

The Contribution of Contact and Non-contact Residues of Antibody in the Affinity of Binding to Antigen

The Interaction of Mutant D1.3 Antibodies with Lysozyme

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We have analysed the contribution of residues of the D1.3 Fv fragment to binding of hen egg lysozyme. We altered residues at the contact interface by site-directed mutagenesis, and determined the affinity of the mutant Fv fragments for lysozyme by fluorescence quench titration. We found that a band of residues at the centre of the contact interface were much more important for binding affinity than those at the periphery. We also subjected the scFv fragment to random mutagenesis to simulate somatic mutation and affinity maturation. By display of the mutants on the surface of filamentous phages, and selection of the phages with biotinylated lysozyme, we were able to select mutants with modest improvements in binding affinity to lysozyme. By combining the mutations we obtained a scFv fragment with a fivefold improved affinity ($K_d \sim 0.6$ nM compared to wild-type $K_d = 3.3$ nM). However, none of the altered residues leading to improved affinity was located in the contact interface. This indicates that the interactions of a few residues at the centre of the contact interface are responsible for the binding affinity to antigen, but that these interactions can be modulated by alterations of residues outside the binding site. This may represent a typical mechanism for the affinity maturation of antibodies.

Keywords: antigen binding; affinity maturation; filamentous phage; mutagenesis

In vivo, antibodies are derived by selection from repertoires of antigen-binding sites displayed on the surface of a population of B-cells. The rearranged V-genes, encoding the heavy and light chain variable domains responsible for binding to antigen are created by combinatorial assembly of V-gene elements (heavy chain VH, D and JH; light chain VL and JL, or VK and JK) during lymphocyte development. Binding of antigen and help from T-cells triggers differentiation of the B-cell to plasma cells and production of soluble antibody (for review, see Kocks & Rajewsky, 1989; Vitetta *et al.*, 1989). In the mouse, there are approximately 2.5×10^6 B-cells (for review, see MacLennan & Gray, 1986) displaying a large repertoire of approximately 10^7 antigen binding sites (Winter & Milstein, 1991). Each antigen binding site is selected from this large repertoire of structures and, typically, the antigen binding affinities obtained are in the range 10^5 to 10^7 M⁻¹ (Winter & Milstein, 1991). The affinities

can be improved by random mutation, by which variants are created with an estimated mutation rate of 10^{-3} to 10^{-4} point mutations per base-pair per generation (Griffiths *et al.*, 1984; Berek & Milstein, 1987; Cumano & Rajewsky, 1986; Allen *et al.*, 1987), and those with higher affinity of binding are selected on further immunization with antigen. "Affinity maturation" often leads to improvements in affinity of five- to tenfold (Cumano & Rajewsky, 1986; Berek & Milstein, 1987; Foote & Milstein, 1991).

Although the recognition of antibodies and antigen is based on random encounter, the selected antibody-antigen complexes reveal extensive and highly complementary surfaces with multiple contacts, involving several hypervariable loops of the antibody (for review, see Poljak, 1991). To help dissect the energetics of this recognition, we have exploited the X-ray crystallographic structures of the D1.3 antibody (Amit *et al.*, 1988; Bhat *et al.*, 1990; Fischmann *et al.*, 1991). The monoclonal antibody D1.3 is derived from the secondary immune response (made by immunizing a Balb/c mouse 3

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times over a period of 36 days; Harper *et al.*, 1987). Close germ-line genes are known (VH = PJ14 (Sakano *et al.*, 1980) and V κ = K2 (Nishioka & Leder, 1980)) suggesting that D1.3 contains five somatic mutations: Ser56 $\rightarrow$ Asn in VH CDR2 and Asn50 $\rightarrow$ Tyr, Ala51 $\rightarrow$ Thr, Lys52 $\rightarrow$ Thr in V κ CDR2 and also a framework mutation Gln86 $\rightarrow$ His in FR3 of the VH. The Fab and Fv fragments have been solved free, and in complex with hen egg lysozyme: only one of the somatically mutated residues makes direct contact to antigen (VL Tyr50). Here we first tried to identify the contribution of the contact residues defined by X-ray crystallography to the affinity of binding of the Fv fragment.

