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N-terminal mutations in the anti-estradiol Fab 57-2 modify its hapten binding properties

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Abstract

Recombinant antibodies often contain N-terminal mutations arising from the use of degenerate cloning primer sets and/or the introduction of restriction sites in the framework 1 regions. We studied the effects of such mutations in a recombinant anti-estradiol Fab fragment derived from the hybridoma cell line 57-2. The 5′ ends of the heavy and light chain genes were originally modified to introduce the restriction sites *Xho*I and *Sac*I, respectively, for cloning purposes. However, the affinity and specificity of the recombinant Fab were lowered compared to the proteolytic Fab′ fragment of the parental hybridoma IgG. Replacing the mutated sites with authentic amino acid coding sequences restored the binding properties as well as increased the bacterial production levels fivefold and 10-fold at 30 and 37 °C, respectively. Local changes in the antigen binding site were probed by determining the affinity constants (K_a) for estradiol and four related steroids. It was found that the mutated heavy chain amino terminus specifically increased the K_a for testosterone whereas the mutated light chain amino terminus decreased the K_a for all of the steroids to the same extent; the heavy and light chain effects were additive. Analysis of a newly determined crystal structure of the authentic Fab 57-2 in complex with estradiol suggests that mutations in the residue 2 in V_H, and 2 and 4 in the V_L domain were those responsible for the observed effects. Their general roles as structure-determining residues for the CDR3 loops imply that similar effects can occur with other recombinant antibodies as well.

Keywords: estradiol; N-terminus; recombinant antibodies; steroid recognition

Estradiol (17β-estradiol, E_2), the principal female sex hormone, is an important regulator of the female physiological cycle as well as a factor in the maintenance of the body tissues such as bone and muscle. The status of the estrogen-dependent functions is reflected in the concentration of estradiol in the blood circulation, forming the basis for the determination of serum estradiol in clinical laboratories. The development of immunoassays for estradiol is, however, complicated by the fact that the human serum contains a variety of structurally similar steroid hormones that differ only at a few sites around the common steroid nucleus. Moreover, estradiol is present at much lower concentrations than some of the other steroids, setting a high demand for the specificity of the antibody used in the immunoassay. Although a few rabbit polyclonal antibodies have shown reasonably good discrimination for these steroids, there are no such mouse monoclonal antibodies available to date.

To study the molecular recognition of estradiol by mouse antibodies, we have recently cloned the genes of three anti-E_2 Mabs from the hybridoma cell lines 57-2, 8D9, and 11B6 (Pajunen et al., 1997). Mab 57-2 was particularly interesting as it showed high specificity for the D ring of estradiol: cross reactivities with the clinically relevant analogs estriol and estrone were less than 0.2% (Lamminmäki et al., 1997). By contrast, the A ring was poorly recognized, as exemplified by a 37% cross reactivity with testosterone (TES). In a homology modeling study of 57-2, this high cross reactivity was associated to the scarcity of contacts with the A ring, as seen by molecular docking of the steroid to the binding site (Lamminmäki et al., 1997). On the other hand, we were able to decrease the TES cross reactivity of 57-2 by a factor of 20 by error-prone polymerase chain reduction (PCR) mutagenesis of the V_H region followed by phage display selection of improved mutants in the presence of a large excess of TES (Saviranta et al., 1998). The occurrence of mutational hot spots in both CDRH1 and CDRH2 as well as a few "accessory" mutations in the framework regions indicated that the V_H domain was actually quite readily amenable to the modulation of its cross reactivity with TES (Saviranta et al., 1998).

The recombinant Fab 57-2 slightly differed from the proteolytic (papain-digested) Fab′ fragment of the parental hybridoma IgG

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with respect to affinity and specificity: the K_a for a labeled E_2 derivative was decreased by 40% and the cross reactivity for TES was increased twofold (Lamminmäki et al., 1997; Pajunen et al., 1997). In these studies, Fab was expressed with the phagemid vector pComb3 (Barbas et al., 1991), which necessitated the creation of the restriction sites *Xho*I and *Sac*I in the framework 1 regions (FR1) for the insertion of the heavy (Fd) and the light chain genes, respectively, to the vector. Even if the amino termini could not be in direct contact with the hapten, their location closely behind the CDR3 loops raised the question whether some of the necessary FR1 mutations could have indirect effects on the steroid binding. Moreover, considering the fact that several positions in the V_H domain had already been shown to be prone to mutations affecting the TES cross reactivity (Saviranta et al., 1998), we set out to clarify the significance of the mutated heavy as well as the light chain amino termini. In this study, we show that the amino termini of both chains contribute to the steroid recognition of Fab 57-2 and that authentic binding properties could be re-established on restoring the original FR1 sequences. With the aid of a 2.15 Å crystal structure of the authentic Fab 57-2 in complex with estradiol (U. Lamminmäki, unpubl. data), we discuss possible structural explanations for the observed N-terminal effects. The proposed mechanisms are based on the roles of residues 2 and 4 as structure-determining residues (SDRs) for the CDR3 loops (Martin & Thornton, 1996); therefore, similar effects may occur with other N-terminally modified antibodies as well.

Results and discussion

The significance of authentic amino termini in recombinant antibodies has been addressed in several recent studies (Benhar & Pastan, 1995; McCartney et al., 1995; Short et al., 1995; Kipriyanov et al., 1997; de Haard et al., 1998, 1999; Langedijk et al., 1998). Although it is now generally accepted that some N-terminal mutations can deteriorate the folding and/or stability of recombinant Fab or ScFv fragments, there is not much information on how they affect the antigen binding properties. In this study, we analyze in detail the effects of N-terminal mutations on the fine specificity and affinity of a hapten-binding Fab fragment. Our results imply that the amino termini in recombinant antibodies deserve more attention as potential fine modulators of the antigen recognition process.

