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Contribution of a single heavy chain residue to specificity of an anti-digoxin monoclonal antibody

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Abstract

Two distinct spontaneous variants of the murine anti-digoxin hybridoma 26-10 were isolated by fluorescence-activated cell sorting for reduced affinity of surface antibody for antigen. Nucleotide and partial amino acid sequencing of the variant antibody variable regions revealed that 1 variant had a single amino acid substitution: Lys for Asn at heavy chain position 35. The second variant antibody had 2 heavy chain substitutions: Tyr for Asn at position 35, and Met for Arg at position 38. Mutagenesis experiments confirmed that the position 35 substitutions were solely responsible for the markedly reduced affinity of both variant antibodies. Several mutants with more conservative position 35 substitutions were engineered to ascertain the contribution of Asn 35 to the binding of digoxin to antibody 26-10. Replacement of Asn with Gln reduced affinity for digoxin 10-fold relative to the wild-type antibody, but maintained wild-type fine specificity for cardiac glycoside analogues. All other substitutions (Val, Thr, Leu, Ala, and Asp) reduced affinity by at least 90-fold and caused distinct shifts in fine specificity. The Ala mutant demonstrated greatly increased relative affinities for 16-acetylated haptens and haptens with a saturated lactone.

The X-ray crystal structure of the 26-10 Fab in complex with digoxin (Jeffrey PD et al., 1993, *Proc Natl Acad Sci USA 90*:10310–10314) reveals that the position 35 Asn contacts hapten and forms hydrogen bonds with 2 other contact residues. The reductions in affinity of the position 35 mutants for digoxin are greater than expected based upon the small hapten contact area provided by the wild-type Asn. We therefore performed molecular modeling experiments which suggested that substitution of Gln or Asp can maintain these hydrogen bonds whereas the other substituted side chains cannot. The altered binding of the Asp mutant may be due to the introduction of a negative charge. The similarities in binding of the wild-type and Gln-mutant antibodies, however, suggest that these hydrogen bonds are important for maintaining the architecture of the binding site and therefore the affinity and specificity of this antibody. The Ala mutant eliminates the wild-type hydrogen bonding, and molecular modeling suggests that the reduced side-chain volume also provides space that can accommodate a congener with a 16-acetyl group or saturated lactone, accounting for the altered fine specificity of this antibody.

Keywords: anti-digoxin antibody; complementarity; fluorescence-activated cell sorting; hapten docking; hybridoma variant

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The X-ray crystal structures of antibodies complexed with antigen or hapten (Amit et al., 1986; Sheriff et al., 1987b; Herron et al., 1989; Padlan et al., 1989; Alzari et al., 1990; Bentley et al., 1990; Fischmann et al., 1990; Stanfield et al., 1990; Brunger et al., 1991; Cygler et al., 1991; Rini et al., 1992; Tulip et al., 1992; Arevalo et al., 1993; Rose et al., 1993; Vix et al., 1993) suggest that the specificity of antibody recognition is reliant upon a high degree of complementarity between the antigen-combining site and antigen. The surfaces of the antibody and antigen often fit together with enough precision to exclude most

water molecules from the interface. The complementarity also extends to the precise positioning of atoms to allow charge-charge interactions and hydrogen bond formation between antibody and antigen. Although it then follows that altering the structure of the binding site will affect recognition, it has proven difficult to predict how a particular amino acid substitution will modulate antibody specificity.

To correlate antibody sequence with binding function, we are studying high-affinity anti-digoxin monoclonal antibodies (Mudgett-Hunter et al., 1982). We previously described variants of anti-digoxin hybridoma cell lines that secrete antibody possessing altered hapten binding due to variable (V) region substitutions (Panka et al., 1988; Schildbach et al., 1991). Here we detail the isolation of 2 V region variants of the anti-digoxin hybridoma 26-10. Each variant produced antibody with drastically reduced affinity for digoxin, due to 1 or 2 heavy- (H) chain V region amino acid substitutions. Because both variant antibodies contained substitutions at H chain position 35 (H35), and because this is a site of recurrent mutation in 26-10 hybridoma variants demonstrating reduced antibody affinity for digoxin (D.J. Panka, S.Y. Shaw, D.R. Parks, & M.N. Margolies, unpubl. obs.), we undertook mutagenesis experiments to define the contribution of this position to 26-10 antibody specificity. First, we established that the position 35 mutations observed in the variants were responsible for the reduced affinity of the variants. To examine the influence of other position 35 substitutions on hapten binding, additional mutants were generated. The 26-10 antibody proved sensitive to position 35 substitutions, with most mutant antibodies displaying not only reduced affinity for digoxin, but also altered fine specificity. Subsequently, the X-ray crystal structure of 26-10 Fab in complex with digoxin was determined (Kinemage 1; Jeffrey et al., 1993) and showed that the H35 Asn not only provides hapten contacts but is also involved in a hydrogen bond network with 2 other contact residues. The structure enabled us to model the mutant antibodies and correlate the models with the results of the binding studies.

The results of the modeling and binding studies indicate that the H35 Asn is an important contact residue and structural element of antibody 26-10, and the identity of the amino acid at this position can greatly affect hapten recognition. In addition, the effect of H35 amino acid substitutions upon specificity in other antibodies suggests that the residue at this position may be important for maintenance of binding site architecture and function for many antibodies.

Results

The 26-10 hybridoma variants L1B1 and R3 were selected by fluorescence-activated cell sorting (FACS) due to their reduced staining by a conjugate of digoxin, human serum albumin (HSA), and phycoerythrin. Antibodies produced by these hybridoma variants had no detectable binding in a saturation equilibrium assay (data not shown). Using a solid-phase assay that is more sensitive to low-affinity binding, both L1B1 and R3 demonstrated some binding above background to a conjugate of digoxin and HSA (Table 1). The binding of L1B1 and R3, however, was much lower than that of a similar concentration of 26-10 antibody (Table 1) and was consistent with affinities lower than can be measured using the saturation equilibrium assay (see below).

Table 1. *Digoxin binding of 26-10 variant antibodies*[a]

Antibody/mutant	CPM bound
26-10	9,705 ± 750
R3[b]	1,050 ± 55
L1B1[c]	538 ± 22
No antibody	215 ± 10

[a] Antibody solutions of similar concentrations were incubated in wells of plates coated with a conjugate of digoxin and human serum albumin, the plates washed, and antibody detected by radioiodinated goat anti-mouse-Fab.

[b] Contains Lys for Asn substitution at H35.

[c] Contains Tyr for Asn substitution at H35 and Met for Arg substitution at H38.

The H chain of secreted L1B1 antibody appeared more acidic, and that of R3 appeared more basic, than the H chain of secreted 26-10 antibody when analyzed by 2-dimensional SDS-PAGE (data not shown). In addition, the H chain of R3 cell surface antibody appears to be of slightly lower molecular weight than that of 26-10, although the cell surface R3 antibody H chain is larger than the secreted 26-10 and R3 H chains (data not shown). The R3 and L1B1 light (L) chains were indistinguishable from that of 26-10 (data not shown).

Sequence analysis of variant antibodies

Amino acid and nucleotide sequencing of R3 and L1B1 antibody V regions were undertaken to determine the basis for the altered hapten binding. Automated Edman degradation of the R3 H chain extended for 58 cycles, with the use of *o*-phthalaldehyde (OPA) treatment at cycle 14 (at which Pro is N-terminal). The sequence was identical to that of 26-10 except for substitution of Lys for Asn at cycle 35 (Fig. 1). This substitution was confirmed by the sequence of the CNBr peptide N-terminal at position 35 (Lys), which extended through position 76 (Kabat numbering; Kabat et al., 1991). This sequence was otherwise identical to 26-10. The CNBr peptide N-terminal at position 101 was also sequenced into C_{H1} and showed no differences from 26-10. The N-terminal sequence of the intact R3 L chain (55 cycles, aided by extended cleavage of the phenylthiocarbamyl at cycle 8 and OPA treatment at cycles 12 and 49) revealed no differences from that of 26-10 (not shown).

Residues 1–61, 81–98, and 101–113 of the L1B1 H chain were also identified by amino acid sequencing. Two substitutions relative to 26-10 were found: replacement of Asn with Tyr at H chain position 35 and Arg with Met at H chain position 38 (H 38). The partial amino acid sequence of L1B1 L chain V region (positions 1–37 and 55–108) was identical to 26-10.