The contacts between D1.3 Fv or Fab fragments in complex with lysozyme that were analysed are noted in Table 1, and mutants of the Fv fragment were constructed by site-directed mutagenesis. (However, the importance of the main-chain contacts from residues VH 31, VH 53 and VL 91 is impossible to analyse by site-directed mutagenesis.) The binding affinities for lysozyme were measured by fluorescence quench titrations, and are summarized in Table 1. This shows that the residues of loops VH CDR2 and 3 and VL CDR1 and 3 are more important for binding than VH CDR1 and VL CDR2. Residues VH Asp54 and Tyr101 and VL Tyr32 are particularly important, and with VH Trp52 and VL Trp92 help form a central band of key contacts, mainly from aromatic side-chains (Fig. 1). Such contacts have been proposed as major determinants of affinity (Mian *et al.*, 1991), and here form binding pockets, setting up multiple, perhaps co-operative interactions, for example around Gln121 and Asp119 of lysozyme (Amit *et al.*, 1986).

Much of the energetics of interaction seem to be driven by the contacts from a small linear epitope, Gly117 to Gln121 of lysozyme to this central band of residues (HEL Gln121 to VH Tyr101, VL Tyr32 and Trp92; HEL Val120 to VH Tyr 101; HEL Asp119 to VH Trp52 and Tyr101; HEL Thr118 to VH Trp52 and Asp54; and HEL Gly117 to Asp54). Other contacts analysed (VH Thr30, Tyr32 and Arg99, VL Tyr50, Thr53 and Ser93) are less important and are located at the periphery of the antibody binding site (Fig. 1). Each of these peripheral side-chains makes only a single contact with antigen, whereas each residue of the central band makes several. Indeed the number of contacts (clearly related to the location at the centre or periphery) appears to be at least as reliable a guide to predicting the energetics of the interaction of the D1.3 antibody and lysozyme as semi-empirical calculations (Novotny *et al.*, 1989). The effects of the mutation of VH D100 and VH R102 are more difficult to analyse. From the structure, these side-chains seem to make a salt-bridge between each other, and the side-chain of R102 seems to make a hydrogen bond to the main-chain of residue VH 99. Thus alteration of these residues may change the structure of the VH-CDR3 loop.

The dissociation constant of the D1.3 antibody

Table 1
Affinity determinations by fluorescence quench titration of mutants of the contact residues

Heavy chain	K_d (nM)	Light chain	K_d (nM)
CDR1			
T30A†	4.3 (0.9)		
Y32F	7.5 (1.8)	Y32F	109.0 (16)
CDR2			
W52A	30.0 (5)	Y50F	15.0 (2)
D54A	103.0 (12)	T53A	2.5 (0.6)
CDR3			
R99A†	8.2 (2.3)	W92A	68.0 (12)
D100A	No binding	S93A	4.5 (1.2)
Y101F	203 (25)		
R102A	No secretion		

Wild-type Fv D1.3 K_d = 3.7 (0.8).

Additional mutations were made in other contact residues (K_d in nM). VH G31A (3.2); VH G53A (43); VL F01Y (22) but the effects are difficult to interpret as these residues are thought to make main chain contacts only. The Fv fragment (version 1) made during the construction of general bacterial expression vectors (Ward *et al.*, 1989) and crystallized by Boulot *et al.* (1990) was used for the site-directed mutagenesis. It was initially subcloned into pUC119 (Vieira & Messing, 1987) to facilitate production of single-stranded DNA for sequencing. Site-directed mutagenesis employed the method described by Hemsley *et al.* (1989). In brief, separate vectors were made containing either the heavy or the light chain cassette as it was found to be impossible to perform inverse PCR on a vector containing repeat *pelB* sequences. Mutants were made to change all the contact residues between D1.3 and HEL (Amit *et al.*, 1986; Bhat *et al.*, 1990; Fischman *et al.*, 1991). PCR primers were made to replace tyrosine by phenylalanine and other amino acids by alanine. Putative mutants were sequenced using the dideoxy method (Sanger *et al.*, 1977) and with a Sequenase kit (USB, Cleveland, OH), and after identification of a clone with the correct mutation (and no other PCR-induced errors), the mutant chain was recombined with the other chain to make an Fv construct. Single-stranded DNA was prepared and sequenced again to confirm the desired mutation. The Fv fragments were expressed in bacteria as previously described (see Hawkins & Winter, 1992) and the culture supernatant applied to a 2 ml HEL-Sepharose column, washed with 10 ml phosphate-buffered saline, then 10 ml 100 mM Tris HCl (pH 8.5), 500 mM NaCl and finally eluted with 5 ml 100 mM triethylamine. The eluted scFv was collected in 1 ml fractions into 0.5 ml ice-cold 1 M Tris-HCl (pH 7.4) and then the peak protein fractions dialysed into phosphate-buffered saline. Affinity measurements were performed by fluorescence quench titration using HEL as described (Foote & Winter, 1992) with ubiquitin added to a final concentration of 25 μ g/ml. The results shown are obtained from the mean of 3 or more determinations and the figures in parentheses are standard errors.

