) 1.56 (0.01)</td>
<td>1–3</td>
<td>1–3</td>
</tr>
</tbody>
</table>

\( \Delta LW \), increase in linewidth; SC, side chain; RA, residue accessibility.

* Differences in chemical shift of resonances between the bound and free forms of the peptide are shown in parentheses. All shifts are referenced to dioxan at 3.741 ppm. No data are given for the glycine residues (residues 67 and 71) as the H<sup>3</sup>H<sup>4</sup> crosspeak is obscured by the diagonal in the COSY spectrum.

† Residue accessibilities are calculated from the protein crystal structure (19) using the method of Novotony et al. (20).
crosspeaks, as would be expected for a molecule of this size ($M_r$, 3200). By contrast, the spectrum of the uncomplexed Fab (Fig. 1B) ($M_r$, 56,000) shows only a small number of crosspeaks. The absence of crosspeaks from the large majority of residues in the protein is a consequence of the broad spectral lines, which are characteristic of the long correlation times associated with slow molecular tumbling. The few intense crosspeaks observed in the spectrum are from residues with substantial independent mobility; such mobility has been observed in a number of other proteins of high molecular weight (22-25). In the present case the mobile regions are likely to derive from the relatively disordered N and C termini of the Fab polypeptide chain. In the 1:1 complex (Fig. 1C) we observe substantially more crosspeaks.

Fig. 1. Region of the DQF COSY spectrum of the reduced loop peptide (A), uncomplexed Fab (B), and an approximately equimolar mixture of Gloop1 Fab and the loop peptide (C). All spectra were obtained under identical conditions at a temperature of 27°C in deuterated phosphate buffer (20 mM phosphate/100 mM NaCl, pH 7.10); sample concentrations were 1.2 mM. Assignments for $\alpha\beta$ crosspeaks (and $\beta\gamma$ crosspeaks in the case of Thr-69) of residues in the free (A) and bound (C) loop peptide are indicated. Amino acids are identified by the single-letter code.
than in the spectrum of the free Fab but only a subset, from 14 of the 28 residues, of those found in the spectrum of the free peptide. In a titration of Fab with peptide, where the ligand is present in excess, we observe a second set of crosspeaks from all of the resonances of the free peptide, with linewidths and chemical shifts identical to those of the free ligand. The linewidth and chemical shifts of both sets of observed peaks are independent of the amount of peptide added over the whole range of the titration. This shows that chemical exchange between the free and bound forms of the peptide is slow on the NMR time scale. We can thus conclude that all of the crosspeaks in the spectrum of the complex are due to either the Fab or to tightly bound peptide (see Fig. 1). The observation of intense crosspeaks corresponding to resonances of the bound peptide indicates that the residues from which these derive have substantial motility.

Analysis of spectra such as those displayed in Fig. 1 has allowed the changes in linewidth and chemical shifts of the peptide crosspeaks upon binding to be characterized (Table 1). Resonances from both the main chain (α) and the side chains of residues at the N and C termini of the peptide (Gln-57, Ile-58, Ser-81, Ala-82, Leu-83, and Leu-84) are largely unaffected by binding and are clearly visible in the spectrum of the 1:1 complex. The crosspeak fine structure and the chemical shift positions of these resonances, and also of a resonance from the side chain of Arg-73, are virtually identical to those in the free ligand (±0.03 ppm shift), and the increase in linewidth is only 1–3 Hz (Table 1). Several other intense, although slightly broader, resonances are observed from the bound peptide, and they fall into two groups. The first comprises residues Ser-60, Pro-79, and Cys-80, which lie adjacent to the ends of the peptide; all these experience very small changes in chemical shift between the bound and free states (±0.03 ppm). The second group is clustered between residues 69 and 76, and their crosspeaks show somewhat larger changes in chemical shift on binding (0.03–0.1 ppm). These residues also experience larger increases in linewidth than is observed at each end of the chain (Table 1). Finally, no crosspeaks are observed from the remaining 14 residues of the peptide in the 2D spectrum of the bound complex; these include all the residues from Arg-61 to Arg-68 and residues Asn-77 and Ile-78. Simulations show that, although in many cases linewidth changes of 3–5 Hz may be sufficient to cause their disappearance, for four of the residues (Arg-61, Trp-62, Trp-63, and Ile-78) an increase in linewidth of >10 Hz above the free peptide value is required to eliminate all of their crosspeaks from the spectrum.

