Site-specific Mutagenesis of a Recombinant Anti-single-stranded DNA Fab

ROLE OF HEAVY CHAIN COMPLEMENTARITY-DETERMINING REGION 3 RESIDUES IN ANTIGEN INTERACTION

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The heavy chain complementarity-determining region 3 (HCDR3) of the anti-oligo(dT) recombinant antibody fragment, DNA-1, contributes significantly to antigen binding (Komissarov, A. A., Calcutt, M. J., Marchbank, M. T., Peletskaya, E. N., and Deutscher, S. L. (1996) J. Biol. Chem. 271, 12241–12246). In the present study, the role of separate HCDR3 residues of DNA-1 in interaction with oligo(dT) was elucidated. Based on a molecular model of the combining site, residues at the base (Arg98 and Asp108) and in the middle (Tyr101, Arg-Pro-Tyr-Tyr105) of HCDR3 were predicted to support the loop conformation and directly contact the ligand, respectively. Twenty-five site-specific mutants were produced as hexahistidine-tagged proteins, purified, and examined for binding to (dT)$_{15}$ using two independent methods. All mutations in the middle of HCDR3 led to either abolished or diminished affinity. Tyr101 likely participates in hydrogen bonding, while Tyr104 and Tyr105 may be involved in aromatic-aromatic interactions with the ligand. The residues Arg102 and Pro103 were not as critical as the tyrosines. It is speculated that HCDR3 interacts with the thymines, rather than the phosphates, of the ligand. A 3-fold increase in affinity was observed by mutation of Asp108 to alanine. The highly conserved Arg98 and Asp108 do not appear to form a salt bridge.

Antibodies comprise a valuable class of protein produced in mammals and other species in response to antigen. The importance of antibodies is highlighted by their vital roles in immune protection. Mammalian immune systems are capable of generating millions of different antibody specificities in response to foreign antigens. For unknown reasons, a breakdown in immune tolerance can occur resulting in activation of self-reactive B and T cells. This abnormal cascade of events leads to autoimmune disease. A hallmark of the autoimmune disorders systemic lupus erythematosus (SLE) and mixed connective tissue disease is the presence of serum antibodies that recognize nucleic acids (1–3). The presence of high levels of circulating antibodies that bind double-stranded (ds) DNA is diagnostic for SLE, and certain anti-DNA immune complexes contribute to disease pathology (4, 5). It is uncertain what are the distinguishing features of pathogenic versus non-pathogenic antibodies, however.

Studies of the binding properties of anti-DNA antibodies have been facilitated by methods to select for DNA-binding antibody fragments (Fab) from bacteriophage display libraries and the ability to produce large quantities of the Fab in Escherichia coli. We previously isolated an anti-single-stranded (ss) DNA-binding Fab, DNA-1, from a bacteriophage display library derived from the immunoglobulin repertoire of an autoimmune MRL/MpJ-lpr/lpr mouse (6). The MRL/lpr murine library was enriched in anti-DNA Fab since these mice spontaneously produce anti-ssDNA and anti-dsDNA antibodies and develop an SLE-like syndrome. DNA-1 was shown to preferentially bind to oligo(dT)$_{15}$ of 15 nucleotides or greater in length, with an equilibrium dissociation constant ($K_{d}$) of 150–200 nM (7, 8). The regions responsible for DNA binding were known to reside in the three heavy (H) and light (L) chain complementarity-determining regions (CDRs) of the Fab. It has been shown that the H chain contributes more to the interaction with DNA than the L chain (9). Comparison of HCDR3 transplantation mutants between DNA-1 and a Fab that bound poorly to DNA demonstrated that HCDR3 of DNA-1 was critical for oligo(dT) binding (7, 8). The importance of HCDR3 in binding to other DNA molecules including Z-DNA (10), dsDNA (11, 12), and ssDNA (13, 14) has been demonstrated. Previous data generated from x-ray analyses of anti-protein antibodies indicated that HCDR3 does not participate in secondary structure formation and represents a loop, often with a putative salt bridge at its base (15) that may function to stabilize loop conformation. That HCDR3 loop residues are critical in antigen recognition has been demonstrated in a limited number of studies (16–18). Nevertheless, the precise function of HCDR3 in DNA interaction has not been elucidated.

