Use of 2D NMR, Protein Engineering, and Molecular Modeling To Study the Hapten-Binding Site of an Antibody F_v Fragment against 2-Phenyloxazolone

Siobhan McManus and Lutz Riechmann*
MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.
Received January 17, 1991; Revised Manuscript Received March 13, 1991

ABSTRACT: Two-dimensional (2D) ^1H NMR spectroscopy was used to study the hapten-binding site of a recombinant antibody F_v fragment expressed in *Escherichia coli*. Point mutations of residues in the CDR loops of the F_v fragment were designed in order to investigate their influence on hapten binding and to make site-specific assignments of aromatic NMR proton signals. Two tyrosines giving NOEs to the ligand 2-phenyloxazolone were identified, residue 33 in CDR1 of the heavy chain and residue 32 in CDR1 of the light chain. The benzyl portion of 2-phenyloxazolone is located between these two residues. The binding site is close to the surface of the F_v fragment. Comparison with a different anti-2-phenyloxazolone antibody, the crystal structure of which has recently been solved, shows that the general location of the hapten-binding site in both antibodies is similar. However, in the crystallographically solved antibody, the hapten is bound farther from the surface in a pocket created by a short CDR3 loop of the heavy chain. In the binding site identified in the F_v fragment studied in this report, this space is probably filled by the extra seven residues of the CDR3.

The development of "triple-resonance NMR spectroscopy" utilizing isotopic labeling of both nitrogens and carbons in the same molecule will make it possible to assign and solve the detailed solution structure of proteins up to 30 kDa (Kay et al., 1990). It therefore seems expedient to begin NMR studies on antibodies. Although the size of a complete antibody (about 160 kDa) still precludes a detailed NMR analysis, it can be dissected into functional domains of smaller size. The most interesting domains of an antibody are those that determine the antigen specificity. The two identical antigen-binding sites of an antibody are located in the paired N-terminal domains (VH and VL)[1] of heavy and light chains, which form the tips of the Y-shaped intact immunoglobulin. This heterodimer of variable domains forms the F_v fragment. It has a size of about 25 kDa and can be expressed in a functional form in both procaryotic (Better et al., 1988; Skerra & Plueckthun, 1988) and eucaryotic (Riechmann et al., 1988) cells.

How suitable the noncovalently associated F_v fragments are for structural studies remains to be seen. One F_v fragment was successfully used for crystallography (Bhat et al., 1990), for which traditionally the F_{ab} fragments (50 kDa) of antibodies are used. F_{ab} fragments have also been used for NMR studies, most successfully by Anglister and co-workers (Levy et al., 1989, and references therein), but they are still too big for a complete assignment. Proteolytically produced F_v fragments were investigated by one-dimensional NMR studies many years ago (Dwek et al., 1977), and recently a recombinant F_v fragment was used in heteronuclear 2D NMR experiments (Wright et al., 1990).

We started the analysis of an F_v fragment using site-directed mutagenesis of aromatic residues to assign and study its hapten-binding site in ^1H 2D NMR experiments. The F_v fragment is based on an antibody (NQ11.7.22, Berek et al., 1985) from the secondary immune response in mice against the antigen 2-phenyloxazolone-conjugated chicken serum albumin. The antibody is derived from a set of germ-line genes, which is used in many secondary response antibodies (Berek et al., 1985, 1987). Recently the crystal structure of the 2-phenyloxazolone–F_{ab} complex of an antibody (NQ10.12.5) with different germ-line genes for both the heavy and light chain was elucidated (Alzari et al., 1990). NQ11.7.22, studied here, and NQ10.12.5 belong, therefore, with respect their genetic origin, to different types of antibodies expressed in the 2-phenyloxazolone immune response. Comparison of their structures may reveal why different types of antibodies are expressed during the immune response. Knowledge of the nature of the hapten-binding site in NQ11.7.22 will further enable us to interpret the structural effects of somatic point mutations in the original germ-line genes during the maturation of the immune response in different antibodies based on the same genes.

Materials and Methods

Cloning. Cytoplasmic RNA from the hybridoma cell line NQ11.7.22 was prepared by lysis of washed cells in 0.15 M NaCl, 0.01 M Tris, pH 7.4, 0.5% Nonidet P40, and 1 mM $MgCl_2$ followed by extraction with 1/8th volume of 10% SDS and 1/2 of volume phenol and by precipitation. The antibody variable-region genes were amplified after cDNA cloning with reverse transcriptase in the presence of RNAsin (Promega) with TAQ polymerase as described for the heavy chain in Ward et al. (1989). For the light chain, the same conditions were used with the forward primer 5′-CCG TTT TAT CTC GAG CTT GGT-3′ and the backward primer 5′-GAT GTT GAG CTC ACC CAA ACT CCA CTC TCC-3′. The backward primer is based on the sequence of mouse κ light-chain subgroup II, which is different from all other light-chain groups in this region.

The amplified DNA was digested with *Pst*I/*Bst*EII for the VH and *Sac*I/*Xho*I for the VL. DNA fragments of the expected size were cloned into M13 for sequencing and afterward

* To whom correspondence should be addressed.

[1] Abbreviations: CDR, complementarity determining region; DQ, double quantum; FR, framework region; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; PCR, polymerase chain reaction; ROESY, rotating frame Overhauser effect spectroscopy; TOCSY, total correlated spectroscopy; VH, heavy-chain variable region; VL, light-chain variable region.