**DISCUSSION**

The results of our NMR studies of the binding of the loop peptide to Gloop1 show that the antigen must have a considerable degree of motional freedom when bound to the antibody and also that the motional behavior differs substantially in different regions of the peptide. The regions with the narrowest resonances in the bound complex (i.e., those comprising the most mobile regions) are primarily from residues of the N- and C-terminal segments of the peptide. The residues whose linewidths indicate a very substantial degree of immobilization when bound in the complex do not, however, correspond to a single, continuous region of peptide sequence. Three (Arg-61, Trp-62, and Trp-63) lie adjacent to one another toward the N terminus of the peptide, but the fourth (Ile-78) is 15 residues further down the peptide chain. Furthermore, several residues that lie in the sequence between Trp-63 and Ile-78 give rise to clearly detectable resonances (Thr-69, Ser-72, Arg-73, Leu-75, and Ala-76; see Fig. 1). Most derive from side-chain protons, but for Ser-72 and Ala-76 resonances from the main chain are also observed. Of this group, it is interesting that Arg-73 was excluded from the protein epitope in serological studies (12). In contrast, the residues that border on either side, Arg-68 and Asn-77, were identified as important in the recognition of the protein antigen, and these show no resonances in the spectrum of the bound peptide.

Recently, the crystal structure of an antibody–peptide complex has been reported. In this 2.8 Å structure, only 9 residues of the 19-residue peptide could be located unambiguously; the remainder of the peptide is presumed to be disordered (6). This observation appears in good accord with the findings of the present study, which indicate that at least half of the 28 residues in the bound loop are highly mobile. It is of particular significance that of the 9 residues located in the crystal structure 7 fall in the region of the epitope. In the absence of any structural data for the specific peptide–antibody complex studied here, but in recognition of this finding and of the high crossreactivity of native lysozyme with Gloop1, we have examined the crystal structure of the protein antigen (19) to explore the environment of the residues observed to have very different mobility in the peptide complex. Nine of the 10 residues at the N- and C-terminal segments, which are associated with high mobility in the bound peptide, are, in the crystal structure of the protein, located in ordered regions with low thermal factors and low accessibility (Table 1). The majority (12 of 17) of residues from Arg-61 to Pro-79, including Arg-61, Trp-62, and Ile-78, identified as having low mobility in the complex are surface residues with high accessibility. Furthermore, the residues Arg-61, Trp-62, Trp-63, and Ile-78, although remote in the sequence, are grouped together in the structure of the native protein, forming a hydrophobic projection on the surface of the molecule (Fig. 2). This suggests that the peptide could bind to the antibody in a conformation that brings these hydrophobic groups together in a manner related to that found in the native protein. This idea is supported by the fact that the peptide against which the antibodies were raised, unlike the peptide studied here, contains the native-like disulfide bond between residues 64 and 80, which serves to link together the two regions of the sequence found to interact most strongly with the antibody. It also offers a straightforward view of the epitope.

![Fig. 2. Conformation of the backbone of the loop peptide taken from the crystal structure (19) of hen egg lysozyme. The side chains of residues Arg-61, Trp-62, Trp-63, and Ile-78 are shown; these residues form a hydrophobic cluster and lie along a ridge on the protein surface.](image-url)
ward explanation of not only the insensitivity of the antibody binding to the presence or absence of the disulfide bond but also the high crossreactivity of the protein to the anti-peptide antibody. Examination of the crystal structure of the protein shows that no major conformational rearrangement of hen egg lysozyme is required to allow an antibody to bind this hydrophobic projection. It is noteworthy that the one region of the peptide antigen, the C terminus, that corresponds in the protein to an element of secondary structure is among the most mobile regions of the bound peptide and by implication does not interact significantly with the antibody. Biochemical studies have shown that deletion of the C-terminal 3 residues has no effect on the measured binding affinity of the peptide (S. Roberts and A.R.R., unpublished results).

It is of interest that hydrophobic clustering involving residues in the region of the disulfide bond between residues 64 and 80 has been proposed from chemical shift and hydrogen exchange measurements in both unfolded and intermediate folded states of lysozyme (26, 27). In particular instances, the observation of crossreactivity between peptide and protein antigens may therefore be of considerable value in studies of the formation and stability of regions of local structure in nonnative states of proteins (28).

Epitopes on proteins are largely discontinuous and are often larger than the short peptides used to raise antibodies (29–31). Our results indicate that much of the 28-residue peptide bound to Gloo1 has substantial independent mobility and therefore probably interacts weakly with residues in the combining site but that the regions where interactions with the antibody appear strongest are well separated in the peptide sequence. These latter regions, however, involve residues that are close together in the structure of the parent protein, providing a possible explanation for the high crossreactivity of the antibody. In the intact protein and in the peptide against which Gloo1 was raised, these regions are linked by a disulfide bond, which may be of significance in the light of previous observations that peptides constrained by disulfide bonds or chemical crosslinks are more likely to lead to crossreactive antibodies (1, 32, 33). The results we describe support the idea that larger peptides may prove more suitable for generating strongly crossreactive antibodies because they are potentially better able to mimic discontinuous epitopes than their shorter analogues. Further, in designing peptides as vaccines, surface hydrophobic patches in the protein epitope may be more judicious targets for inclusion than hydrophilic ones.

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