Structural Requirements for a Specificity Switch and for Maintenance of Affinity Using Mutational Analysis of a Phage-Displayed Anti-Arsonate Antibody of Fab Heavy Chain First Complementarity-Determining Region

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Structural Requirements for a Specificity Switch and for Maintenance of Affinity Using Mutational Analysis of a Phage-Displayed Anti-Arsonate Antibody of Fab Heavy Chain First Complementarity-Determining Region

Yee Wah Wong,* Davinder S. Gill,† Behnaz Parhami-Seren,‡ Mary K. Short,§ Seshi Reddy Sompuram,¶ and Michael N. Margolies2‡

We previously showed that a single mutation at heavy (H) position 35 of Abs specific for p-azophenylarsonate (Ars) resulted in acquisition of binding to the structurally related hapten p-azophenylsulfonate (Sulf). To explore the sequence and structural diversity of the H chain first complementarity-determining region (HCDR1) in modulating affinity and specificity, positions 30–36 in Ab 36–65 were randomly mutated and expressed as Fab in a bacteriophage display vector. Ab 36–65 is germline encoded, lacking somatic mutations. Following affinity selection on Sulf resins, 55 mutant Fab were isolated, revealing seven unique HCDR1 sequences containing different amino acids at position H:35. All Fab bound Sulf, but not Ars. Site-directed mutagenesis in a variety of HCDR1 sequence contexts indicates that H:35 is critical for hapten specificity, independent of the sequence of the remainder of HCDR1. At H:35, Asn is required for Ars specificity, consistent with the x-ray crystal structure of the somatically mutated anti-Ars Ab 36–71, while Sulf binding occurs with at least seven different H:35 residues. All Sulf-binding clones selected following phage display contained H:Gly33, observed previously for Ars-binding Abs that use the same germline VH sequence. Site-directed mutagenesis at H:33 indicates that Gly plays an essential structural role in HCDR1 for both Sulf- and Ars-specific Abs. The Journal of Immunology, 1998, 160: 5990–5997.

When A/J mice are immunized with p-azophenylarsonate (Ars)3 protein conjugates, the majority of the serum Abs in secondary responses express a heritable cross-reactive Id (1), designated IdCR. These Abs are each encoded by a single combination ("canonical") of germline V region gene segments (2–4). These canonical Abs, which differ from each other only in somatic mutations and at heavy (H) chain V region gene junctions, have proved useful models for the analysis of Ag-driven somatic mutation resulting in affinity maturation (2, 5–7). Using site-directed mutagenesis, it has been possible to increase affinity for Ars in vitro through amino acid replacements that are not observed in vivo following immunization (8) and to change

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Materials and Methods

Cell lines

The production of the murine A/J hybridoma cell line 36–65 (IgG1,k) and its H chain loss variant (36–65L) was previously described (5, 16). The hybridoma cell line HP Sulf-1 (11) was a gift of L. Wysocki (National Jewish Center for Immunology, Denver, CO). The Sulf-binding transfec-

toma cell line 36–65 containing the H chain mutations V100H/Y107S/

¶ Abbreviations used in this paper: Ars, p-azophenylarsonate; BGG, bovine γ-globulin; Carb, carbencillin; CDR, complementarity-determining region; GsM, goat anti-mouse Abs; H chain, heavy chain; HCDR1, heavy chain first complementarity-determining region; HRP, horseradish peroxidase; IdCR, dominant cross-reactive idiootype associated with Abs elicited in response to p-azophenylarsonate; IPTG, isopropyl β-D-thiogalactoside; L chain, light chain; LB, Luria-Bertani medium; Phos, p-azophenolphosphonate; PVC, polyvinyl chloride; Sulf, p-azophenylsulfonate; Tet, tetracycline; wt, wild-type.
N35A was described (9, 17). An Ars-binding variant of 36–65 containing the H chain junctional mutations V100H and Y107S, as well as the Sulf-binding mutants derived therefrom containing H:N35A, was described (9). The 36–65 transfected cell line with a polyglycine-replaced HCDR1, as well as the mutant derived therefrom containing the back mutation G35N, has also been described (18).

Synthesis and purification of hapten and hapten conjugates
p-aminobenzylarsonate (Ars), p-aminophenylphosphonate (Phos), and p-aminophenylsulfonate (Sulf) were obtained from Aldrich (Milwaukee, WI). T4-DNA acetylase, I/II, and V100H, Y107S, G35A, and N35A derivatives of Ars, Sulf, and Phos were prepared as described (19, 20). Hapten conjugates of bovine γ-globulin (BGG) were prepared as described (21).