Complete nucleotide sequencing of cloned, PCR-amplified H and L V region cDNA of R3 and L1B1 confirmed and extended these results and those of cDNA sequencing by chemical cleavage (see Materials and methods). No additional differences between 26-10 and the variant antibody V regions were found.

Mutagenesis analysis of variants

Mutagenesis experiments were first undertaken to determine the individual contributions of the 2 amino acid substitutions in the L1B1 H chain to the binding defect. Mutant antibodies with ei-

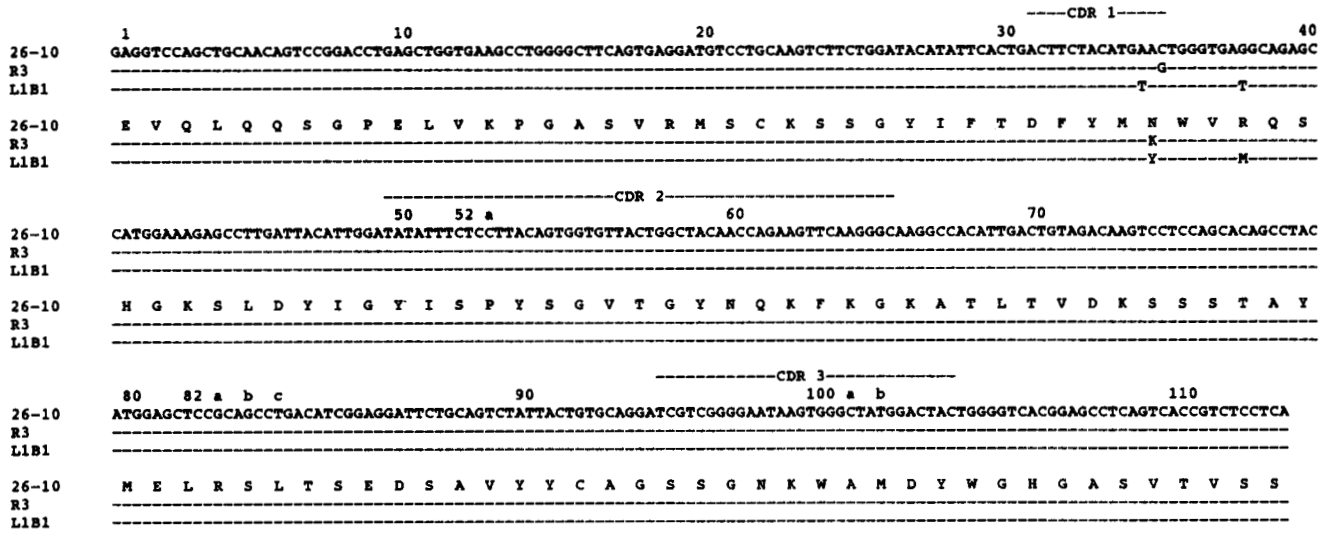

Fig. 1. Nucleotide and amino acid sequences of 26-10, R3, and L1B1 H chains. Amino acid sequences were translated from nucleotide sequences and confirmed in part by protein sequence analysis. Amino acid sequences are given in 1-letter code. Amino acid residue numbering and complementarity determining regions are as defined by Kabat et al. (1991). A dash indicates identity to the topmost sequence. Complete genomic and cDNA nucleotide and partial protein sequences of 26-10 V regions were reported (Novotny & Margolies, 1983; Mudgett-Hunter et al., 1985; Hudson et al., 1987; Near et al., 1990; Schildbach et al., 1991).

ther the H35 Tyr for Asn (H:Asn-35-Tyr) or H 38 Met for Arg (H:Arg-38-Met) substitution were constructed. In addition, antibody H:Asn-35-Lys was engineered to confirm the results for the spontaneous variant R3. The mutagenic oligonucleotides used are listed in Table 2. Both the H:Asn-35-Tyr and H:Asn-35-Lys antibodies demonstrate greatly reduced binding to digoxin–HSA relative to 26-10wt (Table 3). Antibody 26-10wt ($K_a = 9.1 \times 10^9\ M^{-1}$; see below) is the product of expression of the unmutated 26-10 H chain in 26-10κ cells and differs from 26-10 in H chain isotype (γ2a for 26-10 and γ2b for 26-10 wt), but not in hapten recognition (data not shown). Antibodies H:Asn-35-Tyr and H:Asn-35-Lys also have lower binding than the engineered 26-10 mutant H:Tyr-50-Asp, which was used as a control because it has an affinity near the lower limit of measurement of the saturation equilibrium assay ($2.3 \times 10^6\ M^{-1}$; Schildbach et al., 1993a). The low binding of antibodies H:Asn-35-Tyr and H:Asn-35-Lys in the solid-phase assay is therefore consistent with their lack of detectable binding in the saturation equilibrium assay. These results also confirm the observation made of the spontaneous variants: nonconservative substitutions at H35 can greatly reduce (>10^4-fold) the affinity for digoxin of 26-10 mutant antibodies.

Table 2. *Mutagenic oligonucleotides*

Mutant	Sequence 5′ 3′
H:Asn-35-Tyr	CT CAC CCA GTA CAT ATA GAA G
H:Asn-35-Lys	CT CTG CCT GAC CCA TTT CAT GTA GAA G
H:Asn-35-Ala	CT CAC CCA GGC CAT GTA GAA GTC
H:Asn-35-Asp	GCT TTG CCT CAC CCA GTC CAT GTA GAA GTC
H:Asn-35-Val	CT CAC CCA GAC CAT GTA GAA GTC
H:Asn-35-Gln	CT CAC CCA CTG CAT GTA GAA GTC
H:Asn-35-Leu	CT CAC CCA GAG CAT GTA GAA GTC
H:Asn-35-Thr	CT CTG CCT GAC CCA GGT CAT GTA GAA G
H:Arg-38-Met	CC ATG ACT CTG CAT CAC CCA GTT C

[a] Oligonucleotides are anti-sense and complementary, annealing from H chain position 31 to 38 or 40 for H35 mutants, and from position 34 to 42 for H:Arg-38-Met (see Fig. 2). Nucleotides differing from wild type are underlined. Oligonucleotides for H:Asn-35-Asp, H:Asn-35-Lys, and H:Asn-35-Thr also contain silent mutations to increase differences in melting temperatures of mutant oligonucleotides annealed to mutated and unmutated 26-10 H chain sequence during dot blot hybridization screening (Sambrook et al., 1989).

Table 3. *Digoxin binding of engineered 26-10 mutant antibodies*[a]

Antibody/mutant	CPM bound[b]
26-10wt	13,608 ± 82
H:Asn-35-Lys	2,562 ± 72
H:Asn-35-Tyr	445 ± 5
H:Tyr-50-Asp[c]	8,622 ± 112
No antibody	215 ± 10

[a] Antibody solutions of similar concentrations were incubated in wells of plates coated with a conjugate of digoxin and human serum albumin, the plates washed, and antibody detected by radioiodinated goat anti-mouse-Fab.

[b] Discrepancies between CPM listed here and in Table 1 are due in part to different recognition by the radioiodinated goat anti-mouse-Fab of 26-10 and the spontaneous variants (heavy chain isotype γ2a) versus 26-10wt and the engineered mutants (heavy chain isotype γ2b).

[c] The engineered 26-10 mutant H:Tyr-50-Asp is included as a control because its affinity for digoxin (2.3×10^6 M^{-1}) approaches the lower limit of measurement by the solution-phase affinity assay (Schildbach et al., 1993a).

Affinity measurements established that H:Arg-38-Met and 26-10wt have equivalent affinities for digoxin (data not shown). The greatly reduced affinity for digoxin of L1B1 is therefore solely due to the H35 Tyr for Asn substitution.

Mutagenesis analysis of H35

It was reasoned that more conservative substitutions than Tyr (L1B1) or Lys (R3) for Asn may have less drastic effects and would provide additional insight into the role of H:Asn-35 in the hapten binding of 26-10. Therefore, a panel of 26-10 antibodies mutated at H35 was constructed. In antigen combining sites, Asn residues often form hydrogen bonds through their side-chain atoms to other side chains or to the main chain (Padlan, 1990). Therefore Gln, Asp, and Thr, which are capable of forming hydrogen bonds, and sterically similar amino acids incapable of forming hydrogen bonds (Leu for comparison to Asn, Val for comparison to Thr, and Ala) were substituted for Asn. The mutant antibodies and the mutagenic oligonucleotides used in their creation are listed in Table 2.

