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Basis for selection of improved carbohydrate-binding single-chain antibodies from synthetic gene libraries

(phage display/random mutagenesis/ligase chain reaction)

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ABSTRACT **A technique is described for the simultaneous and controlled random mutation of all three heavy or light chain complementarity-determining regions (CDRs) in a single-chain Fv specific for the O polysaccharide of *Salmonella* serogroup B. Sense oligonucleotides were synthesized such that the central bases encoding a CDR were randomized by equimolar spiking with A, G, C, and T at a level of 10% while the antisense strands contained inosine in the spiked regions. Phage display of libraries assembled from the spiked oligonucleotides by a synthetic ligase chain reaction demonstrated a bias for selection of mutants that formed dimers and higher oligomers. Kinetic analyses showed that oligomerization increased association rates in addition to slowing dissociation rates. In combination with some contribution from reduced steric clashes with residues in heavy-chain CDR2, oligomerization resulted in functional affinities that were much higher than that of the monomeric form of the wild-type single-chain Fv.**

The relatively low affinities that are characteristic of carbohydrate binding proteins represent a major challenge in efforts to produce therapeutics designed to intervene in carbohydrate-recognition events central to many disease states. Previous mutation studies with an antibody, Se155-4, specific for the O polysaccharide of *Salmonella* serogroup B (1) showed that rational redesign for improved binding and interpretation of altered binding remain difficult even when the three-dimensional structures of the epitope and antibody are known (2, 3). Phage libraries as a source of diversity for *in vitro* mimicry of the immune system, and its capacity for antigen-driven selection, are a useful alternative to a completely rational approach.

The success of phage display in antibody redesign and in the isolation of different binding activities ultimately depends on the composition of the source of diversity, namely, the antibody gene library (4). Error-prone PCR and chemical mutagenesis of target DNA followed by PCR have been used to randomize antibody genes (5–7) but these procedures have the disadvantages of uncontrolled mutation frequency and the alteration of residues throughout the structure, some of which may compromise protein stability. Such an approach has been used to obtain a 10-fold improvement in antigen binding by Se155-4 (5). In well-designed libraries, however, introduction of diversity should be limited to the complementarity-determining regions (CDRs) and to nearby framework residues that fine tune the conformation of the CDRs. Gene amplification using spiked primers (8, 9) and codon-based mutagenesis approaches (10) have been examined as a means of targeting diversity to specific regions. Barbas *et al.* (11) constructed a semisynthetic library in which a Fab sequence was randomized by replacing the heavy-chain CDR3 gene sequence with randomly synthesized oligonucleotides of the same length. In a similar approach, Hoogenboom and Winter (12) introduced random sequences encoding five or eight heavy-chain CDR3 residues into rearranged gene libraries.

In a preliminary report (13), we described a synthetic strategy by using the ligase chain reaction (LCR) for the simultaneous randomization of the three heavy chain variable region (V_H) CDRs. Here, we report the randomization of all six CDRs to a degree that resulted in amino acid substitution levels approximating those observed in the *in vivo* affinity maturation process (14). From these libraries, we isolated single-chain Fv (scFv) mutants with binding properties that were much superior to those previously isolated from libraries in which randomization was introduced throughout the scFv gene (5).

