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Modulation of antibody affinity by a non-contact residue

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Abstract

Antibody LB4, produced by a spontaneous variant of the murine anti-digoxin monoclonal antibody 26-10, has an affinity for digoxin two orders of magnitude lower than that of the parent antibody due to replacement of serine with phenylalanine at position 52 of the heavy chain variable region (Schildbach, J.F., Panka, D.J., Parks, D.R., et al., 1991, *J. Biol. Chem. 266*, 4640–4647). To examine the basis for the decreased affinity, a panel of engineered antibodies with substitutions at position 52 was created, and their affinities for digoxin were measured. The antibody affinities decreased concomitantly with increasing size of the substituted side chains, although the shape of the side chains also influenced affinity. The crystal structure of the 26-10 Fab complexed with digoxin (P.D.J., R.K. Strong, L.C. Sieker, C. Chang, R.L. Campbell, G.A. Petsko, E.H., M.N.M., & S.S., submitted for publication) shows that the serine at heavy chain position 52 is not in contact with hapten, but is adjacent to a tyrosine at heavy chain position 33 that is a contact residue. The mutant antibodies were modeled by applying a conformational search procedure to position side chains, using the 26-10 Fab crystal structure as a starting point. The results suggest that each of the substituted side chains may be accommodated within the antibody without substantial structural rearrangement, and that none of these substituted side chains are able to contact hapten. These modeling results are consistent with the substituents at position 52 having only an indirect influence upon antibody affinity. The mutagenesis and modeling results suggest that even conservative replacements of non-contact residues can alter affinity indirectly through their impact on contact residue placement.

Keywords: antibody; anti-digoxin; molecular modeling; mutagenesis; non-contact residue

Analyses of X-ray crystal structures of antibody complexed with antigen indicate that although a variety of amino acids are located in each antigen-binding site, certain residues, including tyrosine and tryptophan, are more frequently found (Padlan, 1990; Mian et al., 1991). This finding suggests that these amino acids provide important preferred contributions to high-affinity interactions. Recognition, however, requires not only that proper side chains be present in the binding site and accessible to antigen, but that they also be positioned correctly to contribute to binding.

Experiments using genetic techniques to graft complementarity-determining regions (CDRs) from one antibody onto another antibody framework illustrate this point. Although antibody framework regions (defined by Kabat et al., 1991) are structurally similar (Padlan & Davies, 1975) and can allow successful grafting of CDRs from one framework to another with retention of binding activity (Jones et al., 1986; Verhoeyen et al., 1988), the constructed antibody often has diminished antigen binding (Reichmann et al., 1988; Kettleborough et al., 1991; Tempest et al., 1991). Because framework residues can directly contact ligand (Amit et al., 1986; Sheriff et al., 1987b; Herron et al., 1989; Padlan et al., 1989; Alzari et al.,

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1990), the lowered affinity may often be attributable to loss or alteration of complementarity. In addition, it is possible that differences in framework regions force the same CDR loop to adopt different conformations in the engineered and native antibodies, hampering antigen binding. Engineering of these framework residues of the constructed antibody can restore antigen recognition, presumably by reproducing the native CDR loop structure (Reichmann et al., 1988; Kettleborough et al., 1991; Tempest et al., 1991).

While studies such as these indicate that framework residues affect binding, they often do not establish whether these framework residues contact antigen. These studies also do not include extensive examinations of the effect of amino acid substitutions at these sites. Here we detail mutagenesis and modeling experiments which indicate that substitution of an antibody CDR residue that does not contact hapten may modulate antibody affinity. We previously reported isolation of a variant of the anti-digoxin antibody-producing hybridoma line 26-10 that demonstrates a 150-fold reduction in affinity for digoxin relative to the parent antibody. The reduced affinity is due to a replacement of the Ser at heavy chain (H) variable region position 52 (H:Ser52) with Phe (Schildbach et al., 1991). To examine the basis of the reduced affinity, we constructed a panel of 9 antibodies mutated at H chain position 52 (H 52) and measured their affinities for digoxin. The results reveal a relationship between the size and geometry of the substituted amino acid and the reduced affinities.

The X-ray crystal structure of the 26-10 Fab complexed with digoxin has been determined (P.D.J., R.K. Strong, L.C. Sieker, C. Chang, R.L. Campbell, G.A. Petsko, E.H., M.N.M., & S.S., submitted for publication). The structure reveals that H:Ser52 makes no contact with hapten despite its location within the second complementarity-determining region (CDR2) of the 26-10 heavy chain. The H:Ser52 side chain contacts the Tyr side chain at H chain position 33 (H:Tyr33) (see Fig. 1), which does interact with hapten. The altered affinities of some of the H:Ser52 mutants may therefore result indirectly, perhaps through displacement of H:Tyr33 or other contact residues, rather than by direct contact between H 52 and hapten. To examine this possibility, we modeled the mutant antibodies starting from the 26-10 Fab:digoxin crystal structure, using side-chain conformational searches to position the H 52 side chains. The resulting models suggest that each of the side chains can be accommodated within the 26-10 antibody structure without substantial structural adjustment. In addition, none of the substituted side chains in the models makes contact with the hapten. These results are consistent with an indirect effect mediated through relatively subtle changes in other binding site residues, rather than a direct effect through hapten contact.