The affinities of the mutant antibodies for digoxin are compared to that of 26-10wt in Table 4. Replacing Asn with Gln (H:Asn-35-Gln) causes an approximately 10-fold drop in affinity, whereas replacement with Val (H:Asn-35-Val) reduces affinity approximately 90-fold. Substituting Thr, Leu, or Ala for the H35 Asn (H:Asn-35-Thr, H:Asn-35-Leu, and H:Asn-35-Ala, respectively) reduces affinity for digoxin approximately 200-fold, whereas replacement with Asp (H:Asn-35-Asp) reduces affinity 1,400-fold.

The relative affinities of the antibodies for digoxin analogues were measured using a competition assay (Tables 5, 6). A summary of the analogue structures is given in Table 5, by reference to Figure 2. Analogues chosen included those demonstrating altered binding to the mutant H:Asn-35-Ala in preliminary assays. The values are reported in Table 6 as relative K_i, which is the K_d of an antibody for an analogue as determined by a competition assay, normalized to the K_i of digoxin for that antibody.

Table 4. *Affinity of H35 mutants for digoxin*[a]

Antibody	Affinity ($\times 10^{-5}$ M^{-1})
26-10wt	91,000 ± 10,000
H:Asn-35-Gln	8,400 ± 1,000
H:Asn-35-Val	970 ± 120
H:Asn-35-Thr	440 ± 70
H:Asn-35-Leu	390 ± 50
H:Asn-35-Ala	380 ± 60
H:Asn-35-Asp	66 ± 16
H:Asn-35-Lys	<10[b]
H:Asn-35-Tyr	<10[b]

[a] Affinities for digoxin were measured in a saturation equilibrium assay using filtration through glass fiber filters to separate bound from free tritiated ligand (see Materials and methods).

[b] Affinity below lower limit of this assay (10^6 M^{-1}).

The K_i values for digoxin agreed well with the K_d determined by the saturation equilibrium assay (data not shown).

As described previously for 26-10 (Schildbach et al., 1991), the 26-10wt antibody has lowered affinity for digoxin analogues with 16-position substitutions (see Fig. 2; Tables 5, 6). The affinity decreases as the size of the 16-position substituent increases. Gitoxin, which has a 16-hydroxyl (16-OH) group, is bound by the 26-10wt antibody with 6-fold lower affinity than digoxin. Gitaloxin, with a 16-formyl group, is bound with 32-fold lower affinity. The analogues 16-acetylgitoxin, oleandrin, and oleandrigenin all have 16-acetyl groups and are bound with even lower affinities (250–9,900-fold reductions). The affinities for these 3 congeners, however, are affected by the identity of the substituent at the 3-position: 16-acetylgitoxin, with a 3-tridigitoxose, is bound with the highest affinity of the 3, whereas oleandrin, with a 3-oleandrose, and oleandrigenin, with a 3-OH, are bound with progressively lower affinities. Dihydrodigoxin, which has a saturated C20–C22 lactone bond, is bound by antibody 26-10wt with 1,300-fold lower affinity than digoxin.

Antibody H:Asn-35-Gln exhibits a specificity similar to 26-10wt (Table 6). The only possible difference is in recognition of oleandrigenin, which H:Asn-35-Gln binds with an affinity below the limits of this assay, precluding direct comparison to 26-10wt.

Table 5. *Structure of digoxin and digoxin analogues*

	Substituents at positions[a]			
Analogue	3	12	16	C20–C22 bond
Digoxin	Tridigitoxose	-OH		Unsaturated
Gitoxin	Tridigitoxose		-OH	Unsaturated
Gitaloxin	Tridigitoxose		-CHO	Unsaturated
16-Acetylgitoxin	Tridigitoxose		$-COCH_3$	Unsaturated
Oleandrin	Oleandrose		$-COCH_3$	Unsaturated
Oleandrigenin	-OH		$-COCH_3$	Unsaturated
Dihydrodigoxin	Tridigitoxose	-OH		Saturated

[a] Cardenolide numbering scheme is shown in Figure 2.

Table 6. *Relative K_i values of N35 mutants for digoxin analogues*[a]

Analogue	26-10wt	H:Asn-35-Gln	H:Asn-35-Val	H:Asn-35-Thr	H:Asn-35-Leu	H:Asn-35-Ala	H:Asn-35-Asp
Digoxin	1	1	1	1	1	1	1
Gitoxin	6	11	11	4	13	14	0.9
Gitaloxin	32	51	29	17	41	3	6
16-Acetylgitoxin	250	180	63	68	86	8	49
Oleandrin	920	740	220	>180	260	19	>47
Oleandrigenin	9,900	>7,100	>760	>180	>460	14	>47
Dihydrodigoxin	1,300	2,700	110	94	190	190	>47

[a] K_i of antibodies for digoxin analogues were determined in a solution-phase competition assay (see Materials and methods). K_i is K_d as determined by competition assay. Values were normalized to the K_i for digoxin for each antibody. Values for those haptens that at the highest concentrations used in the assay (10 μM) inhibited less than 50% of binding of [^{3}H]digoxin are denoted as greater than the highest measurable relative K_i.

Antibodies H:Asn-35-Val, H:Asn-35-Thr, and H:Asn-35-Leu all have similar fine specificities, demonstrating relative affinities 3–4-fold higher for 16-acetylated haptens and 7–14-fold higher for dihydrodigoxin compared to 26-10wt. The only difference between the 3 mutants is that H:Asn-35-Thr has a 3-fold higher relative affinity for gitoxin than the others. As is the case for H:Asn-35-Gln, these mutant antibodies bind some of the 16-acetylated compounds with affinities too low to be measured.

Antibody H:Asn-35-Ala also demonstrates a higher relative affinity for dihydrodigoxin than does 26-10wt. More significantly, the relative affinities of H:Asn-35-Ala for gitaloxin and the 16-acetylated haptens were dramatically increased: all are bound with affinities approximately equal to or significantly higher than for gitoxin. The affinity of H:Asn-35-Ala for oleandrigenin (K_i = 360 nM) is actually greater than that of 26-10wt (K_i = 720 nM). Antibody H:Asn-35-Asp also possesses a unique specificity: this mutant binds gitoxin and digoxin equally, and binds gitaloxin and 16-acetyldigoxin with improved relative affinities, compared to 26-10wt. The low affinity of H:Asn-35-Asp precluded measurement of the K_i for oleandrin, oleandrigenin, and dihydrodigoxin.

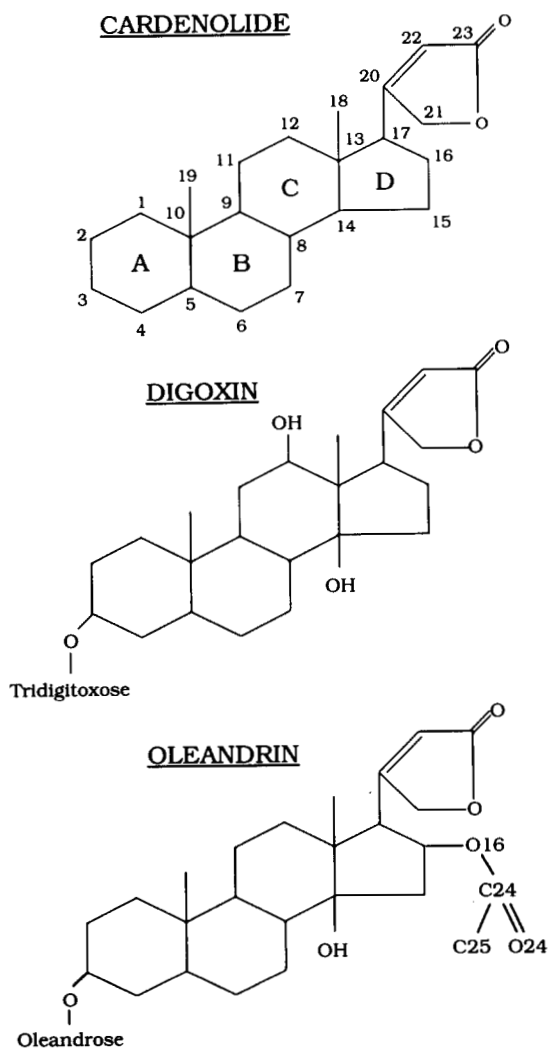

Fig. 2. Diagrams of the cardenolide numbering system, digoxin (digoxigenin tridigitoxose), and oleandrin including the numbering for the 16-acetyl moiety (from Kartha & Go, 1981).