N-terminal mutations

Initially, the genes coding for the Fab fragment of the anti-estradiol antibody 57-2 were subcloned into the phage display vector pComb3 (Barbas et al., 1991) between the restriction sites *Xho*I-*Spe*I (Fd chain) and *Sac*I-*Xba*I (light chain) (Fig. 1A). The 5′ cloning sites (*Xho*I and *Sac*I) were introduced in the framework 1 (FR1) coding regions of each chain via the PCR primers used for the amplification of the genes from first strand cDNA (Pajunen et al., 1997). According to the earlier literature, the introduction of a few changes to the beginning of the FR1 was considered to be of minor significance (Huse et al., 1989; Orlandi et al., 1989; Sastry et al., 1989). In the case of Fab 57-2, four of the first six residues of the Fd chain were mutated, while the light chain was both truncated (residues 1 and 2) and mutated (residues 3 and 4), as shown in the sequence alignment in Figure 2. The N-terminal truncation of the light chain was not necessary for creating the *Sac*I restriction site to the 5′ end

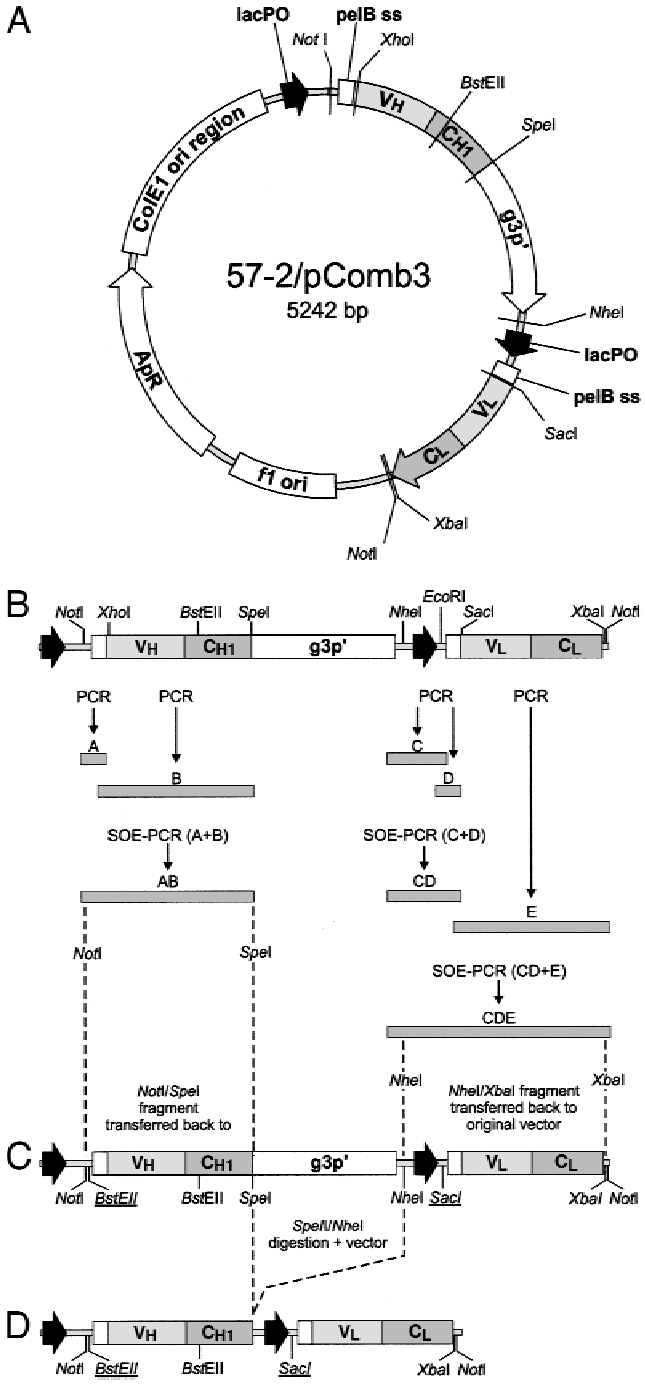

Fig. 1. DNA constructions. **A:** The vector 57-2/pComb3, containing the Fd (V_H + C_{H1} domains) and light chain genes of the anti-estradiol antibody 57-2. The Fd chain is fused to the C-terminal part (Pro198–Ser406) of the minor coat protein (gene 3 protein) of the bacteriophage M13 via a $(Gly)_4Ser$ linker. **B**: Linear view of the Fab expression cassette, extending from the first *lac* promoter to the second *Not*I site. **C:** The N-terminally authentic construct (still a g3p′ fusion). **D:** Soluble Fab expression construct was obtained by cutting the plasmid with *Spe*I and *Nhe*I, followed by religation of the compatible cohesive ends. The Fd gene translation now ends in two in-frame stop codons (TAGTAA) immediately following the *Spe*I/*Nhe*I ligation site. Explanation of labels: *lac*PO, *lac* promoter-operator; pelB ss, signal sequence from the pectate lyase gene of *Erwinia carotovora*; V_H, variable domain of the heavy chain; C_{H1}, first constant domain of the heavy chain; V_L, variable domain of the light chain; C_L, constant domain of the light chain; g3p′, C-terminal part of the gene 3 protein coding sequence.

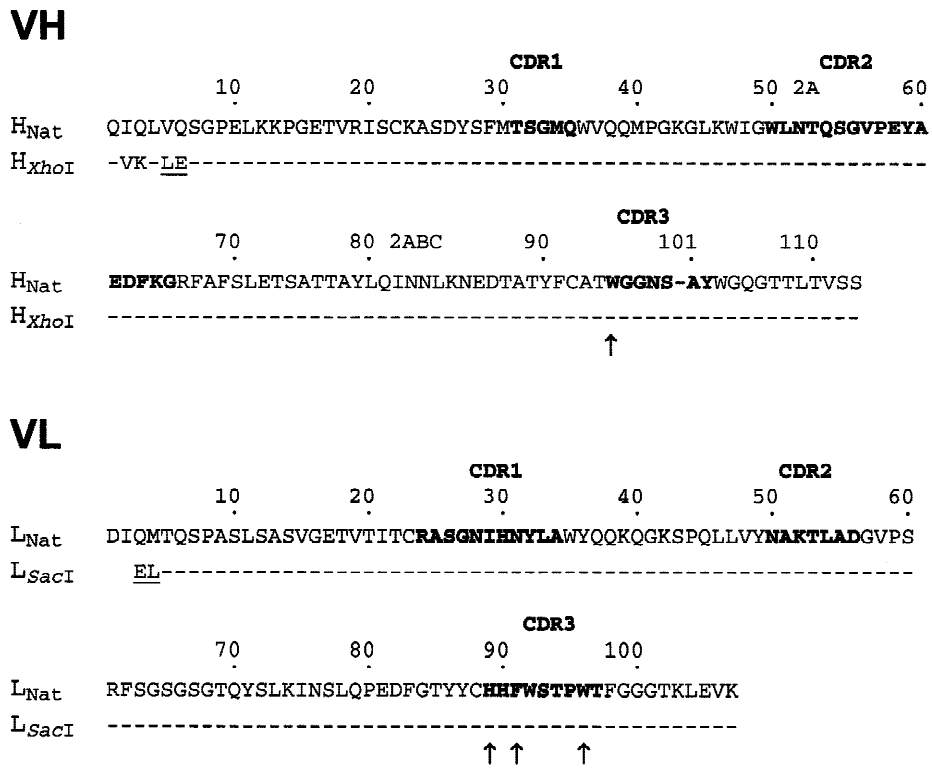

Fig. 2. Alignment of the native and mutated variable regions. The native amino acid sequences are shown in full on the upper lines, while the mutated residues are shown on the lower lines. Identity with the native sequence is indicated by a dash. Positions of the *Xho*I and *Sac*I restriction sites are underlined. The arrows point to the CDR3 antigen contact residues that are discussed in the text. Residue numbering and CDR locations (indicated by bold) follow Kabat's definitions (Kabat et al., 1991). H_{Nat} and L_{Nat}, native heavy and light chains; H_{XhoI} and L_{SacI}, N-terminally mutated heavy and light chains.

of the gene, but was actually a consequence of sequence misalignment in designing the PCR primer. Nevertheless, this truncation serves to increase the knowledge on the significance of the N-terminal residues in antibodies.