Results and discussion

The 26-10 variant LB4 contains an H 52 substitution of Phe for Ser (H:Ser52Phe), which causes a lowered affinity for digoxin but does not appreciably alter recognition of cardiac glycosides and aglycones, relative to digoxin (Schildbach et al., 1991). To explore the basis of the affinity loss, nine antibodies with substitutions at H 52 were engineered. The H 52 mutants are listed in Table 1 with the sequence of the mutagenic oligonucleotides used for their production. The influence of the serine hydroxyl (OH) was tested by making H:Ser52Ala and H:Ser52Cys. Mutants H:Ser52Thr and H:Ser52Val were constructed to compare a larger β-branched side chain, both with and without an OH. The effect of β-branched versus γ-branched residues was examined by making H:Ser52Ile and H:Ser52Leu. Large aromatic side chains were substituted (H:Ser52Tyr and H:Ser52Trp) for comparison with the Phe of LB4 (H:Ser52Phe), and glycine was substituted as well (H:Ser52Gly).

The affinities of the engineered mutants, the spontaneous variant LB4, and 26-10wt (the 26-10 antibody produced by 26-10κ cells transfected with an expression vector containing the 26-10 H variable region) are listed in Table 2. The antibody 26-10wt has a K_A of 9.1×10^9 M^{-1}. This is lower than the value previously reported for

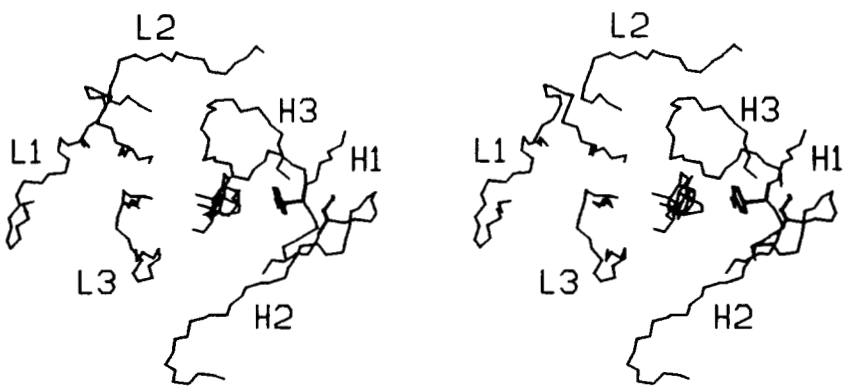

Fig. 1. Stereo view of the 26-10 hypervariable regions (as defined by Kabat et al., 1991) and digoxigenin from the crystal structure of the 26-10:digoxin complex (P.D.J., R.K. Strong, L.C. Sieker, C. Chang, R.L. Campbell, G.A. Petsko, E.H., M.N.M., & S.S., submitted for publication). View is from solvent into the binding site, with digoxigenin at the center. To the right of digoxigenin are the H:Tyr33 (in heavy chain hypervariable region 1, H1) and H:Ser52 (H2) side chains. H:Tyr33 contacts the hapten whereas H:Ser52 does not.

Table 1. *Mutagenic oligonucleotides*

Mutant	Sequence[a]
	5′ 3′
H:Ser52Ala	CTG TAA GGA GCA ATA TAT CC
H:Ser52Cys	CTG TAA GGG CAA ATA TAT CC
H:Ser52Gly	CTG TAA GGA CCA ATA TAT CC
H:Ser52Ile	CTG TAA GGA ATA ATA TAT CC
H:Ser52Leu	CTG TAA GGA AGA ATA TAT CCA A
H:Ser52Thr	CTG TAA GGA GTA ATA TAT CC
H:Ser52Trp	CTG TAA GGC CAA ATA TAT CCA A
H:Ser52Tyr	CTG TAA GGG TAA ATA TAT CC
H:Ser52Val	CTG TAA GGA ACA ATA TAT CC

[a] Oligonucleotides are anti-sense and are complementary to positions 48 (for mutants H:Ser52Leu and H:Ser52Trp) or 49–54 (Kabat numbering; Kabat et al., 1991) of the H chain of antibody 26-10. Differences from the anti-sense complementary 26-10 sequence are underlined.