Molecular modeling analysis

The X-ray crystal structure of the 26-10 Fab complexed with digoxin was determined subsequent to completion of the mutagenesis experiments (Kinemage 1; Jeffrey et al., 1993). The structure shows that the H:Asn-35 contacts (as defined by Sheriff et al., 1987a) the D ring (atoms C16 and C17) and the lactone (atoms C20, C21, and C22) of digoxin (P.D. Jeffrey, unpubl. obs.; Figs. 2, 3; Kinemages 1, 2). In addition, H:Asn-35 forms hydrogen bonds with the hydroxyls of H:Tyr-47 and H:Ser-95, both of which are hapten contact residues. The electron density maps were interpreted as the H:Asn-35 $N_{\delta 2}$ serving as a hydrogen bond donor to the H:Tyr-47 O_η and the H:Ser-95 O_γ acting as a hydrogen bond donor to the H:Asn-35 $O_{\delta 1}$. The electron density maps, however, are consistent with an H:Asn-35 orientation in which the $O_{\delta 1}$ and $N_{\delta 2}$ are rotated 180° about the C_β–C_γ bond and the pattern of hydrogen bond donors and acceptors is altered.

We used the 26-10 Fab:digoxin structure as the basis for modeling the mutants. A description of the modeling procedure, including assumptions made and the resulting limitations of the method, is given below (see Materials and methods). As a control for the modeling procedure, an H:Asn-35 side chain was modeled into the 26-10 structure. The 2 lowest-energy modeled side-chain conformations included a conformation similar to that of the crystal structure (second lowest; Fig. 4) and a conformation rotated approximately 180° about the C_β–C_γ bond

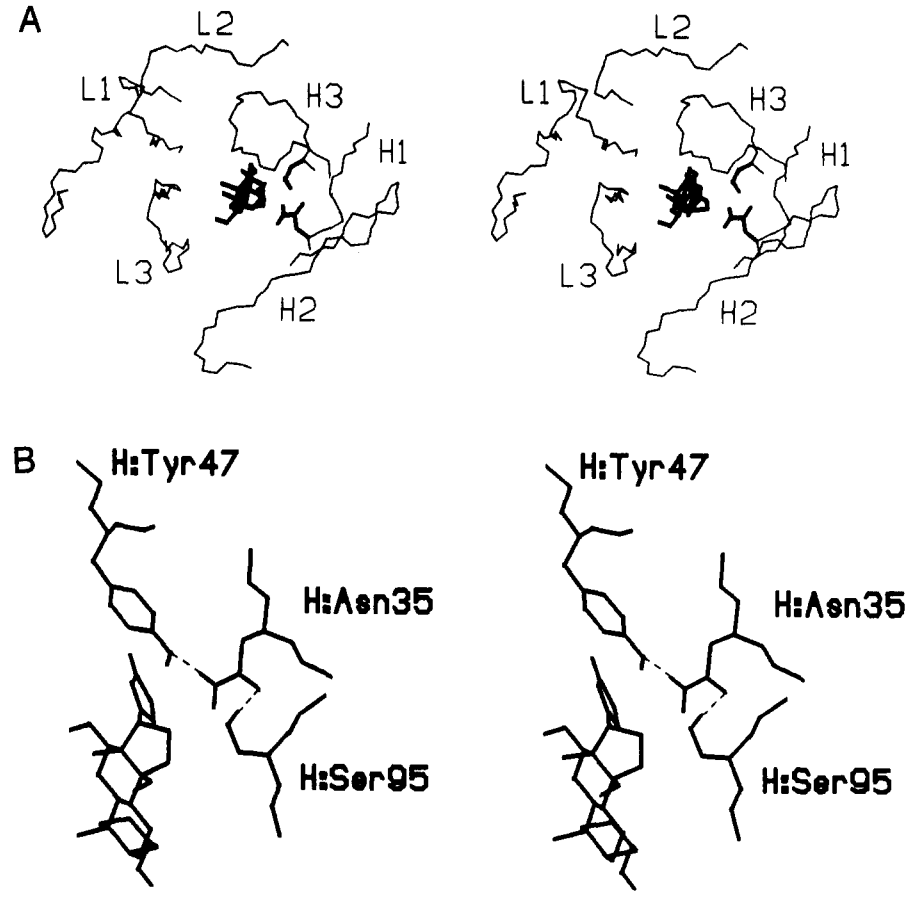

Fig. 3. **A:** Stereo view of 26-10 CDRs and bound digoxigenin from the 26-10 Fab:digoxin crystal structure (Jeffrey et al., 1993). View is from solvent into the binding site. Depicted in bold lines (center of diagram) are digoxigenin and residues H:Asn-35 (in first H chain CDR, H1) and H:Ser-95 (in H3). Hydrogens are shown explicitly only on potential hydrogen bond donors. **B:** Stereo view showing detail of bound digoxigenin, H:Asn-35, H:Tyr-47 (from framework region 2), and H:Ser-95. View is rotated 130° about the horizontal axis from view shown in top panel. Amino acids are shown with the main chain through the C_α atoms of the adjacent residues. Hydrogens are shown explicitly only on potential hydrogen bond donors. Hydrogen bonds are shown as dashed lines.

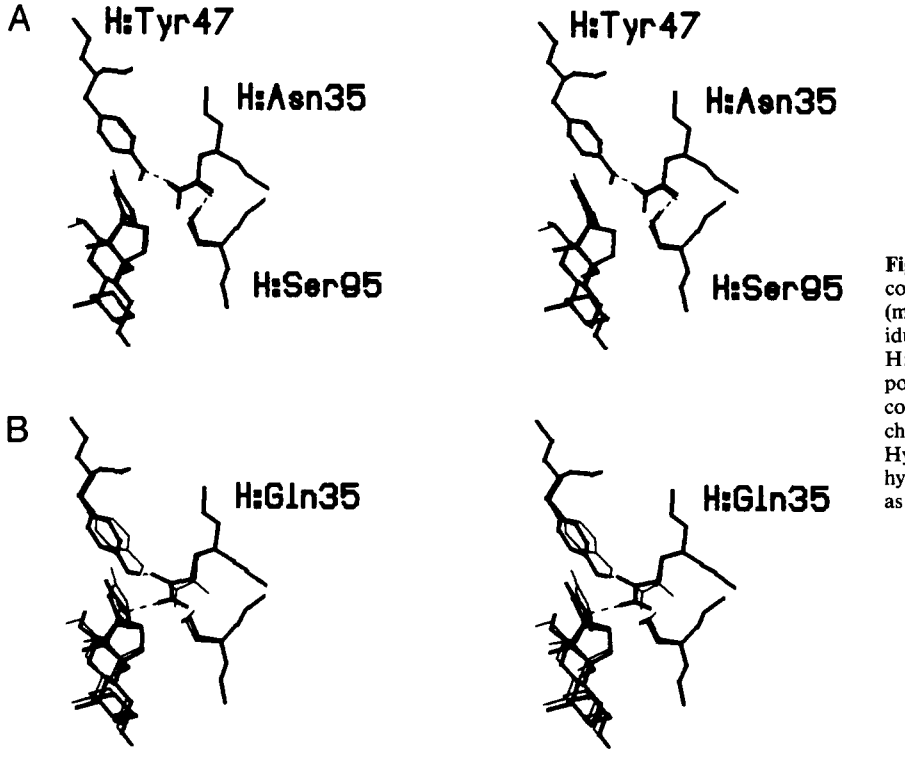

Fig. 4. Stereo views of modeled H35 mutants complexed with digoxigenin. **A:** H:Asn-35-Asn (modeled 26-10). **B:** H:Asn-35-Gln. The H35 residue is shown with digoxigenin, H:Tyr-47, and H:Ser-95 (bold lines). The models are superimposed upon the 26-10 structure (thin lines) for comparison. Amino acids are shown with main-chain atoms through the C_α of adjacent residues. Hydrogens are shown explicitly only on potential hydrogen bond donors. Hydrogen bonds are shown as dashed lines.

