Protein Science (1994), *3*:2351–2357. Cambridge University Press. Printed in the USA.
Copyright © 1994 The Protein Society

Dissecting the energetics of an antibody–antigen interface by alanine shaving and molecular grafting

LEI JIN[1,2] AND JAMES A. WELLS[1,2]
[1] Department of Protein Engineering, Genentech, Inc., South San Francisco, California 94080
[2] Department of Pharmaceutical Chemistry, University of California at San Francisco, San Francisco, California 94143

(RECEIVED August 5, 1994; ACCEPTED September 3, 1994)

Abstract

Alanine-scanning mutagenesis on human growth hormone (hGH) identified 5 primary determinants (Arg 8, Asn 12, Arg 16, Asp 112, and Asp 116) for binding to a monoclonal antibody (MAb 3) (Jin L, Fendly BM, Wells JA, 1992, *J Mol Biol 226*:851–865). To further analyze the energetic importance of residues surrounding these five, we mutated all neighboring residues to alanine in groups of 7–16 (a procedure we call alanine shaving). Even the most extremely mutated variant, with 16 alanine substitutions, caused less than a 10-fold reduction in binding affinity to MAb3. By comparison, mutating any 1 of the 5 primary determinants to alanine caused a 6- to >500-fold reduction in affinity. Replacing any of the 4 charged residues (Arg 8, Arg 16, Asp 112, and Asp 116) with a homologous residue (i.e., Arg to Lys or Asp to Glu) caused nearly as large a reduction in affinity as the corresponding alanine replacement. It was possible to graft the 5 primary binding determinants onto a nonbinding homologue of hGH, human placental lactogen (hPL), which has 86% sequence identity to hGH. The grafted hPL mutant bound 10-fold less tightly than hGH to MAb3 but bound as well as hGH when 2 additional framework mutations were introduced. Attempts to recover binding affinity by grafting the MAb3 epitope onto more distantly related scaffolds having a similar 4-helix bundle motif, such as human prolactin (23% sequence identity) or granulocyte colony-stimulating factor, were unsuccessful. These studies show that only a small number of side chains on hGH confers tight binding affinity to MAb3 and that substituting or transplanting them onto another scaffold has narrow design tolerances.

Keywords: alanine-shave mutagenesis; antibody–antigen interface; energetics; human growth hormone; molecular grafting

An understanding of the forces that drive protein–protein interactions is essential to the rational design of molecules that can bind to these interfaces. Antibody–antigen complexes are particularly attractive for studying protein–protein interactions because of the ease in preparing and assaying these binding partners. X-ray crystallographic studies of these complexes show that the contact interfaces are generally large (650–1,000 Å^2), involving about 14–21 residues on each side, and characterized by a high degree of structural and chemical complementarity (for a review, see Davies et al., 1990). However, theoretical predictions of antibody–antigen complexes (Novotny, 1991) and alanine scanning of all residues that are buried at the hGH–receptor interface (Cunningham & Wells, 1993; Clackson & Wells, 1995) indicate that less than a quarter of the contact residues provide most of the binding energy.

The notion that the so-called functional epitope is smaller than the contact or structural epitope was reinforced by alanine scanning of hGH. Single alanine mutants covering the entire surface of hGH were assayed for their binding to any of 21 different MAbs (Jin et al., 1992). Most alanine substitutions had no effect on binding affinity, but those that did usually caused massive reductions in affinity, typically in the range of 20- to >1,000-fold. These side chains mapped to discrete patches on the folded hormone, and on average only 3–5 side chains could account for more than 80% of the binding free energy to a given MAb. Similar conclusions were reached from alanine scanning the binding loops for the anti-HER-2 antibody, 4D5 (Kelley & O'Connell, 1993). Although no structures have been solved for these complexes, it is striking that the average number of side

Reprint requests to: James A. Wells, Department of Protein Engineering, Genentech, Inc., 460 Pt. San Bruno Blvd., South San Francisco, California 94080.

Abbreviations: hGH, human growth hormone; hPL, human placental lactogen; hPRL, human prolactin; hGM-CSF, human granulocyte–macrophage colony-stimulating factor; MAb, monoclonal antibody; hGHbp, extracellular domain of the hGH receptor, also called the hGH binding protein; HEL, hen egg white lysozyme; ELISA, enzyme-linked immunosorbent assay; mutants are designated by the single-letter code for the wild-type residue followed by its position in the mature sequence and then the replacement residue; multiple mutants are designated by component single mutants separated by slashes.

chains constituting the functional epitope is much smaller than the average number of contact side chains seen in some half-dozen different protein antigen–antibody complexes.

Alanine scanning is a useful tool for identifying functionally important side chains (for review see Wells, 1991). It is possible that side chains flanking the functionally important ones may play little role alone, but together they may affect solvation energies and/or the microdielectric of the medium. Moreover, alanine scanning does not indicate how tolerant a functional residue is to homologous substitutions or how precisely each side chain needs to be displayed from the protein scaffold to achieve tight binding. These issues are important if one would desire to use alanine-scanning data in the design of protein or small-molecule mimics of large binding interfaces.