In the present study, the role of amino acid residues of HCDR3 of DNA-1 in oligo(dT) interaction was examined. Two regions were chosen for mutagenesis experiments based on a molecular model of DNA-1: (i) residues in the middle of HCDR3 (Tyr101, Arg-Pro-Tyr-Tyr105), and (ii) the amino acids Arg98 and Asp108 at the base of the loop, which have been proposed to form a salt bridge. Both fluorescence quenching titration (8)
and a Ni-NTA-agarose radioimmunoassay (19) were used for comparison of the affinities of 25 purified HCDR3 mutants of DNA-1. The data obtained highlighted the critical role of tyrosine residues in the central portion of HCDR3 in proper Fab-oligonucleotide complex formation. A change in the size as well as an increase in the negative charge of the HCDR3 loop resulted in a loss of ssDNA binding, while elimination of the sole carboxylic group resulted in increased affinity for (dT)$_{15}$.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Gamma-bind Sepharose, Sephadex G-50M, and a Mono-S column were purchased from Pharmacia Biotech Inc. Ni-NTA-agarose was purchased from Qiagen Corp. (Chatsworth, CA). [γ-32P]ATP (3000 Ci/mmol) was obtained from NEN Life Science Products. Protein molecular weight standards were from Novex (San Diego, CA). All other reagents were obtained from Sigma or Fisher unless otherwise noted. The (dT)$_{15}$ was synthesized by the University of Missouri DNA Core Facility, using an Applied Biosystems DNA synthesizer (model 380B), reverse-phase column purified and quantitated spectrophotometrically (20), and terminally phosphorylated with [γ-32P]ATP by T4 polynucleotide kinase. Unincorporated radioactivity was removed by separation on Chroma Spin-10 columns (CLONTECH Laboratories, Inc.).

**Molecular Modeling of the Combining Site of DNA-1**—The framework region of DNA-1 was modeled on the crystal structure of the mouse anti-hapten antibody R19.9 because of the high sequence homology with DNA-1 and because five out of six CDRs were identical in length to their counterparts (21). The DNA-1 sequence was aligned with the R19.9 sequence and then substituted into the R19.9 structure using the biopolymer and homology modeling tools present in SYBYL (Tripos, Inc.). Once the framework of DNA-1 was completed, the CDRs were added to the model. The loop structure of the HCDR3 from DNA-1, which differed in sequence length from that of antibody 19.9 by an additional two residues, was generated from the x-ray structure of HCDR3 of an anti-p15(1-27) antibody of similar sequence and identical length (22).

**Selection of HCDR3 Residues for Site-specific Mutagenesis**—In accordance with the molecular model of the combining site (Fig. 1A), HCDR3 represents a loop with the potential for formation of a salt bridge at its base as a result of the interaction of a conserved Arg residue in framework region 3 and Asp$_{32}$ at the C terminus of HCDR3 (Fig. 1B). Results of x-ray analysis together with data from site-directed mutagenesis of different antibodies (15, 16) suggest that residues in the central portion of the HCDR3 loop likely participate in direct interactions with the antigen at the combining site, while residues placed near the base of HCDR3 are more likely to contribute to the conformational support or flexibility of the loop. Therefore, the five residues in the middle of HCDR3 (Tyr$_{55}$ Arg$_{56}$ Tyr$_{57}$) and two residues at the base of the loop (Arg$_{65}$ and Asp$_{66}$) were chosen for site-directed mutagenesis experiments to examine the possible contributions of the separate amino acid residues in complex formation. The mutants generated are shown in Table 1.

**Site-directed Mutagenesis**—VSX, a derivative of the plasmid pBC (Stratagene) containing a chloramphenicol resistance gene and lacking an XhoI restriction enzyme site, was used as an intermediate vectors for mutagenesis experiments: the middle of HCDR3 (Tyr$_{55}$ Arg$_{56}$ Tyr$_{57}$) and two residues at the base of the loop (Arg$_{65}$ and Asp$_{66}$) were chosen for site-directed mutagenesis experiments to examine the possible contributions of the separate amino acid residues in complex formation. The mutants generated are shown in Table 1.

**Expression and Purification of DNA-1 and HCDR3 Mutants**—The recombinant H and L chain DNA-1 and HCDR genes were cloned into pComb3/6-His (24), expressed in E. coli DH12S, and purified as described (7). The Fab preparations were more than 95% pure, as evidenced by SDS-PAGE (25), and had a A$_{280}$/A$_{220}$ ratio $> 1.9$. Protein concentration was determined by a bicinchoninic acid method (26) using bovine serum albumin as a standard.