(not shown). Both of these modeled conformations represent possible orientations for the side chain (see above) and are consistent with the 26-10:digoxin crystal structure.

Noting that the H:Asn-35-Gln mutant antibody retained the wild-type specificity and that Gln, like Asn, may be able to participate in a hydrogen bond network, we modeled the H:Asn-35-Gln mutant to ascertain whether the substituted Gln could maintain the network (Fig. 4). The lowest-energy conformation for the Gln side chain allows formation of a hydrogen bond between H35 and the H:Tyr-47 hydroxyl but does not permit formation of a hydrogen bond with H:Ser-95 without reorientation of the H:Ser-95 side chain. Although the Gln side-chain $N_{\epsilon 2}$ protrudes slightly into the binding site, causing energetically unfavorable contacts with the digoxigenin lactone when the hapten is inserted into the binding site, these van der Waals energies were significantly reduced during an energy minimization step through minor positional shifts by the Gln side chain. This result suggests that these unfavorable contacts may account for the 10-fold lower affinity of H:Asn-35-Gln for digoxin relative to 26-10wt. A hydrogen bond is possible between the Gln amide and the O21 of the digoxin lactone.

Several other mutants were also modeled. The only other mutant that maintained the hydrogen bonds seen in 26-10 was H:Asn-35-Asp (not shown), although the polarity of the hydrogen bond between H35 and H:Tyr-47 was reversed. Although this antibody demonstrates significantly altered hapten binding relative to wild type (Tables 4, 6), the negative charge of Asp rather than a structural effect due to the H35 substitution may be responsible. Modeling experiments suggest that none of the mutants with uncharged H35 residues, with the exception of Gln, are able to reproduce the hydrogen bonding seen in 26-10 and correspondingly do not show wild-type specificity.

To seek explanations for the altered fine specificity of the Ala mutant, hapten docking experiments for both 26-10 and H:Asn-35-Ala were conducted (see Materials and methods). The models of 26-10 and H:Asn-35-Ala each docked with oleandrin differ (Fig. 5). The low-energy position of the oleandrin 16-acetyl group in the H:Asn-35-Ala model is forbidden in the 26-10 model because of unfavorable contacts with the 26-10 H:Asn-35. In addition, the position of the H:Tyr-33 side chain in the 26-10:oleandrin model is shifted relative to the position of the side chain in the 26-10:digoxin crystal structure. The position of H:Tyr-33 does not differ between the models of H:Asn-35-Ala complexed with digoxigenin or with oleandrin.

Results of docking dihydrodigoxigenin into the binding sites of 26-10 and H:Asn-35-Ala are shown in Figure 6 (thick lines),

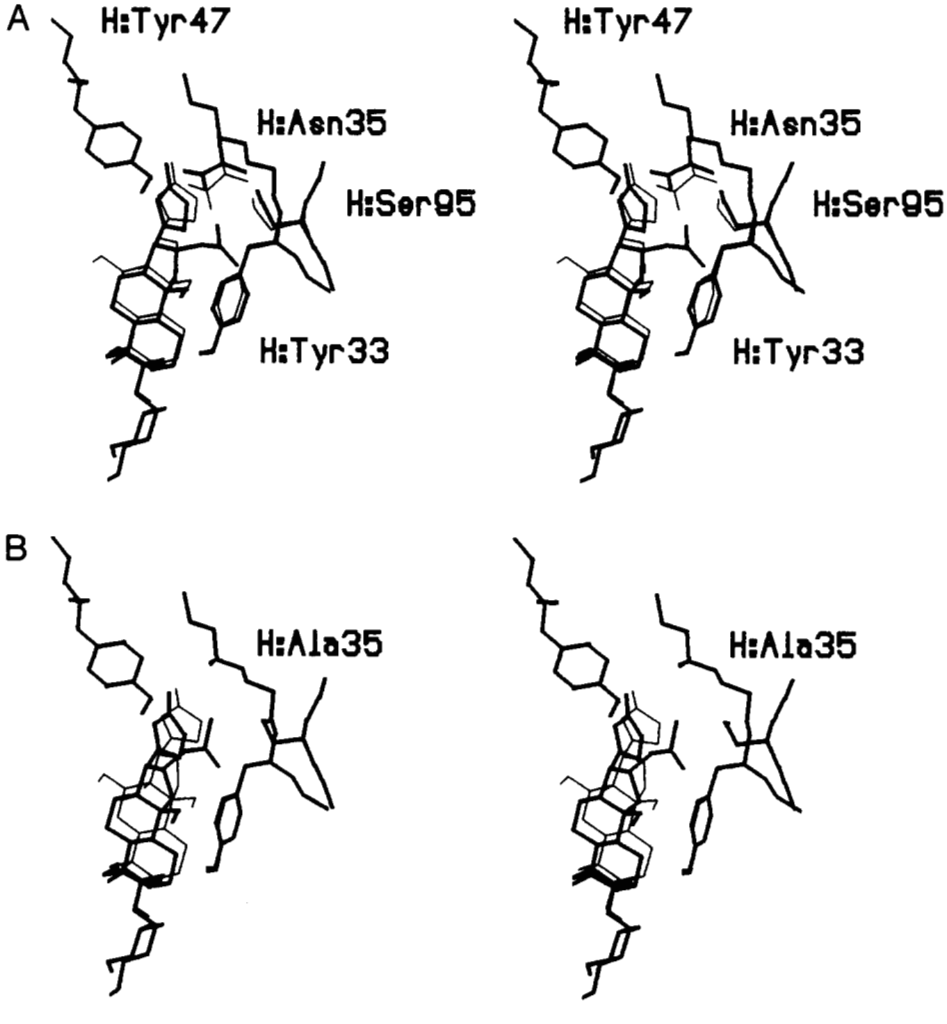

Fig. 5. Stereo views of modeled complexes of (**A**) 26-10 and (**B**) H:Asn-35-Ala with oleandrin. Oleandrin, H:Tyr-33, H:Tyr-47, H:Ser-95, and the H35 residue are shown (thick lines) with the corresponding side chains and hapten molecules from modeled complexes of the antibodies with digoxigenin (thin lines). The main-chain atoms of amino acids are displayed through the C_α of adjacent residues. Hydrogens are shown explicitly only on potential hydrogen bond donors.

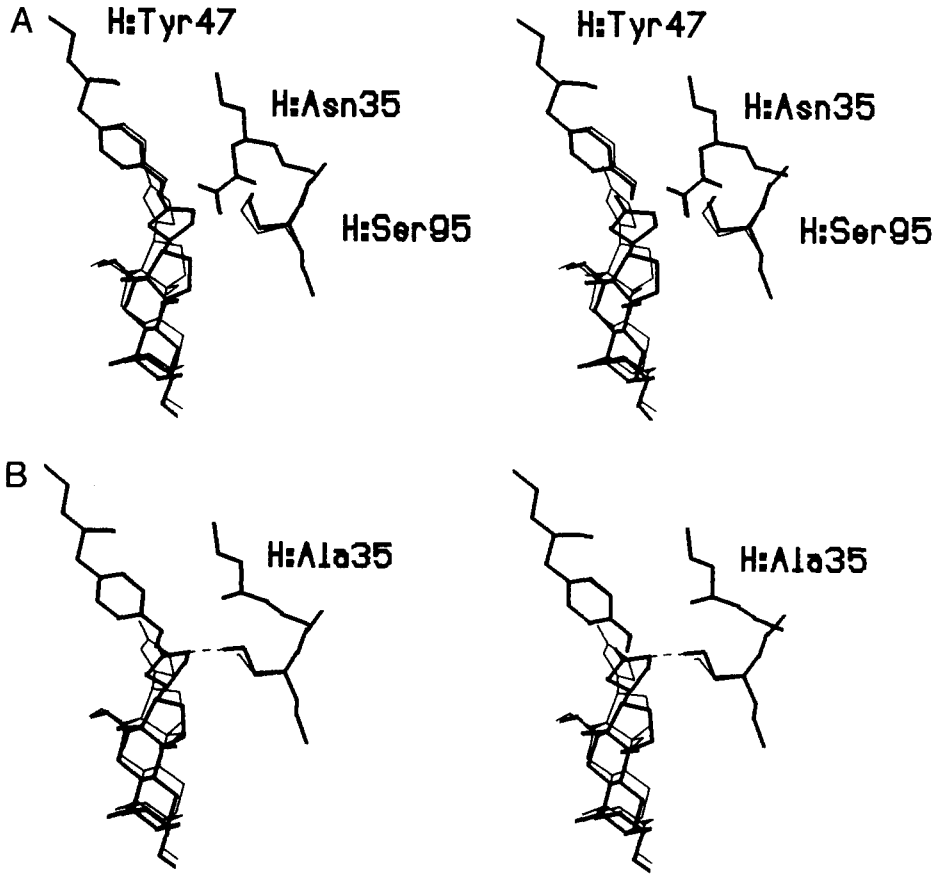

Fig. 6. Stereo views of modeled complexes of (**A**) 26-10 and (**B**) H:Asn-35-Ala with dihydrodigoxigenin (bold lines). Models are superimposed upon those of complexes of their respective antibodies with digoxigenin (thin lines). The main-chain atoms of amino acids shown through the C_α of adjacent residues. Hydrogens are shown explicitly only on potential hydrogen bond donors. Hydrogen bonds are shown as dashed lines.