Construction of library of HCDR1 mutants in bacteriophage
Total mRNA was extracted from 36–65 hybridoma cells, and the cDNA of the Fd (Vα + Cα) and light (Vλ + Cλ) chain of Ab 36–65 was amplified using the following primers: for the H chain, H1, 5′-ATGCGCCGATTCTAAATGCGAGGT; H2, 5′-GCTGTACCTATAGCCTGCTCTAGACCCGAC. For the L chain, L1, 5′-GACTTGCGAATTCGATGATGACACAGACTACCTCC-3′; and L2, 5′-GGCTGATCTAGATATCCAGATGACACAGACTACCTCC-3′ (restriction sites are underlined). The PCR fragments of H and L chains were digested with Mun I and Ncol/XhoI, respectively. The pComb3 vector (Fig. 1, a gift of Dr. Carlos Barbas, The Scripps Research Institute, La Jolla, CA) (22) was modified by adding a Mun I restriction enzyme site, (HCDR1), two restriction enzyme sites, Xba I and Nco I, to confirm the sequence, the H chain was ligated into the pComb3 vector containing the L chain. Primers H1 and L1 contain codons at the H and L chain N termini designed to reproduce the correct 36–65 N-terminal sequences (Fig. 1). To randomly mutate the CDR1 of the 36–65 H chain (HCDR1), two restriction enzyme sites, HindIII and XhoI, were constructed, flanking the HCDR1 sequence using two primers, R1, 5′-ATGGCTGCTGACAACTCCTGTTGAAGCTCTTGAGGTT and R2, 5′-ACAGGGGCTCGAGTGGATTGGATA-CAACCA-3′ as template, in which N represents an equimolar ratio of the four dNTPs and S represents an equimolar ratio of dGTP and dCTP. Primer sequences were synthesized by PCR using these two primers with the oligonucleotide 5′-GCCATGGCCGAGGT-3′ (restriction sites are underlined). The PCR fragments of H and L chains were digested with HindIII and XhoI and ligated into the HindIII/XhoI-digested vector.

The above construct was electroporated into Escherichia coli BB4 cells. After growth at 37°C for 1 h, the culture was supplemented with 100 μg/ml carbenicillin (Carb) and 10 μg/ml tetracycline (Tet) and grown for another hour. The culture was then infected by bacteriophage (1:100,000 dilution, La Jolla, CA), and a fraction of the culture was plated onto LB-ampicillin agar plates to determine the titer of the library, while the rest of the culture was shaker at 2 h before adding 70 μg/ml kanamycin and incubated overnight. The supernatant by affinity chromatography with Sulf-BGG-conjugated Sepharose, as described (9). The purification of the 36–65 mutant with polyglycine-replaced HCDR1 (36–65 H1Gly/G35N has been described (18). The mutants 36–65 H1Gly/G35A, polyglycine-replaced HCDR1 (36–65 H1Gly) and the back-mutant H1Gly/G35N was incubated for 1 h at 37°C. The wells were then washed three times with 200 μl 0.05% Tween-20/PSBA, and 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (GoM) Fab′ (2) (ICN, Costa Mesa, CA) and incubated overnight at 4°C. The wells were rinsed three times with 200 μl 0.05% Tween-20/PSBA; 0.14 M NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 0.02% NaN3, pH 7.4) and then blocked with 150 μl 5% BSA/0.05% Tween-20/PSBA and incubated at 37°C for 1 h. The supernatant was mixed with 100 μl 0.05% Tween-20/PSBA and incubated at 37°C for 2 h. The wells were washed three times with 100 μl 0.05% Tween-20/PSBA, and 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-m13 Abs (Pharmacia, Piscataway, NJ) was added and incubated for 1 h at 37°C. The wells were then washed three times with 200 μl 0.05% Tween-20/PSBA; 100 μl of TMB substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added and the reaction was terminated by adding 50 μl 2 M H2SO4. Color development was determined at 450 nm using a Bio-Tek ELISA reader (Bio-Tek Instruments, Winooski, VT). Clones were selected for further study if the OD was ≥1 for Sulf-BGG and GoM Fab′ (2). All positive clones selected bound equally well to both Sulf-BGG and GoM Fab′ (2), while the negative clones were all at background levels (OD < 0.002). To determine the specificity of the selected SCAB-binding clones, an ELISA was performed on plate-displayed Fab with plates coated with 100 μl/well of 2 μg/ml Ars-BGG, Sulf-BGG, Phos-BGG, GoM Fab′ (2), and BGG (as negative control).

Expression and purification of soluble Sulf-binding Fab fragments
The plasmids of selected Sulf-binding clones were digested with SpeI/XhoI to excise the gene III DNA fragment (Fig. 1). The plasmid was self ligated and electroporated into E. coli BB4 cells. Each selected clone was grown in 1 L Carb/Tet/superbroth at 37°C overnight. The cells and supernatant were separated by centrifugation. The cells were resuspended in 100 ml of lysis buffer (50 mM glucose, 0.5 mg/ml Pefabloc, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin (Boehringer Mannheim, Indianapolis, IN), 25 mM EDTA, and 25 mM Tris-HCl, pH 8.8) and incubated with 2.5 mg/ml lysozyme for 15 min on ice. Brij 58 was added to a concentration of 0.5%, and the mixture was left on ice for 30 min. The supernatant was harvested by centrifugation at 16,000 rpm for 30 min at 4°C. The Sulf-binding Fab was purified from the supernatant by affinity chromatography with Sulf-BGG-conjugated Sepharose, as described (9). The purification of the 36–65 mutant with polyglycine-replaced HCDR1 (36–65 H1Gly/G35N was achieved was used in a competition ELISA assay.

Determination of Ab affinity and avidity
Binding affinity (Kd) of Abs and Fab fragments for Ars-N-acetyl-l-tyrosine and Sulf-N-acetyl-l-tyrosine was determined by fluorescence quenching, as reported (24, 25). Kd values reported are averages of at least three determinations. The Fab obtained by enzymatic digestion from the mAbs 36–65 and HP Sulf-1 were used as controls. The direct binding of Abs and Fab fragments to Ars-BGG and Sulf-BGG was determined in triplicate by ELISA. The 96-well PVC plates were coated with 100 μl of 2 μg/ml Ars-BGG or Sulf-BGG, and 100 μl of serially diluted purified Abs or Fab fragments (0.64–50,000 ng/ml; fivefold dilutions) was added. Binding was detected by adding HRP-conjugated goat anti-mouse IgG Fab′ (2) (Jackson ImmunoResearch, West Grove, PA) and stopped by addition of 50 μl 2 M H2SO4. The end point of the reaction was determined in an ELISA reader at 450 nm. The concentration of each Fab fragment at which 50% binding was achieved was used in a competition ELISA assay.