† Residues were thought to contact the antigen in lower-resolution structures (Amit *et al.*, 1986) but not in higher-resolution structures (Bhat *et al.*, 1990; Fischmann *et al.*, 1991).

(or Fv fragment) for lysozyme has been determined as 3 to 5 nM by both fluorescence quench titrations (Ward *et al.*, 1989; Foote & Winter, 1992) and titration calorimetry (R. Poljak, personal communication). The affinity of binding is characteristic of a good antibody from the secondary immune response (Foote & Milstein, 1991; Cumano & Rajewsky, 1986), and we wondered if the interaction of these highly intermeshed surfaces could be improved further by random point mutation, and if so whether the contact interface would be altered.

In earlier work, we showed that the affinity of a primary response anti-hapten antibody (B1-8) for

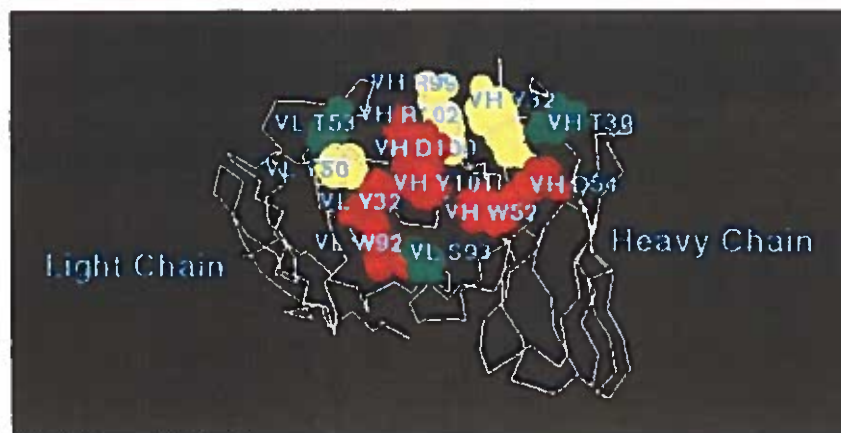


Figure 1. Contact residues of the D1.3 antibody. Conservative mutations in the residues shown in red produce > 8-fold decrease in affinity, those shown in yellow produce a 2 to 8-fold decrease in affinity and those in green < 2-fold decrease in affinity.

the hapten 4-hydroxy-5-iodo-3-nitrophenyl acetate (NIP†) could be matured *in vitro*. We randomly mutated the genes encoding the B1-8 scFv fragment by the polymerase chain reaction (PCR); displayed the population of mutant scFv fragments on filamentous phage; bound the phage to biotinylated NIP-bovine serum albumin and captured the bound phage by streptavidin-coated magnetic beads. This led to the selection of phage bearing antibody fragments with improved affinities of binding to hapten (Hawkins *et al.*, 1992). The affinity improvement obtained was typical of that obtained by the immune system going from the primary to the secondary response (Hawkins *et al.*, 1992; Cumano & Rajewsky, 1986). Here we used the same process of "affinity selection" to isolate mutants of the D1.3 scFv fragment with improved affinity for lysozyme. A library of 2×10^6 members, with an average error rate of 3.8 nucleotide changes per D1.3 scFv gene (42 mutations from 11 random clones sequenced) was displayed on filamentous bacteriophage (McCafferty *et al.*, 1990). After 13 rounds (3 "single" and 5 "double" rounds) of affinity selection, eight mutant clones were sequenced and found to be identical (mutant M1), with residue changes confined to the VH domain (Table 2).