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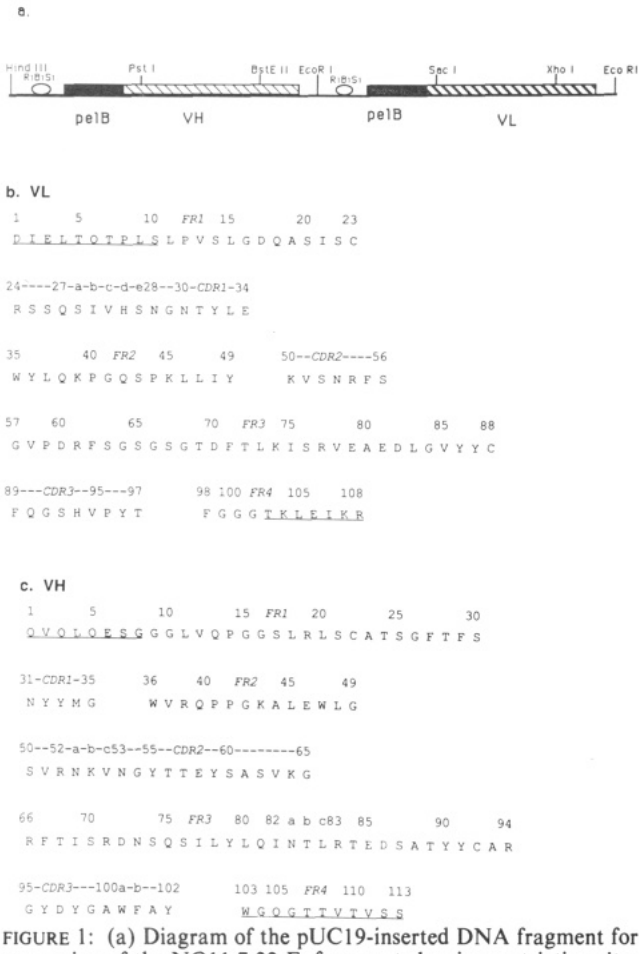

FIGURE 1: (a) Diagram of the pUC19-inserted DNA fragment for expression of the NQ11.7.22 F_v fragment showing restriction sites used for cloning (RiBiSi = ribosome binding site). (b,c) Amino acid sequence of NQ11.7.22 variable regions of the light (b) and heavy (c) chains. Framework (FR) and complementarity determining regions (CDR) are indicated. Sequences originating from PCR primers are underlined. Residues are numbered according to Kabat et al. (1987).

into an expression plasmid. The plasmid for expression is based on pUC19 with an inserted (*Hin*dIII/*Eco*RI) expression cassette providing both variable genes with 5′/3′ flanking regions; a secretory leader peptide that is cleaved off during secretion, a ribosome-binding site, and a termination codon. Transcription is regulated by the *lac*Z promoter in pUC19. The plasmid is shown schematically in Figure 1a. Details can be found in Better et al. (1988), Ward et al. (1989), and Orlandi et al. (1989). Mutants were generated by standard oligonucleotide site-directed mutagenesis in M13.

The sequence of the PCR clones differed in two positions (residues 52c and 82 in the heavy chain) from the previously published RNA sequence (Berek et al., 1985).

Protein Preparation. The F_v fragment was prepared by using the *E. coli* strain BMH 71–18 grown in a rich medium (2×TY) containing 1% glucose and ampicillin (100 mg/L). On reaching the stationary phase, cells were induced for 24 h in fresh medium containing 1 mM isopropyl-β-D-thiogalactopyranoside and ampicillin but no glucose. The F_v fragment from the culture supernatants was adsorbed to EAH-Sepharose 4B (Pharmacia) derivatized with 4-ethoxymethylene-2-phenyloxazol-5-one and was eluted with 1.5 mM 4-γ-aminobutyryl-2-phenyloxazol-5-one. The bound antigen was removed by passing the protein through a PD10 gel filtration column (Pharmacia) at pH 11. The F_v fragment was further purified on a TSK-G2000SW column. Finally, it was transferred into 5 mM phosphate and 200 mM NaCl, pH_{app} 6.2, in D_2O for the NMR experiments at about 0.8 mM and in the presence of a 4-fold excess of the 4-glycyl-2-phenyloxazol-5-one antigen.

Derivatives of phenyloxazolone were synthesized by reaction of γ-aminobutyric acid or glycine with 4-ethoxymethylene-2-phenyloxazol-5-one and repeated recrystallization from dimethylformamide/H_2O.

NMR Experiments. NMR measurements were performed on a Bruker AM500 spectrometer equipped with digital shift hardware and an Aspect 3000 or an X32 computer. 2D NOESY (Jeener et al., 1979, 80-ms mixing time, 298 K), 2D TOCSY (Braunschweiler & Ernst, 1983, 46-ms spin-lock time, 313 K), double-quantum (DQ) experiments (Rance et al., 1989, 22-ms DQ generation time, 313 K), and 2D ROESY spectra (Bothner-By et al., 1984, 40-ms spin-lock time, 298 K) were recorded as described in Neuhaus et al. (1990). The carrier in all experiments was set on the HDO frequency, and the DQ spectra were folded about this point in f_1. Typically, 1024 real points in f_2 (spectral width 8064 Hz) and 250 real points in f_1 (spectral width 6300 Hz) were used for data processing with UXNMR software.