compared to digoxigenin docked into the same binding sites (thin lines). The saturated lactone of dihydrodigoxigenin, unlike that of digoxigenin, is not planar, disrupting the complementarity between antibody and hapten. In both 26-10 and H:Asn-35-Ala, the entire dihydrodigoxigenin molecule, and the lactone in particular, is shifted relative to the position of digoxin bound in the same site. Despite the shift, unfavorable contacts remain between the 26-10 H:Asn-35 side chain and the lactone. Substitution of Ala for Asn avoids these unfavorable contacts. In addition, the lactone of dihydrodigoxigenin bound by H:Asn-35-Ala is rotated approximately 30° about the C17–C20 bond, relative to the lactone of dihydrodigoxigenin docked into the 26-10 binding site. This conformation is lower in energy than the configuration in the 26-10 model and also allows a hydrogen bond to be formed between the hydroxyl of H:Ser-95 and the O21 of dihydrodigoxigenin.

The modeled hapten:antibody complexes therefore suggest that haptens dihydrodigoxigenin and oleandrigenin can adopt lower energy conformations within the binding site of H:Asn-35-Ala that are disallowed in the 26-10 binding site.

Discussion

The high affinity of antibody 26-10 for digoxin is derived largely from shape complementarity and the attendant desolvation of the complementary surfaces; there are no apparent energetic contributions from ionic or hydrogen bonding interactions (Jeffrey et al., 1993). Consistent with this observation, we previously reported that mutations of a noncontact residue can significantly reduce affinity for digoxin, presumably by altering the shape of the binding cavity (Schildbach et al., 1993b).

The binding of many of the 26-10 H35 variant and mutant antibodies described here can also be attributed to the effects of amino acid substitutions on the shape of the binding site. Spontaneous variant antibodies L1B1 and R3, and the engineered mutant antibodies containing their H35 substitutions (H:Asn-35-Tyr and H:Asn-35-Lys, respectively) have greatly reduced affinity for digoxin, due to the introduction of bulky side chains into a region known to be of tight complementarity (Fig. 3; Kinemage 2). The lowered affinities for digoxin of the Val, Leu, Thr, or Ala mutants appear too large to be accounted for by the reduction in contact area alone. Although the H:Asn-35 side chain is buried in the antibody:hapten complex, the side chain has only a 7-Å^2 solvent-exposed surface when uncomplexed (measured using a 1.5-Å diameter probe; Lee & Richards, 1971). The approximately 3 kcal loss in binding energy (where binding energy is calculated by $\Delta G = RT \ln K_d$ and R is the gas constant, T is 293 K, and K_d is the experimentally determined dissociation constant of the antibodies for digoxin) of the Val, Leu, Thr, and Ala mutants, relative to 26-10wt, is far greater than expected according to standard estimates (25–47 cal/Å^2 of surface area of complex; Chothia, 1974; Eisenberg et al., 1989; Sharp et al., 1991). Destabilization of the antibody:hapten complex by an unoccupied volume at the interface, analogous to the reduced stability caused by substitution of small for large amino acids within a protein core (Eriksson et al., 1992), is also an un-

satisfactory explanation for the reduced affinities because H:Asn-35-Leu has a 200-fold lower affinity for digoxin than 26-10wt, despite similar volumes of Leu and Asn. Given the similar affinities for digoxin conferred by dissimilar H35 substitutions (Val, Thr, Leu, Ala), the altered hapten binding of these antibodies may be due largely to loss of the hydrogen bonds formed by the wild-type Asn.

The hydrogen bonds formed by H:Asn-35 may contribute to the binding of 26-10 by stabilizing the side-chain conformations of the contact residues H:Asn-35, H:Tyr-47, and H:Ser-95, reducing the entropic penalty incurred when the mobility of the side chains is limited during complexation with hapten. In addition, the hydrogen bonds may orient the side chains of these residues in positions that optimize the area of contact between antibody and hapten.

Molecular modeling studies of antibodies with substitutions at position H35 suggest that Gln and Asp can maintain one or both of these hydrogen bonds (Fig. 4). The model of H:Asn-35-Gln suggests that the moderately reduced affinity of this antibody is due to unfavorable contacts between hapten and the Gln side chain. A hydrogen bond between the Gln and the O21 of digoxin may partially compensate for these unfavorable contacts. The altered hapten binding of antibody H:Asn-35-Asp, however, is probably due to introduction of the Asp negative charge, which makes desolvating the binding site of the Asp mutant energetically costly, relative to desolvating the 26-10wt binding site. The similarities in the fine specificity of antibodies 26-10wt and H:Asn-35-Gln are consistent with similar binding site structures of these antibodies. These results, taken together with the binding of the other mutant antibodies with neutral H35 substitutions, support the contention that the hydrogen bonding of H:Asn-35 is essential to maintain the local binding site structure and the specificity of the antibody.

Although the altered affinity of the Ala mutant may be the result of the effect of this substitution on other residues (e.g., H:Tyr-47 and H:Ser-95) through hydrogen bond loss, the altered fine specificity appears to be due to the smaller size of Ala compared to Asn. Docking experiments (Figs. 5, 6) suggest that H35 Ala allows more space at the hapten:antibody interface than does H35 Asn, permitting dihydrodigoxigenin and oleandrigenin to adopt low energy conformations within the binding site of H:Asn-35-Ala that are disallowed in the 26-10 binding site. In contrast, the altered fine specificity of H:Asn-35-Asp may be due to the introduction of the negatively charged side chain, which interacts more favorably than the neutral Asn side chain with the 16-hydroxyl of gitoxin. In principle, this model may be tested by pH titration of the negative charge of the Asp side chain in the affinity or specificity assays. Presumably effects due to the charge of Asp would diminish as the side chain is protonated. Under the acidic conditions required to protonate Asp (pH ~3.5), however, the apparent affinity of all antibodies tested was considerably reduced. In particular, mutant antibodies H:Asn-35-Asp and H:Asn-35-Leu had no detectable specific binding of digoxin under these conditions, preventing characterization of their binding as a function of pH.

Not all contact residues in 26-10 are as sensitive to replacement as is H:Asn-35. We previously described mutations of the CDR2 contact residue H:50-Tyr (Fig. 1) that reduce affinity and alter fine specificity due to a reduction in the size of the hapten:antibody interface (Schildbach et al., 1993a). Many of the 26-10 H35 mutant antibodies described here also display reduced affinity and altered fine specificity resulting from substitution of a hapten contact residue. However, mutations at H35 and at H50 exhibit different patterns of constraints on hapten recognition. For example, substitutions larger than Asn at H35 reduced affinity significantly (10-fold for Gln or greater than 4,000-fold for Lys or Tyr), whereas replacement of H50 Tyr with Trp had little effect (Schildbach et al., 1993a). Replacement of H:Asn-35 with smaller residues (Val, Thr, Leu, Ala) resulted in similar reductions in affinity for digoxin (100–200-fold), but substitution of H:Tyr-50 with Asn, His, Leu, Ala, or Gly resulted in a greater range of affinities (reductions of 30–3,000-fold). Furthermore, the difference in affinities for digoxin of antibodies with H35 Asn vs. H35 Asp is 1,400-fold, whereas the difference for antibodies with these residues at H50 is 130-fold.