26-10 as measured by a saturation equilibrium assay using filtration through glass fiber filter to separate free ligand from bound (2.4×10^{10} M^{-1}; Schildbach et al., 1991). The difference in these affinities, however, reflects differences in the methods used (see Materials and methods) rather than differences in hapten recognition between the native and engineered 26-10 antibody (heavy chain isotype γ2a and γ2b for 26-10 and 26-10wt, respectively). The engineered and native 26-10 antibodies do not differ in hapten binding (data not shown).

Replacement of Ser with Ala (H:Ser52Ala) did not result in a significant difference in affinity (Table 2), suggesting that the Ser γ-OH makes no contribution to binding. This conclusion is further supported by the high affinity of H:Ser52Gly. The affinity of H:Ser52Gly is the same as that of 26-10wt, even though the Gly substitution may allow greater CDR2 loop flexibility, at least locally. This result suggests that the substitution either did not affect the stability of the main-chain segment or that the potentially increased flexibility had no impact on binding.

Table 2. *Affinities for digoxin*

Antibody/mutant	K_A ($\times 10^{-6}$ M^{-1})[a]	Amino acid residue volume (Å^3)[b]
26-10wt (H:Ser52)	9,100 ± 1,000	88.6
H:Ser52Gly	10,000 ± 1,000	59.9
H:Ser52Ala	8,000 ± 1,400	88.3
H:Ser52Thr	950 ± 160	115.7
H:Ser52Leu	760 ± 120	166.2
H:Ser52Cys	500 ± 40	108.1
H:Ser52Val	370 ± 40	139.3
H:Ser52Ile	330 ± 90	166.2
LB4 (H:Ser52Phe)	61 ± 10	189.1
H:Ser52Trp	59 ± 7	226.9
H:Ser52Tyr	36 ± 3	192.9

[a] Affinities were measured by an equilibrium saturation assay using filtration through glass fiber to separate bound from free [^{3}H]digoxin (Schildbach et al., 1991). A representative affinity measurement is given with standard error estimates calculated by LIGAND.

[b] Values taken from Zamyatnin (1972).

Substitution of Cys (H:Ser52Cys) caused an 18-fold decrease in the affinity, despite the similar stereochemistry of Cys and Ser. The possibility that the loss of affinity is due to structural effects of an intramolecular disulfide bond involving the substituted Cys is unlikely given the distance of the side chain from the nearest Cys in the properly folded molecule (>10 Å from Cβ of H 52) and the involvement of all other Cys residues in buried disulfide bonds. We cannot, however, formally exclude the involvement of the substituted Cys in an intermolecular disulfide bond or another modification. Cys occupies a greater volume than Ser (Table 2), which could contribute to the difference in affinities via a steric effect. A Ser to Cys substitution within the oxydianion-binding site of sulfate-binding protein substantially reduces its affinity for sulfate (He & Quiocho, 1991), underscoring the different structural and functional roles of Cys and Ser despite their apparent similarities.

The decrease in affinity for digoxin of mutants H:Ser52Thr and H:Ser52Val also suggests an effect resulting from side-chain volume. Substitution of Thr for Ser causes a 10-fold decrease in affinity, while substitution of the similarly shaped Val causes a 25-fold loss. It is possible that through hydrogen bonding or another interaction with surrounding atoms the Thr γ-OH can account for the higher affinity of H:Ser52Thr relative to H:Ser52Val. The Thr γ-OH, however, occupies less volume than the corresponding methyl of Val (Table 2), and the smaller size of Thr seems the more likely cause of the affinity difference given the similarity between the affinities of 26-10wt and H:Ser52Ala.

Replacement of Ser with Leu results in a 12-fold drop in affinity. This contrasts with replacement by Ile, which causes a 28-fold drop despite having similar volume and chemical properties to Leu. The affinities of H:Ser52Ile and H:Ser52Val are similar, indicating that the addition of a δ-methyl group (Ile) has little impact on hapten binding. Val and Ile are β-branched amino acids, whereas Leu is γ-branched, suggesting that both volume and shape of the H 52 side chain can affect hapten recognition.

The 26-10 variant LB4 (H:Ser52Phe) and H:Ser52Trp have similar affinities, which are 150-fold reduced relative to the parent 26-10. Substitution of a Tyr (H:Ser52Tyr) results in a slightly lower affinity. These mutants contain substitutions by the largest of the amino acids and possess the lowest affinities of the antibodies mutated at H 52.