MATERIALS AND METHODS

LCR. For the sense strand, 56- to 85-nt oligonucleotides were synthesized on an Applied Biosystems model 394A automatic DNA/RNA synthesizer with 15–24 of the central bases encoding CDRs spiked at a level of 10% with an equimolar mixture of all four nucleotides, essentially as described by Hutchison *et al.* (15). The antisense oligonucleotides were synthesized so that the spiked regions were paired with inosine. A two-stage library construction strategy was used (Fig. 1). In the first stage, the three V_H CDRs were randomized by using the wild-type scFv plasmid (pSK4) as template, and the second stage involved light chain variable region (V_L) randomization by using mutant B5-1 (Table 1) as template. The LCR-generated libraries were constructed by using the scFv–gene III expression phagemid as described (5). The first-stage LCR mixture contained all eight oligonucleotide building blocks for the V_H, each at 4 pmol (13), in a total volume of 8 μl, 5 μl of 10× reaction buffer (200 mM Tris·HCl, pH 7.6/100 mM KCl/100 mM $MgCl_2$/1% Triton X-100/1 mM ATP/10 mM dithiothreitol), 4 μl of *Pfu* DNA ligase (Stratagene), 5 μl (20 ng) of template DNA, and 28 μl of distilled water. LCR was performed in a GeneAmp 9600 thermocycler (Perkin–Elmer/Cetus) by running step I (92°C for 3 min and 60°C for 3 min) for 1 cycle, step II (92°C for 45 sec and 60°C for 30 sec) for 30 cycles, and step III (92°C for 4 min and 60°C for 3 min) for 1 cycle. The 300-bp LCR product was purified by electrophoresis and removed from the agarose gel. After phosphorylation, half of the V_H product was directly cloned

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Abbreviations: CDR, complementarity-determining region; LCR, ligase chain reaction; LPS, lipopolysaccharide; scFv, single-chain Fv; SPR, surface plasmon resonance; V_H and V_L, heavy and light chain variable regions, respectively; BSA, bovine serum albumin.

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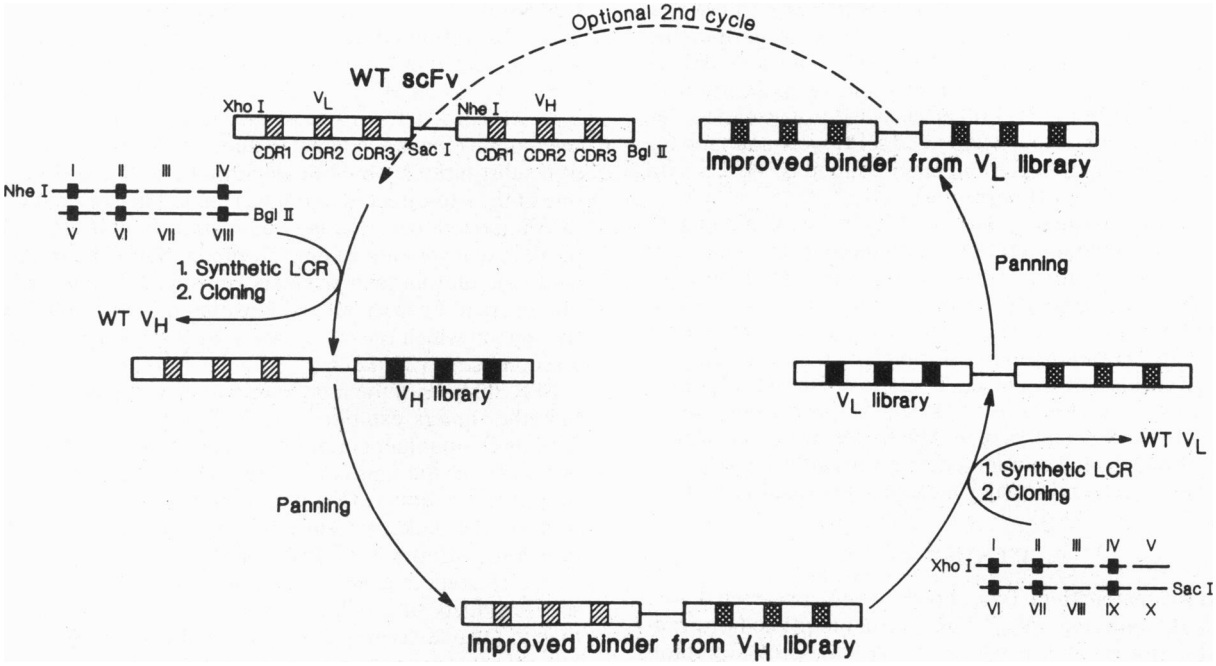