Details of the fluorescence measurements to determine dissociation constants and the on-rate will be described elsewhere (J. Foote and C. Milstein, manuscript in preparation).

Results

Mutants. The variable regions of light and heavy chain of the antibody NQ11.7.22 and its mutants were expressed in *E. coli* by using a vector containing a leader peptide sequence, which facilitates the secretion of the correctly folded F_v fragment and thus enables purification from the culture supernatants. The purification yield for the wild-type F_v fragment and most mutants was about 4 mg/L. A functional F_v fragment was formed, as it could be purified by using phenyloxazolone-Sepharose. Gel filtration experiments gave no indication of dissociation of the (ligand) free F_v fragment.

To determine the hapten-binding site of the F_v fragment, the following strategy was used. Known structures of antigen–antibody complexes show that the binding site is formed principally by three loops of each heavy- and light-chain variable region [roughly corresponding to the so-called complementarity determining regions (CDRs)], which come together at the N-terminal top of the domains. Within the CDRs, aromatic residues and especially Tyr side chains are frequently involved in antigen binding [see Alzari et al. (1988) for a review of the crystal structures].

A panel (Table I) of mainly conservative (e.g., Tyr/Phe, Phe/Tyr, Tyr/Leu) single mutants of aromatic residues was generated to enable the assignment of their proton frequencies in 2D NMR experiments. Conservative mutations should guarantee that the chemical shift changes of nearby residues are only marginally affected and that phenyloxazolone affinity is retained, enabling purification via affinity chromatography. In addition, the influence of some mutations on hapten affinity was measured (see Table I).

The most pronounced effect of these mutations on the general properties of the F_v fragment was a strong decrease in the amount of secreted protein (based on Western-blot analysis) for two of the mutations (VH-Y90F and VH-W100aY), suggesting problems in correct folding. Residue VH-Y90 is part of the framework and is conserved in all antibodies and its side chain is buried in the interface of the two β-sheets of the VH domain (Chothia et al., 1985). The structural function of residue VH-W100a as part of VH-CDR3 is unclear as no structure of an antibody with a CDR3 length of NQ11.7.22 has yet been solved. VH-W100a might

Table I

mutant[a]				K_d Ox-Gaba	K_d Ox-Gly	
VH	VL	Ox-aff	secr	(nM)	(nM)	^{1}H ppm
WT	WT	+	+	205 ± 27	ND	
Y32L	WT	-	+	ND	ND	
Y32F	WT	+	+	597 ± 292	375 ± 95	Y32 ring H, 7.08, 7.56
Y33L	WT	-	+	ND	ND	
Y33F	WT	+	+	249 ± 112	224 ± 97	Y33 ring H, 5.50, 6.19
Y55L	WT	+	+	ND	ND	
F67Y	WT	+	+	ND	ND	
Y79F	WT	+	+	ND	ND	
Y90F	WT	-	(+)	ND	ND	
Y91F	WT	+	+	ND	ND	
R94A	WT	-	+	ND	ND	
Y96F	WT	+	+	593 ± 164	523 ± 222	Y96 ring H, 7.07, 6.65
Y98F	WT	+	+	ND	ND	Y98 ring H, 7.22, 6.87
W100aY	WT	-	(+)	ND	ND	
F100bY	WT	-	+	ND	ND	
Y102F	WT	+	+	435 ± 141	212 ± 49	Y102 ring H, 6.87, 6.98
WT	H27dL	+	+	295 ± 148	443 ± 167	
WT	Y32L	-	+	ND	ND	
WT	Y32F	-	+	ND	ND	Y32 ring H, 7.36, 6.54
WT	Y36L	+	+	ND	ND	
WT	Y49F	+	+	ND	ND	Y49 ring H, 6.91, 6.67
WT	F55Y	+	+	ND	ND	
WT	Y86F	+	+	ND	ND	
WT	Y87F	+	+	ND	ND	
WT	F89Y	+	+	ND	ND	
WT	Y96L	+	+	ND	ND	Y96 ring H, 5.62, 5.34

[a] Single-residue mutants of the NQ11.7.22 F_v fragment (WT = wild-type chain). Phenyloxazolone binding (Ox-aff) is judged by the ability to purify the F_v fragment from culture supernatants with 2-phenyloxazolone-Sepharose. Secretion (secr) refers to the amount of secreted F_v fragment as detected by Western blot analysis of culture supernatants with an anti-D1.3 F_v fragment antiserum (Ward et al., 1989). Brackets indicate poor secretion (less than a tenth of the protein as compared to the wild-type F_v fragment). Dissociation constants (K_d) for 4-γ-aminobutyryl-2-phenyloxazolone (Ox-Gaba) and 4-glycyl-2-phenyloxazolone (Ox-Gly) were determined by fluorescence quenching at 25 °C (ND = not determined). ^{1}H frequencies refer to signals of the respective residues in the wild-type F_v fragment–glycylphenyloxazolone complex.

be important for VH–VL association.