To begin to address these points, we studied the binding of hGH to a particular anti-hGH MAb, MAb3. Previous alanine scanning (Jin et al., 1992) showed binding of MAb3 to hGH was disrupted from 6- to >500-fold for alanine substitutions at any of 5 polar residues (Arg 8, Asn 12, Arg 16, Asp 112, and Asp 116) (Fig 1). Single alanine replacements at surrounding residues caused <2-fold reductions in binding affinity. To further study the role of surrounding surface residues in total, we mutated them to alanine in groups of 7–16, a procedure we term alanine shaving. To evaluate the tolerance of the functional determinants, we mutated them to homologous residues and also grafted them onto more distantly related protein scaffolds. From these studies we conclude that the MAb3 functional epitope is largely confined to the 5 residues defined by the original alanine scan. Surrounding side chains play little role in modulating affinity, although they may be important for specificity. Finally, the functional epitope is very sensitive to homologous substitution or to structural changes in the scaffold from which it is displayed.

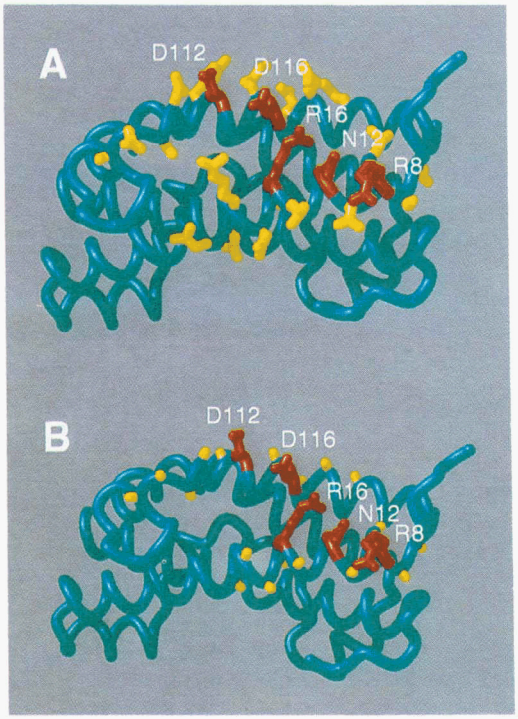

Fig. 1. Structure of hGH. **A:** Five residues constituting the MAb3 functional epitope (shown in red) and residues surrounding it (shown in yellow). **B:** Residues substituted to alanine. (See also Kinemage 1.)

Results

Alanine-shave mutagenesis

We decided to study the functional epitope for MAb3 (Fig. 1) because it is reasonably representative of the epitopes for the 21 MAbs analyzed on hGH (Jin et al., 1992). For example, the MAb3 epitope is discontinuous in that determinants are presented from 2 different helices (helices 1 and 3). Furthermore, Arg and Asp residues were often found in the functional epitopes for these MAbs; of the 5 side chains in the MAb3 functional epitope, 2 are Arg and 2 are Asp. For alanine shaving, we fixed the 5 functional residues and mutated to alanine the 24 surrounding residues (Fig. 1). The surrounding residues were chosen based upon the criteria that they were solvent accessible (i.e., ≥10% accessible to a 1.4-Å radius probe relative to their accessibility in Gly-X-Gly tripeptide [Lee & Richards, 1971]) and within 8 Å of at least 1 of the 5 functional residues in helix 1 or helix 3. For these calculations, we used the X-ray coordinate set of hGH provided by a 1:1 complex of hGH bound to the hGHbp solved at 2.5 Å resolution (A.M. de Vos, M. Ultsch et al., unpubl. results). This is nearly the same as that of the 1:2 hGH–(hGHbp)2 complex solved at 2.8 Å resolution (de Vos et al., 1992).

In general, we found the expression levels for the alanine-shaved mutants were inversely proportional to the number of alanine mutations introduced (data not shown). As a result it was not possible to simultaneously shave all 24 surrounding residues in helices 1 and 3. However, it was possible to shave groups of them and make several combinations that together mutated all of these side chains (Table 1). The conformational integrity of each mutant was tested by measuring the binding affinity of these mutants with 2 MAbs (MAb4 and 7) that have functional epitopes that do not overlap that of MAb3 or each other (Jin et al., 1992). None of the alanine-shave mutants were detectably reduced in binding affinity to either MAb4 or 7 (data not shown).

Affinity and kinetics for binding of each mutant to MAb3 were analyzed by BIAcore™ and ELISA (Table 1). Alanine shaving of helix 1 (h1) or helix 3 (in 2 parts called $h3_N$ and $h3_C$) caused little change in either the affinity or kinetics of binding relative to wild-type hGH. Even the combination $h1_C/h3_N$ (B), containing 16 alanine mutations, was only reduced 8-fold in affinity. These slight reductions in affinity were primarily the result of faster off-rate, not slower on-rate. Relative changes in affinity calculated from BIAcore™ analysis agreed closely with those obtained from ELISA.