The relative affinity was measured in triplicate by competition ELISA. The 96-well PVC plates were coated with 100 μl of 2 μg/ml Ars-BGG or Sulf-BGG. A total of 50 μl of Fab (concentrations as determined above) was added to each well with 50 μl of serially diluted Ars-N-acetyl-l-tyrosine or Sulf-N-acetyl-l-tyrosine (twofold dilutions, 0.8–500 nM) and incubated at 37°C for 2 h. The binding of Fab to the plate was determined by

* Amino acids are denoted in a one- or three-letter code in the text. For amino acid mutations, the amino acid preceding the position number represents the unmutated residue. The amino acid following the number is the engineered mutation.
The resulting construct was transfected into PCR amplification and subcloned into the pComb3 vector (Fig. 1). The cDNA fragment representing 36–65 Fab was generated by Construction of randomized HCDR1 region of 36–65 Fab

Results

Site-directed mutagenesis of mutant Fab in pComb3

For the H:G33A site-directed mutation of both the anti-Ars 36–65 Fab and a Sulf-binding mutant Fab designated S3 (Table III), two oligonucleotides were synthesized with sequences matching a 65-bp region across HCDR1, except for the residue to be mutated. These oligonucleotides were then used as a template in separate PCR reactions using a 26-mer sense primer bearing a HindIII site and a 26-mer antisense primer bearing an XhoI site (Fig. 1). The PCR products were gel purified and digested with HindIII/XhoI. The digested products were repurified and ligated into pComb3 containing the appropriate Fab that had been digested with HindIII/XhoI. The vector containing the site-directed mutation was introduced into XLI-Blue E. coli cells. The presence of the mutations was verified by dideoxy chain termination DNA sequencing.

Mutagenesis and expression of mutant Abs in hybridoma cells

As described previously (25–27), mutations (indicated in parentheses) were introduced into the cloned 36–65 VDJ gene (H:G33A, H:G33L), the 36–65 VDJ gene containing the mutations N35A/V100H/Y107S (9, 17) (H:G33A), or the 36–65 polyglycine HCDR1/G35N (18) (H:G35A). The mutant genes were subcloned into an expression vector (28) and transfected into the L chain-producing hybridoma cell line 36–65 L by electroporation and selected as described (24, 27).

Selection of Sulf-specific phage clones

One milliliter of phage library (>10^{12} plaque-forming units/ml) was used in selection using Sulf-BGG-conjugated Sephacryl resin. After the fourth round of selection, the eluted phage were transfected into bacteria, and independent colonies were picked and grown with helper phage. The phage supernatant was harvested, and binding to Sulf was determined by ELISA on Sulf-BGG-coated plates. Ninety-six clones were screened; 55 bound Sulf-BGG. These positive clones were sequenced, revealing seven different DNA sequences. The amino acid sequences and the frequency of appearance are shown in Table I. Among clones with identical amino acid sequences, in all cases only a single nucleotide sequence was observed. At position 30, six different amino acid residues were observed. At position 31, five different amino acids occurred with three of seven sequences containing Gly. At position 32, four different amino acids were identified, with aromatic residues found in five of seven mutants. At position 33, glycine occurred in all seven sequences, encoded by two different DNA sequences. The amino acid sequences and the frequency of appearance are shown in Table I. Among clones with identical amino acid sequences, in all cases only a single nucleotide sequence was observed. At position 30, six different amino acid residues were observed. At position 31, five different amino acids occurred with three of seven sequences containing Gly. At position 32, four different amino acids were identified, with aromatic residues found in five of seven mutants. At position 33, glycine occurred in all seven sequences, encoded by two different codons. At position 34, three different amino acids were found.

Table 1. Amino acid sequences of the HCDR1 region of Sulf-binding mutants and frequency of their appearance in selected phage library

<table>
<thead>
<tr>
<th>Antibody/Mutant</th>
<th>CDR1</th>
<th>30</th>
<th>31</th>
<th>32</th>
<th>34</th>
<th>35</th>
<th>36</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>36–65 wt</td>
<td></td>
<td>T</td>
<td>S</td>
<td>Y</td>
<td>G</td>
<td>I</td>
<td>N</td>
<td>W</td>
</tr>
<tr>
<td>S3</td>
<td></td>
<td>P</td>
<td>M</td>
<td>H</td>
<td>—</td>
<td>L</td>
<td>I</td>
<td>F</td>
</tr>
<tr>
<td>S22</td>
<td></td>
<td>L</td>
<td>G</td>
<td>—</td>
<td>—</td>
<td>V</td>
<td>V</td>
<td>Y</td>
</tr>
<tr>
<td>S19</td>
<td></td>
<td>G</td>
<td>E</td>
<td>—</td>
<td>—</td>
<td>F</td>
<td>V</td>
<td>Y</td>
</tr>
<tr>
<td>S15</td>
<td></td>
<td>N</td>
<td>W</td>
<td>F</td>
<td>—</td>
<td>L</td>
<td>V</td>
<td>F</td>
</tr>
<tr>
<td>S23</td>
<td></td>
<td>A</td>
<td>G</td>
<td>—</td>
<td>—</td>
<td>L</td>
<td>I</td>
<td>Y</td>
</tr>
<tr>
<td>S4</td>
<td></td>
<td>S</td>
<td>G</td>
<td>N</td>
<td>—</td>
<td>L</td>
<td>A</td>
<td>Y</td>
</tr>
<tr>
<td>E41</td>
<td></td>
<td>A</td>
<td>P</td>
<td>—</td>
<td>—</td>
<td>L</td>
<td>A</td>
<td>—</td>
</tr>
</tbody>
</table>