Five other mutations (VH-Y32L, VH-Y33L, VH-R94A, VH-F100bY, and VL-Y32L) gave good yields of secreted protein but poor or no binding in affinity purification (Table I). With respect to residues VH-Y32 and VH-Y33, Tyr/Phe instead of Tyr/Leu substitutions did restore phenyloxazolone affinity, whereas the Tyr/Phe substitution of VL-Y32 did not restore affinity sufficiently to allow affinity purification. As shown in Table I, some dissociation constants for phenyloxazolone derivatives were measured, showing, however, only small variations in affinity.

In summary, these results suggest that VH-CDR1 (via Y32 and Y33), VH-CDR3 (via R94 and F100b), and VL-CDR1 (via VL-Y32) are directly or indirectly involved in the formation of the binding site. To obtain direct structural evidence for specific hapten–antibody interactions, we then analyzed some mutants by 2D ^{1}H NMR.

^{1}H Assignment via Mutagenesis. The chemical shifts of the aromatic spin systems (especially those in the binding site) in spectra for the free and the complexed F_v fragment are so different that the NMR analysis is at present restricted to the F_v fragment–phenyloxazolone complex. The complexed form also proved less susceptible to aggregation than the free F_v fragment.

For the assignment of aromatic residues of the F_v fragment, TOCSY (τ = 45 ms) and DQ-correlated 2D spectra for the wild-type and seven mutant F_v fragments with Tyr substitutions were recorded in the presence of a 4-fold excess of glycylphenyloxazolone. All assignments therefore refer to the hapten-complexed F_v fragment. Assignments based on the mutant spectra are summarized in Table I.

TOCSY experiments (Figure 2) were more helpful for the identification of those spin systems giving weak (i.e., broad) signals, which were sometimes obscured in the DQ spectra. Because of the antiphase character of the cross peaks and the lack of a diagonal, DQ spectra (Figure 3) were most useful when peaks were found in regions of severe overlap and when protons within one spin system had very similar chemical shifts, i.e., the cross peaks were very close to the diagonal in the TOCSY spectra. As expected, Tyr/Phe mutations had little influence on the chemical shift of other aromatic spin systems. On the other hand, the Tyr/Leu substitution of residue VL-Y96 gave a straightforward identification of this Tyr side chain, and in addition caused a downfield shift of a Trp spin system (VH-W47 in Figure 2a). Although such effects can lead to ambiguous interpretation of point mutation effects, in this case the shift of the Trp signals suggests it to be W47 of the VH, which is a very conserved residue in all antibodies and lies close to the side chain of residue 96 of the VL in known antibody structures. There are also NOE cross peaks between both spin systems.

Antigen–Antibody Interactions. The NQ11.7.22 F_v fragment and the ligand glycylphenyloxazolone have a dissociation constant of about 200 nM, which corresponds reasonably well to that of the intact antibody (100 nM, J. Foote, personal communication). By stopped-flow fluorimetry, the on-rate of the glycylphenyloxazolone was determined as 10^8 M^{-1} s^{-1}. Accordingly, the off-rate (or the exchange rate between bound and free antigen in the NMR experiments) is about 20 s^{-1}. The spin-lattice relaxation time (T_1) of the aromatic residues of the F_v fragment and the hapten was determined as about 1.1 s. Thus, the exchange rate is sufficient to transfer magnetization from the bound to the free ligand (Neuhaus & Williamson, 1989; Anglister, 1988). In a similar way, transferred NOEs were used by Anglister et al. (1988) and Levy et al. (1989) to identify (peptide) antigen–antibody interactions using amino acid specific deuterated F_{ab} fragments.

NOESY spectra (Figure 4) of the F_v fragment/glycylphenyloxazolone complex were recorded in D_2O at 298 K. At the higher temperature of 313 K (used for the TOCSY and DQ experiments), the exchange peaks between free and bound phenyloxazolone protons broadened and interfered with nearby signals. Exchange peaks were identified in 2D ROESY spectra

