

Shotgun Alanine Scanning Shows That Growth Hormone Can Bind Productively to Its Receptor through a Drastically Minimized Interface*

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Jean-Louis K. Kouadio^{‡§}, James R. Horn^{†¶}, Gabor Pal^{‡||}, and Anthony A. Kossiakoff^{‡***‡‡}

From the [‡]Department of Biochemistry and Molecular Biology and the ^{**}Institute for Biophysical Dynamics, the University of Chicago, Chicago, Illinois 60637

The high affinity binding site (Site1) of the human growth hormone (hGH) binds to its cognate receptor (hGHR) via a concave surface patch containing about 35 residues. Using 167 sequences from a shotgun alanine scanning analysis of Site1, we have determined that over half of these residues can be simultaneously changed to an alanine or a non-isosteric amino acid while still retaining a high affinity interaction. Among these hGH variants the distribution of the mutation is highly variable throughout the interface, although helix 4 is more conserved than the other binding elements. Kinetic and thermodynamic analyses were performed on 11 representative hGH Site1 variants that contained 14–20 mutations. Generally, the tightest binding variants showed similar associated rate constants (k_{on}) as the wild-type (*wt*) hormone, indicating that their binding proceeds through a similar transition state intermediate. However, calorimetric analyses indicate very different thermodynamic partitioning: *wt*-hGH binding exhibits favorable enthalpy and entropy contributions, whereas the variants display highly favorable enthalpy and highly unfavorable entropy contributions. The heat capacities (ΔC_p) on binding measured for *wt*-hGH and its variants are significantly larger than normally seen for typical protein-protein interactions, suggesting large conformational or solvation effects. The multiple Site1 mutations are shown to indirectly affect binding of the second receptor at Site2 through an allosteric mechanism. We show that the stability of the ternary hormone-receptor complex reflects the affinity of the Site2 binding and is surprisingly exempt from changes in Site1 affinity, directly demonstrating that dissociation of the active signaling complex is a stepwise process.

The sophisticated sets of protein-protein interactions that govern the regulation of many essential biological functions are

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|| Present address: Dept. of Biochemistry, Eotvos University, 1/C Pazmany P. Street, Budapest, Hungary.

‡‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology and Institute for Biophysical Dynamics, University of Chicago, Cummings Life Science Center, 920 East 58th St., Chicago, IL 60637. Tel.: 773-702-9257; Fax: 773-834-2777; E-mail: koss@cummings.uchicago.edu.

features that have been highly refined through an extensive and dynamic evolutionary process. The class of molecules consisting of polypeptide hormones and their cognate receptors is one example of a highly refined co-evolutionary process (1, 2). In particular, the growth hormone-prolactin system of hormones and receptors is a specific illustration of how related molecules have simultaneously evolved both overlapping and independent specificities, as well as overlapping and independent functions (3).

Extensive functional analyses of the hormone-receptor binding interfaces of human growth hormone (hGH)¹ and human prolactin, using site-directed alanine-scanning mutagenesis, have indicated that molecular specificity and binding affinity of these molecules are driven by a relatively few key residues (4–6). In most cases, the residues essential for binding affinity do not overlap with those that confer specificity and cross-reactivity (7, 8). An important physical characteristic of these traits is that the binding determinants are generally organized in a spatially clustered region, whereas specificity determinants are likely to be charged residues that can be spread throughout the binding surface (3, 9).

Although the site-directed alanine scanning method has proven to be extremely useful in characterizing large protein-protein interactions like the hGH-human prolactin system, in practice this type of analysis is quite labor intensive and not amenable to high throughput approaches. In this regard, a new high throughput method called "shotgun alanine scanning" has proven to be very powerful (10–13). This phage display mutagenesis method is based on a combinatorial strategy where at every position to be analyzed, either the wild type (*wt*) residue or an alanine is introduced into the phage display library. After several rounds of binding selection, DNA sequencing is used to determine the *wt*/Ala ratio at each randomized position. It has been demonstrated that these ratios can be used to calculate the energetics ($\Delta\Delta G$) of each analyzed side chain to the binding interaction at a level comparable to the conventional Ala-scanning method (10, 13).

This technology made it possible for us, using a set of simple phage display selection experiments, to determine the functional binding epitope of a variant of hGH (hGHv) binding through its high affinity site (Site1) to the extracellular domain (ECD) of the hGH receptor (hGHR) (13). This analysis was based on the compilation of the *wt*/Ala ratios from 167 unique input sequences, and ratios were determined for the 35 residues contained in the hGHv Site1 interface (13). Because of the

¹ The abbreviations used are: hGH, human growth hormone; hGHv, high affinity variant of hGH; hGHR, human growth hormone receptor; ECD, extracellular domain; ITC, isothermal titration calorimetry; SPR, surface plasmon resonance; SG, shotgun; *wt*, wild-type.

inherent degeneracy of the “shotgun” Ala-scanning codons used in the analysis, in several instances another amino acid type besides Ala was introduced into the sequence. However, in most cases this amino acid type was non-isosteric with the *wt* residue and could be grouped with the Ala substitutions when assessing the functional importance of the *wt* residue without affecting the results (13).

We had originally assumed that about 5–10 such substitutions might be tolerated without extensively reducing binding to hGHR. However, an examination of the amino acid composition of the sequenced variants from the shotgun analysis led to the surprising finding that many of the binding-selected variants actually contained ~50% of Ala (or non-*wt*) residues among the 35 residues intimately involved in the Site1 binding interface. To our knowledge, these variants represent as a group the largest wholesale introduction of alanine-like substitutions into a binding epitope while still keeping a high affinity interaction. We note that, although our analysis based on 167 sequences produces a good overview of the variety in number and distribution of possible alanine-like substitutions, we would expect that a fully comprehensive analysis of the shotgun data could identify many more unique variants that bind to the wild-type receptor target with reasonably high affinity.