Dash indicates identity with the topmost sequence.
Five of seven mutants contained leucine at position 34, while the other two substitutions were also hydrophobic. At position 35, three different aliphatic residues occurred: isoleucine, alanine, and valine. We had reported previously that single mutations at position 35 to Ser, Thr, Ala, and Cys in Ab 36–65 resulted in a specificity change from Ars to Sulf (9).

The fact that identical nucleotides were used to encode the same amino acid sequences of mutants that were selected more than once raises the possibility that the library size was limiting and/or that screening or selection bias occurred. Nonetheless, the data were sufficient to serve as a basis for the design of site-directed mutants to examine the requirements in HCDR1 for Ars and Sulf binding. The high frequency of clone S3 (42/55) may be related to its higher affinity for Sulf and/or to preferential growth.

The plasmid DNA from each Sulf-binding mutant was digested with SpeI/NheI to excise the gene III coding region. The digested plasmids were religated and electroporated into bacteria. Single clones were selected and grown to generate soluble Fab fragments.

The soluble Fab were purified from both periplasmic space extracts and culture supernatant using Sulf-BGG-conjugated Sepharose. Using SDS-PAGE, protein bands were observed as 25-kDa doublets (reduced) and as 50-kDa single bands (nonreduced) and were indistinguishable from enzymatically generated 36–65 Fab fragments. The total yield of mutant Fab was 1 to 5 mg/L. Neither induction with IPTG nor incubation at 30°C increased the Fab yield. Amino acid sequence analysis of purified bacterial Fab of wt 36–65 resulted in a mixture of H and L chains in a 1:1 ratio with residues identical to the bona fide hybridoma Ab 36–65. This result indicates that cleavage of the pel B leader occurred at a site (C-terminal to Ala in both heavy and light chain; Fig. 1), resulting in chains of native length and with wt N-terminal sequences, indicating that the pComb3 modification was correct.

**Binding affinity of selected Sulf-binding clones**

Bacterially expressed Fab was purified for each mutant, following gene III excision. Affinity-purified Fab were first tested in a solid-phase direct binding assay against Ars-BGG and Sulf-BGG. The Sulf-selected Fab mutants bound strongly to Sulf-BGG-coated plates, but bound poorly to Ars-BGG-coated plates (Fig. 2). Wild-type 36–65 Fab bound Ars-BGG, but not Sulf-BGG-coated plates, as noted previously (9); the binding of 36–65 Fab produced by enzymatic digestion of IgG and bacterially expressed 36–65 Fab was indistinguishable. The control hybridoma Ab HP Sulf-1 Fab binds Sulf-BGG, but also binds Ars-BGG to a small extent (data not shown) (9). It should be noted that the range of concentrations required to detect binding of 36–65 Fab is higher than that for the corresponding Ig owing to differences in avidity in the solid-phase assay. In summary, the direct binding assays indicated that the selected Fab mutants were specific for Sulf-BGG; all seven new HCDR1 sequences resulted in changing the binding specificity from Ars to Sulf (Fig. 2). These seven selected anti-Sulf mutants also did not bind Phos coupled to BGG in a phage ELISA (data not shown).

The binding constants of affinity-purified Fab measured by fluorescence quenching (24, 25) are shown in Table II. Compared with the enzymatically produced HP Sulf-1 Fab (9, 11), five Fab clones, S3, S22, S19, S15, and S23, bound Sulf-N-acetyl-L-tyrosine with affinity up to 8.5-fold higher. All seven selected clones bound Sulf at a level measurable by fluorescence quenching, while the wt 36–65 Ig, Fab produced by enzymatic digestion of 36–65 Ig, and bacterial 36–65 Fab did not bind Sulf (Fig. 2).
of Abs for each hapten were also compared by competition ELISA (data not shown).

In direct binding assays.

tant Fabs, as these Abs/Fabs do not bind Ars in direct binding assays.

averages of three determinations.

Q was determined using HRP-labeled goat anti-mouse Ab.