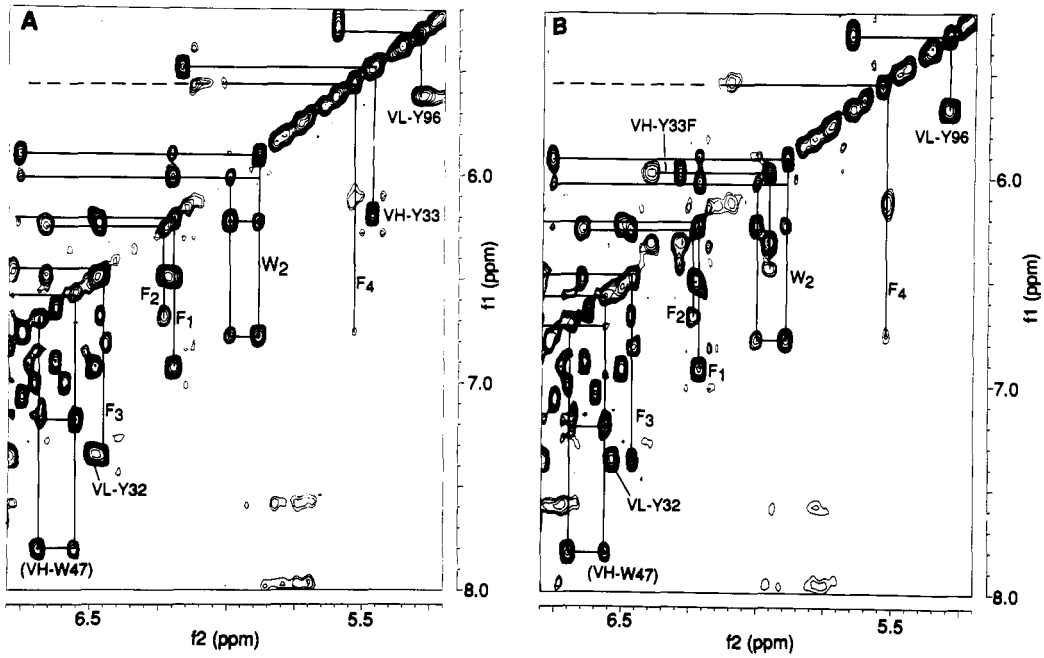

FIGURE 2: 2D TOCSY (τ = 48 ms, 313 K) spectra recorded with wild-type (a) and VH-Y33F F_v fragment (b) of NQ11.7.22 in the presence of a 4-fold excess of glycylphenyloxazolone. The signals for VL-Y96 residues were determined by mutagenesis experiments. Signals for VH-W47 and VL-Y32 are indirectly determined as described in the text. Four Phe spin systems (F1 to F4) are indicated.

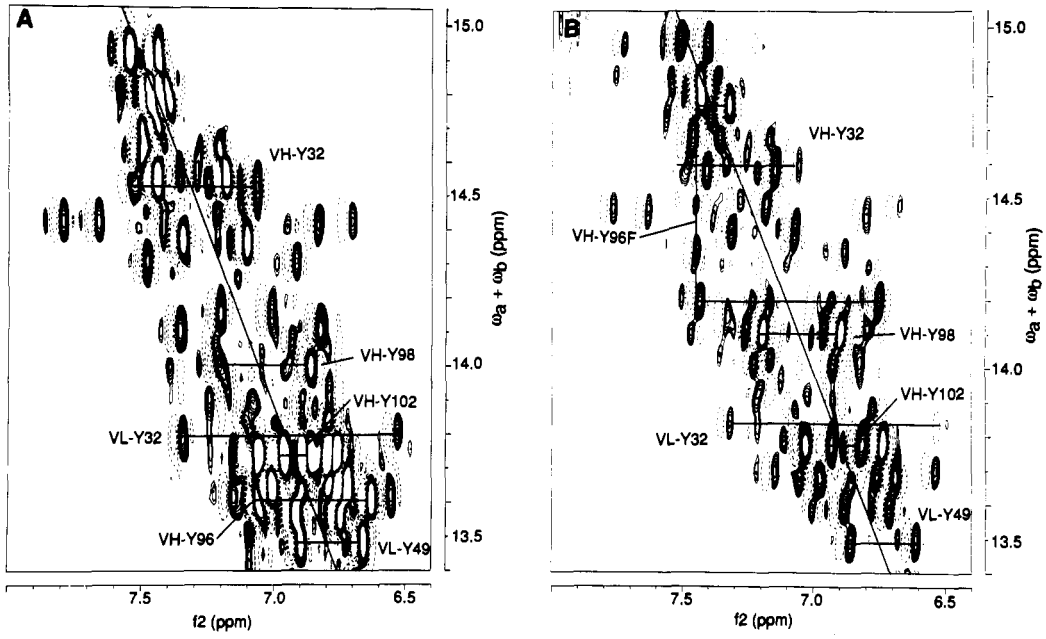

FIGURE 3: 2D DQ (τ = 22 ms, 313 K) spectra folded in f_1 for wild-type (a) and VH-Y96F F_v fragment (b) of NQ11.7.22 recorded with a 4-fold excess of glycylphenyloxazolone. The signals for VH-Y32, VH-Y98, VH-Y102, and VL-Y49 were determined by mutagenesis experiments. Signals for VL-Y32 were indirectly determined as described in the text. Positive and negative (dashed lines) levels are shown. The pseudodiagonal is drawn in to assist orientation.

having the same algebraic sign as the diagonal. The aromatic ^1H frequencies of the free/bound phenyloxazolone are 7.95/6.69 ppm (ortho), 7.58/5.72 ppm (meta), 7.59/5.93 ppm (para), and 7.56/5.76 ppm (olefinic). The free phenyloxazolone can form minor isomers (rotated around the olefinic double bond and the Schiff base C–N bond) with slightly shifted frequences of the ortho and olefinic protons (Titman et al., 1991). These minor forms, however, did not give exchange peaks to any bound form of phenyloxazolone. Therefore, the main isomer (61% in water) (Figure 5) must also be the predominantly bound isomer. Transfer NOEs observed at the frequencies of the free phenyloxazolone protons are due to cross-relaxation processes of the antigen in its bound state. No antibody signals at the frequencies of the ortho and para protons of the free phenyloxazolone were observed in D_2O 2D spectra.

In the NOESY spectra (τ = 80 ms), a set of weak F_v fragment/phenyloxazolone transfer NOEs is observed between residue Y33 of the VH and the ortho and also probably the meta protons of the free phenyloxazolone (Figure 4a). The same weak NOEs are present in the mutant F_v fragment VH-Y33F (Figure 4b). Stronger NOEs from the meta/para phenyloxazolone protons to a proton at 6.52 ppm from another Tyr side chain and from the ortho phenyloxazolone proton to a signal at 3.7 ppm were also measured. All of these NOEs are also seen at a mixing time of 60 ms, suggesting that they are not caused by spin diffusion. In addition, there are intra-phenyloxazolone NOEs between ortho and meta protons.