The amino termini were restored to their authentic forms by splicing by overlap extension PCR (SOE-PCR) (Ho et al., 1989) as outlined in Figure 1. The 5′ primers used for the amplification of the heavy chain (fragment B) and the light chain (fragment E) were designed according to the native N-terminal sequences of the heavy and the light chains of Mab 57-2, as previously determined by protein sequencing (Pajunen et al., 1997). The SOE-PCR products were then cloned back to the phage display vector (Fig. 1C), after which the gene 3 protein fragment (g3p′) was removed to yield the soluble Fab expressing construct (Fig. 1D). This wholly authentic construct was termed H_{Nat}/L_{Nat}. The constructs H_{Nat}/L_{SacI} and H_{XhoI}/L_{Nat} were created by correcting only the heavy chain or the light chain, respectively. Finally, the construct H_{XhoI}/L_{SacI} was made directly from the original plasmid by removing the g3p′ fragment as described above.

Fab production levels

The effects of the amino termini on the production levels and kinetics were studied by measuring functional (i.e., antigen binding) Fab concentrations in shake flask cultures over a period of 18 h after the isopropyl-β-D-thiogalactopyranoside (IPTG) induction. To study if there were temperature-dependent differences in the folding and/or stability between the N-terminal variants, the production levels were monitored at two growth temperatures (37 and 30 °C) after induction. The degree of periplasmic leakage was used as a measure of the physiological condition of the cells and was assessed by determining the Fab concentrations both from sonicated cells (representing the periplasmic fraction) and from the growth medium. Results from these experiments are collected in Figure 3.

At the moment of induction, virtually no Fab was present, indicating that the *lac* promoter was adequately repressed during the pre-induction period; thus, all the clones had equal starting conditions for the production period. The kinetics of production was very similar for all the clones: At 37 °C, the Fab levels reached their maxima and stabilized in 4 h, while at 30 °C, the levels increased further until 6 h postinduction to settle thereafter. The N-terminal mutations, although not affecting the production kinetics, clearly decreased the maximal production levels. At 37 °C, the all-authentic Fab reached a level of 3.4 μg/mL, whereas the variants having one mutated and one native chain (H_{Nat}/L_{SacI} and H_{XhoI}/L_{Nat}) were produced at 58 and 26% of that, respectively, and the Fab with both chains mutated (H_{XhoI}/L_{SacI}) was produced only at 10% of the native Fab level. At 30 °C, all Fab forms were produced at higher levels (1.6- to 3.1-fold more) than at 37 °C. The same ranking order was maintained, but the doubly mutated Fab benefited more than the others from the lower temperature such that it finally reached a level of 1.1 μg/mL, about one-fifth of that of the all-native Fab (5.3 μg/mL). All the produced Fab fragments

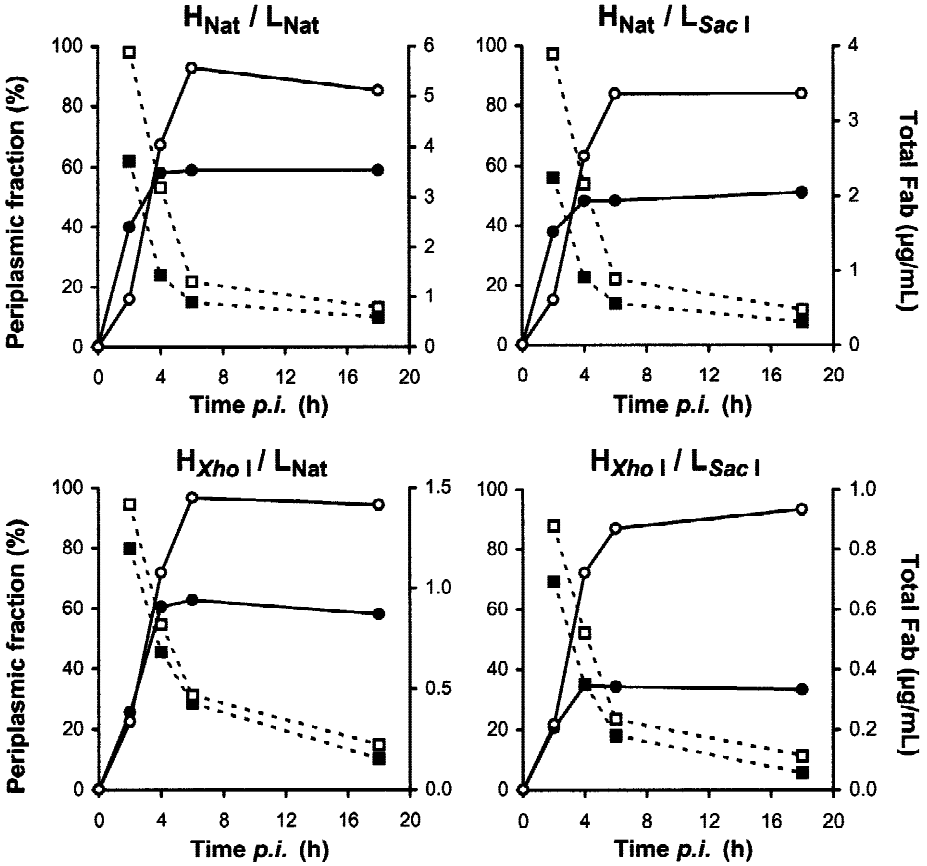

Fig. 3. Fab production kinetics and localization. Functional Fab concentrations were measured at 0–18 h post-induction (p.i.) for cultivation temperatures of 30 °C (open symbols) and 37 °C (filled symbols). Total functional Fab concentrations (circles, connected with solid lines) are plotted on the right-hand *y*-axis, while the periplasmic fractions (squares, connected with dashed lines) are plotted on the left *y*-axis.

were relatively stable even at 37 °C, as the levels attained at 4 h showed no decline through until 18 h. Thus the differences in the measured production levels were apparently not caused by different turnover rates of the fully folded and assembled Fab fragments.

Leakage of the outer membrane, as monitored by the decrease of the fraction of Fab retained in the periplasm, occurred similarly for all the different N-terminal variants (Fig. 3). It started more rapidly at 37 °C than at 30 °C, appearing in concert with the cessation of the Fab production. In all cases, the periplasmic fraction finally (18 h postinduction) settled at ~10% of the total Fab. These results agree with earlier reports in that differences in the production levels between mutant Fab fragments do not necessarily correlate with the extent of periplasmic leakage (Knappik & Plückthun, 1995; Forsberg et al., 1997).