FIG. 1. Two-stage scFv library generation strategy showing the LCR assembly of eight oligonucleotides encoding the CDR-randomized V_H library followed by the LCR assembly of 10 oligonucleotides encoding the CDR-randomized V_L library by using the wild-type (WT) scFv gene as the template DNA in the first stage and mutant B5-1, isolated from the first-stage library, as the template DNA in the second stage.

into the *Nhe* I–*Bgl* II sites of pSK4, the wild-type phagemid vector (5), and half was PCR-amplified with one of the primers containing a diagnostic *Sty* I site not present in the wild-type gene. PCR was carried out with *Taq* DNA polymerase at 94°C for 3 min (1 cycle); 94°C for 35 sec, 50°C for 45 sec, and 72°C for 30 sec (15 cycles); and 72°C for 5 min for the final extension. The PCR product was digested with *Nhe* I and *Bgl* II and cloned into the *Nhe* I–*Bgl* II sites of the phagemid vector pSK4. The ligation mixtures were pooled and electroporated into *Escherichia coli* XL1-Blue resulting in 2×10^7 transformants.

An LCR-generated V_L library containing a diagnostic *Bss*HII site was constructed in an identical manner except that all 10 oligonucleotide building blocks for the V_L domain, each at 5 pmol, were used. After digestion with *Xho* I and *Sac* I, the V_L product was cloned into the *Xho* I–*Sac* I fragment of the phagemid vector encoding mutant B5-1, a V_H mutant (Met34Ile, Gly109Ser) isolated from the first-stage library. The light-chain ligation products were electroporated into *E. coli* XL1-Blue, resulting in 1.2×10^7 transformants.

Phage Panning. Phage were prepared from overnight cultures and panned against *Salmonella* serogroup B lipopolysaccharide (LPS). Selected clones were screened for the diagnostic *Sty* I or *Bss*HII sites followed by an ELISA procedure in which binding of the gene III fusion protein to plates coated with the LPS was detected with an alkaline phosphatase-conjugated anti-mouse λ chain antibody (5). Clones displaying the highest activities were sequenced by using a double-stranded DNA cycle sequencing kit (Life Technologies, Grand Island, NY).

Site-Directed Mutants. Gly109Ser was constructed by subcloning the *Mlu* I–*Hin*dIII fragment from mutant B5-1 into pSK4, the plasmid encoding the wild-type scFv (5). Mutant B5-1/B5-5 was created by two rounds of PCR amplification using B5-5 as the template and mutagenic upper-strand primers.

Expression and Purification. Mutants were digested with *Bgl* II for insertion of a self-complementary terminator sequence (5) between the scFv and gene III regions. Cultures of

Table 1. Amino acid differences of mutants selected from CDR-randomized libraries and generated by site-directed mutagenesis

		V_L			V_H								
scFv type	Clone	54†	94‡	96‡	34*	43	46	55†	56†	57†	77	104‡	109
Wild-type	SK4	N	S	N	M	Q	E	N	S	A	I	Y	G
V_H library	B5-6	—	—	—	—	—	—	—	G	—	T	—	—
mutants	B3-20	—	—	—	—	—	—	V	Y	G	—	T	—
	B3-19	—	—	—	I	—	—	—	—	—	—	—	—
	B3-13	—	—	—	I	—	—	—	—	—	T	—	—
	B5-1	—	—	—	I	—	—	—	—	—	—	—	S
	B4-3	—	—	—	—	—	—	—	N	—	—	—	S
	B5-5	—	—	—	—	—	—	—	G	—	—	—	S
	B5-8	—	—	—	—	—	—	—	—	S	—	F	S
	B5-12	—	—	—	—	—	ΔQ	—	—	G	—	—	S
V_L library	B5-1/B1-4	G	—	—	I	—	—	—	—	—	—	—	S
mutants	B5-1/B2-5	—	Y	D	I	—	—	—	—	—	—	—	S
Site-directed	Gly109Ser	—	—	—	—	—	—	—	—	—	—	—	S
mutants	B5-1/B5-5	—	—	—	I	—	—	—	G	—	—	—	S

*, CDR1; †, CDR2; ‡, CDR3; Δ, deletion. —, No change from wild type.