**Modification of DNA-1 with Tetranitromethane (TNM)**—DNA-1 was modified with TNM as described (27). Portions (10 μl) of a 100 mM stock solution of TNM in acetoneitrile were added to 1.0 ml of DNA-1 (20 μM) in 0.1 M Tris/HC1 buffer, pH 8.0, and the mixture was incubated at room temperature. After incubation for 30 min, excess reagent was removed by gel filtration on a Sephadex G-50M column. The number of modified tyrosine residues was estimated spectrophotometrically using a value of 2750 M$^{-1}$ cm$^{-1}$ for the extinction coefficient of nitrotyrosine at 380 nm (27).

**Purification of Fab-Oligonucleotide Complexes with Ni-NTA Resin**—To examine the ability of HCDR3 mutants of DNA-1 to bind oligonucleotide, a Ni-NTA microtiter plate radioimmunoprecipitation assay was utilized (19). Mixtures (200 μl) of 32P-labeled (dT)$_{15}$, in 0.5 M Tris/HC1, pH 7.0, with 0.1 M NaCl were incubated with increasing concentrations of Fab (5–2000 nM) in the wells of microtiter plates at room temperature for 15 min until equilibrium was established. To separate bound oligonucleotide from unbound, a 40-μl suspension of Ni-NTA-agarose was added to each mixture to trap the Fab-32P(dT)$_{15}$ complex through the hexahistidine tag at the C terminus of the H chain (19). Immobilized complex was quantitatively eluted with 400 mM imidazole. All values were counted using a Tri-Carb 2100 TR automatic liquid-scintillation analyzer (Packard Instrument Co., Downers Grove, IL) for 2 min using an automatic 32P window.

**Fluorescence Quenching Titration**—Changes in protein fluorescence at 346 nm (292 nm excitation) over the course of Fab-(dT)$_{15}$ complex formation were measured as described previously (8). The titrations were performed with varying amounts of oligonucleotide (5–20,000 nM) added to a fixed Fab concentration (10–60 nM) in 2 ml of 0.05 M Tris/HC1, pH 7.0, with 0.1 M NaCl. Fluorescence titration experiments were carried out using a SLM 8100 spectrofluorimeter interfaced to a Dell/433 PC running SLM AMINCO S100 series 2 software. The temperature of the cell compartment (25 °C) was controlled using a constant temperature cell holder connected to a circulating water bath, RTE-100 (Newport, NH). The K$_{i}$ values for Fab-oligonucleotide complexes were determined from binding isotherms using a single binding site curve-fitting procedure as described previously (8).

**RESULTS AND DISCUSSION**

**Choice of HCDR3 Mutants**—Our previous results (7, 8) demonstrated that HCDR3 plays a principal role in the interaction of DNA-1 and oligo(dT). In the present study, HCDR3 site-specific mutants were generated based on a molecular model of DNA-1 to better define amino acid residues important in ssDNA interaction and their possible contribution to the mechanism of antigen recognition. A molecular model for the DNA-1 variable region (Fv) was constructed to assist in the visualization of the amino acids located in the antigen combining site and to integrate experimental data with subsequent interpretation (Fig. 1A). Two regions of HCDR3 were the primary focus for directed mutagenesis experiments: the middle of HCDR3, which was predicted to participate in direct interaction with antigen, and residues Arg$_{55}$ and Asp$_{66}$ at the base of the loop, which may form a putative salt bridge to maintain the conformation of HCDR3 (Fig. 1B) (28).

As shown in Table 1, three of the five residues in the middle of HCDR3 of DNA-1 are tyrosines. Antibody combining sites are often rich in aromatic residues (28–30) that have been shown to be important for the interaction with antigen (14, 31–33). Possible mechanisms for antigen interaction include formation of hydrogen bonds by the phenolic hydroxyl group and aromatic-aromatic or hydrophobic interactions (33). Therefore, each tyrosine studied was first changed to a phenylalanine (Fab mutants Y101F, Y104F, and Y105F) to detect the possible involvement of the phenolic hydroxyl in hydrogen bonding (Table 1). Mutation of Tyr$_{101}$ and Tyr$_{104}$ to His (Fab mutants Y101H and Y104H), was created to test the effects of changing the phenolic ring to an imidazole, on Fab affinity. Mutants Y101D, Y104D, Y101C, and Y105C were produced to study the effects of changing the phenolic ring to an imidazole, on Fab affinity. Mutants Y101H and Y104H, which were created to test the effects of changing the phenolic ring to an imidazole, on Fab affinity.
HCDR3 Residues Involved in ssDNA Interaction

