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Comparison of a Structural and a Functional Epitope

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A comprehensive analysis of the energetic importance of the 31 side-chains buried at the interface between human growth hormone (hGH) and the extracellular binding domain of its receptor (hGHbp) has been carried out to assess the roles of contact side-chains in modulating the affinity and kinetics of binding. Each side-chain in hGH was converted to alanine, and the kinetics and affinity were measured using a biosensor device. This detects binding of the mutated hormones to the immobilized hGHbp by changes induced in refractive index. The data generated on the biosensor match affinities obtained by radio-immune assay in solution. The study shows that only one-quarter of the side-chains buried at the interface can account for the majority of the binding energy. These residues cluster near the center of the structural epitope. The role of these side-chains is predominantly to slow dissociation because most of the effect of the alanine substitutions is to increase the off-rate, not to slow the on-rate. The hormone associates about 10,000 times slower than expected from random diffusion but 1000 times faster than may be expecied if one imposes strict orientation restraints for a productive collision. Electrostatic interactions partly modulate association because mutations at Arg residues most affect association and together contribute a factor of about 20 to the on-rate. The data suggest that the hormone and receptor associate by diffusion and electrostatics to form an ensemble of weak collisional complexes. From these a bound complex is produced that is stabilized by only a small proportion of the contacts. We suggest that solvation energies and/or side-chains interactions within the free hormone or receptor may be so favorable that little energy is gained at most side-chains upon binding. The fact that the functional binding epitope is much smaller than the structural epitope suggests it may be possible to design smaller hormone mimics.

Keywords: hormone-receptor interactions; protein–protein interactions; mutagenesis; structure/function analysis

1. Introduction

There are a large number of high-resolution structures that show the molecular details of protein–protein interfaces (for reviews see Argos, 1988; Alzari *et al.*, 1988; Janin *et al.*, 1988; Miller, 1989; Davies *et al.*, 1990). These define contact residues, but not the energetics for them, nor do they show how docking occurs. A comprehensive understanding of the role of contact residues in affecting association and dissociation is fundamental to molecular recognition processes, and is practically important for rational protein and drug design.

Perhaps the best characterized hormone–receptor complex is that between hGH‡ and the extracellular domain of its receptor, called the hGHbp (for review, see Wells and De Vos, 1993). Mutational (Cunningham & Wells, 1989, 1991; Cunningham *et al.*, 1991*a*,*b*) and structural (De Vos *et al.*, 1992) analyses have shown that two receptor molecules bind to two distinct sites on hGH (called site 1 and site 2). The receptors associate sequentially;

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‡ Abbreviations used: hGH, human growth hormone; hGHbp, the extracellular domain of the hGH receptor otherwise called the hGH binding protein; RU, refractive index unit; RIA, radio-immuno assay; mAb, monoclonal antibody. Mutant proteins are defined by the wild-type residue in single letter code followed by its position in the mature protein sequence and then by the mutant residue. Thus, (S237C)hGHGbp indicates that Ser237 is converted to Cys in the hGHbp.

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receptor 1 binds to site 1 followed by binding of receptor 2 to site 2 (Cunningham *et al.*, 1991).

Here we evaluate the kinetics and affinity of binding for alanine substitutions at contact residues in site 1 of hGH. We utilized a biosensor device, called BIAcore™, that relies upon surface plasmon resonance to measure changes in refractive index upon hormone binding to an immobilized receptor. We find that affinity is dominated by about one-quarter of the 31 contact side-chains, and these cluster in a small patch near the center of the contact epitope. Thus, the "structural epitope" is considerably larger than the "functional binding epitope". Using this information, the accompanying paper shows it is possible to enhance binding affinity dramatically by random mutagenesis of these epitopes and phage display.

2. Materials and Methods

Alanine mutations of residues buried at site 1 in hGH were available from previous studies (Cunningham & Wells, 1989) or newly made by site-directed mutagenesis (Kunkel, *et al.*, 1987). Variant proteins were expressed and purified as described (Cunningham & Wells, 1989). Yields were improved by extending the duration of the ammonium sulfate precipitations to 1 h.

The hGHbp was immobilized on the Pharmacia Biosensor (BIAcore™) and changes in refractive index upon binding of hormone were used for kinetic measurements. The association and dissociation constants were calculated using software provided with the instrument (Karlsson *et al.*, 1991). To immobilize hGHbp in discrete or entations on the sensor chip we fixed the hGHbp *via* a free thiol. This was accomplished by introducing a cysteine residue at 1 of 2 specific sites (S201C or S237C) using site-directed mutagenesis (Kunkel *et al.*, 1987). The thiol variants of the hGHbp were expressed in *Escherichia coli* and purified to homogeneity (Fuh *et al.*, 1990). To couple these proteins to the chip surface the carboxyl-dextran matrix was activated with *N*-ethyl-*N'*-(3-diethylaminopropyl)-carbodiimide (EDC) and reacted with *N*-hydroxysuccinimide (NHS). The NHS-ester was reacted with 2-(2-pyridinyldithio)-ethaneamine (PDEA). Remaining unreacted NHS-ester groups were displaced by addition of ethanolamine. The hGHbp variants were reacted with the matrix (at 50 μg/ml in 50 mM sodium acetate, pH 5) until approximately 1000 refractive index units (RUs) were coupled (1·0 ng/mm^2; see the BIAcore™ manual).