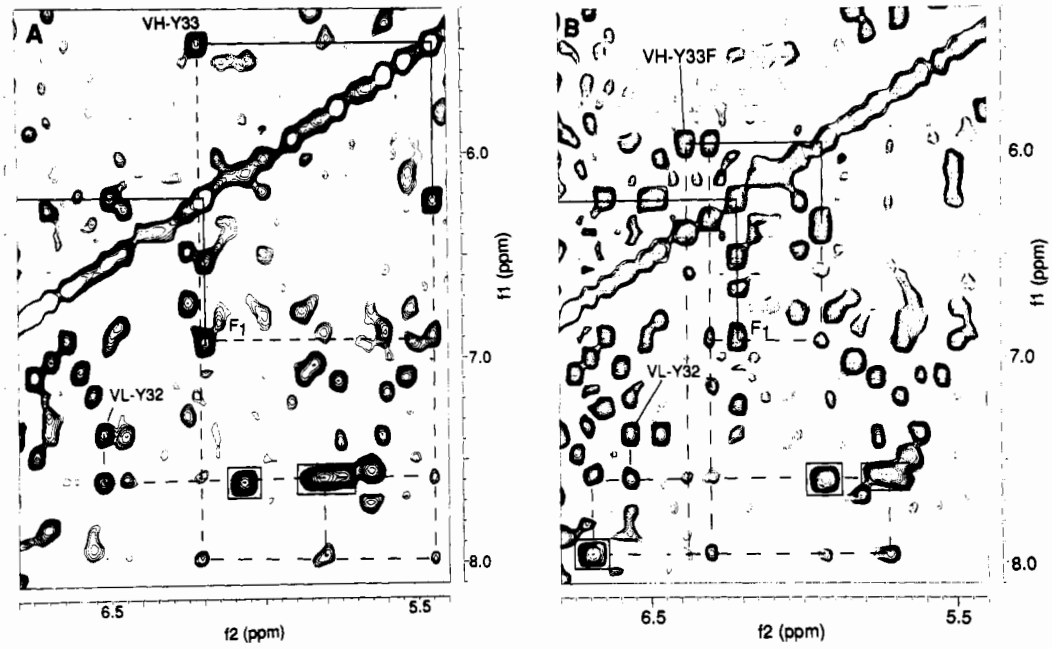

FIGURE 4: 2D NOESY (τ = 80 ms, 298 K) spectra recorded with wild-type (a) and VH-Y33F F_v fragment (b) of NQ11.7.22 in the presence of a 4-fold excess of glycylphenyloxazolone. Signals for VL-Y32 are indirectly determined as described in the text. One Phe spin system (F1) is marked. Exchange peaks observed between free and bound glycylphenyloxazolone are boxed. NOEs between VH-Y33, VH-Y33F, and F1 and transfer NOEs from VH-Y33, VH-Y33F, and VL-Y32 to glycylphenyloxazolone are indicated with dashed lines.

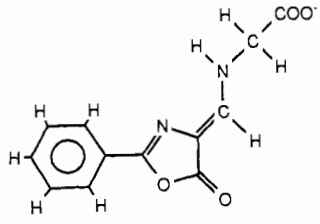

FIGURE 5: Schematic representation of 4-glycyl-2-phenyloxazolone.

Some spin diffusion from the para to the ortho protons of the bound phenyloxazolone is seen in Figure 4b.

We have as yet no NMR evidence for the identity of the proton at 3.7 ppm, but we can identify the phenyloxazolone NOE to the aromatic proton at 6.52 ppm. We know from the DQ and TOCSY spectra that the signal at 6.52 ppm belongs to a Tyr side chain. Also it must be reasonably close (less than 10 Å) to VH-Y33 in order to bind to phenyloxazolone, which leaves only the tyrosines within the CDRs. We have identified all CDR tyrosines with the exception of VL-Y32, as the VL-Y32L and VL-Y32F mutants bound only very weakly or not at all to the antigen affinity column. This suggests that VL-Y32 (part of VL-CDR1) is indeed the residue interacting with the meta/para protons of phenyloxazolone.

The Hapten-Binding Site. The NOEs from residues VH-Y33 and VL-Y32 to the benzyl portion of glycylphenyloxazolone place the ligand in its binding site on the F_v fragment; the glycyl side chain can be safely assumed to point to the exterior of the F_v fragment as phenyloxazolone was linked via this side chain to the carrier immunogen chicken serum albumin (Berek et al., 1985). This is further supported by the observation that the length of this side chain (γ-aminobutyric acid in place of glycine) does not effect the dissociation constant (Table I).

Comparison of known antibody structures reveals that the main-chain conformation of the CDR loops (with the exception of VH-CDR3) in different antibodies depends on a few key residues and enables a reasonable prediction of their structure (Chothia et al., 1990). Thus, a known antibody structure with identical key residues can be used to define the general location of phenyloxazolone in the NQ11.7.22-binding site formed by the loops of VH-CDR1, VH-CDR3, and VL-CDR1. Of the crystallographically solved antibody structures, the most similar in sequence is that of 4-4-20 (Herron et al., 1989), which (with the exception of VH-CDR3) has loops of the same length as NQ11.7.22 and identical key residues for CDR1 in both the light and heavy chains. The conformation of VH-CDR3 is difficult to predict as no antibody with a VH-CDR3 of identical size has yet been structurally studied.