The overall kinetics of Fab production as well as the fractional localization were remarkably similar for all the N-terminal variants, implying that the differences in the production levels were caused neither by variations in the toxicity of the protein products nor by differences in the periplasmic leakage behavior. It appears as if the mutated chains simply failed to form as much functional Fab fragments as the authentic ones. No attempt was made to study the fate of the unproductive translation products.

Hapten binding properties

Each recombinant Fab and the proteolytic Fab′ fragment were profiled for their hapten recognition characteristics with a set of five steroids consisting of analogs differing from estradiol either in the D-ring (Estriol, Estrone) or in the A-ring (Estradiol-3-sulfate, Testosterone) as well as estradiol itself (Fig. 4). As a first step, affinity constants were determined for the labeled tracer, E_2–4–CET-N1[Eu] with a hot saturation assay (Fig. 5A). Affinities for each of the steroids were then determined with a competition assay in which the labeled tracer (fixed concentration) competed with the unlabeled steroid (varied concentrations) so as to get a decrease in the bound tracer concentration as a function of the increasing unlabeled steroid concentration (Fig. 5B). The binding data were analyzed by nonlinear fitting using a model consisting of one binding site and two competitive ligands. The calculated affinity constants (K_a) are shown in Table 1. As the K_a values for the different steroids vary by more than three orders of magnitude, the differences between the Fab fragments can be better perceived by expressing the affinity constants as percentages of the corresponding values of the proteolytic Fab′, as shown in Figure 6. From the column profiles in Figure 6, it can be seen at a glance that the

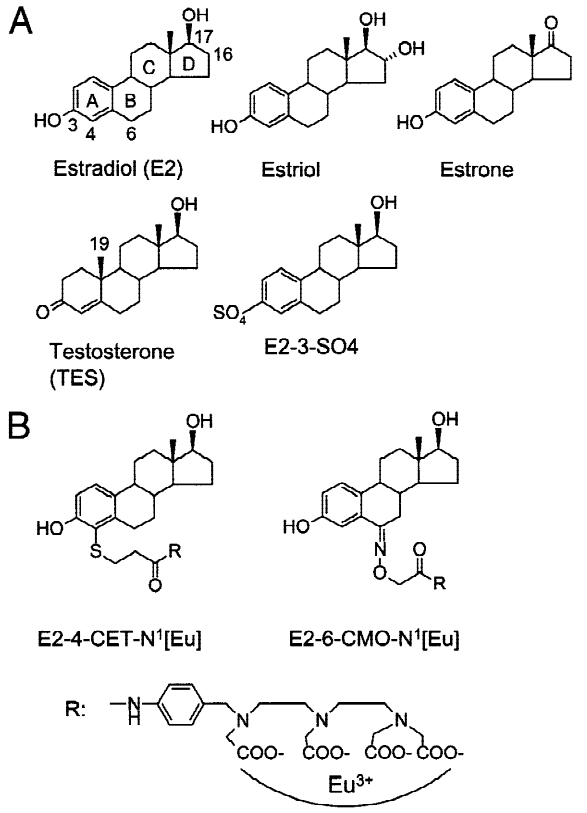

Fig. 4. Structures of the steroids and their derivatives used in this study. **A:** Estradiol and four related steroids. E_2, estradiol; E_3, estriol; E_1, estrone; TES, testosterone; E_2–3–SO_4, estradiol-3-sulfate. The lettering of the steroid rings as well as the numbers of the relevant ring carbons are shown for E_2. The extra methyl group in TES is indicated by its carbon atom number 19. **B:** Europium-labeled estradiol derivatives. On conjugating E_2–4–CET (estradiol-4-carboxyethylthioether) and E_2–6–CMO (estradiol-6-carboxymethyloxime) with the europium chelate of N^1-(*p*-aminobenzoyl)-diethylenetriamine-N^1, N^2, N^3, N^3-tetraacetic acid, a stable amide is bond formed between the carboxyl group of the steroid derivative and the *para*-amino group of the europium chelate. The "N^1[Eu]" suffix comes from the abbreviated name of the europium chelate.

authentic recombinant Fab fragment behaves nearly identically with the proteolytic Fab, while the N-terminally modified variants show different characteristics, depending on whether the light chain, heavy chain, or both are modified. The "*Sac*I" modification in the light chain decreases the affinities for all the steroids more or less unselectively, while the "*Xho*I" modification in the heavy chain generally just slightly increases the affinities (14–27% increase in K_a) except for TES, for which the affinity constant increases by 138%. The specific increase in the binding affinity for only one of the steroids implies that the "*Xho*I" amino terminus has a relatively localized structural effect on the steroid recognition, as will be discussed in the next section.

Combination of the N-terminal modifications in both chains (clone H_{XhoI}/L_{SacI}) results in a kind of binding profile that would be expected for independent mutations, as demonstrated by comparing it with a hypothetical profile calculated for a combination mutant with the assumption of full additivity (Fig. 6). The independence of the heavy and light chain N-terminal mutations suggests that the observed changes in steroid binding do not involve large movement, or shift in the orientation, of the hapten, because otherwise one mutated amino terminus would exert its effect on a different part of the steroid, depending on the presence or absence of mutations in the other amino terminus. Therefore, the changes in the antibody–hapten interaction most likely arise from local conformational changes in the antibody side while the steroid remains relatively stationary.

Structural roles of the mutated N-terminal residues

The amino termini of the V_H and V_L domains in antibodies have similar topologies in which the side chains of the succeeding residues point alternately to the solvent or to the interior of the protein. The odd-numbered residues are solvent exposed, which makes it unlikely that their mutations affect the conformation of the protein. By contrast, the side chains of the even-numbered residues are packed in the hydrophobic interior, and their mutations can potentially change the interactions and positions of the neighboring atoms and groups. Among the *buried residues*, there were mutations in Ile-H2 and Gln-H6 in V_H as well as in Ile-L2 and