These differences in the results of mutagenesis of 2 different contact residues reflect in part their different locations in the binding site. Position H50 is near the mouth of the binding site, whereas H35 is located deep within the binding pocket (Jeffrey et al., 1993). The relative solvent accessibility of H50 compared to H35 in the 26-10:digoxin complex may account for the less detrimental effect of the negative charge of Asp on binding when located at H50 than when at H35. In addition, the location of H35 deep within the antibody:hapten complex places stricter restraints on the size of residues at H35 than on those at H50. The different roles of these side chains in recognition is also responsible for the differences in the effects of mutation. Although H:Tyr-50 contributes far more hapten contact area than does the H:Asn-35 (33 vs. 7 $Å^2$; calculated using a 1.5-Å diameter probe [Lee & Richards, 1971]), the H:Asn-35 contributes to the structure of the binding site via hydrogen bonds to H:Tyr-47 and H:Ser-95, both of which are contact residues. The H:Tyr-50 side chain does not form hydrogen bonds with hapten or with other antibody residues.

The importance of the identity of the H35 residue to hapten recognition has been demonstrated for other antibodies. Substitution of Asp for Asn at H35 of the anti-*p*-azophenylarsonate antibody 36-71 caused a 70-fold decrease in affinity, and substitution of Gln reduced affinity greater than 350-fold (Sompuram & Sharon, 1993). Substitution of Ala, Gln, or Asp for Asn at H35 for the corresponding germline antibody 36-65 caused virtual loss of binding of arsonate (Parhami-Seren et al., 1993). Substitution of Ala at H35 of antibodies 36-65 and 36-71 resulted however in greatly increased affinity for sulfonate (Kussie et al., 1994). As for 26-10, the H:Asn-35 of 36-71 not only contacts hapten in the modeled complex (Strong et al., 1991) but forms hydrogen bonds with contact residues H47 (Trp) and H95 (Ser). In the phosphorylcholine-binding myeloma protein McPC603, H:Glu-35 forms a hydrogen bond with the hydroxyl of the contact residue L:Tyr94. Mutagenesis of H:Glu-35 to Gln in the McPC603 Fv (Glockshuber et al., 1991), or to Ala in the related S107 anti-phosphorylcholine antibody (Rudikoff et al., 1982; Diamond & Scharff, 1984), greatly reduces the affinity for phosphorylcholine. The Ala substitution also confers a different specificity upon the S107 antibody, causing it to recognize double-stranded DNA (Rudikoff et al., 1982). The lost affinity for phosphorylcholine in the McPC603 mutant may be the result of either the lost negative charge of Glu, or the structural effects caused by alteration of the hydrogen bonding of H:Glu-35 (Glockshuber et al., 1991). For the mutants of 36-65 and 36-71, either a lost hydrogen bond between hapten and antibody or more indirect structural effects may have caused the reduced

affinity for hapten. Regardless of the exact cause, however, H35 substitutions in these antibodies and in 26-10 can significantly alter antibody binding.

Although the identity of the residue at H35 is central to the specificity exhibited by 26-10, McPC603, 36-65, and 36-71, there is limited homology among them. The antibodies differ in V region sequence (Kabat et al., 1991) and in the effect of mutation at sites other than H35 (Glockshuber et al., 1991; Parhami-Seren et al., 1993; Sompuram & Sharon, 1993). The same amino acid substitution in these antibodies at a site other than H35 can have substantially different effects on binding function, indicating differences between these antibodies in the modes or mechanisms of hapten binding. Substitutions at H35 in all of these antibodies, however, can alter affinity and specificity. The importance of H35 to binding function in these antibodies probably reflects a shared structural role of the H35 residue. The structure of H chain CDR1 is relatively conserved among antibodies (Chothia et al., 1989); this structure may position H35 in a crucial site at the bottom of the binding site.

Examination of the sequences of antibody V regions (Kabat et al., 1991) shows that Asn occurs frequently at murine H chain position 35 as does His, Glu, and Ser. Each of these residues is capable of forming hydrogen bonds, and therefore H35 may often be involved in maintaining the local structure of the antibody binding site. Certainly, substitutions at this site can profoundly affect antibody function. This position, therefore, is an obvious focus of antibody engineering designed to alter specificity or introduce a new specificity.

Materials and methods

Cell lines

The derivation of the murine anti-digoxin hybridoma 26-10 (IgG2a, κ) was described (Mudgett-Hunter et al., 1982). The isolation of the 26-10 hybridoma subclone 26-10P2 and the procedure used for cloning the spontaneous hybridoma variants 26-10P2.6R3 (referred to herein as R3) and 26-10P2.28L1B1 (referred to herein as L1B1) by 3 cycles of 2-color FACS from the 26-10 hybridoma subclone P2 were described (Schildbach et al., 1991). Cell line 26-10κ, a spontaneous H chain loss variant of 26-10, was subcloned and selected on the basis of H chain isotype loss as determined by screening supernatants using an isotype ELISA. Cell line H:Tyr-50-Asp is an engineered 26-10 H chain mutant that produces antibody with reduced affinity for digoxin, relative to wild-type 26-10 (Schildbach et al., 1993a).

Ascites production and antibody purification

Antibody was produced in ascites as described (Mudgett-Hunter et al., 1982). Antibodies R3 and L1B1 were purified for amino acid sequencing on DEAE-cellulose as described (Smith & Margolies, 1984).

Variable region nucleotide sequencing

Nucleotide sequencing of the V region cDNA of antibodies R3 and L1B1 by chemical cleavage was done as described (Panka & Margolies, 1987) except oligonucleotide primers complementary to L and H chain V region framework sequences were used in addition to those complementary to the V–C junction for synthesis of ^{32}P-end-labeled cDNA. The oligonucleotides hybridized to position 67–73 of the L chain ([5′d(CAGTGTGAAATC TGTCCC)3′]), and positions 40–45 ([5′d(AAGGCTCTTTC CATGGC)3′]), 66–71 ([5′d(CTACAGTCAATGTGGCC)3′]), and 82a–89 ([5′d(AGACTGCAGAATCCTCCGATGTCAGG CTGCG)3′]) of the H chain. Nucleotide sequencing of PCR-amplified V region cDNA was done as described (Schildbach et al., 1991).

Variable region amino acid sequencing

Antibody H and L chains were separated and purified as reported previously (Novotny & Margolies, 1983; Smith & Margolies, 1984). Variant R3 H chain CNBr peptides were prepared and purified by gel filtration and HPLC as described (Smith & Margolies, 1984, 1987). Variant L1B1 H chain CNBr peptides were prepared and purified by HPLC using Vydac C-4 columns (Separations Group, Hesperia, California) eluting with a linear gradient from 100% solvent A (0.1% trifluoroacetic acid [TFA] in water) to 60% solvent B (0.1% TFA in acetonitrile) over 40 or 60 min. Fully reduced and alkylated L chains were citraconylated, digested with trypsin, and purified by HPLC using either described methods (Smith & Margolies, 1984) or Vydac C-4 columns as above. Amino acid sequence analyses of purified peptides were performed on an Applied Biosystems 470A gas phase sequencer (Foster City, California) with phenylthiohydantoin analyses using on-line HPLC. Amino-terminal amino acid sequence analyses of intact H and L chains were done using a Beckman 890C sequencer with OPA treatment at selected cycles where proline was N-terminal (Brauer et al., 1984).

Antibody mutagenesis

Mutagenesis was performed according to Kunkel (1985) on the cloned rearranged 26-10 H chain V region gene as described (Schildbach et al., 1993b). The mutated V regions were cloned into an expression vector, the vectors transferred by electroporation into 26-10κ cells, and antibody-producing clones selected by screening of supernatants for digoxin binding or presence of antibody (Schildbach et al., 1993b).

Digoxin binding assays

Cell culture supernatants of 26-10, variants R3 and L1B1, and engineered 26-10 mutants were tested for the presence of antibody and for antigen binding. Serial 2-fold dilutions of cell culture supernatants in 10% γ-globulin-free horse serum (Gibco, Grand Island, New York) in phosphate-buffered saline (10% HS-PBSA) were added to wells of polyvinylchloride plates coated with goat anti-mouse-Fab antibody (ICN Immunobiologicals, Lisle, Illinois). A solution of 10% HS-PBSA was used as a negative control. The plates were incubated for 60 min at 20 °C and washed repeatedly with distilled water. A solution of goat anti-mouse-Fab antibody that had been radioiodinated by the chloramine T method (Greenwood et al., 1963) was added to the wells, and the plates were incubated for 60 min at 20 °C. The wells were washed with water, cut from the plates, and counted using a gamma counter. The dilution of each cell supernatant giving 70–80% maximal binding to the goat anti-mouse-Fab antibody-coated plates was added to wells of plates

coated with digoxin–HSA, and the plates were incubated and bound antibody was detected with radioiodinated goat anti-mouse-Fab antibody as above.