Tolerance and additive effects for mutations in the MAb3 functional epitope

To investigate the tolerance of the charged residues within the functional epitope to small structural perturbations, we introduced 4 isoionic substitutions (R8K, R16K, D112E, D116E) that altered the side-chain size without affecting the charge (Table 2). In general, the disruptions in affinity measured by ELISA for these substitutions were large (>10-fold), although not quite as dramatic as the corresponding alanine substitutions.

Table 1. *Kinetic and dissociation constants for the binding of alanine-shave hGH mutants to MAb3*

Mutant[a]	No. of Ala mutations	k_{on}[b] ($\times 10^5$) (s^{-1} m^{-1})	k_{on}/k_{on} (wt)	k_{off} ($\times 10^{-3}$) (s^{-1})	k_{off}/k_{off} (wt)	K_d (nM)	K_d/K_d (wt)	EC_{50} (mut)/EC_{50} (wt)[c]
wt hGH	0	5.6	1	3.7	1	0.6	1	1
h1	9	10.3	1.8	9.5	2.6	0.9	1.4	1.0
$h3_N$	9	4.7	0.8	6.7	1.8	1.5	2.3	1.5
$h3_C$	7	10.7	1.9	9.6	2.6	0.9	1.4	1.0
$h1_Ch3_N$ (A)	14	6.7	1.2	19.7	5.3	2.9	4.4	3
$h1_Ch3_N$ (B)	16	3.0	0.54	16.9	4.6	5.7	8.6	5

[a] Mutant notations: wt, wild type; h1, residues mutated to alanine are L6, S7, L9, D11, L15, H18, R19, H21, Q22; $h3_N$, residues mutated to alanine in the N-terminal part of helix 3 are S106, D107, S108, N109, Y111, K115, E118, E119, Q122; $h3_C$, residues mutated to alanine in the C-terminal part of helix 3 are Q122, T123, M125, G126, R127, E129, D130; $h3_Nh3_C$ (A), residues mutated in both the C-terminal part of helix 1 and the N-terminal part of helix 3 are L15, H18, R19, H21, Q22 + $h3_N$; for (B) the residues mutated are L9, D11 + $h3_Nh3_C$ (A).

[b] Rate measurements on BIAcore™ were made using the MAb3 matrix at 25 °C as described in the Material and methods. Average standard errors for on-rate, off-rate, and affinities on the same biosensor chip are about 25%, 22%, and 33% of the value reported.

[c] Relative EC_{50} values were calculated from duplicate binding curves by ELISA (see Materials and methods) and standard deviations are generally ±30% of the value shown. Relative EC_{50} = EC_{50} (mutant)/EC_{50} (wt hGH).

We analyzed how independently these determinants functioned by making multiple alanine replacements and measuring their change in binding affinity for MAb3 using an ELISA (Table 3). The D112A and D116A single mutants caused a 20-fold and 100-fold reduction in affinity, respectively. It was difficult to evaluate accurately extreme additive effects because large amounts of mutant protein were required and reductions in affinity of ~1,000-fold vary in duplicate measurements about 5–10-fold by ELISA. Nonetheless, the double mutant, D112A/D116A, causes ~1,000-fold reduction in affinity, which is roughly the product of the 2 single mutants. In contrast, the triple mutant, R8A/N12A/R16A, is about 1,000-fold weaker than wild type. Nonetheless, from the product of the reductions for each of these single mutants, one would predict binding to be reduced by about 200,000-fold below wild type (undetectable binding) if the effects were entirely additive (Wells, 1990). Thus, the two binding determinants in helix 3 act more independently than the three in helix 1.

Table 2. *Effects of alanine and isoionic substitutions at functionally important determinants for binding MAb3*

Mutant	EC_{50} (mut)/EC_{50} (wt)[a]
R8A	40
R8K	20
N12A	6
R16A	~500
R16K	30
D112A	20
D112E	10
D116A	100
D116E	20

[a] Relative EC_{50} values were calculated from duplicate binding curves (see Materials and methods) and standard deviations are generally ±30% of the value shown. Relative EC_{50} = EC_{50}(mutant)/EC_{50}(wt hGH). The values reported here for alanine substitutions are within ~2 fold the values previously reported (Jin et al., 1992).