Association rates were measured from binding profiles obtained by injecting increasing concentrations of each hGH variant. Five serial dilutions (each 2-fold) were made starting at 200 or 1000 nM hormone, depending on the affinity for the hGHbp. We applied a maximum flow rate of 20 μl/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 0·02% Tween 20 to reduce non-specific binding. The matrix was regenerated by washing for 20 s with 4·5 M $MgCl_2$. Control experiments showed that this was sufficient to remove all the bound hormone, and the matrix could be reused more than 50 times without significant change in the binding kinetics.

Dissociation rates were measured by saturating the biosensor with 5 μM hGH mutant 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 10 min of each dissociation profile were used for calculation of the dissociation constant. Both association and dissociation constants were determined using the Pharmacia Kinetics Evaluation software to solve the rate equations (Karlsson *et al.*, 1991).

The average standard deviation within triplicate determinations of association constants on the same biosensor chip was ±7% of the value reported, and for dissociation constants was ±4% of the value reported. Values determined between different biosensor chips vary up to 60%. 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. The concentration of hGH and variants was determined by densitometry of Coomassie blue-stained proteins after SDS/polyacrylamide gel electrophoresis. This method confirms the purity and integrity of the variant hormones as well as providing a protein concentration independent of the substitution with a precision of ±10% (Cunningham & Wells, 1989). 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 12%, 11% and 16%, respectively.

3. Results

To study binding of hGH to the hGHbp we immobilized a variant of the hGHbp, (S237C) hGHbp, to the thiol-derivatized matrix on the BIAcore™ biosensor *via* a mixed disulfide bond (Fig. 1A). The S237C(hGHbp) mutation does not affect binding affinity to hGH and has been used to attach a single thiol-specific fluorescent probe to follow hGH-induced dimerization of the hGHbp in solution (Cunningham *et al.*, 1991). This attachment ensured uniform orientation of the hGHbp on the matrix, unlike that obtained if we had used random coupling through primary amine groups. From the change in RUs that occurred during the coupling reaction we calculated the amount of attached hGHbp from calibration curves supplied by Pharmacia (see the BIAcore™ manual).

When excess hGH was added to the (S237C) hGHbp-matrix we observed rapid association and extremely slow dissociation (Fig. 1B). From the change in RU we calculated a molar ratio of 0·4 hGH bound per immobilized hGHbp (Table 1). This suggested that hGH dimerized the immobilized hGHbp as it did in solution (Fig. 1A). To test further for dimerization on the matrix we measured the binding of a non-dimerizing mutant of hGH, (G120R)hGH, which is blocked in its ability to bind site 2 (Fuh *et al.*, 1992). When a saturating level of (G120R)hGH was added we found that about twice as much hormone bound (Fig. 1B), with a calculated stoichiometry of 0·7 (G120R)hGH per immobilized hGHbp (Table 1).

Analysis of the on and off-rate profiles showed that both wild-type and (G120R) hGH associate at similar rates (Table 1). However, the off-rate for the wild-type was too slow to calculate a reliable dissociation constant. These data are consistent with the proposed sequential binding mechanism; that is, both hormones bound in the same way to the first

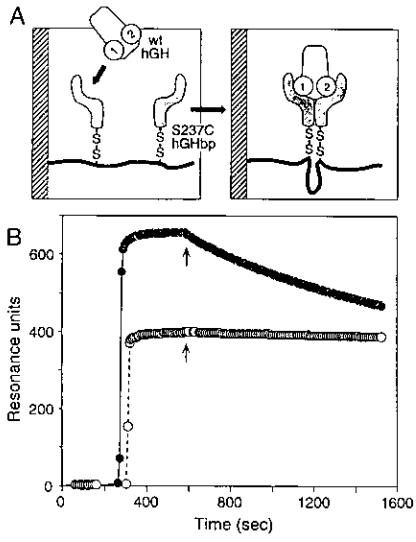

Figure 1. Reaction (A) and kinetics (B) for binding of hGH or (G120R)hGH to the (S237C)hGHbp coupled to the BIAcore™ biosensor. The (S237C)hGHbp was immobilized on the thiol-dextran matrix (A) at a level of 1220 RUs, which corresponds to 1·2 ng/mm^2. In the example, binding profile (B) hGH (open symbols) or (G120R)hGH (filled symbols) was injected at saturating concentrations (5 μM) to follow association and establish the limiting amount of bound hormone from which the stoichiometry was calculated. After saturation, the injector loop was switched to buffer to follow dissociation (indicated by the arrow). See text and Materials and Methods for further details.

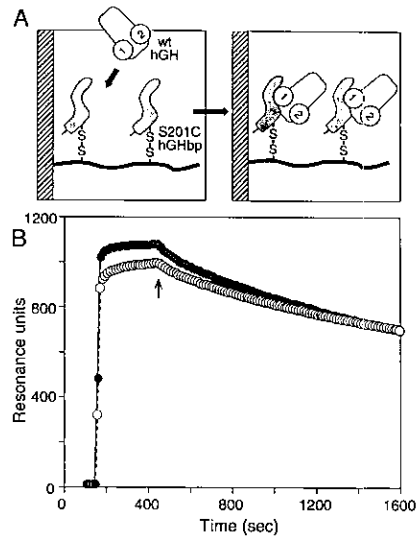

Figure 2. Reaction (A) and kinetics (B) for binding of hGH (open symbols) or the (G120R)hGH (filled symbols) to the (S201C)hGHbp coupled to the BIAcore™ biosensor. The (S201C)hGHbp was immobilized at a level of 1480 RUs (1·48 ng/mm^2) on the biosensor as described in Materials and Methods. Binding conditions and profiles are analogous to those in Fig. 1.

receptor and hence have nearly the same on-rates. However, the wild-type hormone bound to the second receptor and thus was extremely slow to dissociate.