We also deployed the process of "kinetic selection", in which phage with slower dissociation kinetics were selected by loading with biotinylated lysozyme, flooding with unlabelled lysozyme, and then capturing the phage after several lifetimes of the complex (Hawkins *et al.*, 1992). As kinetic selection should be more powerful at discriminating between phage with similar off-rates, mutants were sequenced after five rounds. Of eight mutant clones,

five were identical (mutant M2), with residue changes confined to the Vk domain. The other three mutants contained only silent mutations. A further mutant scFv fragment (M3) was constructed by combining the M1 VH domain with the M2 Vk domain (Table 2).

Table 2
scFvD1.3 mutants selected from phage repertoires

	Amino acid changes in mutants					
	Heavy chain			Light chain		
	40	58†	83	3	29†	97†
M0	P	D	N	E	I	T
M1	L	A	D			
M2				K	T	S
M3	L	A	D	K	T	S

Phage display libraries of random mutants of the scFv D1.3 were made as described (Hawkins *et al.*, 1992). In brief the procedure is as follows: Random mutagenesis was based on the method previously described (Hawkins *et al.*, 1992), except mutating both chains simultaneously using primers VH1BACK (5'-AGG TSM ARC TGC AGS AGT CWG G-3') (Ward *et al.*, 1989) and NotVkJFORA (5'-GAT ATG AGA TAC TGC GGC CGC CCG TTT GAT TTC CAG CTT GGT GCC-3'). The product from a 100 μ l reaction was extracted once with phenol/chloroform, precipitated with ethanol and then resuspended in 90 μ l of water. The vector, Fil.Tet.DOG1, and the PCR product were both digested with *Pst*I (NBL) and *Not*I (NBL) and then gel-purified and the ligation performed.

Biotinylated HEL prepared as described (Hawkins *et al.*, 1992) was used for the selections. The affinity and off-rate selections were performed essentially as described (Hawkins *et al.*, 1992). For affinity selection, the initial HEL concentration used was 2 nM and this was gradually reduced to 0.5 nM as the rounds of selection progressed. For off-rate selection, the concentration of HEL used was 1 nM and with capture 20 min after flooding with unlabelled antigen. The binding of the scFvD1.3 phage to lysozyme was assessed by ELISA (McCafferty *et al.*, 1990). Binding of the soluble scFvD1.3 was assessed in an ELISA by using the C-terminal Myc tag as described (Ward *et al.*, 1989). Sequencing template was prepared from phage as in Clackson *et al.* (1991) and sequenced (Sanger *et al.*, 1977) with phage T7 DNA polymerase (Sequenase, USB, Cleveland, U.S.A.).

† Change in a CDR residue.

† Abbreviations used: NIP, 4-hydroxy-5-iodo-3-nitrophenyl acetate; PCR, polymerase chain reaction; HEL, Hen egg lysozyme; CDR, complementarity determining region; scFv, single chain Fv. The residues are numbered sequentially from the N terminus for both VH and VL.

Table 3
Relative affinities and off-rates for D1.3 scFv fragments on phage

	Titres of scFvD1.3 phage	Titres of M1 phage	Titres of M2 phage	Titres of M3 phage
Input phage	10^9	10^9	10^9	10^9
Affinity selection with 0.2 nM HEL				
Eluted Phage	0.5×10^6	1.3×10^6	1.1×10^6	2.1×10^6
Ratio	1	2.6	2.2	4.2
Off-rate selection using 10 mM HEL to bind and then flooding				
0 min	20×10^6	24×10^6	25×10^6	27×10^6
5 min	10×10^6	19×10^6	19×10^6	25×10^6
15 min	3×10^6	11×10^6	13×10^6	21×10^6
30 min	0.6×10^6	5×10^6	6×10^6	12×10^6
60 min	0.3×10^6	2×10^6	2×10^6	5×10^6
120 min	0.2×10^6	0.5×10^6	0.7×10^6	1×10^6
Approx half-life of dissociation (min)	5	15	15	25

The methods are as described previously (Hawkins *et al.*, 1992). For each mutant a phage stock of standard concentration was made. The relative affinity is determined using a limiting concentration of biotinylated HEL (less than the lowest anticipated K_d) and estimating the ratio of phage eluted. For off-rate estimation the phage were saturated with biotinylated HEL and then diluted into an excess of unlabelled HEL and then captured at different times. The apparent half-life of dissociation can be estimated.