To test the correlation between the volume of each H 52 residue and the affinity of the corresponding antibody for digoxin, the energies of complexation (E_C) were calculated and plotted against the H 52 residue volume (Fig. 2). The E_C values were obtained according to the equation

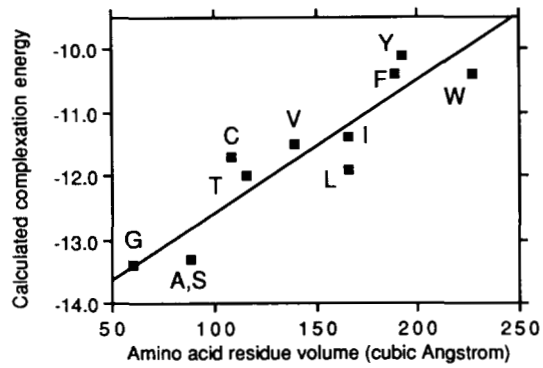

Fig. 2. Plot of the energy of complexation of digoxin and with each antibody versus the volume of the amino acid residue at H 52. The points are identified by the one letter code for the amino acid at H 52. The energies were calculated from the experimentally determined K_D for digoxin according to $E_C = RT \ln K_D$, where R is the gas constant and T is 293 K. The amino acid residue volumes were taken from Zamyatnin (1972). The formula of the line fit to the data is $y = -14.7 - 0.02x$ ($R = 0.92$).

$E_C = RT \ln K_D$, where R is the gas constant, T is 293 K, and K_D is the reciprocal of the experimentally derived K_A for digoxin (Table 2). The correlation of the volume of the H 52 amino acid residue to the E_C of the antibody is good ($r = 0.92$ for a line fitted to the data), despite side-chain shape not being accounted for in this analysis (compare affinities of H:Ser52Leu and H:Ser52Ile, Table 2).

Following completion of the mutagenesis studies, the X-ray crystal structure of 26-10 complexed with digoxin was determined at 2.5 Å resolution (P.D.J., R.K. Strong, L.C. Sieker, C. Chang, R.L. Campbell, G.A. Petsko, E.H., M.N.M., & S.S., submitted for publication). Examination of the structure reveals H:Ser52 is not a contact residue but instead abuts H:Tyr33, which does contact hapten (see Fig. 1). The H:Tyr33 side chain contacts (as defined by Sheriff et al., 1987a) digoxin at positions C3, C4, C7, and C15 (Figs. 3, 4; P.D.J., unpubl.). Because of the size of H:Tyr33 and its position relative to H:Ser52 and the hapten, it seems unlikely that any except the largest side chains of substituted residues could directly contact hapten without significant local rearrangement, but even relatively conservative substitutions (H:Ser52Thr, H:Ser52Val) adversely affected binding. In addition, H 52 has not been identified as a determinant of CDR loop structure (Chothia et al., 1989), and most substitutions here are not expected to alter the loop conformation.

To study the possible structural impact of these substituted side chains, computer models of the mutants were made using a procedure that combines side-chain conformational search with energy minimization. This procedure has been used to successfully model an H:Asn35His mutation in 26-10 (Schildbach, 1992) that is contained in 26-10R9, a mutant of 26-10 for which the crystal structure has been determined at 2.5 Å resolution to an R-value of 0.176 (R.K. Strong, P.D.J., L.C. Sieker, C. Chang, R.L. Campbell, G.A. Petsko, E.H., M.N.M., & S.S., in prep.). The procedure also reliably positioned side chains, including H:Asn35 of 26-10, in agreement with their positions in the crystal structure (Schildbach et al., in prep.).

As a test of the modeling procedure, the wild-type Ser at H 52 was removed from the structure and modeled using the procedure used for the substituted side chains (see Materials and methods). The modeled H:Ser52 (thick lines; Fig. 4) differs from the 26-10 crystal structure (thin lines) by its OH being in gauche (55°) as opposed to trans (178°) configuration relative to the N-Cα-Cβ-Oγ angle (Fig. 4). The modeled conformation provides a lower energy, when analyzed by CONGEN, due to more favorable electrostatic energy and to hydrogen bonding between the H:Ser52 γ-OH and the H:Ser52 carbonyl and between the H:Val56 amide and the H:Ser52 γ-OH (not shown). In contrast, the crystal structure suggests that the H:Ser52 γ-OH participates in a single hydrogen bond, interacting with the H:Ser54 γ-OH. The significance of the difference between the positions of the modeled Ser side chain and that found in the crystal structure is unknown. The electron density at position H:Ser52 does not strongly distinguish the modeled conformation from the crystal structure conformation and therefore does not necessarily indicate a failure of the modeling procedure.

At least one conformation for each modeled side chain was allowed. The models of H:Ser52Cys and H:Ser52Trp are shown in Figure 4 as examples. The relative positions of H:Tyr33, portions of the main chain of CDR1 (resi-

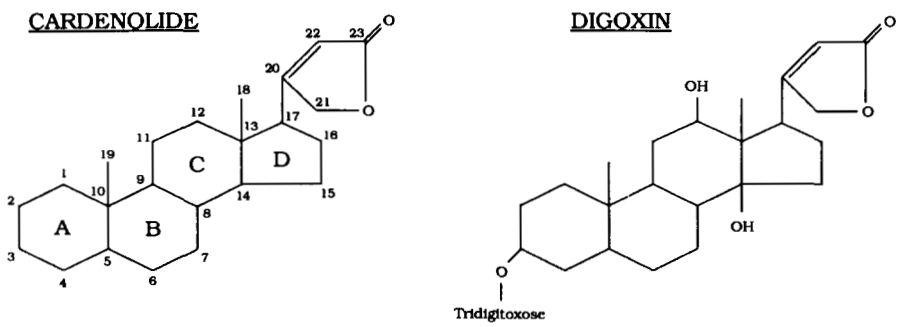

Fig. 3. Schematic diagrams of the cardenolide numbering system (left) and digoxin (right). Digoxigenin is the aglycone of digoxin and lacks the tridigitoxose moiety.