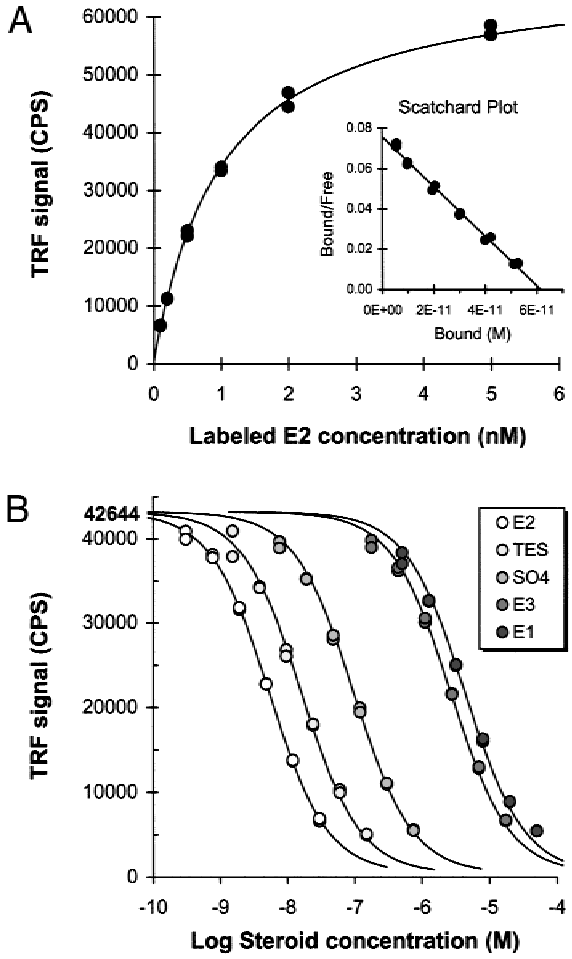

Fig. 5. Hapten binding assays. Typical binding curves, showing the time-resolved fluorescence (TRF) signal of bound tracer at varying steroid concentrations. Individual data points from replicate measurements are shown to demonstrate the reproducibility of the assays. The plots are for the authentic recombinant Fab. **A:** Hot saturation assay. **B:** Competition assay.

Table 1. *Affinity constants of the Fab variants for different steroids*

	K_a (M^{-1}) ± SD[a]				
Fab variant	E_2 ($\times 10^{-8}$)	TES ($\times 10^{-8}$)	E_2–3–SO_4 ($\times 10^{-7}$)	E_3 ($\times 10^{-5}$)	E_1 ($\times 10^{-5}$)
Fab′ 57-2[b]	4.62 ± 0.05	1.22 ± 0.04	2.40 ± 0.03	8.25 ± 0.11	3.87 ± 0.35
H_{Nat}/L_{Nat}[c]	5.18 ± 0.11	1.40 ± 0.04	2.47 ± 0.03	8.38 ± 0.11	4.07 ± 0.33
H_{Nat}/L_{SacI}	1.52 ± 0.02	0.52 ± 0.01	0.76 ± 0.02	2.94 ± 0.04	1.19 ± 0.12
H_{XhoI}/L_{Nat}	5.67 ± 0.08	2.92 ± 0.07	2.76 ± 0.03	9.44 ± 0.20	4.92 ± 0.50
H_{XhoI}/L_{SacI}	2.09 ± 0.04	1.40 ± 0.04	1.11 ± 0.02	4.25 ± 0.05	1.85 ± 0.18

[a] K_a ± SD were calculated from the corresponding K_d values obtained with the Radlig program.
[b] Proteolytic (papain-digested) Fab fragment of Mab 57-2.
[c] Heavy/light chain combination.

Met-L4 in V_L (Fig. 2). The roles of these residues are discussed in more detail below, with the aid of a newly determined 2.15 Å crystal structure of the authentic Fab 57-2 in complex with estradiol (U. Lamminmäki, unpubl. data).

Ile-H2 → Val

The conservative hydrophobic change of Ile to Val reduces the residue volume from 124 Å^3 to 105 Å^3. This either creates a cavity or changes the local packing, as the neighboring residues might readjust their conformations to fill the cavity. One of the neighboring residues, Tyr-H102, being the last residue of CDRH3, lies between Ile-H2 and the rest of the loop (Fig. 7A,B). The sequence of CDRH3 (WGGNSAY) contains a pair of glycines, which gives it flexibility and may allow it bend in the direction of the new cavity, along with Tyr-H102. This scheme could provide an explanation for the specific increase in the affinity for TES with the H_{XhoI}/L_{Nat} clone. In the complex of E_2 and Fab 57-2, the β face of estradiol has contacts mainly with Trp-H95 of CDRH3. Assuming that TES is bound in the same orientation as E_2, there must be a slight shift in the position of either Trp-H95 (especially the Cβ atom of it) or the steroid in order avoid clash with the "extra" methyl group (C19) of TES. Movement of the hapten outward is unlikely because the O17 of the steroid is fixed at the bottom of the binding site by a hydrogen bond network between Gln-H35, Tyr-L36, and His-L89. However, the CDRH3 loop, containing two consecutive glycine residues, is probably flexible enough to allow the binding of TES, albeit with a lowered affinity. The accommodation capability of the wild-type antibody might be further facilitated by the loss of one methyl group in the hydrophobic core underlying the CDRH3 loop as a result of the Ile-H2 → Val mutation; thus, the created cavity would compensate for the strain caused by the extra methyl group of TES. In a sense, the suggested scheme is analogous to a situation in which a protein has to accommodate a mutation introducing a bulky residue in its core. In a recent study with T4 lysozyme, it was found that experimental "small-to-large" mutations within the core of the enzyme were tolerated better at positions where there was a pre-existing cavity near the mutated residue (Liu et al., 2000).

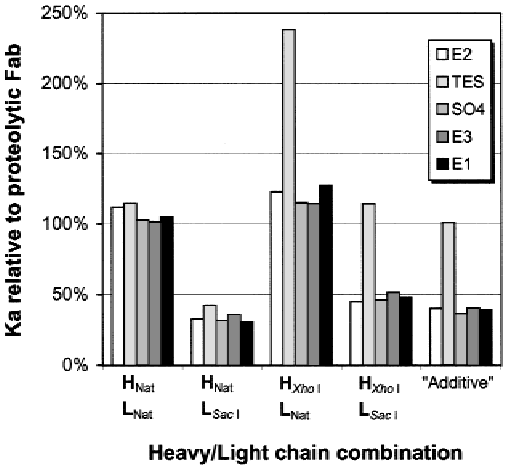

Fig. 6. Relative changes in steroid affinities. Each column represents the ratio of the K_a of the recombinant Fab fragment to that of the proteolytic Fab′. The steroids are indicated by the color (gray shade) of the column as shown in the legend. The label "Additive" refers to a hypothetical, fully additive combination mutant, whose relative steroid affinities were calculated by multiplying together the corresponding values of the individual light and heavy chain N-terminal mutant clones (H_{Nat}/L_{SacI} and H_{XhoI}/L_{Nat}, respectively).

Although the residue H2 has been recognized as belonging to the "Vernier zone" underlying the CDR loops (Foote & Winter, 1992), there is little direct evidence on its significance in any particular antibody. Yet, Ping et al. (1993) found that one to three N-terminal residues could be deleted from the heavy chain of the anti-digoxin antibody 40–150 with very mild effects on the antigen binding affinity. Deletion of the first residue only did not affect the affinity at all, while the deletions of either two or three residues both decreased the affinity to about 55% of the wild-type. The results of Ping et al. (1993) thus corroborate the view that the residue H2 can modulate the hapten binding properties while the solvent-exposed residues H1 and H3 are less significant.