Binding affinities of HCDR1 site-directed mutants

Table II. Binding constants of Abs and mutants

<table>
<thead>
<tr>
<th>Ab/Mutant</th>
<th>Affinity (K&lt;sub&gt;a&lt;/sub&gt; × 10&lt;sup&gt;6&lt;/sup&gt; M&lt;sup&gt;-1&lt;/sup&gt;) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulf-N-acetyl-l-tyrosine</td>
<td></td>
</tr>
<tr>
<td>HPSulf-1 Fab</td>
<td>8.1 ± 2.1</td>
</tr>
<tr>
<td>S3</td>
<td>68.1 ± 16.5</td>
</tr>
<tr>
<td>S22</td>
<td>43.3 ± 21.1</td>
</tr>
<tr>
<td>S19</td>
<td>35.7 ± 16.5</td>
</tr>
<tr>
<td>S15</td>
<td>24.7 ± 14.3</td>
</tr>
<tr>
<td>S23</td>
<td>13.0 ± 5.8</td>
</tr>
<tr>
<td>S4</td>
<td>3.5 ± 0.6</td>
</tr>
<tr>
<td>E41</td>
<td>1.8 ± 0.8</td>
</tr>
<tr>
<td>36–65 Ig (wt)</td>
<td>NM&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>36–65 Fab (enzymatic)</td>
<td>NM</td>
</tr>
<tr>
<td>36–65 Fab (bacterial)</td>
<td>NM</td>
</tr>
<tr>
<td>Ars-N-acetyl-l-tyrosine&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>36–65 Ig (wt)</td>
<td>5.8 ± 0.6</td>
</tr>
<tr>
<td>36–65 Fab (enzymatic)</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>36–65 Fab (bacterial)</td>
<td>5.5 ± 1.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Binding affinity (K<sub>a</sub>) was determined by fluorescence quenching. The data are averages of three determinations. Q<sub>100</sub> was greater than 30% in all cases. Relative affinities of Abs for each hapten were compared by competition ELISA (data not shown).

<sup>b</sup> Affinity was not measurable (NM), as 36–65 Ig and Fab do not bind Sulf in direct binding assays.

<sup>c</sup> Affinity for Ars-N-acetyl-l-tyrosine was not measurable for HP Sulf-1 and mutant Fab, as these Abs/Fabs do not bind Ars in direct binding assays.

Site-directed mutagenesis of HCDR1

Previous mutagenesis experiments indicated that replacement of H:Asn35 by Ala, Ser, Thr, or Cys resulted in Sulf-specific forms of Ab 36–65 (9). The phage-selected Sulf-binding mutants reported in this study exhibit even further diversity at position H:35, i.e., Ile and Val are also observed (Table I), albeit in the context of non-wt HCDR1. Replacement of all five residues in 36–65 HCDR1 with glycines (36–65 H1Gly) resulted in complete loss of affinity for Ars (18) (Table III). However, restoration of the wt residue (Asn) at position 35 (36–65 H1Gly/G35N) resulted in Ars binding (18).

Because Asn35 appeared important for Ars specificity, we wondered whether replacement with Ala at position 35 in a polyglycine HCDR1 would result in a shift in fine specificity of the Ab from Ars to Sulf, independent of the identity of other HCDR1 residues. We constructed this mutant as a transfectoma using site-directed mutagenesis. Ab 36–65 H1Gly/G35A bound Sulf, but not Ars, in a direct binding ELISA (Table III), in contrast to Ab H1Gly/G35N, which is Ars specific. The affinity of Ab H1Gly/G35A for Sulf was 6.3 × 10<sup>7</sup> M<sup>-1</sup> (Table III). We also assayed the 36–65 mutant containing a polyglycine HCDR1 (36–65 H1Gly) (18), which contains H:Gly35; for Sulf binding. This mutant binds Sulf with an affinity of 1.6 × 10<sup>7</sup> M<sup>-1</sup> (Table III).

The data reported in this work for phage-selected Fab and site-directed mutants, as well as previous mutagenesis data (9), indicate that at least seven different amino acid replacements at H:Asn35 result in a change in specificity from Ars to Sulf. Furthermore, the results suggest that the specificity change is independent of the context of the remainder of HCDR1. Because all seven phage-selected Sulf mutants contained Gly at position 33, and all Ars-binding canonical Abs also express Gly at this position (30, 31; data summarized in 12, 13), we constructed mutants at H:33 to determine whether other residues were permissive here for Sulf and/or Ars binding. Thus, H:Gly33 was mutated to Ala in both 36–65 and the Sulf-binding mutant Fab S3 (Table III). The 36–65 G33A mutant showed virtually no binding to Ars-BGG and to goat anti-mouse F(ab′)<sub>2</sub> in a phage ELISA, indicating poor expression of the mutant in functional form on phage. Control experiments were done to test the integrity of the pComb3 36–65 H G33A construct. The region of plasma DNA encoding the Fd, gene III, and L chain is flanked by two NorI restriction sites in this construct. A NorI restriction digest showed bands of the expected size. A fragment including the entire Fd was obtained by restriction digestion with MunI/SpeI (see Fig. 1). The fragment was ligated back into the parental vector that had been predigested with the same enzymes, and the correct placement was verified by DNA sequencing. However, an ELISA on the reengineered mutant plasmid again showed virtually no binding. As bacterial production of this Fab mutant did not appear possible, the mutation was produced instead in hybridoma cells. The mutants 36–65 H:G33A and 36–65 H:G33L were constructed by oligonucleotide-directed mutagenesis, inserted into an expression vector, and electroporated into 36–65 L chain-producing cells (see Materials and Methods).