We wished to investigate in greater detail the binding of mutants in the site 1 contact epitope alone without the complication of the hGHbp dimerizing on the matrix. According to the X-ray structure of the hGH(hGHbp)$_2$ complex (De Vos *et al.*, 1992) the two hGHbps contact each other at Ser201. Therefore, to block dimerization on the matrix we replaced Ser201 with Cys and attached the S201C variant *via* its single thiol to the activated-thiol matrix (Fig. 2A). Indeed, when saturating levels of hGH were added (Fig. 2B) we calculated a maximum stoichiometry of 0·84 hGH per immobilized (S201C)hGHbp (Table 1). The (G120R)hGH bound with a stoichiometry of 0·94 per (S210C)hGHbp. By proper placement of the thiol-coupling it was possible to orient the hGHbp on the matrix to allow for either 1 : 1 complex formation or 1 : 2 complex formation. Thus, the solution binding properties of hGH for the hGHbp can be reproduced on the BIAcore™. The (G120R)hGH had virtually the same kinetics as hGH on the (S210C)hGHbp-matrix and the same as that of (G120R)hGH on the (S237C)hGHbp-matrix (Table 1). Together these data indicate that the (S201C)hGHbp-matrix is a reliable means of testing variants of hGH for binding to site 1 alone.

We define a buried side-chain on hGH as one that contains side-chains atoms beyond the β-carbon atoms whose accessibility to solvent changes when

Table 1

Kinetic constants for binding of wild-type (WT) or (G120R)hGH to (S237C)hGHbp or (S201C)hGHbp immobilized on the thiol-matrix of the BIAcor™ biosensor

Hormone	Matrix	Stoichiometry (hormone : hGHbp)	On-rate (s^{-1} M^{-1})	Off-rate (s^{-1})	K_d (nM)
WT	(S237C)hGHbp	0·40	$4{\cdot}0 \times 10^5$	$<1{\cdot}0 \times 10^{-5}$	ND
G120R	(S237C)hGHbp	0·70	$2{\cdot}6 \times 10^5$	$4{\cdot}3 \times 10^{-4}$	1·6
WT	(S201C)hGHbp	0·84	$3{\cdot}0 \times 10^5$	$2{\cdot}7 \times 10^{-4}$	0·9
G120R	(S201C)hGHbp	0·92	$1{\cdot}4 \times 10^5$	$3{\cdot}7 \times 10^{-4}$	2·7

On-rate and off-rate profiles were measured at 25°C and analyzed for hGH and (G120R)hGH. Average standard errors for on-rate, off-rate and affinities on the same biosensor chip are 12%, 11% and 16% of the value reported, as described in Materials and Methods. Stoichiometries of binding were calculated from data in Figs 1B and 2B according to the following formula:

$$\left(\frac{RU_{(max)}\ \text{hormone}}{RU_{(attached)}\ \text{hGHbp}} \times \frac{M_{r\ \text{hGHbp}}}{M_{r\ \text{hormone}}}\right)$$

bound to the hGHbp site 1. Solvent accessibilities were calculated by rolling a 1·4 Å radius probe (Lee & Richards, 1971) over the surface of hGH when free or bound on one hGHbp through site 1. For these calculations we used the X-ray co-ordinate set for the hGH(hGHbp)$_2$ complex (De Vos *et al.*, 1992). By this criterion there are 31 side-chains, all larger than alanine, which are buried to some degree upon complexation (Table 2).

The (S201C)hGHbp-matrix was used to measure the affinities for alanine mutants at 30 of the 31 buried residues in the site 1 interface (Table 2). K41A could not be expressed and so was not tested. We have previously used an RIA to measure the binding constants for many of these mutants (Cunningham & Wells, 1989; Cunningham & Wells, 1991; Lowman & Wells, 1993). A plot of the change in free energy relative to wild-type for the alanine mutants

Table 2

Relative on-rates, off-rates and affinities for alanine substitutions at residues in hGH that are buried to varying degrees at the site 1 interface

Site 1 contact residue	Changes in accessible area upon binding† (Å^2)	VDW contacts‡	Changes in kinetics from WT§			ΔΔ*G* (kcal/mol)
			Off-rate	1/on-rate	Off/on	
WT	–	–	(1)	(1)	(1)	(0)
M14	0·5 (0·6)	0	1·2	1	1	+0·1 (+0·5)
H18	23 (63)	24 (hN218)	0·41	1·1	0·44	−0·5 (−0·7)
H21	3·7 (27)	11	1·3	1·0	1·3	+0·2 (+0·3)
Q22	−2 (5·8)	1	0·62	1·1	0·69	−0·2
F25	44 (63)	21	0·47	1·0	0·47	−0·4 (−0·2)
D26	0 (0·1)	0	0·79	0·89	0·7	−0·2 (−0·3)
Q29	4·2 (4·4)	0	0·38	0·97	0·37	−0·6
K41	−2·7 (24)	6 (sE127)	ND	ND	ND	ND (+1·1)
Y42	60 (88)	30	1·2	1·2	1·4	+0·2
L45	−1·6 (44)	7	4·3	1·8	7·9	+1·2 (+1·4)
Q46	53 (88)	16 (hE120)	0·9	1·4	1·2	+0·1 (0)
P48	3·8 (5·1)	4	1·2	1·7	2·0	+0·4
S51	0 (0)	0	1·2	1·4	1·8	+0·3
E56	0·5 (0·9)	0	2·1	0·97	2·0	+0·4 (+0·8)
P61	0 (5·1)	0	7·2	1·1	7·7	+1·2
S62	1·8 (14)	1 (hS102)	1·6	0·8	1·3	+0·1
N63	7·1 (17)	2	1·2	1·4	1·7	+0·3 (+0·7)
R64	57 (101)	24 (sD164, sE44)	7·9	2·1	16	+1·6 (+1·8)
E65	3·3 (3·3)	0	0·69	0·66	0·45	−0·5 (−0·3)
Q68	6·4 (26)	2	3·3	0·8	2·7	+0·6 (+1·0)
Y164	−5·7 (24)	4	2·1	0·9	1·8	+0·3 (+0·8)
R167	5·9 (32)	8 (sE127)	0·49	3·3	1·6	+0·3 (−0·2)
K168	15 (60)	12 (hW104mc)	0·64	1·2	0·77	−0·2 (+0·1)
D171	19 (50)	16 (sR43)	4·6	0·83	3·8	+0·8 (+1·2)
K172	−6·5 (27)	15	20	1·5	30	+2·0 (+1·6)
E174	17 (25)	4 (hN218)	0·33	0·61	0·21	−0·9 (−0·9)
T175	−2·1 (47)	9 (hR43)	25	1·0	25	+2·0
F176	−14 (5·8)	4	22	1·1	2	+1·9 (+1·6)
R178	41 (70)	8 (hI165mc, hM170mc)	24	2·5	60	+2·4 (+2·4)
I179	−10 (26)	9	2·9	1·3	3·9	+0·8 (+0·6)
R183	1·2 (1·5)	0	1·4	1·8	2·5	+0·5 (+0·4)
E186	3·4 (5·6)	0	0·97	1·0	0·98	0 (−0·1)