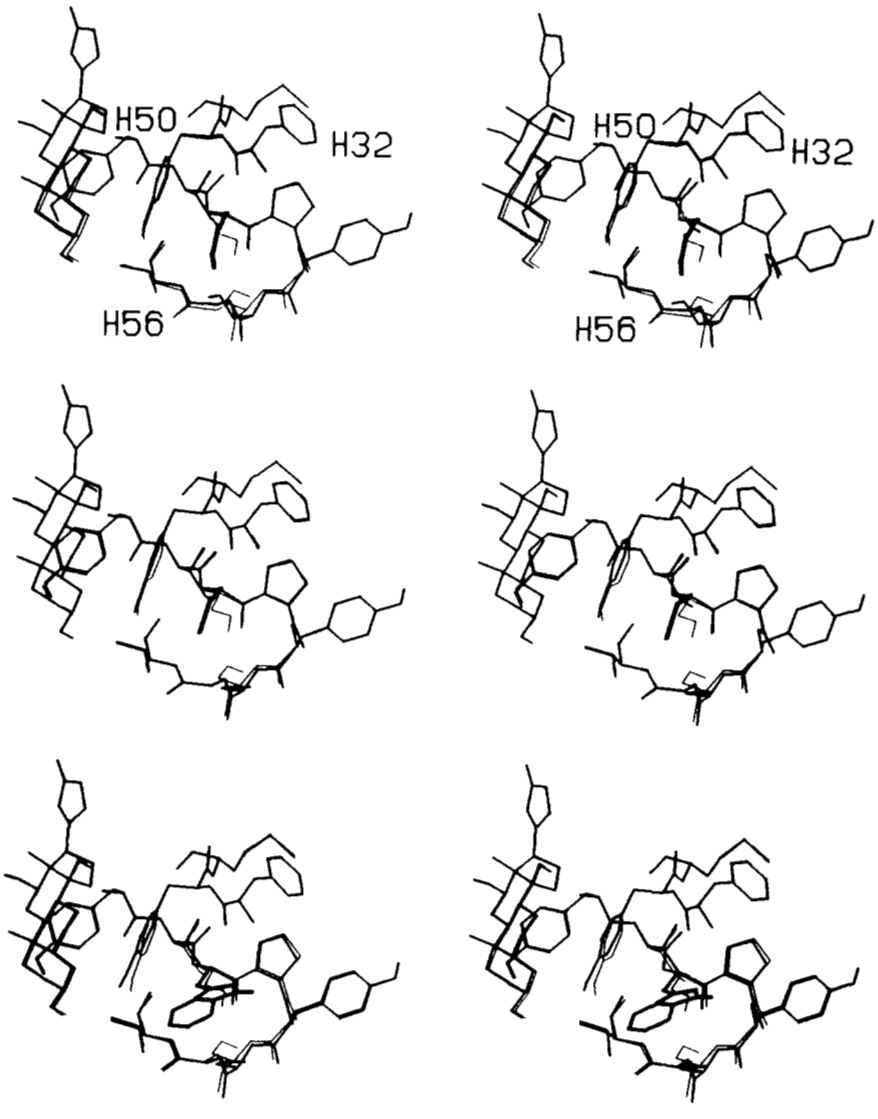

Fig. 4. Stereo views of the modeled complexes of digoxigenin and 26-10 (upper), H:Ser52Cys (middle), and H:Ser52Trp (lower). Shown are digoxigenin, the H chain residue 52, and H chain residues 32–34 and 50–56. Residues H:Phe32 (H32), H:Tyr50 (H50), and H:Val56 (H56) are labeled. The model (thick lines) is shown with the corresponding structure from the 26-10 structure (thin lines) superimposed. The modeled H 52 side chain is in bold. Hydrogens are shown on atoms capable of being hydrogen bond donors.