**Fig. 1. Molecular model of the combining site of DNA-1.** A, the H chain is shown as a dark gray ribbon and the L chain as a light gray ribbon. HCDR3 is shown as a black wire frame. The framework region and five out of six CDRs of DNA-1 Fv were modeled on the crystal structure of the mouse antibody R19.9 due to high sequence homology and identical CDR length (21). The DNA-1 sequence was aligned with the R19.9 sequence and then substituted into the R19.9 structure using modeling tools present in SYBYL. B, HCDR3 depicted as a wireframe. The backbone is traced as a light gray tube.

idue or group with reduced pKₐ to the top of HCDR3. In addition, two point mutants in position 101 (Y101S and Y101N) were created to explore the possible role of size and hydrophobicity of the side chain on ssDNA binding. The double mutant Y105F/A106V was generated to examine the influence of an increase in potential for hydrophobic interactions in this region of the HCDR3 loop.

In accordance with the molecular model of DNA-1, HCDR3 was predicted to form a reverse turn at Pro¹⁰³ (Fig. 1). Four substitution mutants (P103G, P103T, P103C, and P103R) were produced to explore the influence of the change in size and charge at this position on ssDNA affinity. Insertion and deletion mutations (mutant Fab -P103 and P103AA) were also created to study the role of the size of HCDR3 in the interaction with oligo(dT) (Table I).

Combining sites of anti-DNA antibodies often contain arginines and lysines that can interact with the negatively charged phosphates of the antigen (2, 5, 10, 34–37). Three mutant variants with replacement of Arg³⁹⁸ (R102K, R102M, and R102T) and double mutant R102A/P103R were generated in an effort to identify the possible role of the positive charge at the top of HCDR3 loop and the effect of the shift of the arginine residue on the Fab-ssDNA complex formation, respectively (Table I).

Finally, the role of the Arg³⁹⁸ and Asp¹⁰⁸ residues in supporting the functional integrity of the DNA-1 combining site was examined by generation of a “double mutant cycle” (38–40) consisting of two point mutants and one double mutant (R98A, D108A, and R98A/D108A, respectively). Taking advantage of the principle of additivity (38–40), the comparison of the changes in ΔG° for point mutations with the values obtained for the double mutant and wild type Fab may indicate the possible interaction between Arg³⁹⁸ and Asp¹⁰⁸ and its role in formation of the Fab-ligand complex.

**Purification of HCDR3 Mutant Variants of DNA-1**—Individual HCDR3 site-directed mutant versions of DNA-1 were produced in *E. coli* DH12S (8) and purified to homogeneity using affinity and ion-exchange chromatography (Fig. 2A) as described under “Experimental Procedures.” Results of SDS-PAGE analysis of the Fab employed in this study are shown in Fig. 2 (B and C). Samples of DNA-1 and all 25 HCDR3 mutants purified demonstrated neither a decrease in affinity or proteolysis after 6 months of storage at 4 °C. Changes in fluorescence emission induced by denaturation of Fab after incubation for 15 min at 35–60 °C (41) were used as an indicator of the influence of HCDR3 alterations on the overall structure of the molecule. Using this method, the thermostability of each mutant studied was similar to DNA-1 (data not shown). These results were in agreement with the supposition that HCDR3 plays a negligible role in the support of Fab structure.