Rate measurements were made using the hGHbp(S201C) matrix at 25°C as described for Table 1. Preparation of the mutants and their analysis is given in the Materials and Methods.

† Accessible surface area to a 1·4 Å probe was calculated (Lee & Richards, 1971) for each side-chain in the wild-type (WT) hormone and for wild-type lacking atoms beyond the β-carbon (to mimic the alanine mutant), and for their corresponding complexes with the hGHbp using X-ray coordinates (De Vos *et al.*, 1992). The change in area buried attributed to the alanine mutation is the difference in accessible area of $(\text{free-bound})_{WT} - (\text{free-bound})_{Ala}$. We only used the area buried beyond the β-carbon because this is the portion of the side-chain removed upon alanine substitution. Shown in parentheses is the area of each side-chain for atoms beyond the β-carbon in hGH that becomes inaccessible to solvent once the receptor binds.

‡ Total number of van der Waals' (VDW) contacts is the number of receptor atoms within 4·4 Å of any atom beyond the β-carbon of the contact side-chain, based on inspection of the hGH(hGHbp)$_2$ complex. Over 80% of the contact distances are 3·8 to 4·2 Å. Groups making hydrogen bonds (h) or salt-bridge(s) are determined by donor–acceptor or complementary charge pairs within 3·3 Å of each other between hGH and the hGHbp. For example, hN218 next to H18 indicates a H-bond between H18 on hGH and N218 of the hGHbp. mc indicates an H-bond to a main-chain amide.

§ The relative change in off-rate was calculated from $\frac{k_{off}\ \text{WT}}{k_{off}\ \text{Ala mut}}$ and for 1/on-rate by $\frac{k_{on}\ \text{Ala mut}}{k_{on}\ \text{WT}}$. The change in K_d from wild-type (WT) was calculated as $\frac{K_d(\text{Ala mut})}{K_d(\text{WT})} = \frac{k_{off}/k_{on}\ (\text{mut})}{k_{off}/k_{on}\ (\text{WT})}$.

‖ The ΔΔ*G* values were calculated as $+\text{RT ln}\ \frac{K_d(\text{Ala mut})}{K_d(\text{WT})}$ from BIAcore™ data or, in parentheses, from radioimmunoassay data that were reported previously (Cunningham & Wells, 1989; 1991; Lowman & Wells, 1993).

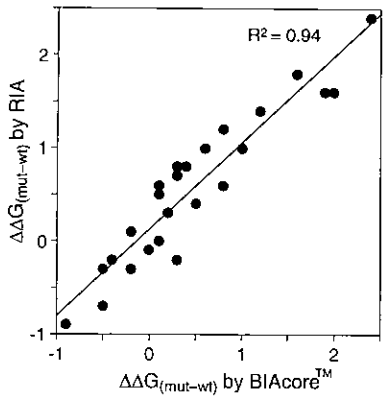

Figure 3. Correlation between the change in the free energy of binding ($\Delta\Delta G_{(mut-wt)}$) calculated for alanine mutants of hGH relative to wild-type hGH when forming a 1 : 1 complex with the hGHbp from data obtained by RIA (y-axis) or BIAcore™ (x-axis). Values were taken from Table 2.

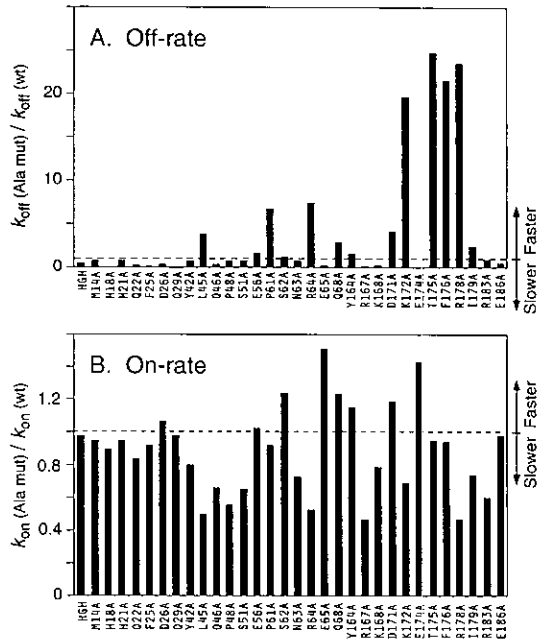

Figure 4. Relative change in off-rate (A) or on-rate (B) for alanine mutants at contact residues. Data taken from Table 2.