dues 32–34) and CDR2 (residues 50–56), and digoxigenin from the model are shown (heavy lines) superimposed upon these same portions of the 26-10 Fab (thin lines) for comparison. The 26-10 structure was subjected to the same hapten docking and energy-minimization procedure as the mutants (see Materials and methods). The H 52 side chain is in bold. The structural differences between the minimized 26-10 structure and the models are small (the root mean square [r.m.s.] difference between the side-chain atoms of H:Tyr33 in the minimized 26-10 structure, and the H:Ser52Trp model is 0.23 Å, within error of the crystal structure refinement), suggesting even the largest of amino acid side chains may be accommodated at H 52 within the 26-10 antibody without substantial rearrangement. The accommodation of large H 52 side chains apparently does not incur large energetic penalties. For example, the overall energy of the van der Waals contacts of H:Tyr33 and H:Trp52 of the H:Ser52Trp model is negative (as calculated by the CHARMM potential; see Materials and methods). No pairwise atom contact between H:Tyr33 and digoxigenin in the modeled complex of H:Ser52Trp and digoxigenin has an energy greater than +0.32 kcal/mol, and no contact between H:Tyr33 and H:Trp52 has an energy greater than +0.40 kcal/mol. The H 52 side chain does not make contact (as defined by Sheriff et al., 1987a) with hapten in any of the models (Fig. 4 and data not shown).

The small structural differences between the models is counterintuitive, given the substantial (3 kcal/mol; see Fig. 2) differences between the calculated E_C of 26-10wt and H:Ser52Trp. The shape complementarity seen in the crystal structure between the 26-10 antibody and digoxin is extensive, however. A substantial shift in the position of, for example, H:Tyr33 in the H:Ser52Trp antibody: hapten complex relative to the H:Tyr33 position in 26-10: digoxin complex may be prevented because of the tight packing of H:Tyr33 with the surrounding residues and hapten in the complex. Even if the structural differences

between 26-10 and the mutant antibodies are small, unfavorable energies would be expected to be present in the modeled antibodies with large H 52 side chains, perhaps in positive van der Waals contact energies between hapten and contact residues. The van der Waals energies (as calculated by CONGEN from the modeled complexes of the H 52 mutants and digoxigenin) between hapten and the H:Tyr33 side chain do, in general, become increasingly unfavorable as the affinities for digoxin of the corresponding antibodies decrease. These calculated van der Waals energies, however, account for only one-tenth the decrease in the E_C (data not shown). No other sources of unfavorable energy in the modeled complexes were obvious. Perhaps the modeling procedure minimizes the structural and energetic differences between the modeled complexes. The accuracy of the modeling may also be insufficient to describe the differences between the energies of the 26-10 and mutant antibody:hapten complexes. Therefore, the conclusions that may be drawn from the models are, in this respect at least, limited. The modeled structures, however, indicate that it is possible for even the largest side chains to occupy the H 52 position without substantially reordering the local structure and without contacting hapten.

Mutations of known non-contact residues that affect affinities have been described previously. A light chain mutation in anti-hen egg lysozyme monoclonal antibody HyHEL-10, suggested by the light chain sequence of the clonally related anti-hen egg lysozyme antibody HyHEL-8, conferred an improved relative affinity for duck lysozyme (Lavoie et al., 1992). The crystal structure of HyHEL-10 complexed with hen egg lysozyme shows that this light chain residue does not contact the antigen (Padlan et al., 1989). The physical basis of the altered antigen recognition of the mutant antibody is unknown. The U10 antibody, produced by a variant of the anti-phosphocholine hybridoma S107, demonstrates no hapten binding due to a single heavy chain substitution (Chien et al., 1989). The crystal structure of the homologous McPC603 myeloma protein (Satow et al., 1986) suggests the substituted amino acid is a non-contact residue. Chien and coworkers proposed that the mutation disrupted a hydrogen bond network involving an Arg residue, permitting the Arg side chain to occupy the hapten binding site, blocking hapten binding. Deletion of N-terminal heavy chain residues, which presumably do not contact hapten, have also been shown to affect the affinity of an anti-digoxin antibody variant (Panka et al., 1988).

The above results, as well as those arising from the mutagenesis experiments described here, indicate that non-contact CDR residues can dramatically affect the affinity or specificity of an antibody. The mixed success of CDR-grafting experiments (Reichmann et al., 1988; Kettleborough et al., 1991; Tempest et al., 1991) suggest that presumably non-contact framework residues may also alter antibody recognition. These latter experiments clearly demonstrate that the amino acids at these positions must be considered when transferring an antibody specificity from one antibody framework to another. The sum of these experiments suggests that non-contact residues are a potential target of efforts to modify antibody binding.

Materials and methods

Cell lines

The derivation of the murine anti-digoxin hybridoma 26-10 (IgG2a, κ) has been described (Mudgett-Hunter et al., 1982). The hybridoma variant 26-10P2.26LB4 (referred to herein as LB4) was isolated by fluorescence-activated cell sorting from a population of 26-10 hybridoma cells (Schildbach et al., 1991). Selection was based on increased relative binding of a conjugate of digoxin, human serum albumin, and phycoerythrin, following a preincubation with digitoxin. Cell line 26-10κ, a spontaneous heavy chain loss variant of 26-10, was subcloned and selected on the basis of heavy chain isotype loss as determined by screening supernatants using an isotype enzyme-linked immunosorbent assay.