Gln-H6 → Glu

The interactions of the buried but polar sixth residue have been discussed in detail by Langedijk et al. (1998). By comparing 58 antibody crystal structures, they divided the V_H domains into two structural subclasses based on whether the residue at position H6 was a Gln or a Glu. The hydrogen bond requirements of the side-chain polar atoms were satisfied in both cases, but the resulting H-bond networks were slightly different. Glu at H6 was more

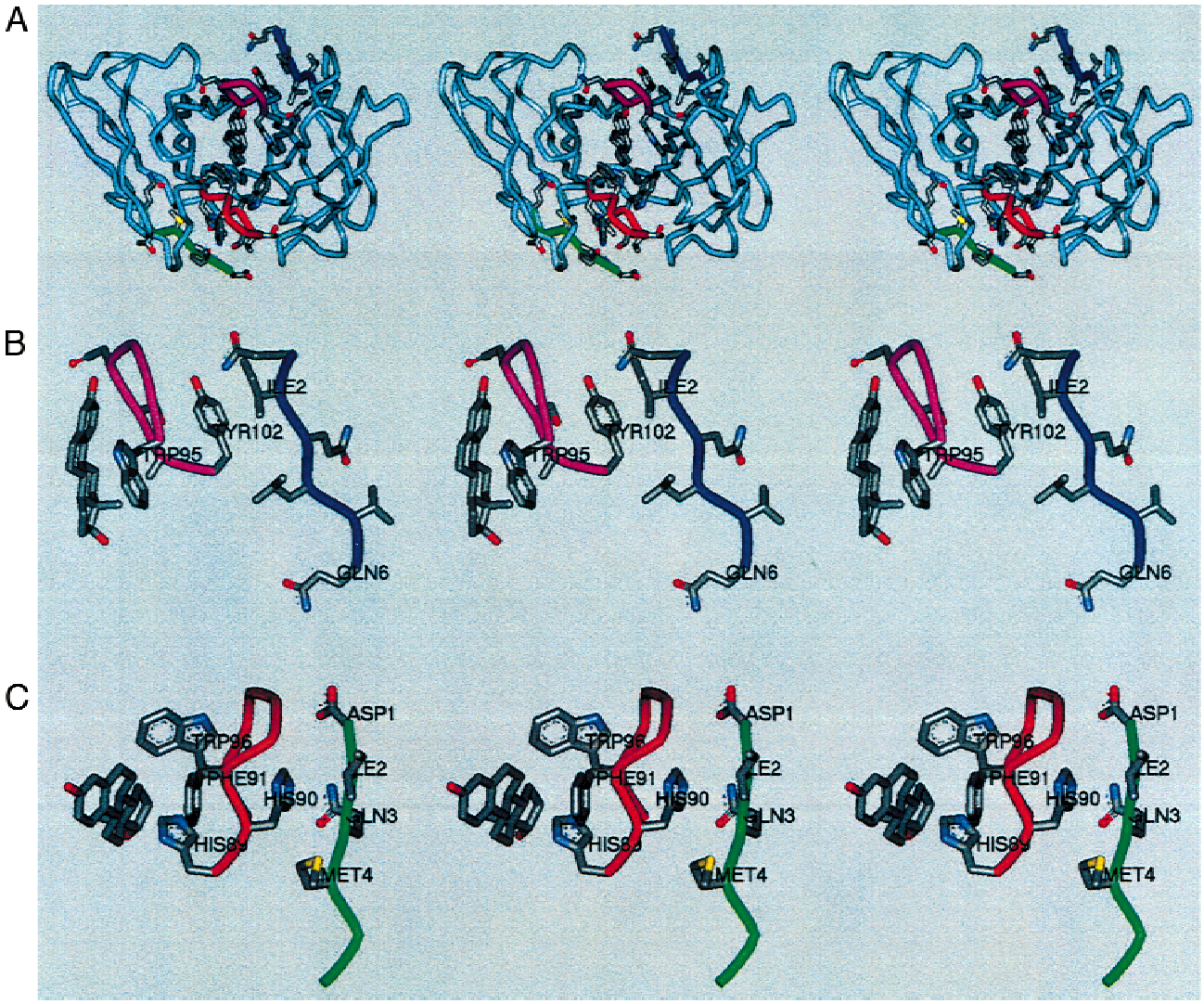

Fig. 7. Spatial relationships between estradiol, CDR3 loops and the amino termini of authentic Fab 57-2. **A:** Top view of the Fv fragment in complex with estradiol; V_L domain on the left, V_H domain on the right. The protein backbone is specially colored at light chain N-terminal residues 1–4 (green), CDRL3 (red), CDRH3 (purple), and heavy chain N-terminal residues 1–6 (blue). **B:** Side view of estradiol, CDRH3, and residues H1–H6. **C:** Side view of estradiol, CDRL3, and residues L1–L4. In each panel, the left stereo pair is divergent while the right pair is convergent.

restrictive of the main-chain conformation between H6 and H10 and, consequently, called for a preferred set of residues there. The authors thus provided a structural explanation for the previous observations that the presence of Glu in a "Gln-type" sequence context results in reduced levels of correctly folded protein (e.g., McCartney et al., 1995; Kipriyanov et al., 1997). The requirement of either Gln or Glu at H6 has also been connected with the proposed family specificity of a hypothetical folding nucleus of the V_H domain (de Haard et al., 1998). Consequently, a "wrong" residue at position H6 would have varying effects on folding efficiency depending on the family, or the Kabat's subclass (Kabat et al., 1991), of the V_H domain. For V_H subclasses IIA and IIB, the single Gln-H6 → Glu substitution has been reported to lower the soluble antibody production to less than 10% of the wild-type (Kipriyanov et al., 1997; de Haard et al., 1998). The V_H of Fab 57-2 falls into the Kabat's subclass "MISC" (Kabat et al., 1991) and seems to tolerate the Gln-H6 → Glu substitution comparably well, as the Fab production level is still about one-fourth that of the authentic Fab (Fig. 2). Antigen binding affinity of the H_{XhoI}/L_{Nat} mutant of Fab 57-2 remains essentially unchanged, except for the increased affinity for TES. The latter, highly specific effect has, however, a plausible explanation in the context of the Ile-H2 → Leu mutation, as discussed above. The role of the Gln-H6 → Glu substitution in Fab 57-2 as well as in other recombinant antibodies may be connected more with the overall ability to adopt the correct fold than with the fine-tuning of specific antigen–antibody interactions. A kind of all-or-nothing behavior with respect to antigen binding was also found with two subclass IIA, anti-hCGβ antibodies, in which the Gln-H6 → Glu substitution resulted in a total loss of antigen binding activity (de Haard et al., 1998), while for a subclass IIB, anti-human CD3 antibody, the affinity remained unchanged (Kipriyanov et al., 1997).