calculated by RIA data *versus* BIAcore™ data shows a tight correlation ($R^2 = 0{\cdot}94$) with a slope near unity and an intercept close to zero (Fig. 3). Thus, the affinity data acquired on the biosensor matrix closely match those measured in solution by the RIA. This indicates the matrix is not causing systematic binding artifacts. The average standard errors in affinity constant is ~20% for using the BIAcore™ versus ~30% for the RIA (see Materials and Methods). It is also possible some dimerization of the hGHbp can occur in the RIA, which would lead to systematic errors in affinities; this is prevented using the (S201C)hGHbp-matrix.

Of the 31 buried side-chains, only eight (K41, L45, P61, R64, K172, T175, F176 and R178) are needed to account for ~85% of the total change in binding free energy resulting from the alanine substitutions. Although we could not test K41A because of expression difficulties, K41Q was expressed and caused a +1·1 kcal/mol reduction in affinity as measured by RIA (Lowman & Wells, 1993). Another six side-chains (P48, E56, Q68, D171, I179 and R183) can essentially account for the remainder (Table 2). Eleven other side-chains (M14, H21, Q22, D26, Q46, S62, N63, Y164, R167, K168 and E186) have essentially no effect on overall affinity (each causing less than 2-fold reduction in affinity). Five side-chains (H18, F25, Q29, E65 and E174) actually hinder binding because when they are converted to alanine we see enhancements in affinity of 2 to 5-fold. The sum of the reductions in free energies caused by the alanine substitutions and K41Q (−15·3 kcal/mol) is comparable to the total free energy of binding between hGH and the hGHbp (−12·3 kcal/mol) measured by the BIAcore™.

The off-rate effects are much larger than the on-rate effects (Table 2; Fig. 4). Thus, the same residues that most affect affinity account for most of the increase in off-rate. Some cause up to a 25-fold increase in off-rate. The conversion of three Arg side-chains (R64, R167 and R178) to Ala produced the greatest reductions in on-rate, but only ~2-fold. The conversion of two Glu side-chains (E65 and E174) to Ala caused the greatest increases in on-rate (nearly 2-fold improved). This suggests that electrostatic interactions are the most important side-chain determinants in guiding the hormone to the receptor.

The side-chains that most affect on-rate are not all the same as those that most affect off-rate (Fig. 4). For example, R167A causes the largest decrease in on-rate but leads to a compensating decrease in off-rate. Many of the alanine mutations at side-chains that dominate the affinity (P61A, K172A, T175A and F176A) have virtually no effect on the association rate. The side-chains that most affect affinity and on-rate are shown mapped upon the structure of hGH in Figure 5 and 6, respectively.

4. Discussion

(a) *The structural epitope is much bigger than the functional epitope*

Our data indicate that only a small set of the buried side-chains at the interface are functionally crucial in binding (Fig. 5). We believe that this is not an artifact of the method of analysis. Firstly, the structure of the 1 to 1 hGH·hGHbp complex has recently been solved (B. de Vos, M. Ultsch & A. Kossiakoff, unpublished results) and the residues buried in site 1 are virtually identical to those seen in site 1 for hGH in the 1 to 2 hGH·(hGHbp)$_2$

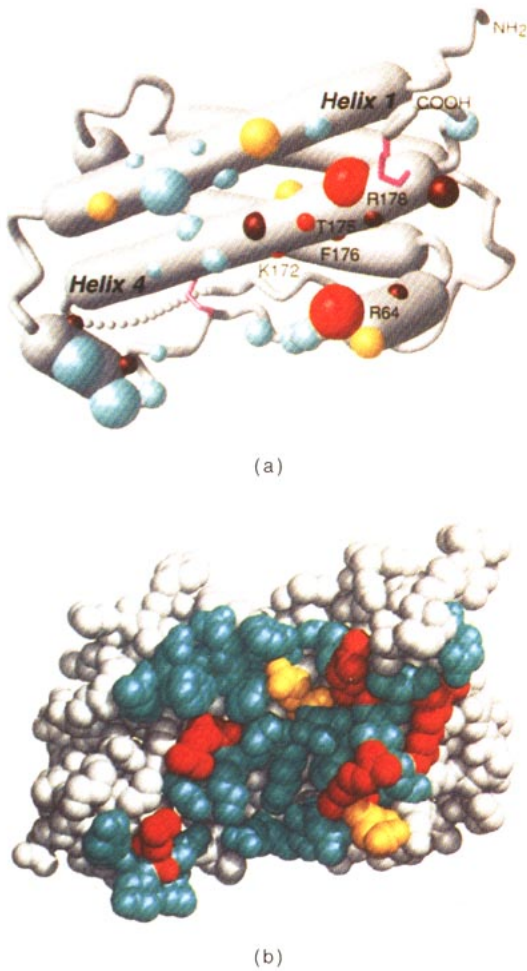

Figure 5. A, The change in area buried at contact residues mapped upon the structure of hGH and colored according to the corresponding effect on binding affinity when residues are converted to alanine. ●, ●, ●, ●, indicate a change in area buried of 40 to 60 Å^2, 20 to 40 Å^2, 0 to 20 Å^2 and −20 to 0 Å^2, respectively, at each residue when converted to alanine upon complex formation (see column 2 in Table 2). The color of the residue indicates its functional impact when converted to alanine. Light aqua residues indicate $\Delta\Delta G_{(mut\ wt)} = -0{\cdot}49$ to $-0{\cdot}49$ kcal/mol; bright red to dull red indicate $\Delta\Delta G_{(mut\ wt)} = 2{\cdot}5$ to 1·5 kcal/mol, and 1·5 to 0·5 kcal/mol, respectively; yellow residues indicate $\Delta\Delta G_{(mut\ wt)} = -1$ to $-0{\cdot}5$ kcal/mol (see column 7 in Table 2). B, A space-filling model of the hormone with residues colored (as in A) according to their impact on binding when converted to alanine. Result for K41Q is based on RIA measurement (Lowman & Wells, 1993).