E. coli TG1 transformed with these plasmids were grown as described (5, 16), and scFvs were isolated from periplasmic extracts by affinity chromatography (5, 17). The scFv linkers were cleaved by digestion for 3 h at room temperature with subtilisin at 5 μg/ml (18). Linker cleavage was examined by SDS/PAGE and intact and hydrolyzed scFvs were analyzed for monomer, dimer, and higher oligomer content by HPLC with a Superdex 75 column (Pharmacia).

Affinity Measurements. The kinetics of scFv binding to bovine serum albumin (BSA)-O-polysaccharide conjugates were determined by surface plasmon resonance (SPR) using a BIAcore biosensor system (Pharmacia Biosensor) as described (5). Rate constants were calculated by using BIAEVALUATION 2.0 software. Titration microcalorimetry of the binding of methyl glycosides of α-D-Gal-(1→2)-[α-D-Abe-(1→3)]-α-D-Man and α-D-Gal-(1→2)-[α-D-Abe-(1→3)]-α-D-Man-(1→4)-α-L-Rha-(1→3)-α-D-Gal (where Abe is abequose and Rha is rhamnose) by selected mutants was performed using a Microcal (Amherst, MA) microcalorimeter as described (19, 20).

RESULTS

Library Construction. Two libraries were constructed, one in which the wild-type V_H CDRs were simultaneously randomized and a second in which the V_L CDRs of a mutant, B5-1, isolated from the V_H library were randomized (Fig. 1). The equation

$$P = \frac{n!}{k!\,(n-k)!} m^k (1-m)^{n-k},$$

where P is the probability of a specified number of residue substitutions, m is the mutation frequency of each amino acid at a selected spiking level, ignoring unequal triplet redundancy, k is the number of amino acid substitutions obtained, from 0 to 19 in V_H and V_L libraries, and n is the number of randomized residues, 19, in V_H and V_L libraries was used to predict the probabilities of residue substitutions at different spiking levels. At a spiking level of 10%, mutants with one to four point mutations in the CDRs should dominate the randomized V_H and V_L libraries. The mutation rate approximates the rate resulting from somatic hypermutation during the affinity-maturation stage of the immune response (14), resulting in functional library sizes of 1×10^8 different sequences.

Heavy Chain Mutants. Based on ELISA screening of fusion protein binding to the LPS, 50 clones from the panned V_H library were sequenced, giving rise to nine sequences (Table 1). The scFv products were characterized by SPR using a BSA-O-polysaccharide surface (Table 2). Two site-directed mutants were constructed and characterized, one with the single amino acid substitution Gly109Ser and a second in which the amino acid substitutions from two mutants were combined (Table 1).

Size-exclusion HPLC analyses showed that, while the wild-type scFv product was almost exclusively monomeric, many of the mutants had a tendency to dimerize (Table 2). Two amino acid substitutions, Met34Ile in mutant B3-19 and Gly109Ser in one of the site-directed mutants, resulted in a dramatic shift to the dimerized form and in combination (mutant B5-1) gave a product that was almost entirely dimer. Subtilisin treatment of wild-type and mutant products converted dimers and higher oligomers to Fv monomers, indicating that the dimers were of the type in which the V_L of one scFv interacts with the V_H of a second scFv (21, 22).

SPR analyses of the monomer and dimer fractions indicated that the dimers exhibited much higher functional affinities than their monomer counterparts and that the higher affinities of many of the mutants were due primarily to a higher proportion of dimer. Oligomer elimination by protease digestion of the linkers resulted in a significant reduction in functional affinity in all instances.