Presumably, the high percentage of substitutions among the residues that are known to play an integral role in the interaction result in a structurally compromised binding interface. Therefore, we decided to investigate how such a high percentage of the functional binding interface could be eliminated, while still preserving substantial affinity to hGHR. Are there definitive patterns for how the mutations are spatially organized? Are they organized in a few groups or are they distributed throughout the interface? Are there regions of the interface that remain highly conserved with the *wt* residues? How are the thermodynamics of binding affected by the extensively perturbed binding interface?

To address these issues and others we describe here a detailed binding analysis based on eleven hyper-mutated hGH variants isolated from the shotgun Ala-scanning analysis of a high affinity Site1 variant of hGH (hGHv). These eleven variants, referred to here as shotgun (*SG*) variants, contain 14–20 substitutions in the binding interface and have binding affinities between 1 and 100 nM.

We find that the substituted residues in the *SG* variants are distributed throughout the Site1 interface with the exception of helix-4, which is more highly conserved. Overall, we find that the binding affinities of the *SG* variants do not track with decreased surface burial or loss of H-bonds and do not appear to follow other general trends associated with kinetic and thermodynamic properties for protein-protein interactions. The measured binding thermodynamics indicate that correlations between ΔS and the hydrophobic effect and conformational dynamics deviate from some of those reported for other systems (14). In fact, the large ΔC_p values determined for the variants cannot be easily rationalized using any of the physical properties generally ascribed to producing ΔC_p values of the magnitudes observed.

Additionally, we find that most of the measured k_{on} rates of the *wt*/Ala variants are generally the same as that measured for the *wt* interaction, suggesting that features such as electrostatic attraction and conformational changes required for binding are similar among the variants, even though the binding surfaces of the variants are highly modified compared with *wt*-hGH. Furthermore, we show that the Site1 mutations produce an allosteric effect on the binding energetics of the second receptor at Site2, and that the stability of the active 1 hGH-2 hGHR ternary complex is dependent on the affinity of Site2 binding, not Site1.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were from Qiagen (Alameda, CA). Plasmids harboring recombinant hGHv, *wt*-hGH, and the truncated hGHR ECD (amino acid residues 29–238) were from Genentech (San Francisco, CA). The dNTP mix was from Amersham Biosciences; XL1-blue and BL21 were from Stratagene. T7 polymerase, T4 ligase, T4 polynucleotide kinase were from New England Biolabs. HEPES, *N*-hydroxysuccinimide, and *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide were from Sigma. 2-(2-Pyridinyldithio)ethaneamine hydrochloride was from Biacore.

Sample Preparation—Mutants were made in the hGHv background using Kunkel mutagenesis (15) or QuikChange PCR (Stratagene). Desired mutations were confirmed by DNA sequencing. For the kinetic measurements, all proteins were expressed in the periplasm of *Escherichia coli* BL21 as described previously (16). BL21-expressing hGHv and the shotgun variants were grown at 20 °C, whereas BL21-expressing hGHR ECD (29–238) and *wt*-hGH were grown at 29 °C. Periplasmic expressed proteins were then obtained by osmotic lysis, precipitated with 45% ammonium sulfate, desalted, and subjected to fast protein liquid chromatography purification on anion exchange resource Q (Amersham Biosciences), followed by size exclusion chromatography on a Superdex 75 HR 10/30. The hGHR ECD was purified on an hGH affinity column. Purity of proteins was assessed by analytical high-performance liquid chromatography, and the molecular weight of the mutants was confirmed by electro-spray mass spectrometry. Sample purity was usually >90%. Protein concentrations were determined by using molar extension coefficients at 280 nm of 16.2 mm⁻¹·cm⁻¹ for hGH and 55 mm⁻¹·cm⁻¹ for hGHR ECD. For the shotgun mutants, molar extension coefficients at 280 nm were adjusted as described by Demchenko *et al.* (17).

Surface Plasmon Resonance—Purified proteins were used for surface plasmon resonance (SPR) measurement on a Pioneer C1 sensor chip using a Biacore 2000 instrument (Biacore, Piscataway, NJ) at 25 °C as described by Walsh *et al.* (18). The C1 sensor chip was first washed with 15 μl of 100 mM glycine-NaOH, 0.3% Triton X-100, pH 12.0, solution, then the surface was activated at a flow rate of 5 μl/min with 25 μl of *N*-hydroxysuccinimide:*N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (75 mg/ml:11.5 mg/ml) and 40 μl of 2-(2-pyridinyldithio)ethaneamine hydrochloride (18 mg/ml). S237C-hGHR ECD (50–100 ng/ml) was coupled to the activated surface through its engineered cysteine near the C terminus. Uncoupled functional groups were blocked with 30 μl of 50 mM reduced glutathione in 50 mM sodium acetate, 1 M NaCl, pH 4.5. Sensor chip coupling and kinetic analysis were performed in HBS buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20, pH 7.4).

All SPR experiments were carried out at a flow rate of 50 μl/min. The surface of the chip was regenerated with 5 μl of 4.5 M MgCl₂. The association (k_{on}) and dissociation (k_{off}) rate constants for Site1 binding were determined by using the 1:1 Langmuir model from the BIAevaluation program (Biacore). For Site2 binding by the second receptor (hGHR ECD2), the kinetic parameters were determined by a Trimolecular surface plasmon resonance (TM-SPR) method (19) using the decaying surface model in the CLAMP (20) program. For each protein, reported kinetics data were the average of triplicate measurements.