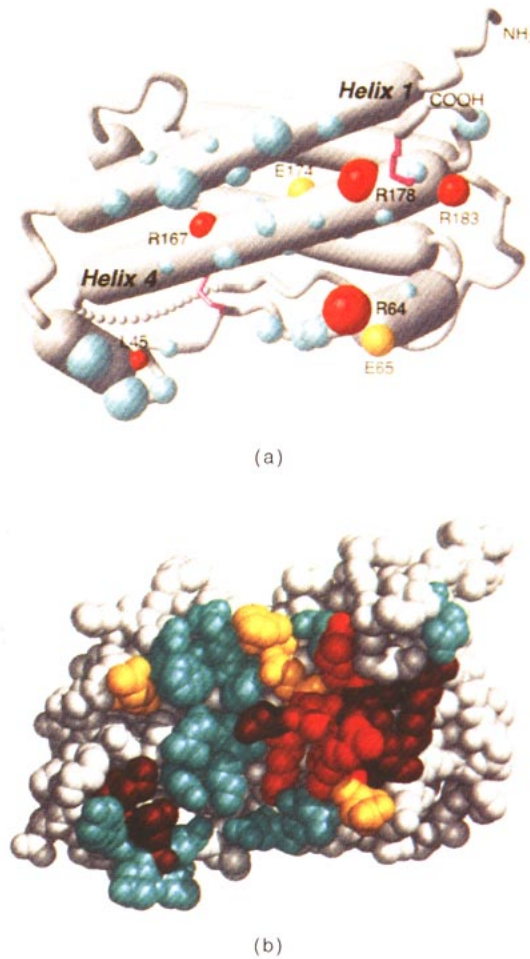

Figure 6. A, The change in buried surface area for residues as shown in Fig. 5A but colored according to the effects alanine substitutions have on on-rate. Bright red residues indicate a ≥1·8-fold reduction on-rate and yellow indicates ≥1·3-fold increase in on-rate for alanine substitutions. B, A space-filling model of the hormone with residues colored (as in A) according to their impact on association rate when converted to alanine. Data are taken from Table 2. Result for K41Q are not shown because kinetics constants were not measured.

complex (De Vos *et al.*, 1992). Thus, the fact that the structural epitope is much smaller than the functional epitope is not because of contact differences in binding in the 1 to 1 *versus* the 1 to 2 complex (which is the coordinate set used to define the contact epitope).

Secondly, analysis of the functional importance of any side-chain by mutational study has the caveat that the mutant protein may exaggerate the effect by imposing a structural disturbance or an unusual steric, electrostatic or hydrophobic interaction. We feel that systematic replacements of side-chains with alanine is least disruptive to the structure (for review, see Wells, 1991). The alanine mutation is the simplest to interpret because it removes atoms without introducing new ones that may create additional unfavorable or favorable interactions. We presume that in some cases solvent occupies the place of the missing side-chain, but we cannot be sure without additional structural studies of the mutated complex. Nonetheless, the finding that only a small set of the alanine substitutions at residues buried at the interface disrupt binding affinity significantly suggests these are not causing large structural effects. We also find that the sum of all the disruptive effects caused by the alanine substitutions plus K41Q (−15·3 kcal/mol) does not

dramatically exaggerate the total binding free energy (−12·3 kcal/mol). This suggests that the effects of the mutations are localized to the individual binding determinants and do not grossly change the whole protein structure or the mode of binding. Given the large number of contact residues it also is unlikely that single alanine substitutions would change the mode of binding in the complex. This is supported by the fact that a number of double alanine substitutions have additive effects on binding (Cunningham & Wells, 1991), indicating that the sites act independently.

We have identified some alanine mutations that affect affinity, which are buried in the hormone and do not become further buried when the receptor binds (Cunningham & Wells, 1989). For example, P5A, L6A, F10A and V185A each disrupt affinity by 2 to 4-fold. Each of these side-chains makes contacts between helix 1 and helix 4, which dominate the site 1 epitope but are not directly involved in binding. Similarly, F54 and I58 disrupt affinity and are buried in the loop region that positions the second mini-helix. This mini-helix contains R64 and other important binding determinants. Thus, some minor effects on binding can result from structural perturbations that are propagated from alanine mutations near but not at the structural epitope. Even so, the vast majority of residues tested away from the site 1 structural epitope have no detectable effect on binding when converted to alanine (Cunningham & Wells, 1989).

The mutational data show only eight of 31 side-chains buried at the interface account for ~85% of the binding energy. Virtually all of the rest can be accounted for by six other side-chains. We have tried to correlate a number of structural parameters that may explain why some residues are critical for binding and others are not. We find the residues important for binding cluster in a small region near the center of the structural epitope (mostly toward the end of helix 4) as shown in Figure 5. The functionally "null" contact residues tend to be near the periphery, in the center of helix 1 and the beginning of helix 4. This is a region that is critical for binding of hGH to the hPRL receptor (Cunningham & Wells, 1991) and for forming a $(Zn^{2+} \cdot hGH)_2$ storage complex (Cunningham *et al.*, 1990). Thus, while this area has little apparent role in binding to the hGH receptor it does have other important functions.