Next, we analyzed the functional effects of these mutations in the context of several of the alanine-shave variants. Single or multiple mutants in the 5 binding determinants caused much larger reductions in affinity when made in the context of the alanine-shave variants (Table 3). For example, whereas D112E and D116E caused 10–20-fold reductions in EC_{50}, respectively (Table 2), their affinity was reduced ~200-fold when combined with the helix 1 variant, h1, which contained 9 alanine mutations. Larger than additive effects were also seen for the R8K single mutant and multiple mutants in these binding determi-

Table 3. *Additivity of functional effects for single and multiple mutations at binding determinants in wild-type hGH and in the context of alanine-shaved variants*

Mutant	EC_{50} (mut)/EC_{50} (wt)[a]	Reduction in affinity predicted[b]
D112A/D116A	~1,000	2,000
D112A/D116A/$h1_C$	$>10^4$	2,000
D112E/h1	200	10
D116E/h1	200	20
R8A/N12A/R16A	~1,000	10^5
R8A/N12A/R16A/$h3_N$	$>10^4$	10^5
R8K/$h3_N$	200	40
R8K/$h3_C$	80	20
G120A	4	–
G120A/$h3_C$	20	5
G120A/$h3_N$	100	8

[a] EC_{50} were determined by ELISA. Reductions in EC_{50} of over 1,000-fold have large errors so that the values can vary by a factor of 5–10 in independent determinations. The detection limit of our assay precluded us detecting drops of more than 10^4.

[b] Calculated from the product of the reduction in affinities of the component mutants described in Tables 1 and 2. This assumes the interactions are functionally independent.

[c] The alanine-shave mutants $h3_C$, $h3_N$, h1, and $h1_C$ are described in Table 1.

nants. These data suggest that the functional determinants take on more importance once the surrounding residues are shaved.

Further support for this observation is provided by the G120A variant (Table 3). Position 120 is 1 helical turn away from the binding determinant, D116. The G120A causes only a 3-fold reduction in affinity. However, when this is combined with the $h3_C$ or $h3_N$ alanine-shave mutant, binding is further reduced by a factor of 5 and 25, respectively. It is likely that the α-carbon of G120 is very near MAb3 because a much bulkier substitution, like G120R, virtually obliterates binding (data not shown). It is also likely that MAb3 interacts with atoms other than those removed by alanine substitution because when all five of the binding determinants are mutated to alanine at the highest hormone concentration (50 μM), we could detect some binding, although not enough to obtain an estimate of the EC_{50}. Nonetheless, if we were to assume an EC_{50} of ~50 μM for this penta-mutant, then relative to wild-type hGH (K_d = 0.6 nM; Table 1) binding would be reduced $>10^5$-fold.

Grafting of the functional epitope from hGH to hPL

hPL shares 86% sequence homology with hGH (27 changes of 191 total; Fig. 2) and the 3-dimensional structure of hPL is virtually identical to that of hGH (W. Somers, B. de Vos, & T. Kossiakoff, unpubl. results). MAb3 shows no detectable binding to hPL ($>10^4$-fold reduced from hGH) and, of the 5 most important binding determinants for MAb3 on hGH, 3 positions are different in hPL (H12, Q16, and H112). We introduced these mutations in a sequential fashion into the cloned and expressed gene for hPL (Lowman et al., 1991) and measured their effects on the kinetics and affinity for binding to MAb3 (Table 4). Binding affinity was below our level of detection for BIAcore™ analysis (~100 nM) for the single mutant (H112D) or the double mutant (H12N/Q16R). However, the affinity for the combined triple mutant was within 10-fold of hGH. This hPL mutant had virtually the same on-rate as hGH but a reduced off-rate. In an effort to improve binding further, we introduced 2 additional mutations at the beginning of helix 3 that are different between these molecules (D109N/D110V). This penta-hPL variant had virtually the same binding affinity and kinetics as hGH for MAb3.

To further probe the sensitivity of the functional epitope toward structural change, we tried to graft it onto hPRL, which has a sequence homology of 23% with that of hGH (Fig. 2). Similar grafting effort was attempted in hGM-CSF, which has no apparent sequence homology with hGH but shares similar folding pattern, particularly in helix 1 and helix 3 region (Diederichs et al., 1991). Although it was possible to produce a number of helix 1 and 3 variants in these 2 proteins (which continued to bind or activate their respective receptors), none of these grafted mutants gave any detectable binding to MAb3 by BIAcore™ analysis (data not shown).

Discussion

Alanine-shaving and alanine-scanning results are consistent

The alanine-shave mutagenesis experiments indicate that the functional epitope for MAb3 is small and largely confined to the 5 side chains identified by alanine scanning (Fig. 1). Shaving any of the 24 surrounding residues in groups of 7–9 caused almost no effect on binding. Even combinations including as many as 14–16 alanine mutants caused only 4–8-fold reductions in affinity, respectively. The fact that binding affinity for MAb3 could be imparted into a nonbinding homolog, such as hPL, by altering the functional residues identified by the alanine scan, provides additional support that these are the primary determinants.