Isothermal Titration Calorimetry—Isothermal titration calorimetry (ITC) experiments were performed using a MicroCal VP-ITC titration calorimeter (MicroCal, LLC, Northampton, MA). The calorimeter was routinely calibrated with 2'-CMP with RNase A titrations. The *wt*-hGH and its variants used for ITC carried a G120R mutation conferring only Site1 binding to the hGHR ECD1 in a 1:1 complex. For titrations of hGH variants with hGHR ECD, either 20 mM HEPES (pH 7.4), 150 mM NaCl, or 20 mM sodium phosphate (pH 7.4), 150 mM NaCl buffers were used. At least two buffers with different enthalpies of ionization were used to assay for the presence of proton linkage. Although the enthalpy of hGHv binding displayed a slight buffer dependence ($n = 0.6$), experiments at pH 6.5 removed this linkage, and the corresponding enthalpy remaining the same as pH 7.4, suggesting that the proton binding equilibrium does not contribute significantly to the observed enthalpy at pH 7.4. The heat capacity (ΔC_p) was determined by performing ITC experiments at 15, 25, and 35 °C. Buffers for experiments run at temperatures other than 25 °C were made so that the appropriate pH was attained at the desired temperature. This was achieved using the known values for ΔH° and ΔC_p for buffer ionization (21). All samples were dialyzed overnight at 4 °C against 4 liters of buffer and were de-gassed prior to use. A typical experiment included 25–30 injections of 10 μl of hGH or variants (20–50 μM) into 1.4 ml of hGHR ECD (2–5 μM). The heats of dilution and viscous mixing were taken into account by subtracting the average of the post-saturation heats. In addition, due

to dilution across the needle during equilibration, the initial injection was set to 1 μ l and was routinely discarded. The single binding site model was used for data fitting in Origin version 7 (MicroCal, LLC) program.

RESULTS

Shotgun Ala Scanning: Library Design and Binding Selections

The sequence data used in this analysis were taken from the previous shotgun Ala-scanning analysis of the high affinity variant of hGH (hGHv) (13). This variant was derived from an affinity maturation process using phage display mutagenesis. hGHv contains 15 mutations in its Site1 binding interface and binds to the hGHR receptor with about 400-fold higher affinity than the wild-type hormone (*wt*-hGH) (22, 23). This analysis was performed using special shotgun Ala-scan codons to vary 35 interface residues; the 15 hGHv mutations and 20 additional positions within the hormone-receptor contact interface (13). The shotgun codons were designed to give either Ala or *wt* for each of the 35 targeted residues in the Site1 interface in the unselected starting library, although the redundancy in the genetic code in some cases produces two other amino acid substitutions (10). We note that Pal *et al.* (13) showed that in most cases the additional amino acid types that might be introduced instead of alanine could be grouped with the Ala substitutions. Thus, the term non-wild type (*non-wt*) is used here to include both Ala and other amino acid types that occur due to the degeneracy of the shotgun codons.

The hGHv phage display libraries were subjected to two independent selection protocols (13). For one of the selections, the hGHR ECD was immobilized on a plate and used as the capture target. The hGHv phage display variants captured in this step formed the basis for determining the *wt*/Ala ratios to calculate the changes in binding free energies (13). In the other independent selection, a monoclonal antibody that recognized an epitope on hGHv outside the area of mutation was used to capture a general population of the displayed library phage. This step quantified the decreases (or increases) in the level of hGHv variants displayed on phage considering the overall pool of *wt*/Ala at each mutated position, either because of expression or stability. Correcting for expression bias is an essential normalization factor that is applied to the results of the hGHR ECD capture to ensure that a certain amino acid type is not over or undercounted at any of the sites of mutation due to an expression or stability bias (10, 13).

Distribution of Non-*wt* Residues in the Site1 Binding Interface

From the shotgun (SG) Ala-scanning library selections produced in the biopanning against the immobilized hGHR ECD, 167 unique clones were isolated and sequenced (13). The term "mutations" is defined as the difference in each of these clones compared with the parental high affinity hGHv molecule instead of the *wt*-hGH sequence. To draw clear distinctions when comparing the sequence of an SG clone to either the hGHv or the native hGH sequence, we have adopted the following nomenclature: *wt_v* to represent the amino acid types based on the hGHv sequence and *wt_N* to represent the amino acid types in the native hGH molecule. It is noteworthy that, although the shotgun Ala-scanning is based on substitutions in the hGHv sequence, there are in most cases so many substitutions overall that it is probably not justified to assign the hGHv sequence as the parental sequence. Rather the extensive substitution of non-homologous residue types in the binding interface of this molecule (and by inference the *wt*-hGH molecule) indicates that tight binding can be accomplished in these 4-helix bundle scaffolds even if the

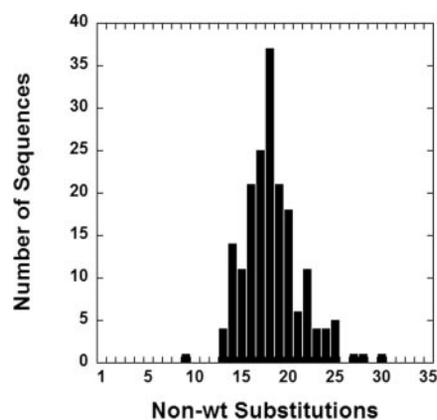


FIG. 1. Distribution of non-*wt* substitutions in SG variants. The distribution of the number of sequences containing a specific number of non-*wt* substitutions was established from the sequences of 167 unique shotgun clones. The three sequences that have more than 25 non-*wt* substitutions are probably an artifact due to nonspecific binding effects.

native binding contacts are significantly compromised.

Fig. 1 shows the composition of *wt_v*/*non-wt_v* ratios in these clones based on the hGHv sequence. The distribution of non-*wt_v* substitutions is reasonably symmetrical centered around 18 non-*wt_v* residue types over 35 positions analyzed by the shotgun Ala-scan of hGHv. In fact, over 70% of the sequenced clones contained between 16 and 20 non-*wt_v* residues types. Of the non-*wt_v* residues, about two-thirds are Ala with the remaining one-third being another amino acid type resulting from the degeneracy of the codons. If these comparisons are made to the native *wt_N* sequence, the number of non-*wt* residues increases typically up to 20–25 non-*wt_N* residues.