In all instances, the dissociation data for the unfractionated scFv products fit much better to the two-component model than to the one-component model, making it possible to derive two off rates in each instance (Table 2). The fast off rates fell in a narrow range around 0.25 s^{-1} (Table 2) and were attributed to monomeric attachment. The presence of this rate in all instances is thought to reflect the fact that steric hindrance would always lead to some monovalent binding. With the exception of mutant B5-8, the slow off rates were attributed to the dimerized forms. The particularly slow k_{off} for B5-8 is probably due to a significant amount of higher oligomer in this product. The observed association and dissociation rate constants translate into major differences in affinity, giving, for example, a functional K_d of 33 μM (calculated by using k_{off}fast) for monomeric wild-type scFv and low nanomolar affinities (calculated by using k_{off}slow) for mutants that existed primarily as dimers.

Analysis of trisaccharide and pentasaccharide binding by titration microcalorimetry indicated that the affinities of the wild type and some of the best V_H mutants, as identified by SPR, did not dramatically differ (Table 3). With the trisaccharide ligand, the mutants all showed slightly weaker affinities than the wild type, while with the pentasaccharide, the representative heavy-chain CDR2 mutant (B5-1/B5-5) exhibited a 2-fold stronger affinity relative to the wild type. Major differences in enthalpy and entropy changes were associated with binding of the two oligosaccharides. The wild-type (SK4) affinity for pentasaccharide (K_d = 20 μM) is in reasonable

Table 2. Kinetics of BSA-O-chain binding by wild-type and mutant scFvs

Clone	M/D	k_{on}, $M^{-1}s^{-1}$	k_{off}slow, s^{-1}	k_{off}fast, s^{-1}
SK4	37	4.3×10^3 (±0.5)	2.2×10^{-3} (±1.2)	0.14 (±3.0)
B5-6	20	3.0×10^4 (±6.7)	3.2×10^{-3} (±4.4)	0.30 (±3.6)
B3-20	39	2.1×10^4 (±0.9)	2.7×10^{-3} (±0.9)	0.15 (±3.6)
B3-13	13	1.0×10^4 (±0.9)	3.5×10^{-3} (±1.3)	0.21 (±4.1)
B3-19	0.7	1.4×10^5 (±0.8)	2.3×10^{-3} (±6.1)	0.38 (±2.2)
B5-1	0.05	1.5×10^5 (±1.8)	2.3×10^{-3} (±1.4)	0.38 (±3.0)
B4-3	0.3	2.3×10^5 (±0.9)	1.5×10^{-3} (±3.1)	0.38 (±3.0)
B5-5	0.6	4.6×10^4 (±10.1)	2.4×10^{-3} (±3.2)	0.42 (±2.4)
B5-8	0.8	7.5×10^4 (±0.3)	6.2×10^{-4} (±1.5)	0.30 (±2.6)
B5-12	0.5	4.0×10^4 (±2.3)	1.9×10^{-3} (±1.1)	0.26 (±5.1)
1-4	0.6	1.6×10^5 (±2.7)	1.2×10^{-3} (±3.2)	0.25 (±5.1)
2-5	0.04	2.1×10^5 (±1.5)	2.1×10^{-3} (±2.0)	0.26 (±3.2)
G109S	0.4	1.5×10^5 (±0.9)	2.8×10^{-3} (±2.1)	0.35 (±6.1)
B5-5/5-1	0.05	1.7×10^5 (±0.9)	2.3×10^{-3} (±1.5)	0.28 (±3.4)

M/D, monomer/dimer ratio as estimated by peak heights in size-exclusion chromatograms. Data in parentheses are the SEM.