Δ(Asp-L1, Ile-L2) and Met-L4 → Leu

The deletion of two residues from the light chain N-terminus is apparently not fatal for the correct folding of Fab 57-2, as the production level of the H_{Nat}/L_{SacI} clone was only 30−40% lower than that of the authentic one (Fig. 3). Moreover, the produced Fab fragments were stable during the 18 h induction period even at 37 °C, implying that the light chain adopts a reasonably compact structure in spite of the N-terminal truncation. The other mutation present in the L_{SacI} N-terminus, a conservative Met-L4 → Leu substitution, is not likely to have any dramatic effect on the light chain folding efficiency or stability; actually, this mutation has been described to slightly stabilize an aggregation-prone scFv-immunotoxin (Benhar & Pastan, 1995). Only a more radical substitution of Met-L4 by a Ser has been recently reported to decrease the production level as well as to abolish the antigen binding activity, when introduced to two anti-hCG scFv fragments (de Haard et al., 1999).

The N-terminal truncation removes Asp-L1 and Ile-L2, of which the former is solvent exposed and the latter is buried in a similar fashion as Ile-H2 in the heavy chain. Deleting Ile-L2 from the light chain leaves behind a cavity much larger than that formed by the size-decreasing Ile-H2 → Val mutation in the heavy chain, and it should be expected that also in the light chain some sort of repacking of the neighboring residues occurs to fill the empty space. From the crystal structure of the authentic Fab fragment, it can be seen that one of the residues surrounding Ile-L2 is His-L90 of CDRL3 (Fig. 7A,C). The CDRL3 of Fab 57-2 can be assigned to the canonical class 1/9A, for which L90 is a key residue (Martin & Thornton, 1996). The removal of Ile-L2 adjacent to the key residue His-L90 can thus potentially lead to a conformational shift in the CDRL3 loop such that some of its hapten-contacting residues (His-L89, Phe-L91, and Trp-L96; Fig. 7C) are affected. His-L90 is also in close proximity to Met-L4, so also the Met-L4 → Leu mutation can have an effect on the CDRL3 conformation. The crystal structure of Fab 57-2, therefore, is in agreement with the general notion that the residues L2 and L4 have roles as structure determining residues (SDRs) for CDRL3 (Martin & Thornton, 1996). The question of which of the three contact residues in CDRL3 could mediate the N-terminal effects into changes in the hapten binding properties may be approached by examining the binding profile of the H_{Nat}/L_{SacI} mutant (Fig. 6; Table 1). The affinity constants were decreased in a fairly uniform fashion (2.4- to 3.2-fold) for all of the steroids, suggesting that the site of the altered contact between the antibody and the hapten was one in which the steroids were alike. With this in mind, the residue Phe-L91 seems the most likely mediator of the N-terminal effects, as it forms extensive contacts with the B, C, and D rings on the alpha side of the steroid (Fig. 7C), covering a region that is invariant among the set of steroids used in this study. On the other hand, the observed drops in the binding affinities might involve different mechanisms with different steroids, in which case the apparent uniformity is only coincidental. Therefore, the contribution(s) of His-L89 and Trp-L96 cannot be excluded, even if these residues interact with the steroid D ring positions 16 and 17, where E_1 and E_3 differ from E_2.

Conclusion

Although the framework residues 2 and 4 in V_H and V_L have been recognized as "Vernier zone" residues (Foote & Winter, 1992) or as structure-determining residues for the CDR3 hypervariable loops (Martin & Thornton, 1996), there is not much information on how mutations at these positions affect the antigen binding properties. As a large number of recombinant antibodies have been cloned with degenerate "family-specific" primers, there are potentially many N-terminally mutated antibodies available for comparison with the parent monoclonal antibody. However, in many cases the lack of accurate enough determination methods may have lead to a failure to detect subtle changes in the antigen recognition properties. In applications where high affinity and specificity are needed (e.g., steroid immunoassays), the possible N-terminal mutational effects should not be ignored; rather, these effects could even be utilized for the fine tuning of the binding characteristics.

Based on the structure of the authentic Fab 57-2 complexed with estradiol, we suggest that mutations in FR1 residues 2 and 4 cause the altered steroid binding properties by interacting with conformational key residues in the CDR3 loops (Tyr-H102 in CDRH3 and His-L89 in CDRL3) through which the steroid contact residues are affected. In more general terms, the side chains of residues 2 and 4 are oriented toward the hydrophobic core between the inner and the outer beta sheets of the immunoglobulin variable domain, and changes in their volume and/or shape can slightly push or pull the CDR3 either toward or away from the antigen. Hapten-specific antibodies typically contain a concave binding site or cleft, and their antigen recognition is largely based on shape complementarity (Wilson & Stanfield, 1993; MacCallum et al., 1996). Conceivably, they can be sensitive to even subtle loosening or tightening of the cleft. Therefore, mutations in FR1 residues 2 and 4 may have more radical effects on hapten-binding antibodies than on protein-binding ones, in which the antigen binding site is generally more planar and covers a much larger area (Wilson & Stanfield, 1994; MacCallum et al., 1996).

Materials and methods

Strains and plasmids

The strain *Escherichia coli* XL1-Blue (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*, [F′ *proAB*, *lacI*^{q}*ZΔM15*, Tn*10* (Tet^r)], Stratagene, La Jolla, California) was used as host for cloning as well as for recombinant Fab expression. The plasmid 57-2/pComb3, containing the Fd (γ1) and kappa chain genes of the anti-estradiol antibody 57-2 (Pajunen et al., 1997), was used as the basic construct to which the different N-terminal changes were made.

DNA constructions

The nucleotide changes needed to restore the authentic amino termini for V_H and V_L were carried out by SOE-PCR, i.e., splicing by overlap extension PCR (Ho et al., 1989), using oligonucleotide primers containing the desired mutations. All PCR reactions were performed with Vent Polymerase (New England Biolabs, Beverly, Massachusetts) in the standard buffer as suggested by the manufacturer. Individual DNA fragments were amplified by PCR from the plasmid template 57-2/pComb3, separated by agarose gel electrophoresis and purified with the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany), after which they were combined by SOE-PCR. The DNA products were purified with the QIAquick PCR purification kit (Qiagen), digested with the appropriate restriction enzymes, then gel-purified and finally ligated with the

gel-purified vector fragment (digested with the corresponding restriction enzymes). After electroporation to XL1-Blue cells, the resulting clones were checked for the correct inserts by test digestions of the plasmid minipreps. The constructs were finally confirmed by nucleotide sequencing over the gene-manipulated areas.