Other systematic structural correlations are less obvious. Chothia & Janin (1975) found that a change in buried surface area generally correlated with the free energy of association between two proteins. We calculated the change in buried surface area that would occur upon complex formation for each of the alanine mutants. This was done by calculating the difference in accessibility in the free and bound states between hGH and the alanine mutant (Table 2). However, a plot of the change in buried surface area upon binding *versus* the change in the free energy of binding when the side-chain is converted to alanine gives a very poor correlation

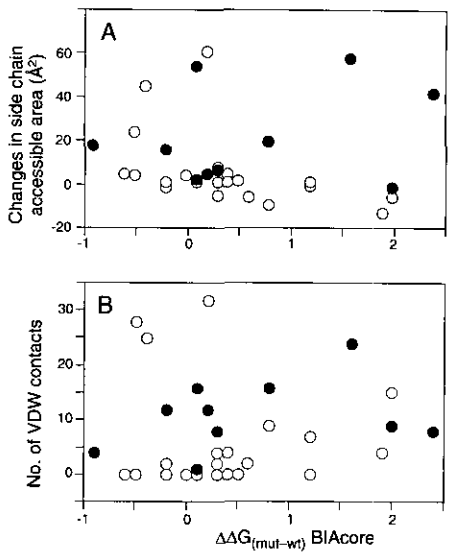

Figure 7. Relationship between the change in binding affinity upon alanine substitution and the change in buried surface area (Å²; A) or number of van der Waals' (VDW) contacts (B) for atoms in contact side-chains beyond the β-carbon. Filled circles show residues buried at the interface that make hydrogen bonds or salt bridges with the receptor at site 1, and open circles show residues that do not. Data are plotted from Table 2.

(Fig. 7A). In some cases negative values for change in accessibility were obtained. This is because the missing side-chain in the alanine mutant creates a cavity at the interface, and hence more surface area would be covered upon complex formation. We also calculated the change in side-chain accessibility that occurs upon binding for atoms beyond the β-carbon, which was our criterion for defining buried side-chains (see values in parentheses in column 2 of Table 2). Yet a plot of these values *versus* the change in free energy gives no better correlation (not shown). A plot of the number of van der Waals' contacts made by atoms of hGH beyond the beta-carbon *versus* the change in affinity when the side-chain is converted to alanine (Fig. 7B) does not show a good correlation either. Neither correlation improves by considering separately the side-chains that are capable of electrostatic interactions.

Horton & Lewis (1992) were able to predict affinities for 15 different protein–protein pairs using a semi-empirical method based on buried surface area and functional scaling of atomic solvation parameters (Eisenberg & McLachlan, 1986) for the contact side-chains. We therefore evaluated how well these scaled atomic solvation parameters can predict the free-energy changes resulting from the individual alanine substitutions. Again we found that there is little correlation (not shown). Thus, while buried surface area, number of van der Waals' contacts and scaled atomic solvation calculations are useful correlates for general binding affinity, we find that these are poor predictors of the role of individual side-chains in this epitope. Perhaps a

great deal of microscopic heterogeneity exists over the surface, so the chemical environment of any residue varies widely. This would tend to average out when the whole surface is considered but not when evaluating individual contacts.

On average the energetics for electrostatic interactions are considerably weaker than estimates made from mutagenesis of enzyme–substrate complexes. From mutational analysis of tyrosyl-tRNA synthetase, it was estimated that the free energy loss for disrupting a charged hydrogen-bond pair is 3·5 to 5 kcal/mol and for a neutral pair is 0·5 to 1·5 kcal/mol (Fersht *et al.*, 1985). Seven side-chains from hGH form hydrogen-bonds with the hGHbp (H18, Q46, S62, K168, E174, T175 and R178). Five of these are charged hydrogen bonds (Q46, K168, E174, T175 and R178), yet the change in binding free energy when they are converted to alanine is only +0·1, −0·2, −0·9, +2·0 and +2·0 kcal/mol, respectively, giving an average value of +0·6 kcal/mol. The change in affinity for mutating the two neutral hydrogen-bonding side-chains (H18 and S62) is only −0·5 and +0·1, respectively. Four other side-chains form salt-bridges with the hGHbp (K41, R64, R167 and D171), yet these cause reductions of only +1·1, +1·6, +0·3 and +0·8 kcal/mol, respectively. These values are less than those estimated for two engineered salt bridges in subtilisin, which range from +1·8 to +2·3 kcal/mol (Wells *et al.*, 1987). Thus, the strength of the contacts varies widely in the hGH·hGHbp interface and the interactions are considerably weaker when compared with those of small molecule binding sites.

From mutational studies of protein interiors it has been estimated that each buried methylene group contributes −1·0 to −1·5 kcal/mol to the overall free-energy of folding (for recent review, see Shortle, 1992, and references therein). Converting a number of hydrophobic side-chains in hGH to alanine caused effects that were very much weaker than would be expected from these studies. For example, the largest effects we see for mutations at hydrophobic side-chains are for L45A, K172A (only the aliphatic portion makes contact with the receptor), F176A and I179A, which cause reductions in affinity of +1·2, +2·0, +1·9 and +0·8 kcal/mol, respectively. Moreover, several other hydrophobic groups that are more highly or comparably buried upon complex formation (F25, Y42 and Y164) have almost no effect when mutated to alanine.