Table 3. Thermodynamics of trisaccharide and pentasaccharide binding by wild-type and mutant Se155-4 scFvs

Ligand	Clone	K, M^{-1} ($\times 10^{-5}$)	Value, kJ/mol		
			$\Delta G°$	$\Delta H°$	$-T\Delta S°$
Trisaccharide	Wild type	1.3 ± 0.2	−29.1 ± 0.4	−28.5 ± 1.2	−0.5 ± 1.3
	B5-1	0.8 ± 0.1	−27.9 ± 0.1	−24.7 ± 0.3	−3.2 ± 0.3
	B5-8	1.0 ± 0.1	−28.5 ± 0.3	−26.7 ± 0.6	−1.8 ± 0.7
	B5-1/B5-5	1.0 ± 0.1	−28.5 ± 0.3	−25.0 ± 0.3	−3.5 ± 0.3
Pentasaccharide	Wild type	0.5 ± 0.05	−27.0 ± 0.3	−36.3 ± 0.7	9.3 ± 0.7
	B5-8	0.4 ± 0.03	−26.4 ± 0.2	−44.8 ± 0.7	18.4 ± 0.7
	B5-1/B5-5	0.9 ± 0.03	−28.2 ± 0.1	−35.9 ± 0.2	7.7 ± 0.3

agreement with the 33 μM value obtained with BSA-O polysaccharide for the monomeric form by SPR.

Light Chain Mutants. Mutants selected from the panned V_L library were screened by ELISA for clones superior to B5-1. Two sequences, one with a single CDR2 mutation and another with two CDR3 mutations, emerged from the eight clones that were sequenced (Table 1). Relative to B5-1, mutant B5-1/B2-5 displayed a slightly faster k_{on} value whereas mutant B5-1/B1-4 had a somewhat slower k_{off} slow (Table 2), probably due to the higher oligomers observed by size-exclusion HPLC.

DISCUSSION

The LCR strategy described here, and applied to improving the functional affinity of an anti-carbohydrate antibody, should have broad applicability in antibody engineering. It provides a fast and effective means of achieving *in vitro* affinity enhancement and of generating synthetic antibody gene libraries to replace animal immunization in antibody production. By permitting controlled and targeted randomization, the synthetic LCR provides an effective means of constructing customized V gene repertoires. Specialized synthetic V gene libraries designed to encode structures with broad complementarity to different classes of antigens are superior to naive repertoires derived from the immune system and are an alternative to generating very large libraries from natural repertoires by CDR randomization and bacterial recombination (23).

Earlier experiments with this scFv using libraries created by error-prone PCR and chemical approaches yielded mainly mutants with improved functional production in *E. coli* (5). The Ile77HThr mutation, previously correlated with for improved scFv yield, was present in three of the nine mutants isolated from the LCR-generated V_H library described here. However, in addition to being good producers (10–20 mg/liter), all of the mutants isolated from the LCR libraries were better binders.

Five of the nine mutants from the V_H library had CDR2 mutations. There was a particularly strong bias for substitution of Ser56H, which was changed to Gly in four and Asn in one of the nine V_H mutants. This is an area of the molecule that does not contact the trisaccharide epitope α-D-Gal-(1→2)-[α-D-Abe-(1→3)]-α-D-Man. It was previously proposed (5) that such changes may remove some steric clashes between this loop and parts of the LPS adjacent to the epitope (5). The SPR data presented here support this interpretation. For example, two of the mutants with CDR2 mutations, B4-3 and B5-1/B5-5, displayed an affinity increase that was higher than that attributable to dimerization alone. The thermodynamic data presented here also support this conclusion. While mutant B5-1/B5-5, which has a Ser56HGly mutation, has an affinity