**Direct Comparison of Fab Affinities by Precipitation with Ni-NTA-Agarose—Ni-NTA precipitation of Fab-oligonucleotide complexes (19) was employed for the rapid and facile analysis of the binding properties of HCDR3 mutants and for the direct estimation of Kd values.** All Fab studied contained a hexahistidine tag at the C terminus of their H chain (24), which did not interfere with oligonucleotide interaction (19, 41). Since previous studies have shown that DNA-1 exhibits preferential specificity for oligo(dT) and has maximal affinity for a ligand 15 or more bases in length (7, 8), all binding experiments were performed with (dT)₁₅. The Fab²⁻P⁻(dT)₁₅ complexes were separated from unbound ligand by precipitation with Ni-NTA-agarose and quantitatively eluted with imidazole (19). The relative affinities of the Fab (500 nM) to (dT)₁₅ (5 nM) presented in Fig. 3 exhibited specificity for oligo(dT) and has maximal affinity for a ligand 15 or more bases in length (7, 8), all binding experiments were performed with (dT)₁₅. The Fab²⁻P⁻(dT)₁₅ complexes were separated from unbound ligand by precipitation with Ni-NTA-agarose and quantitatively eluted with imidazole (19). The relative affinities of the Fab (500 nM) to (dT)₁₅ (5 nM) presented in Fig. 3. As shown, modification of DNA-1 with TNM and 11 of 25 HCDR3 mutations in DNA-1 resulted in a dramatic decrease in the affinity of the Fab to bind (dT)₁₅. All alterations that resulted in a significant decrease in the pKₐ, accompanied with an increase in the negative charge of the residue (Y101D, Y104D, Y101C, Y105C, P103C, modification with TNM), led to a significant decrease in binding ability. Mutants with a change in the length of HCDR3 (–P103 and P103AA) as well as both double mutants directed to the middle of the loop (R102A/P103R and Y105F/A106V) were also greatly diminished in their oligo(dT) binding ability. Therefore, neither the appearance of negatively charged groups (or groups possessing a lower pKₐ than tyrosine) at the top of HCDR3 nor alterations in the size of the loop were tolerated without a significant reduction in binding. The estimated affinities from titrations of (dT)₁₅ with Fab are shown in Tables II and III. These results demonstrated that any alteration to the top of HCDR3 resulted in decreased affinity, underscoring the importance of this region in-ligand interaction (Table II). On the other hand, two of the three mutant variants with a change in the charge at the base of the loop (R98A and R98A/D108A) had a moderate loss in binding ability (Table III). Moreover, the mutation of Asp¹⁰⁸ to Ala, which resulted in removal of the only negative charge from HCDR3, resulted in an approximate 3-fold decrease in the Kd value (Table III).

**Measurement of Affinities of HCDR3 Mutants by Equilibrium Fluorescence Titration—Ni-NTA precipitation is a direct method for the detection of Fab-oligonucleotide complex formation.** However, the corresponding set of Kd values was calculated from data obtained under non-equilibrium conditions. Therefore, equilibrium fluorescence quenching titration (8) was employed as an independent method for the measurement of the affinities of the Fab (Fig. 4). Additional advantages of this method included the higher upper limit of Kd detection (approximately 20 μM) and the possibility to obtain an independent set of Kd values by titration of Fab with oligonucleotide (in contrast to Ni-NTA precipitation). In general, the results of the direct binding measurements by Ni-NTA precipitation were consistent with the results of equilibrium fluorescence quenching titration (Table II). Moreover, the Kd values of the mutants Y101D and R102T, which were beyond the detection limits of the Ni-NTA technique, were measured successfully by fluorescence titration (8 and 10 μM, respectively; Table II).
In accordance with the $K_d$ values (Table II), all mutants directed to the middle of the HCDR3 loop demonstrated a loss in DNA affinity in comparison with DNA-1 and could be divided into three groups: (i) mutants with moderate loss in affinity (less than 1 order), (ii) mutants with significant loss in affinity (more than 1, but less than 2 orders), and (iii) mutants with a $K_d$ above the detection limit (20 μM).

**Possible Role of Tyrosine Residues—**Analysis of the results obtained (Table II) and comparison with published data of studies of antibody combining sites allowed for the supposition or in interaction with the hydrophilic region of the ligand.

Mutagenesis of the other HCDR3 tyrosines to phenylalanine, resulted in Fab (Y104F and Y105F) with moderately reduced affinity (increase in $K_d$ values of 6.1- and 3.7-fold, respectively, in comparison with DNA-1). The lower $K_d$ value for Y105F may indicate a distal position of this residue relative to the ligand (Fig. 1B). Both mutants Y104D and Y105C possessed an increase in negative charge and simultaneous elimination of the aromatic ring and demonstrated a complete loss in oligonucleotide binding ($K_d > 20 \, \mu M$). On the other hand, the phenolic group of Tyr$^{104}$ could be exchanged for a hydrophilic imidazole with only a modest (8.3-fold) decrease in affinity. An attempt to increase the hydrophobicity in this region by creation of the double mutant Y105F/A106V completely abolished binding. Therefore, the tyrosine residues at position 104 and 105 likely participate in aromatic-aromatic interactions with thymine rings that require a circular π-electron system. This type of interaction with ssDNA has been directly shown for the antibody BV04-01(dT)$_3$ complex (36). Mutation of Tyr$^{32}$ to phenylalanine in the L chain of the single chain antibody BV04-01 was accompanied by a 2.7-fold increase in the $K_d$ value (16) that was comparable to the results of mutations of Tyr$^{104}$ and Tyr$^{105}$ to phenylalanine in HCDR3 of DNA-1.