If the same analysis is applied to the smaller 15-residue subset of sites that were mutated in the original phage display libraries that produced hGHv (22), the so-called hot-spot residues, again about half the groups (~8) were found on average to be non-*wt_v* residue types. This indicates that even the group of residues most responsible for Site1 binding can be significantly altered, yet still produce a reasonably tight binding molecule.

Binding of Selected SG Sequences

Eleven SG variants were chosen for detailed study to determine the binding characteristics of their highly modified binding interfaces. The selection criteria were such as to ensure a broad sampling of binding affinities and spatial distribution of the mutations. These variants contained 14–20 non-*wt_v* residues in their Site1 binding site (Fig. 2A). The binding kinetics of these variants were determined by surface plasmon resonance (Tables I and II). The measured equilibrium dissociation constants (K_d) range from 1.5 nM, which is close to *wt*-hGH, to ~100 nM for a SG variant that contained 20 non-*wt_v* residues in its sequence. The differences in binding affinities were generally characterized by changes in the k_{off} step (Table I). However, whereas in most previous studies the k_{on} values of mutated *wt*-hGH variants were remarkably similar (24), several of the weaker binding SG variants analyzed here displayed association rate constants that were 5- to 10-fold lower than that observed for the *wt*-hGH hormone (~2- to 3-fold lower than hGHv).

The expected changes in binding free energies ($\Delta\Delta G$) for each of the different variants were calculated from the statistical analysis of the shotgun Ala-scan data from Pal *et al.* (Table I) (13). The calculated changes in $\Delta\Delta G$ for these variants range from 0.3–4.2 kcal/mol. These calculated values track qualitatively with the measured dissociation constant values. For instance, variants SG1, SG2, SG3, and SG4 are predicted to

A

	Helix 1	Mini-Helix 1	Loop	Helix 4
	14 18 21 22 25 26 29	41 42 43 44 45 46 47 48	60 61 62 63 64 65 67 68 69	164 167 168 171 172 174 175 176 178 179 183
wt-hGH	M H H Q F D Q	K Y S F L Q N P	T P S N R E E T Q	Y R K D K E T F R I R
hGHv	W D N Q F D Q	I H S F W W N P	T P S N K E E T Q	Y N K S K S T Y R T R
SG1	W D A A S A Q	I A A A G G A P	A P A N K A E T E	D N K S K S T Y R A R
SG2	S A A Q V A Q	A A A F S W A A	A P S T K A E T Q	Y T E S K A T Y R T R
SG3	W D A Q F A E	A P S A S W N P	T P A D K A E T Q	A A E S K S T Y R A R
SG4	W A T Q V A Q	A A S A W S D P	A P S T K A E T A	S A K S K S T Y R T R
SG5	W D A Q F A A	V A A S G W A P	T P A D K A E T E	A A T S K A T Y R A R
SG6	W D A A A A A	V A S A W G A P	A P A N T A E T Q	Y A A S K S T Y R A G
SG7	W D A Q V D Q	T H A S G W N P	A A A D K A A T Q	Y T K S K S T Y R A R
SG8	S A A E S A A	V P A F S G A A	T P A T K A E T Q	A N K S K S T Y R A A
SG9	W D A Q F A A	T A A F W A N P	A A S D K A A T A	S N E S K S T Y R A R
SG10	S A D Q S D E	V H S F W G A P	T A A N K E E T A	Y A A S K S T Y R A R
SG11	W D A A F A Q	T H S F G W N A	T A A T K E E T A	A A K S K S T S R A R

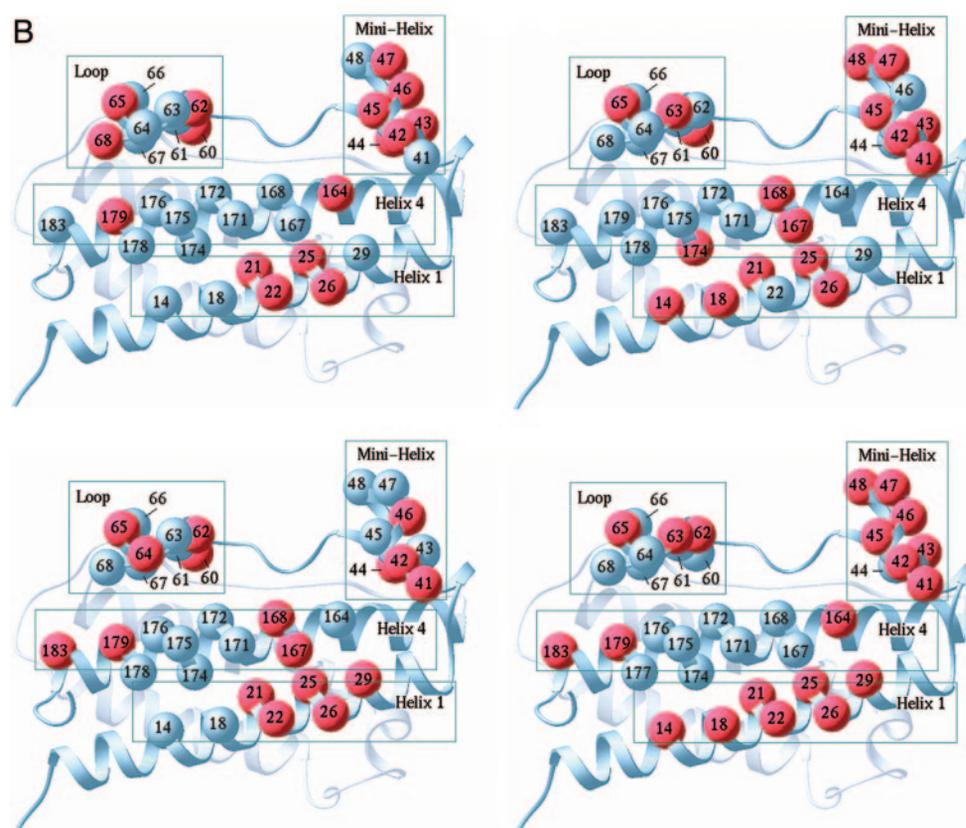


FIG. 2. A, amino acid sequence alignment of residues in the Site1 binding interface of *wt*-hGH, hGHv, and shotgun mutants. Alanine substitutions are in blue, non-*wt* substitutions are in red, unchanged *wt*_v residues are in bold; secondary structure elements that contain the mutations are color-coded. B, spatial distribution of the non-*wt* residues (red) in the binding interface from representative clones (*SG1*, upper left; *SG2*, upper right; *SG6*, lower left; and *SG8*, lower right) superimposed on the ribbon diagram of hGHv. Ribbon figures were generated by using the RIBBONS program (50).