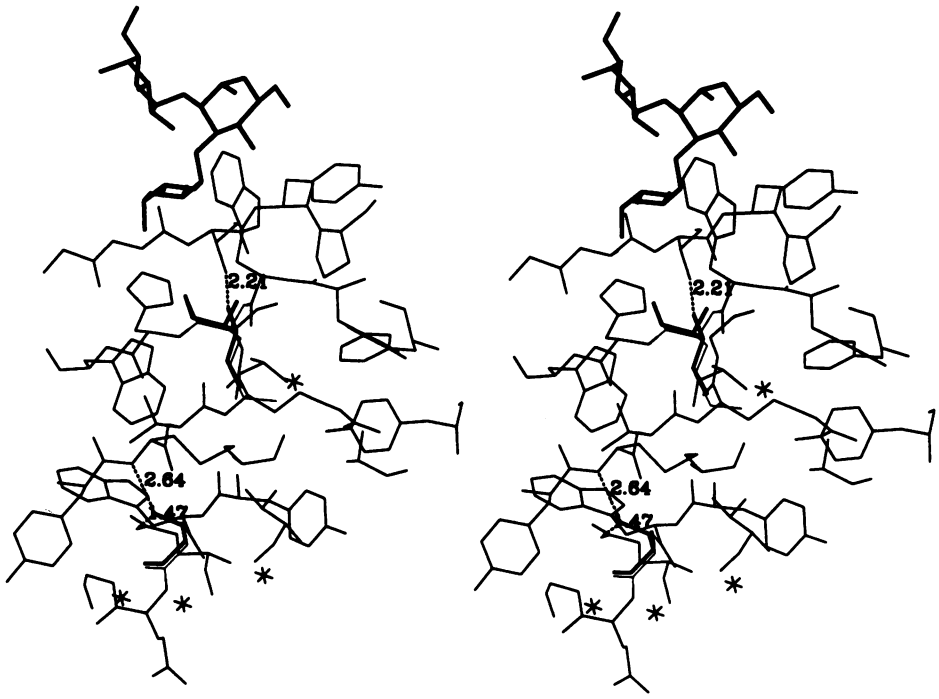

FIG. 2. Stereo view of part of the antibody–trisaccharide complex (2, 3) showing β-sheet stabilization by the Gly109Ser mutation and the proximity of the Met34Ile mutation to two heavy-chain CDR1 residues (Trp-33 and His-35) that contact abequose and to Ile-51 of the heavy-chain CDR2. The mutated form is shown in bold lines.

for trisaccharide that is slightly lower than that of the wild type, its affinity for pentasaccharide is double that of the wild type.

Other mutational hot spots in the mutants described here were Gly109HSer and Met34HIle. Gly109HSer was present in five of the nine V_H mutants and is thought to introduce additional hydrogen bonds that may stabilize the structure (Fig. 2) in the region where the linker approaches the V_H domain (3, 5). Residue 34H, with its side chain pointing away from the binding site, is adjacent to His35H (Fig. 2), a key CDR1 residue that participates in the hydrogen-bond network around the ligand, which includes a water molecule at the base of the binding pocket (2, 3). Residue 34H is also adjacent to Trp33H, one of several aromatic amino acids that form the hydrophobic binding pocket (2). The Met → Ile substitution, which is found at this position in many antibodies, may fine-tune these interactions but the primary reason for the dramatic effect of the Met34Ile and Gly109Ser mutations on functional affinity is that they cause scFv dimerization. Dimerization of scFv molecules has been reported (18, 21, 22, 24, 25), but it is remarkable that single mutations, particularly when they are not in the linker, can change the monomer/dimer ratio so significantly. The fact that these residues are found at the V_L–V_H interface (26, 27) may form the structural basis for this effect.

The effect of dimerization on functional affinity is presumably related to the repetitive nature of polysaccharide antigens. While the slower off rates associated with the dimers are in the range expected with a classical avidity effect, the increased on rates are surprising and not so easily explained. A similar but less dramatic effect was reported (28) for IgG3 binding to a multivalent carbohydrate ligand. The binding reaction between the multivalent polysaccharide antigen and scFv monomers or dimers is complex and a more rigorous analysis of the association phases of scFv preparations that are well defined in terms of their oligomerization state is required. A theoretical treatment by Crothers and Metzger (29) of the effect of polyvalency on antibody binding indicated that dimerization of antibody molecules results in an affinity gain that is the product of the binding constants of the two sites but that this only occurs if the antigen is polyvalent or attached to a surface.

The results presented here indicate that dimerized single-chain antibodies with low nanomolar affinities for carbohydrates can be elicited by *in vitro* randomization and selection techniques. It is possible that further cycles of mutation by the library construction technique described here could yield mutants with even higher affinities.

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