Virutally all of the contacts from hGH to the hGHbp in site 1 are from side-chains (De Vos *et al.*, 1992). Yet it appears that the energetic importance of these contacts is much lower than those between the enzyme and substrate pairs or within a tightly packed protein interior. Perhaps only a few good and/or many weak contacts are required to produce a binding free energy of −12·3 kcal/mol because the hormone–receptor interface is so large (~1300 Å^2 buried in the site 1 interface of hGH; De Vos *et al.*, 1992). Indeed, mutagenesis of the hGH·hGHbp interface by alanine-scanning (Cunningham & Wells, 1989) or phage display (Lowman *et al.*, 1991) can isolate mutants with much higher affinity, indicating that the interface is not optimized for binding to the hGH receptor.

Binding affinity is the difference in free energy between the bound and free states. It is possible that the energy required to desolvate a functionally silent residue is as costly as the free energy gained upon its binding. It is also possible that the interface between hGH and the hGHbp is poorly packed so that inter-residue contacts, bond geometries and bond angles are less optimized for residues that do not affect binding when converted to alanine. At present we cannot evaluate packing imperfections accurately because in the current structure (R-factor of 19% and resolution of 2·8 Å; De Vos *et al.*, 1992) side-chain torsional angles and contact distances are not defined to high enough precision. It is also possible that regions of the hormone that are functionally inert for affinity may be disordered in the free hormone, so that much of the energy of binding is used in freezing them in the bound form. The functionally silent residues at the interface could be making good interactions with other side-chains on hGH that would need to be broken to interact with the receptor. Further structural analysis of the free hormone and receptor as well as higher resolution structures of the complex should help to clarify some of these points.

Several reports have suggested that only a small number of contact residues may be crucial for binding between an antibody and antigen. Novotny and co-workers (1989) made semi-quantitative estimates of binding free-energies for residues in known antibody–protein complexes. They predicted that only a few of the 15 contact side-chain residues would be critical for binding. Alanine-scanning to define the functional epitopes for binding of monoclonal antibodies (mAbs) to hGH showed that on average only three to four side-chains dominate the binding affinity (Jin *et al.*, 1992); however, structures of these complexes were not available. Similar conclusions were reached from alanine-scanning of residues involved in binding an antibody to the Her 2 receptor (Kelley & O'Connell, 1993). Recently, Nuss *et al.* (1993) produced some natural and site-directed mutations at some of the contact residues between neuraminidase and NC41 mAb. They found that replacements at three sites completely blocked mAb binding whereas substitutions at other contacts had much less impact. The alanine-scanning mutagenesis studies presented here corroborate those above and show clearly that only a small set of the residues within a large and defined structural epitope are functionally critical for binding.

(b) *Association is modulated partially by electrostatic interactions*

Generally, the alanine replacements have much larger effects on off-rate than on-rate. The fact that single side-chain replacements cause only small

changes in association rate suggests there are many paths leading to association. The fact that the alanine replacements at specific residues can dramatically affect off-rates suggests that these interactions maintain the complex. Thus, there appear to be many weak collisional complexes and only one (or a few) tightly bound complex(es). In these respects the association mechanism may be similar to ones proposed for protein folding (Ptitsyn *et al.*, 1990; Kuwajima, 1989; Jeng & Englander, 1991); that is, there is an initial weak and fairly non-specific complex that undergoes further desolvation and cooperative rearrangement to yield a more compact and specific structure. We look upon the binding reaction as a multistep process.

The associations rate constant for hGH (3×10^5 s^{-1} M^{-1}) is comparable to many other protein–protein complexes (for review, see Berg & von Hippel, 1985). The rate is nearly 10,000-fold slower than the diffusion limit ($\sim 7 \times 10^9$ s^{-1} M^{-1}) but about 1000-fold faster than the rate expected if the hormone needed to collide within 2 Å of the binding site on the hGHbp ($\sim 7 \times 10^2$ s^{-1} M^{-1}; Northrup & Erickson, 1992). Northrup & Erickson (1992) suggested that much of the 1000-fold increases in rate, beyond that expected when strict orientation factors are considered, derives from random Brownian dynamics of two proteins when they collide.

Our data indicate that specific electrostatic interactions are also important in guiding hormone–receptor association. Generally, the largest reductions in on-rate occur for substitutions at specific Arg residues (R64, R167, R178 and R183; Fig. 5B). The biggest increases in on-rate occur for alanine replacements of nearby Glu residues (E65 and E174). From the summation of the alanine substitutions at R64, R167, R178 and R183 we estimate that the total electrostatic effect contributes a factor of ~20 to the association rate. These are not general electrostatic effects because a number of other charged groups within the site 1 epitope (D26, E56, K168, K172 and E186) or outside the epitope (G120R) have little or no effect on on-rate when mutated. The importance of positively charged groups in hGH in modulating affinity complements alanine-scanning studies of the hGHbp, which shows that negatively charged groups on the receptor affect affinity (Bass *et al.*, 1991). In addition to the electrostatic effects, we find that systematic reductions in on-rate occur for residues in the first mini-helix of hGH (L46, Q46 and P48), suggesting that this structure is recognized early in association.

Understanding the basis for protein–protein association is critical for understanding molecular recognition processes and for rational protein and drug design. The fact that only a small set of contact residues may be functionally important for association and dissociation indicates that high-resolution functional analysis together with structural analysis are necessary to give a realistic picture of a binding epitope. The observation that the functional epitope is much smaller than the structural epitope offers the possibility of mimicking it using smaller molecules.

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