Antibody mutagenesis

Mutagenesis was performed according to Kunkel (1985) on a 1.8-kb Xba I genomic fragment containing the rearranged 26-10 heavy chain variable region gene (Near et al., 1990) that had been subcloned into M13 Phage. The Muta-Gene kit (Bio-Rad, Richmond, California) was used. Mutagenic oligonucleotides (see Table 1) were made using an Applied Biosystems 380B DNA Synthesizer. Mutated variable regions were initially identified by hybridization of mutagenic ^{32}P-end-labeled oligonucleotides to phage DNA present in phage-laden bacterial supernatants blotted on nitrocellulose (Sambrook et al., 1989). Results of mutagenesis were confirmed by dideoxy chain-termination nucleotide sequencing using Sequenase (United States Biochemical, Cleveland, Ohio) and 5′[α-^{35}(S)thio]triphosphate (Amersham Corp., Arlington Heights, Illinois). The mutated fragments were subcloned into the pExpMutVH expression vector, which contains a heavy chain enhancer, a 3.6-kb Xba I–Bgl II genomic fragment encompassing the γ2b heavy chain, and the Ecogpt gene for selection (Near et al., 1991). The vector was introduced into 26-10κ cells (see above) by electroporation and selected on the basis of resistance to mycophenolic acid as described (Near et al., 1991). Resulting colonies were expanded and their supernatants were screened for antibody binding to wells of polyvinyl chloride plates coated with a digoxin–bovine serum albumin conjugate (Dig-BSA) (Mudgett-Hunter et al., 1982). Antibody bound to Dig-BSA was detected by binding of affinity-purified goat anti-mouse-Fab antibody (ICN ImmunoBiologicals,

Lisle, Illinois) radioiodinated by the chloramine T method (Greenwood et al., 1963).

Affinity determinations

[^{3}H]digoxin used for affinity measurements was purchased from New England Nuclear (Boston, Massachusetts). Affinities using cell supernatants were measured by a saturation equilibrium assay using filtration through glass fiber filters to separate bound and free ligand as described (Schildbach et al., 1991), except a Brandel harvester (Bethesda Research and Development, Bethesda, Maryland) was used for filtration instead of a Millipore 1225 manifold previously used (Millipore, Bedford, Massachusetts). Using the harvester, 80% of 26-10 antibody was retained on the filter as measured by retention of ^{125}I-labeled antibody (Schildbach et al., 1991) compared with 90% for the sampling manifold previously used (data not shown). Affinity data were analyzed using LIGAND (Munson, 1983). Incubation volumes used in the assays varied according to antibody affinity, ranging from 2 mL for high-affinity interactions to 0.3 mL for low-affinity interactions (Schildbach et al., 1991). This was done to ensure acceptable signal-to-noise ratios while maintaining antibody concentrations below 0.1 K_D and for economy of the tritiated ligand. The K_A value reported here for LB4 (6.1×10^7 M^{-1}; Table 2) is lower than a previously reported measurement using the glass fiber filter method with the Millipore manifold (8.6×10^7 M^{-1}; Schildbach et al., 1991). The separation of bound from free ligand is more quickly and efficiently accomplished with the Brandel harvester (12 tubes simultaneously compared to 1 tube at a time), resulting in less variability in the measurements. Individual experiments performed with the Millipore manifold (Schildbach et al., 1991) and analyzed by LIGAND had an average residual mean square of 116, whereas for comparable experiments using the Brandel harvester (this paper and J.F.S., unpubl.) the average is 40. Although less of the antibody is retained by the Brandel harvester (80% vs. 90% for the Millipore manifold), the antibody concentration is kept below 0.1 K_D, and the impact on the apparent concentration of free ligand is minimal, thus there is little effect upon apparent affinity. In addition, the values reported in this paper are from a single representative experiment. The previously reported values (Schildbach et al., 1991) were derived from fitting multiple experiments with a single model, a method found to be inadequate due to its handling of the nonspecific binding parameter.