Thus we can assume that 2-phenyloxazolone binding in NQ11.7.22 is similar to the model shown in Figure 6, where VL-CDR1 and VH-CDR1 from the antibodies 4-4-20 (serving as a starting model for NQ11.7.22) and from NQ10.12.5 (the other 2-phenyloxazolone response type) are superimposed. Phenyloxazolone (upper molecule) was modeled arbitrarily between residues VL-Y32 and VH-Y33 of 4-4-20 so that the glycyl side chain (not shown) points into the solvent (top) to illustrate how the hapten might be bound in NQ11.7.22. The NQ10.12.5 model contains the ligand (lower 2-phenyloxazolone molecule) as determined in the crystal structure. Hapten and hapten-binding residues are distinguished by the attached van der Waals surfaces.

The sides of the NQ11.7.22 phenyloxazolone-binding site are formed by the loops of VH-CDR1, VH-CDR3, and VL-CDR1. Within VH-CDR3, residue F100b could be involved in formation of the binding site. This assumption is based on the loss of antigen affinity in the mutant VH-F100bY and on the strong ring current shift effect on one Phe spin system (F1 in Figures 2 and 4), which also has an NOE cross peak to VH-Y33. The two other phenylalanines (VL-89 and VL-98), which lie near or within the CDRs, are too distant (their Cβs are 14.15 and 17.23 Å apart from the Cβ of VH-33, when pointing toward each other) in the structure of 4-4-20, which leaves only VH-F100b for this Phe spin system. However, no NOEs from this Phe spin system to phenyloxazolone are observed. A more detailed analysis in the form of distance geometry calculations based on intra-F_v-fragment NOEs is impossible at present due to the severe overlap of the aromatic signals, which will only be resolved by ^{13}C labeling of the F_v fragment.

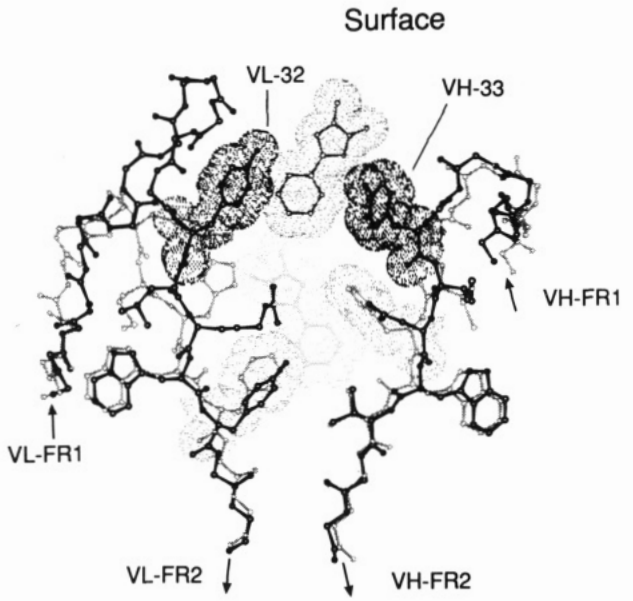

FIGURE 6: Superposition of VL-CDR1 (left) and VH-CDR1 (right) from the crystal structures of the phenyloxazolone complex with the F_{ab} fragment (black) of NQ10.12.5 (Alzari et al., 1990) and of the F_{ab} fragment (grey) of 4–4–20 (Herron et al., 1989). Residue VH-33 in 4–4–20 was mutated from Trp to Tyr to represent the VH-CDR1 to NQ11.7.22. VL-CDR1 of 4–4–20 and NQ11.7.22 are identical. Residues binding to the benzyl portion of phenyloxazolone and phenyloxazolone itself are surrounded by a van der Waals surface. The lower phenyloxazolone is bound to NQ10.12.5 as determined in the crystal structure. The upper phenyloxazolone is modeled between residues VL-32 and VH-33 of the 4–4–20 loops to propose a model for antigen binding in NQ11.7.22. The loops were superimposed with respect to the backbone of four neighboring residues from the frameworks flanking CDR1 of the heavy and light chain in both structures. Only side chains between residues 32 and 36 for VL-CDR1 and residues 33 and 37 for VH-CDR1 are shown. Arrows indicate the N- to C-terminus direction of the polypeptide chains. All manipulations were performed with INSIGHTII.

DISCUSSION

In the early immune response of BALB/c mice to phenyloxazolone, most antigen-specific antibodies consist of a heavy chain derived from the germ-line gene VH-Ox1 and a light chain derived from the germ-line gene VK-Ox1 (Kaartinen et al., 1983). As the immune response diversifies, heavy chains originating from other VH families combined with the VK-Ox1 light chain are also found (Berek et al., 1985, 1987). On the basis of the crystal structure of the complexed F_{ab} fragment from one antibody of this group (having a VH-MOPC21-like heavy chain and VK-Ox1 light chain) and on sequence comparison, it was argued that all anti-phenyloxazolone antibodies with a VK-Ox1 light chain form similar hapten-binding sites (Alzari et al., 1990). This binding site, in which the ligand is well buried, is formed by an internal cavity between CDRs 1 and 3 of both VH and VL. However, in the later stages [6–8 weeks after boosting, (Berek et al., 1985)] of the immune response many anti-phenyloxazolone antibodies are found that use a completely different set of germ-line genes. The antibody NQ11.7.22, the F_v fragment of which was used in this study, is a typical member of this group. It is derived from the germ-line gene VH11 and the presumed germ-line gene VK45.1 (Berek et al., 1985). The antigen interactions in the F_v fragment of NQ11.7.22 reveal differences in the binding mode of this antibody compared with NQ10.12.5, a representative of the first group of anti-phenyloxazolone antibodies (Alzari et al., 1990).