The binding affinities and kinetics of the parent scFv fragment (M0), and mutants M1, M2 and M3 were first estimated as displayed on phage. After a single round of affinity selection with 0.2 nM biotinylated lysozyme, the relative yields of phages (M0 = 1; M1 = 2.6; M2 = 2.2; M3 = 4.2) after capture were consistent with two- to fourfold improved affinities (Table 3). Likewise, after a single round of kinetic selection, the time taken for the number of bound phage to fall to half the initial number (M0 = 5 min; M1 = M2 = 15 min; M3 = 25 min), suggested a three- to fivefold slower dissociation (Table 3). After recloning into a vector for soluble expression, the mutant scFv fragments were prepared and purified on lysozyme-Sepharose. The affinities of the fragments (K_d) were measured by fluorescence quench titration, on-rates (k_{on}) by stopped-flow kinetics; off-rates were deduced from $k_{on}K_d$ (Foote & Winter, 1992). The results (Table 4) agree with the values estimated from phage.

As shown previously for an antibody of the mouse primary response (Hawkins *et al.*, 1992), and here for an antibody of the secondary response, it is

possible to improve the affinity several-fold *in vitro*. However, here none of the set of mutations leading to an increased affinity (and slower off-rate) was a contact residue. Although we have not attempted to dissect the contributions of each of the mutations, three of the residues VH (Pro40 → Leu and Asn83 → Asp) and VL (Glu3 → Lys) are framework residues and relatively distant from the binding site (Fig. 2(a)). Any effect on binding of lysozyme must be indirect, for example in helping to accommodate the flexible linker polypeptide joining the VH and V_L domains. The mutation VL (Thr97 → Ser) is located in VL CDR3, but also lies well outside the contact interface (Fig. 2(a)).

By contrast, the mutations VH (Asp58 → Ala) and V_L (Ile29 → Thr) alter interactions with contact residues. In the structure of the complex of Fab with lysozyme (Fischmann *et al.*, 1991), VH Asp58 forms a hydrogen bond to VH Trp52, an important contact residue. VL Ile29 fits between two important contact residues VL Tyr32 and VL Trp92, and may also help to maintain the structure of L1 loop (Chothia *et al.*, 1989; and see Fig. 2(b)). Indeed,

Table 4
Dissociation constants and kinetic constants of selected mutants

Mutant	K_d (nM)	On-rate ($\times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$)	Off-rate (s^{-1})
Starting antibody (M0)	3.3 (0.8)	1.8	0.006
Selected by affinity (M1)	1.1 (0.2)	1.6	0.0018
Selected by off rate (M2)	1.2 (0.3)	1.5	0.0018
Combined (M3)	0.6 (0.1)	1.6	0.00086

The affinity measurements represent the values obtained from the average of 12 sets of fluorescence quench titrations. Figures in parentheses are the standard errors. Affinity measurements were determined by fluorescence quench titrations. On-rates were determined from a series of stopped flow measurements under pseudo-first-order conditions as described and the off-rates calculated from the K_d and on-rate (Foote & Winter, 1992).