Table III. Binding affinities of HCDR1 site-directed mutants

<table>
<thead>
<tr>
<th>Ab/Mutant</th>
<th>Sequenced&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ng Ab Required for 50% Binding&lt;sup&gt;a&lt;/sup&gt;</th>
<th>(K&lt;sub&gt;a&lt;/sub&gt; × 10&lt;sup&gt;6&lt;/sup&gt; M&lt;sup&gt;-1&lt;/sup&gt;) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>36–65 HCDR1</td>
<td>30 33 36 Fab/IgG&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ars</td>
<td>Sulf</td>
</tr>
<tr>
<td>36–65</td>
<td>T S Y G I N W</td>
<td>IgG</td>
<td>18</td>
</tr>
<tr>
<td>G33A</td>
<td>– – – – – – –</td>
<td>IgG</td>
<td>NA</td>
</tr>
<tr>
<td>G33L</td>
<td>– – – – L – – –</td>
<td>IgG</td>
<td>NA</td>
</tr>
<tr>
<td>S3</td>
<td>P M H G L I F</td>
<td>Fab</td>
<td>NA</td>
</tr>
<tr>
<td>G33A</td>
<td>– – – – – – –</td>
<td>Fab</td>
<td>NA</td>
</tr>
<tr>
<td>36–65 H1Gly</td>
<td>T G G G G G W</td>
<td>IgG</td>
<td>NA</td>
</tr>
<tr>
<td>G35N</td>
<td>– – – – N – – –</td>
<td>IgG</td>
<td>250</td>
</tr>
<tr>
<td>G35A</td>
<td>– – – – – – A</td>
<td>IgG</td>
<td>NA</td>
</tr>
<tr>
<td>36–65 V100H/Y107S</td>
<td>T S Y G I N W</td>
<td>IgG</td>
<td>6</td>
</tr>
<tr>
<td>N35A</td>
<td>– – – – – – –</td>
<td>IgG</td>
<td>NA</td>
</tr>
<tr>
<td>N35A/G33A</td>
<td>– – – – – – –</td>
<td>IgG</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup> Binding affinity (K<sub>a</sub>) was determined by fluorescence quenching. The data are averages of three determinations. Q<sub>100</sub> was greater than 30% in all cases. Relative affinities of Abs for each hapten were also compared by competition ELISA (data not shown).

<sup>b</sup> Affinity was not measurable (NM), as 36–65 Ig and Fab do not bind Sulf in direct binding assays.

<sup>c</sup> Affinity for Ars-N-acetyl-l-tyrosine was not measurable for HP Sulf-1 and mutant Fab, as these Abs/Fabs do not bind Ars in direct binding assays.

<sup>d</sup> The sequence of 36–65 was previously reported (2,6); S3 is from Table I, H1Gly and H1Gly/G35N are from Ref. 18, and 36–65/V100H/Y107S from Refs. 9 and 17. Dashes indicate identity with the topmost sequence in each set.

<sup>e</sup> S3 is phage-derived and therefore assayed as Fab, as is the S3/G33A mutant (see text for details). The other Ab/ mutants are intact IgG.

<sup>f</sup> In a direct binding assay, wells of microtiter plates were coated with either Ars or Sulf coupled to BGG. Binding of various concentrations of affinity-purified Abs or Fab was determined using HRP-labeled goat anti-mouse Ab.

<sup>g</sup> Fifty percent binding was not achieved at Ab concentrations as high as 10 μg/ml.

<sup>h</sup> Binding constants could not be measured (NM), as this assay has a lower limit of measuring K<sub>a</sub> = 5 × 10<sup>8</sup> M<sup>-1</sup>.
A direct binding GzELISA was used to establish satisfactory expression of these mutants. Direct binding ELISA demonstrated that neither the 36–65 G33L mutant nor the G33A mutant bound Ars or Sulf. These data are summarized in Table III.

The mutation H:G33A introduced into the Sulf-binding mutant S3 in pComb3, in contrast to that for 36–65, showed expression of Fab using goat anti-mouse F(ab′)2 in a phage ELISA. Soluble Fab was produced from the mutant S3 H:G33A following gene III excision and purified by affinity chromatography, and binding assays were performed. A direct binding assay (Table III) showed that this mutant bound Sulf specifically, but not Ars; the binding was reduced compared with S3. Inhibition ELISA using Sulf-N-acetyl-L-tyrosine as competitor indicated an IC50 approximately 50-fold less than that for wt S3 (data not shown) and no measurable binding in the equilibrium fluorescence-quenching assay (Table III).

We previously reported that a single mutation in 36–65 at position H:N35 to Ala resulted in Sulf binding (9). To assess the role of Gly33 in such a Sulf-specific mutant Ab, we mutated H:G33 to Ala in a 36–65 Ab containing the D gene junction replacements V100H/Y107S (9, 17) and the H:Asn35 to Ala mutation (9) (Table III). This Ab binds Ars with higher affinity than 36–65 (9). The junctional residues 100H/107S were observed in Abs elicited in response to both Ars and Sulf (11–13), and did not affect the specificity dictated by residue H:35 (9). In the direct binding ELISA for the mutant 36–65/V100H/Y107S/N35A/G33A, the binding to Sulf was reduced approximately 100-fold compared with the parent mutant 36–65/V100H/Y107S/N35A (Table III). Furthermore, this G33A mutant had no measurable affinity using fluorescence quenching.