have relatively small changes in $\Delta\Delta G$ compared with *SG6*, *SG8*, *SG10*, and *SG11*, which is what is observed. We note that the calculated changes in $\Delta\Delta G$ assume that the residues contribute to binding in additive fashion, and it has been shown that residues in some regions of the hGHv interface work through cooperativity. Therefore, although a trend is indicated, it should be viewed as very qualitative in nature.

Buried surface area contribution of the 35 amino acids mutated by shotgun alanine scanning was assessed by using the AREAIMOL program on CCP4I version 5.0 (Table I). Overall, the *SG* variants display a net loss of buried surface area. The

decreases in surface area relative to hGHv vary from 64 Å² for *SG11* to 221 Å² for *SG1* and 223 Å² for *SG8*. These changes do not appear to correlate with the respective equilibrium dissociation constants (K_d) for *SG11* (52 nM), *SG1* (1.5 nM), and *SG8* (100 nM). Fig. 3 displays a model of *SG8* and the surface area lost.

Fig. 2B illustrates the spatial distribution of the *wt* and Ala mutation sites in the binding interface. It had been determined in earlier studies with *wt*-hGH that, within the binding epitope, helix-4 residues have a disproportionately important influence on Site1 binding (24). This trend is also recapitulated

TABLE I
Site1 binding of SG variants to the hGHR ECD1

SRP measurements were done in 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20 at 25 °C.

Hormones	k_{on} $M^{-1} s^{-1} \times 10^5$	k_{off} $s^{-1} \times 10^{-4}$	K_d^a nM	Relative K_d mut/wt	$\Delta\Delta G$ kcal/mol	Buried surface area \AA^2
wt-hGH ^b	3.12	3.80	1.2	1.0		
hGHv	1.20	0.002	<0.014	<0.0025		960
SG1	0.50	0.74	1.5	1	0.5	750 (210)
SG2	0.60	1.94	3.3	3	0.7	820 (140)
SG3	0.71	12.30	17.3	14	0.5	860 (100)
SG4	1.70	11.03	6.5	5	0.3	839 (121)
SG5	1.16	24.40	21.0	17	1.6	816 (144)
SG6	0.32	16.90	53.0	44	3.5	838 (122)
SG7	1.06	31.20	30.0	25	2.6	884 (76)
SG8	0.24	25.00	100.0	83	4.0	737 (223)
SG9	0.73	20.80	28.5	24	1.6	885 (75)
SG10	0.15	12.00	80.0	66	3.4	851 (109)
SG11	0.12	6.21	52.0	43	4.2	896 (64)

^a Equilibrium dissociation constant, $K_d = k_{\text{off}}/k_{\text{on}}$.

^b Values were taken from Bernat *et al.* (24). Buried surface areas contributed by the 35 amino acids were calculated by using AREAIMOL on CCP4I version 5.0 (51). Side-chain conformations for the substituted residues in the variants were taken to be the same as those in hGHv. Values in parentheses are the loss in buried surface area relative to hGHv. Changes in binding free energies ($\Delta\Delta G$) were calculated using data from Pal *et al.* (13). SPR measurements of variants SG1, SG2, and SG10 were made with an additional mutation, G120R, which prevents potential dimerization on the analysis chip. The measured K_d values for these variants were essentially identical to those reported in the table indicating the reported values accurately describe the 1:1 interaction.

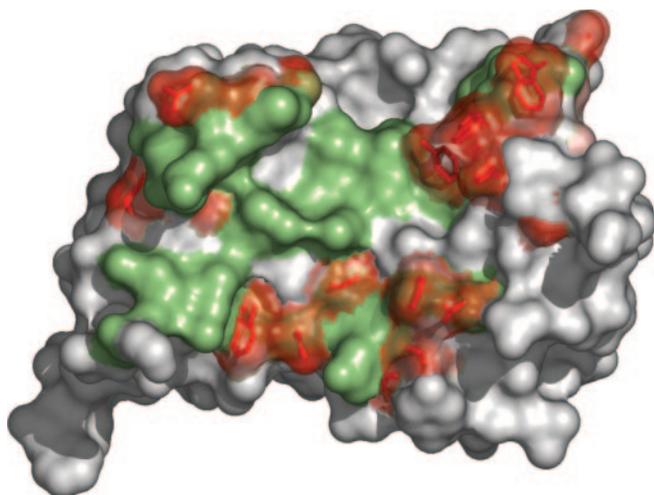


FIG. 3. Surface area representation demonstrating the surface area removed of a SG variant. Accessible surface area of modeled SG8 is shown in white (non-interface residues) and green (interface residues). hGHv residues that are lost are represented in red with the corresponding surface area shown as transparent red.

in Fig. 2, and although there are some non-*wt*_v helix-4 residues in each of the variants, the overall percentage is significantly lower than that found for the other groupings. In particular, the mini-helix grouping shows extensive non-*wt*_v character in most of the variants. In fact, for SG variant 1 (SG1), essentially all the mini-helix side chains that are known to contribute to binding are either Ala or Gly. The character of the mini-helix sequence in this variant would almost ensure that it does not contribute to binding. In other Ala-scanning studies it was found that the mini-helix residues make measurable contributions, especially the two Trp residues at positions 45 and 46 in hGHv that form an extensive van der Waals interaction with a Trp side chain (Trp-72) in the receptor (24, 25). Surprisingly, removing them and making the polypeptide chain presumably much more flexible along with 13 other mutations still allows the hormone to bind with an equivalent affinity as the *wt* hormone.