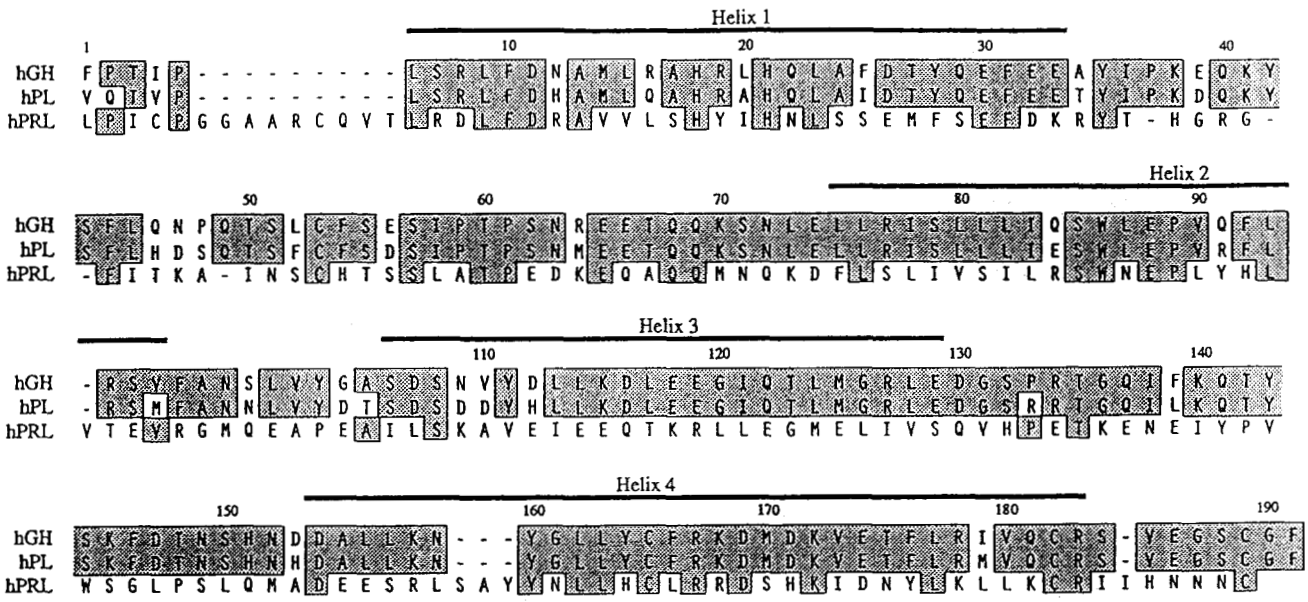

Fig. 2. Sequence comparison of hGH, hPL, and hPRL. Residues identical with hGH are shaded and the segments in helices are indicated by the bars.

Table 4. *Grafting of the hGH MAb3 functional epitope onto hPL*

Mutant[a]	k_{on} ($\times 10^5$) (s^{-1} m^{-1})	k_{on}/k_{on} (hGH)	k_{off} ($\times 10^{-3}$) (s^{-1})	k_{off}/k_{off} (hGH)	K_d (nM)	K_d/K_d (hGH)
wt hGH	5.6	1	3.7	1	0.6	1
wt hPL	ND[b]	ND	>100	>100	>500	$>10^4$
A	ND	ND	>100	>100	>500	$>10^4$
B	ND	ND	>100	>100	>500	$>10^4$
C	5.7	1.1	39.2	10.6	6.1	10.2
D	8.4	1.5	5.9	1.6	0.55	1.1

[a] Mutant hPL notations: A, H12N/Q16R; B, H112D; C, H12N/Q16R/H112D; D, H12N/Q16R/H112D + D109N/D110V.
[b] ND, not determined. There was not sufficient binding for these mutants for their on-rates to be determined.

Although we do not have a structure for the hGH-MAb3 complex, it is probable that the contact epitope includes at least some of the 24 surrounding side chains that were mutated. Mutations at G120 on hGH, 1 helical turn from D116, were disruptive to binding, suggesting that MAb3 does make contact here. The 5 residues in the functional epitope plus the 24 surrounding ones cover a patch of about 12 Å in radius, which is about the size of a typical antibody structural epitope. Structures of antibody-antigen complexes show the protein antigens provide 14-21 contact side chains at the interface (Davies et al., 1990). Thus, it is likely that the contact interface is larger than the functional epitope.

It is possible that functionally "silent" surrounding residues are important for binding affinity, but when they are removed, new complementary side-chain interactions form that are of equal energetic contribution. We think it is improbable that, with each single or multiple alanine substitution, new interactions form with nearly the same energetics as those that were removed. In this analysis we do not mean to imply that contact residues that do not contribute to net binding affinity have no role. These contact side chains may, for example, play a role in preventing interactions with other proteins—that is, they may affect specificity but not affinity.

These alanine-scanning and alanine-shaving experiments do not probe main-chain interactions. About 10-20% of the antigen contacts seen in antibody-antigen complexes are main-chain (Janin & Chothia, 1990). We attempted to probe for these by mutating all 5 MAb3 determinants in hGH simultaneously. In fact, at the highest hormone concentration tested (~50 μM), some binding was detected by ELISA, but it was too low to estimate a binding constant. Thus, although binding is dominated by just 5 side chains, backbone interactions also play a role. In our case it is unlikely that interactions could involve the helical amide bonds in helix 1 and 3 because they would be tied up in hydrogen bonding.