**Proline 103—**Substitution of Pro$^{103}$ with amino acids of different size and charge (mutants P103G, P103T, and P103R) resulted in approximately the same increase in $K_d$ value (6.7-, 5.8-, and 11.1-fold, respectively; Table II), reflecting the absence of strong sterical requirements for the top of the pre-
predicted reverse turn in HCDR3. These data demonstrated that
the side chain of the residue at this position did not interfere
significantly with complex formation and, probably, could be
either exposed to solution or may have formed an interface of
the combining site. The deletion and insertion mutants (P103
and P103AA) exhibited a dramatic (more than 100-fold) reduc-
tion in affinity for (dT)15, suggesting that changes in the length
of HCDR3 led to severe structural alterations of the Fab-oligo-
nucleotide complex. A similar loss of ability to bind oligonucleo-
tide was obtained earlier for the Fab, RNA-1, that was identical
to DNA-1 except for deletion of six HCDR3 residues (6).

Arginine 102—Unexpectedly, the positively charged Arg 102
was not absolutely required for interaction with the negatively
charged (dT)15. Mutation of Arg 102 to either lysine or methio-
nine resulted in approximately the same changes in
$K_{d}$ values (2.9- and 2.8-fold, respectively) (Table II). Substitution of
Arg102 with threonine led to a more significant decrease in
affinity (55.6 times), however. The double mutant R102A/
P103R, which was generated in an effort to move the positive
counter from position 102 to the top of HCDR3 to explore the possibility for contact with the negatively charged phosphates of
the ligand, was unable to bind (dT)15. This result is in
agreement with the suggestion that HCDR3 of DNA-1 interacts
with thymines of the ligand through non-ionic interactions.
However, this finding is in contrast to studies that have shown that electrostatic interactions occur through arginines of
HCDR3 of the anti-dsDNA antibody, D42, and are involved in
complex formation (12).

Arginine 98 and Aspartic Acid 108 at the Base of HCDR3—
Residues Arg 98 and Asp 108, which potentially form a salt
bridge, are most likely buried deep in the Fab molecule (28).
Nevertheless, there are numerous data demonstrating the im-
portance of this structural element in ligand binding (17, 18,
42–45). Despite the alteration of charged residues to alanine
(that was accompanied with a dramatic change in the nature of
the side chain group), all mutations directed to the base of
HCDR3 influenced affinity less than those directed to the pre-
dicted top of the loop (Table III). The Fab with mutation of
Asp108 to alanine demonstrated a decrease in the maximal
fluorescence quenching value (Table III), indicating changes in
the environment of the tryptophan residues. This result also
led to the suggestion that Tyr109 and Trp 110, which follow
Asp108 (6), may contribute to the fluorescence quenching de-
tected. Mutation of Asp 108 to alanine, which eliminated the
only carboxyl group of HCDR3, demonstrated a 3-fold in-
creased affinity for (dT)15. Removal of the positively charged
Arg98 caused an opposite effect: approximately a 1-order in-
crease in $K_{d}$ value in comparison with DNA-1. The double
mutant R98A/D108A demonstrated an affinity ($K_{d}$
970 nM)
intermediate between the corresponding single mutants R98A
and D108A (2000 and 60 nM, respectively; Table III). These
results indicated that Arg 98 and Asp108 most likely independ-
tently stabilize HCDR3 conformation through interactions with
other residues in the Fab molecule rather than through forma-
tion of a salt bridge.