Affinity and specificity determinations

[^{3}H]digoxin used for affinity and specificity measurements was purchased from New England Nuclear (Boston, Massachusetts). Affinities were measured with a saturation equilibrium assay using filtration through glass fiber filters to separate bound and free ligand (Schildbach et al., 1991) as modified (Schildbach et al., 1993b). Specificities were determined by a solution-phase competition assay also using glass fiber filtration (Schildbach et al., 1993a). Cell supernatants were used in assays for all antibodies except mutant H:Asn-35-Asp, for which diluted ascites was used. As H:Asn-35-Asp has a relatively low affinity, higher concentrations of antibody are required for measurements.

Molecular modeling and docking

The modeling procedure (Schildbach et al., 1993b) is an adaptation of the minimum perturbation approach (Shih et al., 1985) that combines a side-chain conformational search with energy minimization to locate the lowest energy conformation of a mutated side chain. The modeling procedure has been used to successfully predict the structure of an H:Asn-35-His 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. Jeffrey, L.C. Sieker, C. Chang, R.L. Campbell, G.A. Petsko, E. Haber, M.N. Margolies, & S. Shaw, in prep.).

In modeling the mutants, we assumed that significant structural changes would be restricted to the area near the H35 residue. The results of comparison of mutants of T4 lysozyme (Eriksson et al., 1992) and chymotrypsin inhibitor 2 (Jackson et al., 1993) to their respective wild-type structures are consistent with this assumption. In the hapten docking procedure used here, we assume that the antibody does not undergo any significant rearrangement upon binding. Although such rearrangements have been noted in other antibodies (Rini et al., 1992), comparison of the X-ray crystal structures of 26-10 Fab and the 26-10 Fab–digoxin complex reveals no significant differences in antibody structure upon binding (Jeffrey et al., 1993). The only observed difference between the crystal structure of digoxin (Go et al., 1980) and the structure of digoxin complexed with 26-10 (Jeffrey et al., 1993) is a rotation of the lactone ring of approximately 180° about the C17–C20 bond. Both of these lactone orientations have been observed in other cardiac glycoside crystal structures (Go et al., 1980), but the high degree of complementarity between antibody and lactone allows only a single lactone conformation when complexed (Jeffrey et al., 1993). We assume digoxin will complex with the mutants in the same orientation as it does with 26-10. For docking experiments using dihydrodigoxigenin and oleandrin, we assumed that the steroid moiety maintains a similar orientation when complexed with antibody, but groups that differ from digoxin that are capable of free rotation (the saturated lactone of dihydrodigoxigenin and the 16-acetyl and oleandrose groups of oleandrin) were positioned by conformational search (see below). The structure of the steroid moiety of cardiac glycosides (Go & Bhandary, 1989), even when bound by antibody (Jeffrey et al., 1993), varies little, supporting this approach. The above assumptions may not be valid in all cases, causing inaccuracies in the modeling.

Parameters used for amino acids, including partial atomic charges, were as described (Novotny et al., 1989). The partial atomic charges of the hydroxyl oxygens and hydrogens and the hydroxyl-bearing carbon atoms (−0.65e, 0.4e, and 0.25e, respectively) of digoxin are taken from those for serine (Novotny et al., 1989). For the O-linked acetyl group of oleandrin, the partial atomic charges were C16 = 0.4e, O16 = −0.44e, C24 = 0.35e, O24 = −0.365e, and C25 = 0.055e (see Fig. 1 for numbering). Partial atomic charges for C17 (0.112e), C20 (0.052e), C21 (0.392e), O21 (−0.543e), C22 (−0.284e), C23 (0.873e), and O23 (−0.602e) were calculated from ab initio computations as described (Schildbach et al., 1993a). The values for bond lengths, bond angles, torsion angles, and improper torsion angles of the lactone and hydroxyl atoms were averaged from the X-ray crystal structures of digoxin and several digoxin analogues as described (Schildbach et al., 1993a).

Modeling was based on the crystal structure of the 26-10 Fab:digoxin complex (Jeffrey et al., 1993), which has been determined to a 2.5-Å resolution with an *R*-value of 0.171 and an RMS deviation from ideal bond lengths of 0.013 Å. The program CONGEN (Bruccoleri & Karplus, 1987) was used. Prior to modeling, a 26-10 Fv fragment was constructed from the 26-10 Fab:digoxin coordinates (Schildbach et al., 1993b). Digoxin was removed from the structure during the modeling procedure. A mutant side chain was introduced into the structure using the SPLICE command of CONGEN. For the Ala mutant, the model was minimized (see below) after introduction of the amino acid. For all other side chains, a conformational search using a 30° grid was performed. Maximum allowable van der Waals energy limits were +200 kcal/atom, and conformations in which this limit was exceeded were discarded. Each of the allowed conformations was subjected to a 4-step energy minimization procedure. The hydrogens of the substituted side chains were deleted, replaced with the HBUILD command of CONGEN, and energy-minimized (200 steps adopted basis Newton Rapheson minimization [ABNR]; Brooks et al., 1983) while all other atoms were fixed. This established hydrogen bonds for the substituted side chain. The modeled side chain was then fixed while the surrounding atoms were minimized (10 steps ABNR), and the modeled side-chain atoms and hydrogen atoms were then minimized while all other atoms were fixed (50 steps ABNR). The final step was a minimization (500 steps ABNR) with no restraints or constraints on the modeled side chain or on side-chain atoms with 7.5 Å of the modeled side-chain β-carbon (C_β) and moderate (4 kcal/Å) harmonic restraints on main-chain atoms with 7.5 Å of the modeled side-chain C_β. Increasing restraints 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. During minimization, the 1–4 nonbonded interactions were excluded from the calculated energies. 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 4× the distance between atom pairs. No wa-

ter molecules were included. The lowest energy conformations were then selected for docking experiments.

Digoxin, dihydrodigoxigenin, or oleandrin was inserted into the models in the same orientation as digoxin in the X-ray crystal structure. The structure of digoxigenin was taken from the 26-10 Fab:digoxin complex crystal structure (Jeffrey et al., 1993) and oleandrin (Kartha & Go, 1981) and dihydrodigoxigenin (S-isomer; Mostad, 1982) from their crystal structures. All hapten structures were energy-minimized. Prior to minimization, the lactone of oleandrin was rotated, relative to the steroid moiety, to the position of the lactone of digoxin in the 26-10 Fab:digoxin crystal structure. The new position of the oleandrin lactone represents an allowed conformation for cardiac glycosides, but not the orientation seen in the oleandrin crystal structure. During minimization of digoxigenin and oleandrin, the lactone ring position relative to the steroid moiety was maintained using harmonic constraints. After insertion of the hapten into an antibody binding site, the lowest energy positions for hydroxyl hydrogens at the 12 position (for digoxin and dihydrodigoxigenin) and 14 position (for all haptens), and for the 16-acetyl and 3-oleandrose moieties of oleandrin, were determined by a conformational search using a 30° grid over all dihedral angles. The position of the lactone ring of dihydrodigoxigenin was determined by conformational search using a 15° grid over the C13-C17-C20-C21 dihedral angle. The antibody:hapten complex model was then energy-minimized in a 2-step process. First, the H35 side chain was minimized while all other atoms were fixed (500 steps ABNR). Then, all main-chain atoms within 12.5 Å of the hapten C8 were restrained (8 kcal/Å), all other atoms within 12.5 Å of the hapten C8 were unrestrained, and all atoms greater than 12.5 Å from C8 were fixed, and the structure was minimized (500 steps ABNR). The hapten C8 was arbitrarily chosen as center of the minimization because it is near the center of the cardenolide structure.

The diagrams of the 26-10:digoxin structure and the models were made using the plotting program PLT2 (R.E. Bruccoleri & D. States, unpubl.). Both PLT2 and CONGEN are available by request to R.E.B.

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