Our data suggest that these multiple mutated hGH variants are not misfolded. Although we did find that the most extensively mutated variants were expressed at much lower levels, perhaps suggesting that they were less stable, the alanine-shave mutants bound 2 other conformationally sensitive MAbs as tightly as wild-type hGH. This suggests that the expressed protein recovered for these mutants is folded like hGH. Moreover, what little effect the alanine-shave mutants had on binding to MAb3 showed up as affecting off-rate, not on-rate. This suggests that the hormones did not require a conformational rearrangement to bind that was much different from wild-type hGH. Furthermore, we did not expect that the packing between the helices 1 and 3 would be changed much because only the surface side chains were altered. Alanine is not expected to disrupt these helical segments because it is one of the best helix-forming residues (e.g., see Chakrabartty et al., 1991). Several studies have shown that helical proteins are remarkably tolerant toward multiple alanine substitutions. For instance, 7 alanine residues have been successfully engineered into a single α-helix of T4 lysozyme (Heinz et al., 1992). Also, functional λ-repressors with 6-7 alanine substitutions have been isolated (Gregoret & Sauer, 1993).

Functional and structural evidence for interaction among the binding determinants

Mutations at molecular interfaces often exhibit additive effects on the free energy of binding (multiplicative effects on their relative binding affinities) when they are far apart and nonadditive effects when they are close to each other (for review, see Wells, 1990). The double mutant in helix 3 (D112A/D116A) showed roughly additive effects, whereas the binding affinity for the triple mutant in helix 1 (R8A/N12A/R16A) was less than additive. This can be partly rationalized by inspection of the structure of hGH (Fig. 3). Side chains D112 and D116 do not contact each other, whereas the central N12 side chain hydrogen bonds to both R8 and R16. Thus, alanine substitutions at any of these side chains in helix 1 break a hydrogen bonding network, whereas those in helix 3 do not.

Greater than additive effects were seen for combining a number of the alanine-shave mutants with alanine replacements at functionally important residues. For example, R8K was 20-fold reduced in affinity alone, but when it was combined with the alanine-shave variant, $h3_C$, it was reduced 200-fold in affinity. Similarly large and exaggerated effects were seen for D112E or D116E when combined with the h1 alanine-shave variant. One possibility is that shaving surrounding residues to alanine allows for better contacts to be made at the functionally important side chains. This is further suggested by the G120A variant, which causes only a 4-fold reduction in affinity but, when combined with $h3_N$, is reduced 100-fold in affinity. Perhaps the alanine shave allows the functionally important side chains to move closer to MAb3 and thereby improve their interactions. In this sense, mutations made in the surrounding residues are compen-

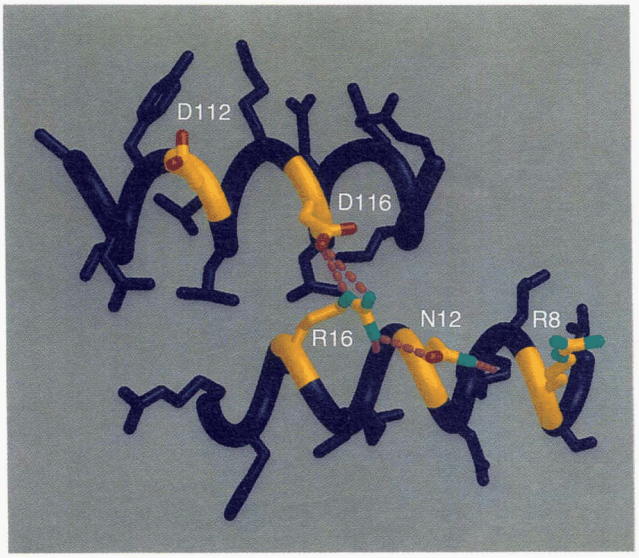

Fig. 3. Hydrogen bonding network within the functional epitope of MAb3. Helix 1 is shown in white, helix 3 in blue; the 5 residues constituting the functional epitope are in yellow and labeled. Hydrogen bonds were schematically shown by red dotted lines.

sated for by improved contacts at the functionally important side chains. In any case, these studies indicate that one only requires the 5 determinants identified by alanine scanning for tight binding. A high-resolution crystal structure of the alanine-shave hGH–MAb3 complex and free alanine-shave hGH variant would help clarify some of these points.