A similar conclusion follows from the analysis of changes in
$\Delta G^{\circ}$ of binding in comparison with DNA-1 ($\Delta G^{\circ}$) induced by
the three mutations formed in “the double mutant cycle” (Table
III). The sum of $\Delta G^{\circ}$ for both point mutants (0.8 kcal/mol) was
only 0.2 kcal/mol less than the value for the double mutant
R98A/D108A (Table III), indicating that the interaction be-
tween these residues did not contribute significantly to ligand
binding.
Table II
Properties of mutants with alterations directed to the middle of HCDR3

| Position of mutation at HCDR3 | Method | Fluorescence quenching titration | Kd (nM) | Q (% of maximal value) | Decrease in affinity (kcal/mol) |
|-------------------------------|--------|----------------------------------|---------|------------------------|-------------------------------|
|                               | Fab    | Kd (nM)                          |         |                        |                               |
|                               | Ni-NTA precipitation |                            |         |                        |                               |
| Wild type                     | DNA-1  | 140 ± 40                         | 180 ± 30| 20 ± 2                 | 5.2                           |
| 101                           | Y101H  | 1700 ± 200                       | 930 ± 150| 22 ± 3                 | 15.6                          |
|                               | Y101N  | 2500 ± 1300                      | 2800 ± 450| 23 ± 3                 | 5.4                           |
|                               | Y101S  | 950 ± 250                        | 970 ± 50 | 21 ± 3                 | 5.4                           |
|                               | Y101D  | >5000                            | 8000 ± 1100| 18 ± 2               | 44.4                          |
| 102                           | R102K  | 520 ± 250                        | 530 ± 80 | 21 ± 3                 | 2.9                           |
|                               | R102M  | 470 ± 110                        | 510 ± 60 | 20 ± 3                 | 2.8                           |
| 103                           | P103G  | 1500 ± 130                      | 1200 ± 150| 22 ± 3                 | 6.7                           |
|                               | P103T  | 1500 ± 550                      | 1050 ± 120| 23 ± 3                 | 5.8                           |
|                               | P103R  | 1900 ± 400                      | 2000 ± 220| 22 ± 3                 | 11.1                          |
| 104                           | Y104F  | 1350 ± 150                      | 1100 ± 90| 23 ± 3                 | 6.1                           |
|                               | Y104H  | 1100 ± 250                      | 1500 ± 300| 21 ± 3                 | 8.3                           |
| 105                           | Y105F  | 760 ± 120                       | 670 ± 50 | 22 ± 3                 | 3.7                           |

Table III
Comparison of the mutants with alterations at the base of HCDR3

| Position of mutation at HCDR3 | Method | Fluorescence quenching titration | Kd (nM) | Q (% of maximal value) | ΔG°Dissociation (kcal/mol) |
|-------------------------------|--------|----------------------------------|---------|------------------------|------------------------------|
|                               | Fab    | Kd (nM)                          |         |                        |                              |
|                               | Ni-NTA precipitation |                            |         |                        |                              |
| Wild type                     | DNA-1  | 140 ± 40                         | 180 ± 30| 20 ± 2                 | -9.20 ± 0.10                 |
| 98                            | R98A   | 1500 ± 300                      | 2000 ± 250| 20 ± 3                 | -7.77 ± 0.08                |
|                               | D108A  | 50 ± 15                         | 60 ± 10 | 14 ± 2                 | -9.83 ± 0.10                |
| 98/108                        | R98A/D108A | 1100 ± 700                      | 970 ± 110| 17 ± 2                 | -8.20 ± 0.07               |

Table II: Titration of [32P]-labeled (dT)15 (2–10 nM) with Fab (2–2000 nM) was carried out in 50 mM Tris/HCl buffer (pH 7.0), 100 mM NaCl, room temperature as described previously (19).

Table III: Values of changes in the Gibbs standard free binding energy (ΔG°) were calculated for each mutant from results of fluorescence quenching titration (ΔG° = -RTlnKd, where R = 1.987 kcal/mol, T = 298 K and association constant Kd = 1/Kd).

Figure 4: Equilibrium fluorescence quenching titration of Fab. Changes in fluorescence upon titration of DNA-1 (■), D108A (●), R98A (□), Y101D (○), and P103AA (▲) with (dT)15. Fab samples (10–60 nM) were titrated with increasing concentrations (5–20,000 nM) of oligonucleotide in 50 mM Tris/HCl, pH 7.0, with 100 mM NaCl at 25 °C. Complex formation was detected by quenching of fluorescence at 346 nm (excitation at 292 nm). Changes in fluorescence were expressed as percent of maximal value. Calculated Kd values were included in Table II.

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