**Discussion**

Immunization of AJJ mice with arsonate, phosphonate, or iodoarsonate-protein conjugates results in each case in the selection of canonical Abs associated with the Ars response (10, 32). In contrast, immunization with sulfonate does not elicit Abs using these canonical germline genes. However, when sulfonate was given as a second immunization shortly after primary immunization with Ars, canonical Abs could be recruited that contained somatic mutations resulting in a switch to Sulf binding (11). We previously used site-directed mutagenesis to localize the structural basis for the specificity change (9). Replacement of N35 in H chain CDR1 with Ser, Thr, Ala, or Cys abolished detectable Ars binding in the unmutated canonical Ab 36–65, associated with acquisition of Sulf binding. However, introduction of Gln or Asp into 36–65 at H:35 abolished binding for both Ars and Sulf. Introduction of Ala at H:35 into the somatically mutated higher affinity canonical anti-Ars Ab 36–71 resulted in higher affinity Sulf binding as well (Kd = 3.4 x 10−7 M−1) with virtual loss of Ars binding. Evidence was adduced using site-directed mutagenesis that the geometries of the hapten contact residues in both 36–65 and 36–71 are similar (20, 28). In the model of the crystal complex of Fab 36–71 with phenylarsionate (12, 13), the side chain of H:Asn35 is a contact residue to Ars. H:Asn35 forms a hydrogen bond with an arsenate oxygen and also is part of a hydrogen bond network with H:Ser99 and H:Trp47. We proposed that the specificity shift to Sulf binding was due to interruption of this hydrogen bond network by replacements at H:35, resulting in a decrease in the binding site size (9). In the new binding site, the putative hydrogen bond geometry was optimal for Sulf as distinct from Phos and Ars. The hapten Ars, Phos, and Sulf differ from each other by only a single atom. The small molecule crystal structures (summarized in Ref. 9) show that the maximal difference in solvent-accessible surface is only 14 Å2, and that the bond lengths involving sulfur, phosphorus, or arsenic vary up to a maximum of 0.29 Å. Remarkably, Sulf and Phos are more similar to each other than to Ars, yet Ars and Phos elicit canonical Abs. The pattern of somatic mutations acquired during immunization with Phos is not distinguishable from that of Ars (32). Ab 36–65 and other anti-Ars Abs bind Ars and Phos in a similar manner. It has long been known that (polyclonal) antisera raised to Ars-protein conjugates bound Ars and Phos, but bound poorly to Sulf (33). When mice were immunized first with Ars, then 1 mo later with Sulf, the resulting canonical Abs bound Ars; they also bound Sulf, although generally with lower affinity (10). For the site-directed mutants at H:35 that resulted in Sulf binding, none bound Phos (9). The seven-phage library-derived anti-Sulf Fab mutants described in this study demonstrated the same remarkable specificity difference. In addition, we screened all mutants previously produced in our laboratory in the anti-Ars 36–65 background that were mutated at positions other than H:35, for Ars, Phos, and Sulf binding in direct binding assays (data not shown) (9, 17, 20, 26, 27, 34). All Abs bound Phos as well as Ars, but not Sulf.

Acquisition of Sulf binding is consistent not only with Ala, Thr, Ser, or Cys at position H:35 (9), but in this study we present evidence, based on Sulf selection of phage-displayed HCDR1 mutants, that Val and Ile at this position also confer specificity for Sulf. H:Val35 has been observed independently in Sulf-binding canonical Abs in transgenic mice (L. Wysocki, personal communication). In addition, even Gly at H:35 is associated with Sulf binding, as a mutant 36–65 with polyglycine-substituted HCDR1 binds Sulf (Table III) (18). Thus, Sulf binding is compatible with at least seven different amino acid side chains at position 35, in contrast to the striking requirement for Asn at H:35 for Ars binding (at least seven different replacements for Asn at H:35 abolish Ars binding) (9, 12, 13, 20, 28). In previous mutagenesis experiments, we showed that Sulf specificity arises from mutations at H:35 in both the germline canonical anti-Ars Ab 36–65 and the somatically mutated anti-Ars canonical Ab 36–71 (the sequence of 36–71 in this region differs from 36–65 only by a Tyr/Asn difference at position 32, a mutation found frequently in other anti-Ars Abs). In addition, we show that Sulf binding can occur in H:35 mutants containing a variety of sequences at other HCDR1 positions (Table I). Moreover, Sulf binding also occurs (increased 3.9-fold) when the mutation H:Gly35Ala is introduced into a polyglycine-replaced HCDR1 (Table III).

The physicochemical conservation at residues 33–36 (Table I) in the segment randomized in HCDR1 among Sulf-binding Fab mutants, as well as the conserved residues as defined by Chothia et al. (35, 36) for canonical HCDR1 loop conformation, which are present in 36–65, 36–71, and in the Sulf mutants (residues 26, 27, 29, 34, and 94 in Vκ1), suggests that among this set of Sulf-binding mutants, the HCDR1 loop conformation is not significantly different from that of 36–65 or 36–71. Moreover, it was shown that a polyglycine-substituted HCDR1 in the unmutated canonical Ab does not bind Ars, but can be restored to Ars binding by changing H:35 to the wt Asn (18). Furthermore, mutation of H:Asn35 to Ala in this construct, as noted above, switches fine specificity from Ars to Sulf. These results are consistent with an extended conformation of the HCDR1 loop in the 36–71 crystal structure (12, 13), in which the backbone is relatively solvent inaccessible and rigid, independent of the identity of the side chains. This hypothesis is strengthened by the results of mutagenesis at position H:33 in the CDR1 loop described below. In addition, since 1) among HCDR1 residues, only the side chain of residue 35 is in the binding pocket (Fig. 3), and 2) Gly is conserved at position 33 in both Sulf-binding and Ars-binding Abs (see below), the evidence indicates that
specificity for both Ars- and Sulf-binding canonical Abs is dictated by the identity of the H:35 side chain. The affinity of Sulf-specific canonical Abs can, however, be modulated by other mutations in HCDR1 (Tables II and III) (11, 25), and was noted previously for Ars-specific canonical Abs (25).