MAb3 functional epitope is not very tolerant to substitution or transplantation

Homologous substitutions for any of the charged functional determinants had nearly as large an effect on binding as the corresponding alanine change. Similar results have been seen for mutations at antibody–antigen interfaces where the structures are known. In mapping the epitope on HEL for MAb HyHEL-5, the R68A or R68K mutations in HEL totally disrupted the binding affinity for MAb HyHEL-5 (Lavoie et al., 1990). In a mutational study of the binding of HEL to MAb HyHEL-10 (Kam-Morgan et al., 1993), it was found that substitutions to any of 7 different residues at either of 2 positions had roughly the same disruptive effect on binding affinity. Finally, in neuraminidase from N9 subtype influenza virus, very subtle changes in the shape or chemistry of the antigen were found to abolish antibody binding (Tulip et al., 1991). Charged residues frequently show up in functional epitopes (Jin et al., 1992) and charge–charge complementarity is often observed in antibody–antigen binding interfaces (for review see Davies et al., 1990). Charge–charge interactions have a weaker distance dependence than either van der Waals or hydrogen bonding interactions. Perhaps the extreme sensitivity to an isoionic substitution reflects the fact that sterically repulsive interactions are introduced as one introduces a longer charged side chain, or that important van der Waals and/or hydrogen bond interactions are broken when one substitutes a shorter charged side chain. In any case, the structural tolerances appear to be narrow.

Similar conclusions were reached from the molecular grafting experiments. No binding was detectable in hPL until all five of the functionally important side chains were in place. Two additional mutations (D109N/D110V) allowed hPL to achieve binding affinity and kinetics equivalent to hGH. We believe these mutations alter the packing of helix 1 and 3 so that their effects are indirect. Valine 110 in hGH is completely buried (Fig. 3) and the main-chain structure of hGH and hPL differ markedly in this region (W. Somers, B. de Vos, & T. Kossiakoff, pers. comm.). Moreover, we were unable to recruit detectable binding affinity into more distantly related 4-helix bundle scaffolds like hPRL and hGM-CSF, indicating that larger deviations for the GH structural scaffold are not tolerated easily.

The intolerance of the MAb3 functional epitope to substitution and grafting is in contrast with that of receptor Site 1 on hGH ($K_d \sim 0.3$ nM). Alanine replacements in receptor Site 1 caused more more modest effects on binding (typically 5–50 fold [Cunningham & Wells, 1989, 1993] versus 20–500-fold for MAb3). More side chains were involved in strongly modulating binding affinity (at least 8 hGH versus 5 for MAb 3). The Site 1 epitope was more tolerant to homologous replacements at charged residues. For example, R64A reduces affinity for Site 1 by 20-fold, but R64K was *improved* in affinity by 2-fold (Cunningham & Wells, 1990). Moreover, it has been possible to graft hGH-binding determinants from Site 1 into hPRL and hPL to bind the hGHbp (Cunningham & Wells, 1990; Lowman et al., 1991). The sharper design tolerances for the MAb3 epitope may reflect the smaller number of critical side chains and thus their greater individual importance to binding.

Implications for rational drug design

These experiments were carried out, in part, to better understand the feasibility of transferring a discontinuous antibody–antigen epitope to other molecular scaffolds. The alanine-scanning and shaving results suggest that most of the binding energy between hGH and MAb3 derives from 5 side chains on hGH. Is it possible to transfer these determinants to another, perhaps smaller molecular scaffold? Our results suggest that this may be possible but challenging because it is not simply the nature of the binding determinants, but their precise display that is important to achieve a tight binding mimic. Finally, we suggest that both structural and functional information are optimal in these endeavors. Although structural analysis can define the molecular contacts, it cannot define their importance to binding and nor their tolerance to slight changes.

Materials and methods

All mutagenesis reactions were carried out following the procedure described by Kunkel et al. (1987) and sequences were confirmed by dideoxy sequencing (Sanger et al., 1977). Variants of hGH and hPL were expressed and purified as described (Cunningham & Wells, 1989; Lowman et al., 1991). Concentrations of variant proteins were determined by Coomassie-stained SDS/PAGE and are accurate to about ±20% of the actual of protein concentration (Cunningham & Wells, 1989). ELISA was carried out as described (Jin et al., 1992). Standard deviation of the measurement based on triplicate determinations of EC_{50} was generally about ±30% reported, in the range of 0.5–100

nM. Much larger derivations occurred (5-10-fold) for EC_{50} values ≥ 1 μM.

The anti-hGH MAb3 was immobilized on the Pharmacia Biosensor BIAcore™ by random amine coupling and changes in refractive index upon binding of hGH (or hPL) were used for kinetic measurement. The association and dissociation constants were calculated using software provided with the BIAcore™ instrument (Karlsson et al., 1991). Association rates were measured from binding profiles obtained by injecting increasing concentrations of each hormone variant. Five serial dilutions (each 2.5-fold) were made starting at 1.5-50 μM mutant hormone, depending on the affinity for the MAb3. We applied a maximum flow rate of 20 mL/min to minimize potential mass transport effects. High salt buffer (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) was used to prevent long-range electrostatic effects and to mimic physiological ionic strength. We also included 0.05% Tween 20 to reduce nonspecific binding. The matrix was regenerated by washing for 20 s with 5 mM HCl. Control experiments showed that this was sufficient to remove all the bound hormone, and the matrix could be reused more than 100 times without significant change in the binding kinetics.