Thermodynamic Analysis of Binding

Characterization of the Thermodynamic Partitioning in Binding—Isothermal titration calorimetry was performed on

wt-hGH, hGHv, and three SG variants to compare the energetic partitioning of the thermodynamic contributions governing their Site1 binding (Fig. 4). The calorimetry data show that *wt*-hGH Site1 binding is driven by favorable contributions from both ΔH° (−9 kcal/mol) and $-T\Delta S^\circ$ (−3.2 kcal/mol) (Table III). In contrast, the binding of the high affinity variant, hGHv, shows a significant disproportional partitioning of the enthalpy/entropy contributions featuring a highly favorable ΔH component ($\Delta H^\circ = -36$ kcal/mol) with an accompanying large unfavorable $-T\Delta S^\circ$ term (21 kcal/mol). The ΔH° and ΔS° partitioning determined for the other SG variants is not as extreme, although in each case the data indicate that binding is enthalpically driven, as is the case for hGHv (Table III).

Measurement of Heat Capacity of Binding (ΔC_p)—To further characterize the thermodynamic properties leading to Site1 binding, the changes in heat capacity (ΔC_p) were determined by monitoring the temperature dependence of the enthalpy using isothermal titration calorimetry (Table II). The ΔC_p measured for the *wt*-hGH-hGHR Site1 interaction was -0.76 kcal·mol^{−1}·K^{−1}, which is virtually identical to the previously reported value (26). Additionally, this value was reasonably close to those measured for three of the SG variants (Table III). The measured ΔC_p value for hGHv was -1.0 kcal·mol^{−1}·K^{−1}, which represents a significant difference compared with the other variants measured. Interestingly, a general observation is that all these molecules have ΔC_p values that are ~2-fold greater than those typically seen for protein-protein interactions (see below) (27).

Influence of Multiple Site1 Mutations on the Kinetics of Receptor Homodimerization

A comprehensive mutational analysis has recently shown that there is allosteric coupling between the Site1 and Site2 binding sites of hGH that influence the kinetics of receptor homodimerization (28). A relatively small set of mutations in the hormone's Site1 has been shown to produce large changes in both the structure and energetics of the hormone and receptor at the Site2 interface on the opposite face of the hormone. Consequently, we believe that it was important to determine whether the more or less random mutations in the SG variants will likewise elicit an allosteric effect on Site2 binding and, if so, what is its magnitude?

The measurements of Site2 binding for five SG variants

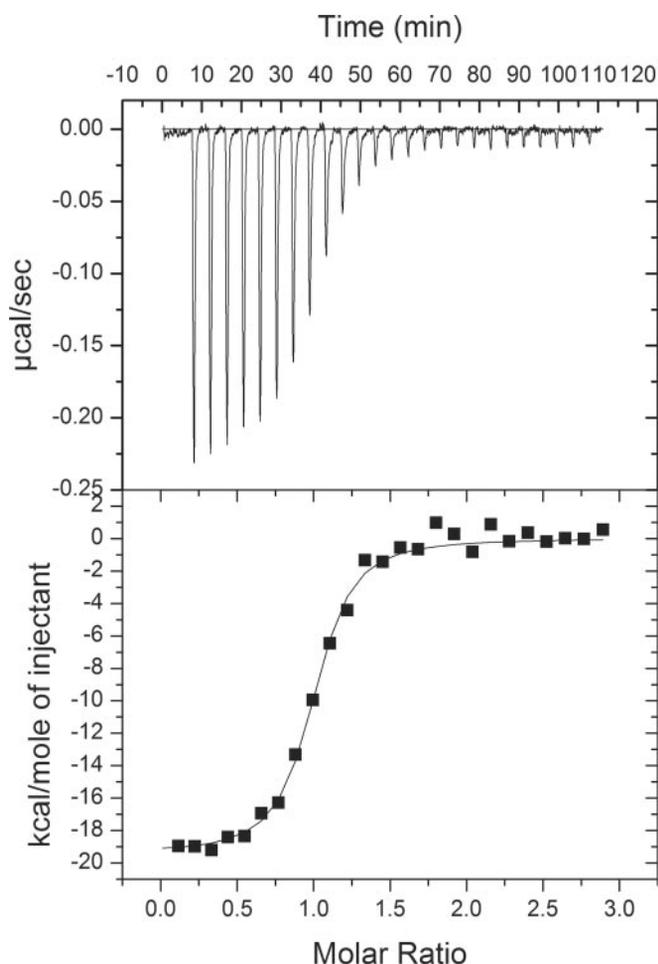


FIG. 4. Example of calorimetric titration of hGHR ECD1. The hGHR ECD ($2.4 \mu\text{M}$) was titrated with *SG8* ($26.8 \mu\text{M}$) in 20 mM HEPES (pH 7.4), 150 mM NaCl, pH 7.4 at 25°C . The raw data with integrated base line are represented in the upper panel. In the lower panel, the integrated peaks are represented by a fitted curve, using the “one set of sites model” from Origin 7 (MicroCal).