Glycine is found at position 33 in the sequences of all seven Sulf clones selected from HCDR1-mutagenized phage-displayed 36–65 Fab (Table I). Using the statistical analysis described by Mathews and coworkers (37), the observed frequency of glycine differs from the expected frequency (based on the NNS mutagenesis scheme; see Materials and Methods) by 12.2 SDs. Thus, glycine appears to be highly selected. That glycine is conserved not because it is required for Sulf specificity is indicated by the fact that H:Gly33 is also conserved among more than 227 anti-Ars mAbs utilizing the canonical gene segment combinations that have been sequenced in this region (30, 31, 38; summarized in 12 and 13). We wondered whether H:Gly33 is conserved in canonical Abs binding Ars or Sulf because it is required for proper HCDR1 loop conformation, i.e., because the $\phi, \psi$ angles at H:33 do not permit substitution of other side chains. However, Ramachandran plots for the crystal structure of the canonical anti-Ars Fab 36–71 (12, 13) indicate that the $\phi, \psi$ angles of H:33 do not preclude other side chain substitutions at this position. In the 36–71 Fab structure, H:Gly33 is within 5 Å of H:Tyr50, a putative hapten contact residue (Fig. 3). Substitutions of residues with longer side chains at H:33 potentially cause steric clash with H:Tyr50, affecting Ars binding. The 36–65 H:Gly33Ala and H:Gly33Leu mutations ablated Ars binding (Table III). In addition, the mutation H:Gly33Ala reduced Sulf binding substantially in the phage display-derived mutant S3 (Table III) and in the background of 36–65 containing the specificity-switch mutation Asn35Ala (Table III). Thus, the mutations at H:33 consistently reduce binding for both Ars and Sulf-specific canonical Abs. These data are in agreement with the conservation of Gly at position 33 in all of the Sulf-binding phage-derived clones reported in this work, as well as all Ars-binding canonical Abs found in vivo. In both in vitro selection by panning and in vivo Ag-driven selection of somatic mutants, selection is based on affinity for Ag.

The side chain at position H:34 in the 36–71 crystal structure packs into the hydrophobic interior (Fig. 3), and all of the substitutions at this position among Sulf-binding Fab and other Fab with this particular canonical HCDR1 loop (35, 36) are hydrophobic. At position 36, Trp is invariant among all mouse VH sequences (29); however, Sharon showed that the engineered mutation H:Trp36Ala results in retention of Ars binding and Id in Ab 36–65 (24). Among the Sulf-binding mutants, Phe and Tyr are found at H:36, as well as Trp (Table I). The side chain of Trp36 packs into the hydrophobic core in the crystal structure of 36–71; the aromatic residues Phe and Tyr can substitute for Trp, consonant with Sulf binding. One factor that may account for the failure to observe Phe or Tyr at position 36 in vivo is that these two replacements require that more than a single base change occur during somatic mutation.

Because the in vivo hypermutation process resulting in somatic mutations may not be random, the observation that among somatically mutated hybridomas certain residues are conserved cannot be taken as firm evidence that such conservation reflects a required structural and/or functional role. Random mutagenesis of phage-displayed Ab fragments or site-directed mutagenesis provides the potential for changes in specificity and affinity not constrained by the bias of germline gene codons and the in vivo somatic mutation process. On the other hand, there are sequence-specific constraints on expression of Fab in bacterial systems that may not be observed in Abs recruited following somatic cell fusions. Nonetheless, for H chain CDR1 residue 33, the conservation of Gly among anti-Ars and anti-Sulf canonical Abs is related to steric requirements for folding, and the conservation of Asn 35, to arsonate specificity.

Among Abs that display improved affinity following somatic hypermutation and Ag selection, certain point mutations were observed repeatedly, and in some instances were shown to be responsible for the increase in affinity (reviewed in Ref. 39). V region hypermutation does not appear to be random in distribution; not only are there mutational hot spots, but the pattern of nucleotide substitutions is characteristically skewed. Such intrinsic base substitution preferences were used by Betz et al. (39) to distinguish intrinsic mutational hot spots from those that are Ag selected, based on examination of sets of sequences from Ag-selected V genes, as a first step in the elucidation of the (enzymatic) mechanisms of hypermutation. The data reported in this work, in which at least seven amino acid substitutions at the same position significantly increase affinity for sulfonate, suggest that searches for recurrent identical mutations that enhance affinity may fail to identify other unique classes of mutants. Furthermore, the distinction between an intrinsic somatic hypermutation hot spot and a hot spot resulting from selection by Ag (39) cannot necessarily be made if several different amino acid substitutions at the same position can be selected by Ag. The anti-Sulf hybridomas that utilize the Ars-associated canonical set of V genes are to date a small set that includes H:Ser35 substituted for the germline H:35 Asn (11). Whether the other Sulf-selected mutations obtained in vitro occur
among in vivo hybridomas is not yet known. It may prove instructive to determine, in a sufficiently large sample, whether certain mutations that are functional in vitro are not observed in vivo. Failure to observe these could be due to 1) a requirement for more than one base change; 2) a locus that is not hypermutable; or 3) the fact that the resultant mutant may be selected against in vivo, as, for example, through autoreactivity.

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References