Dissociation rates were measured by saturating the biosensor with 5-50 μM mutant hormone and switching to buffer without hormone. Buffer flow rates and regeneration conditions were identical to those used to measure the association profiles. To minimize potential rebinding effects, only the initial 0.5-5 min of each dissociation profile were used for calculation of the dissociation constant, depending on the rate of dissociation for each mutant. The average standard deviation within triplicate determinations of association constants on the same biosensor chip was about $\pm 15\%$ of the value reported and for dissociation constants about $\pm 10\%$ of the value reported. Values determined between different biosensor chips varied up to 70%. However, because we always included a wild-type reference, the standard errors for the relative values that we report are the same as determinations made on the same chip. Thus, the average cumulative errors in relative association, dissociation, and affinity constants when calculated as the square root of the sum of the squares are about 25%, 22%, and 33%, respectively.

Acknowledgments

We thank Brian Fendly for providing the antibodies; Mark Vasser and Peter Ng for preparation of oligonucleotides; Will Somers, Mark Ultsch, and Bart de Vos for making coordinates of hPL and hGH-hGHbp complex (1:1) available prior to publication; and Kerrie Andow for graphics.

References

Chakrabartty A, Schellman JA, Baldwin RL. 1991. Large difference in the helix propensities of alanine and glycine. *Nature 351*:586-588.

Clackson T, Wells JA. 1995. A hot spot of binding energy in a hormone-receptor interface. *Science 267*:383-386.

Cunningham BC, Wells JA. 1989. High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science 244*: 1081-1085.

Cunningham BC, Wells JA. 1990. Engineering human prolactin to bind to the human growth hormone receptor. *Science 247*:1461-1465.

Cunningham BC, Wells JA. 1993. Comparison of a structural and a functional epitope. *J Mol Biol 234*:554-563.

Davies DR, Padlan EA, Sheriff S. 1990. Antibody-antigen complexes. *Annu Rev Biochem 59*:439-473.

de Vos AM, Ultsch M, Kossiakoff AA. 1992. Human growth hormone and extracellular domain of its receptor: Crystal structure of the complex. *Science 255*:306-312.

Diederichs K, Boone T, Karplus PA. 1991. Novel fold and putative receptor binding site of granulocyte-macrophage colony-stimulating factor. *Science 254*:1770-1782.

Gregoret LM, Sauer RT. 1993. Additivity of mutant effects assessed by binomial mutagenesis. *Proc Natl Acad Sci USA 90*:4246-4250.

Heinz DW, Baase WA, Matthew BW. 1992. Folding and function of a T4 lysozyme containing 10 consecutive alanine substitutions illustrates the redundancy of information in an amino acid sequence. *Proc Natl Acad Sci USA 89*:3751-3755.

Janin J, Chothia C. 1990. The structure of protein-protein recognition sites. *J Biol Chem 265*:16027-16030.

Jin L, Fendly BM, Wells JA. 1992. High resolution functional analysis of antibody-antigen interactions. *J Mol Biol 226*:851-865.

Kam-Morgan LNW, Smith-Gill SJ, Taylor MG, Zhang L, Wilson AC, Kirsch JF. 1993. High-resolution mapping of the HyHEL-10 epitope of chicken lysozyme by site-directed mutagenesis. *Proc Natl Acad Sci USA 90*: 3958-3962.

Karlsson R, Michaelsson A, Mattson A. 1991. Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system. *J Immunol Methods 145*:229-240.

Kelley RF, O'Connell MP. 1993. Thermodynamic analysis of an antibody functional epitope. *Biochemistry 32*:6828-6835.

Kunkel TA, Roberts JD, Zakour RA. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol 154*: 367-382.

Lavoie TB, Kam-Morgan LNW, Mallett CP, Schilling JW, Prager EM, Wilson AC, Smith-Gill SJ. 1990. Analysis of antibody-protein interactions utilizing site directed mutagenesis and a new evolutionary variant of lysozyme. In: Laver WG, Air GM, eds. *The use of X-ray crystallography in the design of antiviral agents*. New York: Academic Press. pp 213-232.

Lee BK, Richards EM. 1971. The interpretation of protein structures: Estimation of static accessibility. *J Mol Biol 55*:379-400.

Lowman HB, Cunningham BC, Wells JA. 1991. Mutational analysis and protein engineering of receptor-binding determinants in human placental lactogen. *J Biol Chem 266*:10982-10988.

Novotny J. 1991. Protein antigenicity: A thermodynamic approach. *Mol Immunol 28*:201-207.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA 74*:5463-5467.

Tulip WR, Varghese JN, Baker AT, Van Donkelaar A, Laver WG, Webster RG, Colman PM. 1991. The refined atomic structures of N9 subtype influenza virus neuraminidase and escape mutants. *J Mol Biol 221*:487-497.

Wells JA. 1990. Additivity of mutational effects in proteins. *Biochemistry 29*:8509-8517.

Wells JA. 1991. Systematic mutational analyses of protein-protein interfaces. *Methods Enzymol 202*:390-411.