(*SG1–5*) were carried out using tri-molecular surface plasmon resonance (TM-SPR) as has been previously described (19). The Site1 binding affinities for these mutants ranged from being virtually identical to *wt*-hGH to having a 17-fold decrease in binding relative to *wt*-hGH. Interestingly, even though the Site1 affinities of these *SG* variants have a large dynamic range, the resulting Site2 affinities are almost identical (4–14 nM); for instance, the Site1 affinities of *SG1* and *SG5* differ by about 17-fold, yet the measured Site2 affinities are virtually identical (Table II). For variants with low Site1 affinities (>50 nM), the Site2 binding data could not be fitted accurately with the CLAMP software algorithm (data not shown). However, qualitative inspection of the TM-SPR sensorgrams suggested that, even for the poor Site1 binders (*SG10* ~80-fold decrease in binding), a stable ternary complex could be formed. Taken together these data indicate that the stability of the tertiary complex is dominated by the energetics of the Site2 association, which stems from the thermodynamic linkage of the two binding events.

DISCUSSION

The analysis of the binding kinetics for the *SG* variants indicates that a remarkably large number of non-*wt* type substitutions can be accommodated in the Site1 interface without vastly compromising binding affinity compared with the *wt*-hGH molecule. The 11 *SG* variants analyzed in Table I averaged 16 non-*wt*_v substitutions distributed over the whole binding epitope.

The difference in the approach used here and that used for the original conventional Ala-scanning analysis, which established the presence of the focused Site1 binding hot-spot (6), is worth noting. The conventional analysis was based on a set of single Ala substitutions in the background of the parental *wt* sequence. In contrast, the shotgun sequences analyzed here are altogether different in that each sequence contains multiple mutations with no true parental sequence for the binding epitope residues, the binding contributions of individual residues are established by averaging a large number of disparate sequence variants. Thus, we find it rather remarkable that these two fundamentally different approaches to characterizing the energetic contributions of individual residues in a binding epitope result in virtually the same energy pattern.

Thermodynamic Analysis and Binding Properties

Based on the number of non-*wt*_v substitutions and their location in the binding interface, the *SG* variants have a number of altered physicochemical properties in their Site1 binding site. Therefore, it is almost a certainty that the packing complementarity in the interface between the *SG* variants and the receptor will be compromised to some extent. The thermodynamic analysis of the variants was undertaken to determine whether there were features in the binding thermodynamics among *wt*-hGH, the high affinity variant hGHv, and the highly modified *SG* variants that would distinguish the different binding interactions.

The finding that there are differences in the observed $\Delta H^\circ/\Delta S^\circ$ partitioning between *wt*-hGH and hGHv binding to hGHR was expected based on comparisons of the characteristics of the energy epitopes determined for these two molecules. Although the Site1 binding footprint of *wt*-hGH is slightly bigger than that of hGHv (1300 \AA^2 versus 1180 \AA^2), the residues buried by hGHv contribute 10% more hydrophobic content to the interface (25). It is interesting to note that, despite having a slightly reduced and more apolar interface, hGHv binding is a more enthalpically favored (-36 kcal/mol) versus *wt*-hGH (-9 kcal/mol). Additionally, the *wt*-hGH interaction to hGHR has an energy epitope characterized by a dominant hot-spot; the few residues (5–6) contained in this hot-spot contribute a large fraction of the total binding energy (7).

In *wt*-hGH this hot-spot is spatially focused on the conformationally rigid helix-4; that is, the major set of interactions is on a preorganized helix scaffold presumably minimizing entropic penalties for binding. In contrast, the hGHv binding hot-spot is considerably more diffuse with several major binding contributors being located on the loop connecting helices 1 and 2 (13, 24). Additionally Ala-scanning mutagenesis showed that the Site1 residues in *wt*-hGH act in additive fashion, whereas the binding in hGHv is characterized by sets of residues that interact with each other in a more cooperative fashion (24). However, it is not known how or to what extent, if any, differences in additive-cooperative binding effects would play a role in enthalpic-entropic partitioning.

Although the differences in the general character of the binding epitopes suggest a rationale for why the thermodynamics of binding of these two hormones might be different, it was the actual magnitude of these differences that is surprising. In particular, the large difference in the relative $T\Delta S$ contributions to binding is not easily explained, because to a first approximation the reduction of the conformational flexibility of the hormone and receptor ECD groups should be affected in similar ways in both cases. Certainly there is no evidence that in the conformational transition going from its unbound to a bound state hGHv loses appreciably more conformational freedom than its *wt*-hGH counterpart (25, 29). Few if any of the

TABLE II
Site2 binding for SG and D116A variants to the hGHR ECD2

SRP measurement were done in 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20 at 25 °C.

Hormones	k_{on} $M^{-1} s^{-1} \times 10^5$	k_{off} $s^{-1} \times 10^{-4}$	K_d^a nM	Relative K_d mut/wt
wt-hGH ^b	2.0	7.0	3.5	1
SG1	1.17	11.40	9.7	3
SG2	1.45	5.46	3.7	1
SG3	0.89	12.30	13.8	4
SG4	1.16	7.12	6.1	2
SG5	0.76	7.39	9.7	3
D116A_wt-hGH ^b	2.0	6.20	3.1	1
D116A_hGHv ^b	0.58	155	266	75
D116A_SG1	1.27	253	199	56
D116A_SG2	0.72	443	615	174
D116A_SG3	2.40	340	141	40

^a Equilibrium dissociation constant $K_d = k_{\text{off}}/k_{\text{on}}$.

^b Values were taken from Pal *et al.* (13) and Walsh *et al.* (28).

TABLE III
Thermodynamic parameters of the Site1 binding of hGH variants to the hGHR ECD1 at 25 °C

Hormones	ΔG^{oa}	ΔH° kcal mol ⁻¹	$-T\Delta S^{\circ}$	ΔC_p^b kcal/mol/K
wt-hGH	-12.2	-9	-3.2	-0.76 ± 0.05
hGHv	-15.0 ^c	-36	21	-1.00 ± 0.04
SG1	-12.0	-16	4.0	-0.63 ± 0.09
SG2	-11.1	-20	8.9	-0.65 ± 0.04
SG8	-10.0	-20	9.3	-0.53 ± 0.02

^a Changes in entropy were calculated from $-T\Delta S^{\circ} = \Delta G^{\circ} - \Delta H^{\circ}$.