In the model shown in Figure 6, the side chains of VH-Y33 and VL-Y32, the two residues with NOEs to phenyloxazolone in NQ11.7.22, lie very close to the surface of the antibody. This location is not very different from the binding site in the first group (Alzari et al., 1990), where residues from VL-CDR1 and VH-CDR1 are also within NOE distance to the benzyl portion of phenyloxazolone (Figure 6). In the first group, however, phenyloxazolone is located much farther away from the surface. The space for the binding pocket in NQ10.12.5 is created by the short VH-CDR3 (five residues). This space is probably filled by the long VH-CDR3 (12 residues) in NQ11.7.22 and perhaps by the side chain of VH-F100b. The long VH-CDR3 in NQ11.7.22 could form one side and the bottom of the phenyloxazolone binding site, while the loops of VH-CDR1 and VH-CDR1 form two more sides of the binding site.

On the basis of this model, can we speculate whether the differences between the binding sites of these two anti-oxazolone antibodies have any consequences for the mode of antigen binding? The answer lies in the dynamics of their binding interactions. The location of the binding site nearer to the surface of the molecule in the NQ11.7.22-type (Figure 6) causes a faster exchange of free and antibody-bound hapten molecules. Thus we observed exchange peaks between free and bound 2-phenyloxazolone. In contrast, the binding site in NQ10.12.5 is comparatively deep, and access of the hapten is likely to be more hindered. In accordance with this, no exchange peaks between free and bound ligand could be observed in 2D NOESY spectra of the 2-phenyloxazolone–NQ10.12.5 F_v fragment complex (unpublished results), indicating a slower exchange rate compared with that of the NQ11.7.22 F_v fragment. These findings show how solution 2D NMR studies of antibody F_v fragments can yield information about the nature of antigen-binding sites that cannot be obtained from crystal structures.

We can further analyze the structural information about the 2-phenyloxazolone binding site with respect to naturally occurring mutants (somatic maturation) in the NQ11.7.22-type antibodies in different stages of the immune response. Comparison of the 13 sequences determined for anti-phenyloxazolone antibodies derived from the VH11 and the VK45.1 germ-line genes (Berek et al., 1985, 1987) shows that only two light-chain residues near the hapten-binding site are affected by mutational events: residues 96 and 98 as part of the J region (Berek et al., 1985, 1987). The present model of the binding site does not allow us to interpret their role on phenyloxazolone binding as we have as yet no indication for a direct involvement of VL-CDR3.

In the VH11-derived heavy chains, mutations are spread throughout CDR2 and FR3, all of which are unlikely to affect hapten binding because of their distance from the actual binding site. No mutation was found in residue VH-Y33, and VH-Y32 was mutated to Phe in one case, which does not affect affinity significantly in the case of NQ11.7.22 (Table I). VH-CDR1 is expected to be important for antigen binding on the basis of the NMR data. It may be nearly optimal and may only tolerate minor changes while retaining or increasing antigen affinity. Thus Tyr/Leu mutation of either residue 32 or 33 abolished hapten binding completely (Table I).

Major differences between antibodies within the NQ11.7.22 group are found in residues 96–98 of VH-CDR3, which originate from the D segment in the VDJ-joined heavy-chain variable regions (Tonegawa, 1983). VH-Y96 and VH-Y98 have both been assigned and are not directly involved in binding, as no NOE to phenyloxazolone can be observed for either of them. We would predict the side chain of residue 97 (Asp in NQ11.7.22) to be involved in binding, although

we have not yet assigned the ^1H resonances of this residue. All earlier antibodies in this group (up to six weeks after the second injection) (NQ7.47.1 and all six NQ10, Berek et al.,1985, 1987) have a Tyr in position 97. All later ones (eight weeks after the second injection and after the third injection) have a nonaromatic side chain in position 97, some also at position 96. A Tyr at position 97, which is the residue encoded by the germ-line D segment, may obstruct binding and affinity is improved with a smaller side chain. Whether these substitutions in the region of the D segment are due to somatic mutation or to differential processing during rearrangment is impossible to tell, but we predict this region of the antibody to be the main location for affinity enhancement by some sort of affinity maturation in the NQ11.7.22-type anti-phenyloxazolone antibodies.

Acknowledgments

We thank David Neuhaus, Cesar Milstein, John Cavanagh, Jefferson Foote, and Greg Winter for advice and the IRC for Protein Engineering Cambridge for use of its NMR facilities. L. R. is a "Leukemia Society of America Special Fellow." S.M. is a recipient of an MRC studentship.

Registry No. 2-Phenyloxazolone, 1199-01-5.

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