Fab production and sample preparation

Fresh colonies of *E. coli* XL1-Blue cells containing the desired plasmids were inoculated in 5 mL of SB medium (30 g/L Tryptone, 20 g/L Yeast extract, 10 g/L MOPS, pH 7.0) supplemented with glucose (0.2%), ampicillin (100 μg/mL), and tetracycline (10 μg/mL), and grown at 300 rpm, 37 °C until the OD_{600} of ~0.5 was reached. Main cultures (50 mL of the same medium except that glucose concentration was 0.05%) were inoculated with appropriate volumes of pre-cultures to adjust the initial OD_{600} to 0.01. The cultures were shaken at 37 °C /300 rpm until the OD_{600} of either 0.8 or 1.0 was reached. Fab expression was induced by the addition of IPTG to a final concentration of 100 μM, and the cultivation was continued at either 37 °C/300 rpm or 30 °C/240 rpm for 18 h. At 0, 2, 4, 6, and 18 h postinduction, samples of 1 mL were taken from the cultures, chilled on ice for 5 min, and centrifuged at 6,000 × *g*/+4 °C for 5 min. The supernatants were transferred to new tubes, after which the supernatants and the cell pellets were frozen at −20 °C. To prepare the samples for Fab determination, the supernatants were thawn and used as such, whereas the cell pellets were sonicated in 1 mL of ice-cold Assay buffer (see below) using the Brown Labsonic U homogenizer fitted with the probe "T." Each sample was sonicated for 60 s, with a duty cycle of 1.0 and power level of 100 using a cooled metal block to control the sample temperature. The sonicated samples were centrifugated at 10,000 × *g*/+4 °C for 10 min, after which the supernatants were transferred to new tubes and used for the Fab determination.

Immunoassay reagents and equipment

All immunochemical measurements were performed with time-resolved immunofluorometric assays in microtitration wells using the DELFIA label technology (Perkin-Elmer Wallac, Turku, Finland). The labeled estradiol tracers were prepared by covalent coupling with a nonfluorescent europium chelate (see below). After all incubations and final washes, the bound tracer concentrations were measured by adding the specially formulated Enhancement solution in which the europium ions were dissociated from the original, nonfluorescent chelates to the solution phase where new, highly fluorescent β1-diketone complexes were formed (Hemmila et al., 1984). These complexes were then measured by time-resolved fluorometry. The following DELFIA assay reagents and equipment were used: (rabbit) anti-mouse IgG coated microtitration strips, Assay buffer, Washing solution, Enhancement solution and Delfia Research Fluorometer (model 1234).

Europium-labeled estradiol derivatives

The labeled antigen tracers (E_2–6–CMO-N1[Eu] and E_2–4–CET-N1[Eu], Fig. 4B) were gifts from H. Mikola, Perkin-Elmer Wallac, Turku, Finland. E_2–6–CMO-N1[Eu] was prepared from 6-oxoestradiol 6-(O-carboxymethyl)oxime (abbreviated E_2–6–CMO), and the europium chelate of N^1-(*p*-aminobenzoyl)-diethylenetriamine-N^1, N^2, N^3, N^3-tetraacetic acid (Mukkala et al., 1989), as described by Mikola et al. (1993). E_2–4–CET-N1[Eu] was synthesized similarly from estradiol-4-carboxyethylthioether (E_2–4–CET) and N^1-(*p*-aminobenzoyl)-diethylenetriamine-N^1, N^2, N^3, N^3-tetraacetic acid as described by Meltola et al. (1999).

Determination of Fab concentrations

Fab concentrations were determined with labeled antigen immunoassays as described previously (Saviranta et al., 1998), except that 5 nM E_2–6–CMO-N1[Eu] was used as the tracer. At 5 nM concentration, this tight-binding E_2 analog saturates all the studied mutants to a degree of 96–98%; thus, the determination of functional antibody concentration was not significantly affected by differences in the affinity constants. Purified recombinant Fab 57-2 was used as standard.

Affinity and cross-reactivity determinations

Antigen binding properties of the Fab fragments were studied with microtitration plate-based competitive immunoassays using E_2–4–CET-N1[Eu] as the labeled tracer. All incubations were carried out at 25 °C/900 rpm in the Labsystems iEMS shaker (Labsystems, Espoo, Finland); the microtitration strips were sealed with adhesive tape during the incubations. The following basic procedure was used in all assays: Fab samples (200 μL of a 10 ng/mL dilution in Assay buffer) were added to the wells of the anti-mouse IgG antibody-coated microtitration strips, incubated for 2 h, and washed four times. Steroid/tracer solution (200 μL) was added, the strips were incubated for 2 h, and washed four times, after which Enhancement solution (200 μL) was added, and the shaking was continued for 30 min to develop the fluorescent complexes. The fluorescence signals were then measured with time-resolved fluorometry using standard settings for europium. The constitution of the steroid/tracer solution was varied in the different assays as follows: (1) the hot saturation experiment was performed with tracer concentrations of 0.1, 0.2, 0.5, 1, 2, and 5 nM with two replicates; background controls were done for each tracer concentration by omitting the Fab, i.e., by using the plain Assay buffer in the first incubation. (2) The competition assays were done with mixtures of tracer and unlabeled steroid (either E_2, TES, E_2–3–SO_4, E_3, or E_1). A fixed tracer concentration (2 nM) was used while the concentration of the unlabeled (competing) steroid vas varied (E_2: 0.307, 0.768, 1.92, 4.8, 12, and 30 nM; TES: 1.54, 3.84, 9.6, 24, 60, and 150 nM; E_2–3–SO_4: 7.68, 19.2, 48, 120, 300, and 750 nM; E_3: 0.179, 0.448, 1.12, 2.8, 7, and 17.5 μM; E_1: 0.512, 1.28, 3.2, 8, 20, and 50 μM). The measured signals (CPS) were converted to molar concentrations of bound tracer, after which the affinity constants were calculated with the RADLIG program of the KELL package (version 5.0.2, Biosoft, Cambridge, United Kingdom).

Molecular graphics

The coordinates of the 2.15 Å crystal structure of Fab 57-2 in complex with estradiol (U. Lamminmäki, unpubl. data) were saved in Protein Data Bank format, and the protein structure was inspected with the programs Protein Explorer and Noncovalent Bond Finder (Copyright Eric Martz, University of Massachusetts, Amherst, Massachusetts). These programs were downloaded from http://www.umass.edu/microbio/chime/, and they were used from within the Netscape Communicator version 4.51 with the Chime

plugin installed (MDL Chemsacape Chime version 2.0.3, Copyright MDL Information Systems, Inc., San Leandro, California; downloaded from http://www.mdli.com). Molecular graphics illustrations were produced with the program Weblab Viewer Pro version 3.2 (Copyright Molecular Simulations Inc., San Diego, California).

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