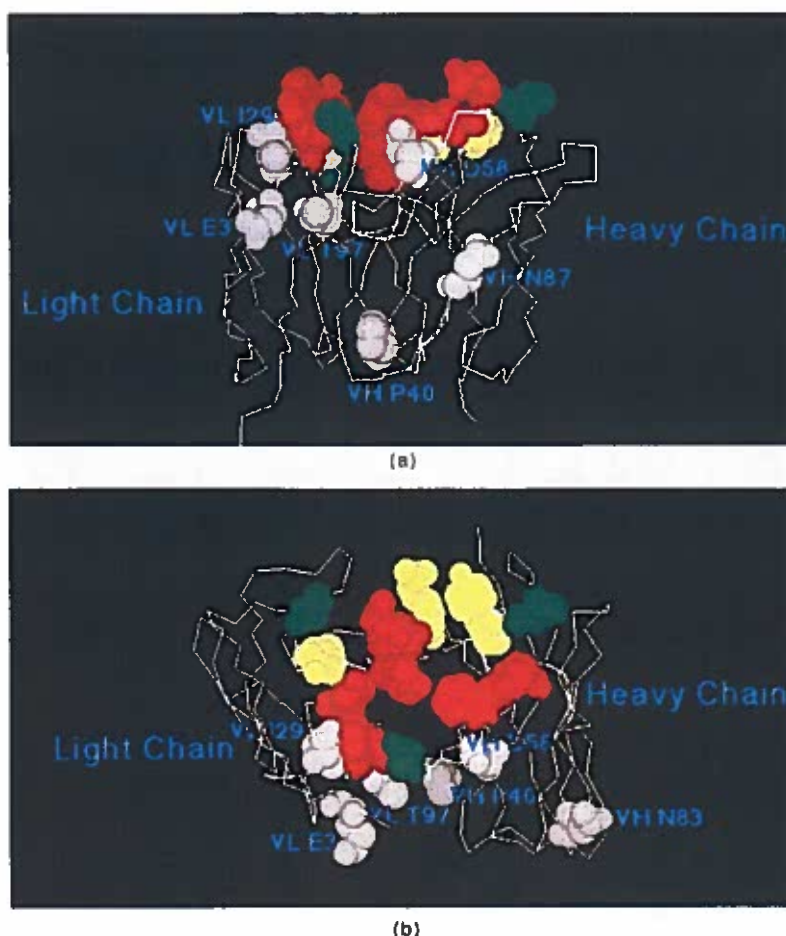


Figure 2. Two views of the binding site of the antibody D1.3 to indicate the position of the mutated residues in the higher-affinity mutant selected from a phage library. (a) The binding site seen from the side and showing how residues VH D58 and VL I29 interact with important antigen contact residues. Residue VL Thr97 although it is in VL CDR3 is not in the binding site and the others are distant from the binding site. (b) The binding site seen from above. Again the interactions of VH D58 and VL I29 with residues important for antigen binding are shown. VL I29 rests in the groove between VL Y32 (CDR1) and VL W92 (CDR3) and VH D58 makes a hydrogen bond with VH W52 across the base of the CDR2 loop.

earlier work has showed that alterations in residues that pack against the CDR loops can modulate the binding affinity for antigen (Riechmann *et al.*, 1988; Foote & Winter, 1992).

We conclude that random mutation of the D1.3 scFv fragment *in vitro* has elicited mutants with improved affinity, but that the residues contacting antigen were unchanged. The improvement in affinity must therefore be mediated by altered residues outside the antigen binding site, some of which interact with the contact residues. Such indirect interactions appear to underpin much somatic mutation *in vivo*. For example, residues of the monoclonal antibodies HyHEL-10 (anti-lysozyme) and 38-71 (anti-*p*-azophenylarsonate) arising by somatic mutation and important for the affinity of binding proved to lie outside the contact interface (Lavoie *et al.*, 1992; Sharon, 1990). However, direct interactions can play a role in affinity maturation, as with the secondary immune response to the

hapten pHx in which a new contact appears to be introduced into the binding pocket for the hapten (Alzari *et al.*, 1990).

The outcome of mutating direct contacts or other residues will depend on the detailed interactions of the antigen-antibody complex. Random mutation of the contacts is likely to destroy antigen binding, but there must also be a slim chance of making greatly improved interactions. For the residues that pack against these contacts, the effects of mutation should be less extreme; indeed high affinities can be built *in vivo* in a step-wise manner (Kocks & Rajewsky, 1988; Sharon, 1990; Foote & Winter, 1992) by accumulating mutations that contribute to a slight improvement in affinity (or as above *in vitro* by combining mutants). Our work provides evidence that in the complex of the D1.3 antibody with lysozyme, the key binding contacts are provided by a band of residues at the centre of the interface, but that the affinity of binding is most

readily improved by altering other residues. Furthermore, it shows that the binding affinity of a high-affinity antibody can be improved further by mutation and selection *in vitro*. This process may be important for clinical applications as experimental evidence suggests that higher affinity antibodies can lead to improved therapeutic effects (Schlom *et al.*, 1992).

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