Molecular modeling

Modeling was based on the crystal structure of the 26-10 Fab:digoxin complex (P.D.J., R.K. Strong, L.C. Sieker, C. Chang, R.L. Campbell, G.A. Petsko, E.H., M.N.M., & S.S., submitted for publication). The structure has been refined at 2.5 Å resolution to an R-value of 0.171 and an r.m.s. deviation from ideal bond length of 0.013 Å. Side-chain conformational searches were done using the program CONGEN (Bruccoleri & Karplus, 1987). The protein atom parameters used in the empirical energy calculations were those of Brooks and colleagues (Brooks et al., 1983) except for modifications of the partial atomic charge set and van der Waals radii (Novotny et al., 1989). Prior to modeling mutants, an Fv was constructed by deleting the constant regions of the Fab, and the heavy chain N-terminal residue, which is disordered in the crystal, was positioned by conformational search using CONGEN. The N-terminus construction was done because N-terminal deletions have been shown to affect hapten recognition (Panka et al., 1988). Prior to the addition of the N-terminal residue, hydrogens were added to polar side-chain and main-chain amide groups using the HBUILD command. Hydrogen bonds were calculated for a distance of 5 Å and a donor-H$\cdots$acceptor angle of 70°, with cutoffs smoothed using a switching function (Brooks et al., 1983) in the ranges of 4.5–5 Å and 50–70°. Nonbonded interactions were calculated over an 8-Å range, with a switching function used from 7.5 to 8 Å. The dielectric constant was set to four times the distance between atom pairs. A 15° search grid was used for main-chain dihedral angles, and a 30° grid was used for side-chain dihedral angles. Maximum allowable van der Waals contact energies were +20 kcal/atom for main-chain atoms and +5 kcal/atom for side-chain atoms, and the van der Waals avoidance option of CONGEN (Bruccoleri & Karplus, 1987) was used for the side chains. Following the search, the lowest energy conformation for the N-terminal residue was chosen, and the hydrogens and heavy chain N-terminus were energy minimized using 200 steps of adopted basis Newton Rapheson (ABNR) minimization while all other atoms were fixed (Brooks et al., 1983).

The modeling procedure is an adaptation of the minimum perturbation approach (Shih et al., 1985). Digoxin was removed from the structure prior to modeling of the mutants by deleting its coordinates. The mutant side chains were introduced using the SPLICE command of CONGEN. For modeling proteins with Gly or Ala substitutions, the models were minimized (see below) after introduction of the amino acid. For all other side chains, a conformational search using a 30° grid was performed. The conditions of the searches were those for the N-terminal residue search (see above), except van der Waals avoidance was not used and van der Waals energy limits were raised to +200 kcal/atom. Each of the allowed conformations from the CONGEN search was subjected to a three-step energy minimization procedure. The procedure included fixing the modeled side chain while minimizing the surrounding atoms (10 steps ABNR), then fixing all but the modeled side-chain atoms and hydrogen atoms while minimizing (50 steps ABNR). The final step was a minimization (500 steps ABNR) with no constraints

on the modeled side chain or on side-chain atoms within 7.5 Å of the modeled side-chain β-carbon (C_β) and moderate (4 kcal/Å) harmonic constraints on main-chain atoms within 7.5 Å of the modeled side-chain C_β. Increasing constraints were placed on all atoms except hydrogens as their distance from the modeled side-chain C_β increased (4 kcal/Å for 7.5–10 Å, 8 kcal/Å for 10–12.5 Å, and 16 kcal/Å for 12.5–15 Å). Atoms 15 Å or more from the modeled side-chain C_β were fixed. For modeling the Gly mutant, the minimization was instead centered around the α-carbon (C_α) of the modeled amino acid. During minimization, the 1–4 nonbonded interactions were excluded from the calculated energies. The lowest energy conformation was then selected for docking experiments.

A digoxigenin molecule was then returned to the original hapten position in the binding site. The digoxigenin coordinates used had been taken from the 26-10:digoxin complex and energy minimized using parameters derived from cardiac glycoside and aglycone crystal structures (Schildbach et al., in prep.). Digoxigenin differs from digoxin only by lacking the tridigitoxose moiety (Fig. 3). Both haptens are bound with similar affinities by the 26-10 antibody (Mudgett-Hunter et al., 1982; Schildbach et al., 1991). Following reintroduction of digoxigenin, the complex was minimized by 500 steps of ABNR with digoxigenin atoms, hydrogen atoms, and H:Tyr33 and H 52 side-chain atoms unconstrained, moderate constraints (4 kcal/Å) on all other atoms within 7.5 Å of H:Tyr33 C_ζ, and increasing constraints on all but hydrogen atoms as their distance from H:Tyr33 C_ζ increased (6 kcal/Å for 7.5–10 Å, 8 kcal/Å for 10–12.5 Å, 16 kcal/Å for 12.5–15 Å). Atoms beyond 15 Å of the H:Tyr33C_ζ were fixed. The C_ζ was arbitrarily chosen as the center of minimization from the list of H:Tyr33 atoms contacting digoxin (see Results and discussion). Van der Waals energies between H:Tyr33 and digoxigenin were detected by the SEARCH function of the ANALYSIS facility of CONGEN and summed. As a control, the 26-10:digoxin Fab crystal structure was subjected to the docking and minimization procedure (see above) prior to use in figures and in van der Waals calculations. The diagrams of the 26-10:digoxin structure and the models were made using the plotting program PLT2 (David States & R.E.B., unpubl.).

Version 2 of CONGEN and PLT2 are available from R.E.B.

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