^b Values were derived from the linear regression of the temperature dependence of ΔH° .

^c Value was estimated from SPR measurements (24).

differences can be assigned to features in the receptor, because based on structural data its unbound to bound conformational transition should be virtually identical for its binding to both hormones (25). The possibility that the large unfavorable entropy for hGHv binding is due to unfavorable desolvation effects is also unlikely, because its Site1 buries significantly more hydrophobic surface, an effect that would tend to produce a favorable entropy contribution.

Kinetic-Thermodynamic Relationships

It might be anticipated that a kinetic-thermodynamic relationship may exist in some binding pathways where slow k_{on} rates correlate with large unfavorable ΔS terms. The conceptual underpinnings of this picture is that if a large structural ordering (large $-\Delta S$) is required to form an interaction, this process would be inherently slower than one where a binding surface(s) was structurally preformed to its binding partner, as is the case for binding between the GP120 and CD4 receptors (30) along with T cell receptor binding peptide-major histocompatibility complex (31). On this point, our data suggest that this relationship is not as clear-cut in the hGH system presented here.

Based on the above arguments and the fact that the $T\Delta S^{\circ}$ terms for wt-hGH and hGHv are so different (differences in $T\Delta S^{\circ} = 23$ kcal/mol), it might be expected that a rate-limiting conformational change necessary for binding exists with hGHv and not with wt-hGH so that these two molecules would greatly show different k_{on} rate constants. However, their respective k_{on} values are remarkably similar (to within 3-fold) (Table I). The three SG variants that were analyzed, which had on average $T\Delta S^{\circ}$ values intermediate between the extremes of wt-hGH and hGHv, have k_{on} values between 5- and 13-fold of wild-type and 2- and 5-fold of hGHv. Although as a group these variants display both decreased k_{on} rates and larger entropic penalties of binding, a clear correlation between the unfavorable entropy of binding and the observed on-rates is not observed for these variants.

This suggests that, whatever features of the interaction that are responsible for such large differences in the $T\Delta S^{\circ}$ components of the binding energy, they have a minimal influence on the kinetics of the initial binding event, but occur later along the binding coordinate. Using a reaction funnel analogy to describe the binding thermodynamics, the data suggest that the path of approach to the transition state (TS^{\ddagger}) at the top of the funnel can be similar for most of the variants; however, the energy landscape that leads from the TS^{\ddagger} to the stable complexes at the bottom of the energy funnel must differ significantly among the variants and shows up as a large difference in the partitioning of their enthalpic/entropic contributions to the binding thermodynamics. This may stem from conformational flexibility of the protein (*e.g.* side-chain rotations) that is explored on a timescale faster than that observed for binding and are thus only energetically realized as an entropy penalty post- TS^{\ddagger} . Alternatively, it should be noted that these differences in association rates may also originate from disruption of electrostatic contributions to binding (32). Previous alanine scanning experiments discovered specific residues that can either slightly decrease (R167A) or increase (E65A and E174A) the association rate when independently mutated to alanine (33). Unfortunately, with the current data and the large number of mutated residues in these variants, it is not feasible to meaningfully interpret these specific electrostatic effects.

Interpretation of Measured ΔC_p Values

Based on a compilation of published thermodynamic data (27), the wt-hGH·hGHR interaction results in a much larger ΔC_p value (-0.76 kcal·mol⁻¹·K⁻¹) than typical protein-protein interactions reported. (The average value for the protein-protein interactions in the data set is $\Delta C_p = -0.33$ kcal·mol⁻¹·K⁻¹ (27).) The ΔC_p of -1.0 kcal·mol⁻¹·K⁻¹ measured for hGHv·hGHR is even a larger outlier from the average value than the wt-hGH value. To put the following discussion into context, we note that the normal dynamic range for the measurements of ΔC_p values is usually small; *i.e.* with accurate data differences

in ΔC_p of even 0.1–0.2 kcal·mol⁻¹·K⁻¹ is considered significant.

Surface Burial Relationships—It has been proposed that the measured ΔC_p of binding can be correlated with the amount of hydrophobic and hydrophilic surface buried in the resulting protein-protein interface (34). An empirical algorithm based on measured relationships between ΔH° and ΔC_p has been able to recapitulate the amounts of observed buried surface area that occur during protein folding (34, 35). As a first approximation it would be expected that at the level of factors contributing to the energetics there would be distinct similarities between protein folding and protein-protein association events.

The ΔC_p -surface area burial relationship has been confirmed in some studies, particularly involving protein-peptide (small protein) interactions (36–39). However, when applied to larger protein-protein interactions the algorithm frequently overestimates the surface area actually buried, often a result of additional surface area burial (*e.g.* folding) upon binding, a common occurrence for larger proteins (40). In this regard, the discrepancies found in this study are striking. Using the determined ΔH° and ΔC_p values for the *wt*-hGH interaction, the predicted buried surface area in the hormone-receptor interface gives an overestimation of about 2-fold (data not shown). The predicted/observed ratio for the buried surface in hGHv interaction results in an even larger discrepancy, predicting a buried surface area that is over three times what is actually observed in the crystal structure. In systems where these large discrepancies exist, it has been suggested that the binding event involves additional linked equilibria. Such contributions to the observed ΔH° and ΔC_p values (whether calorimetrically or Van't Hoff derived) have been illustrated by simulations (41, 42) and have been experimentally assigned to originating from conformational change (30, 43), non-local solvent re-ordering (44), ion linkage (45), and proton binding (36, 37, 46) making the ΔC_p – ΔH relationship much more complex.

Conformational Ordering—Spolar and Record (14) have pursued a different interpretation of thermodynamic components that are embodied in the ΔC_p term. Although the above empirical approach links ΔC_p with ΔH° to estimate burial of hydrophobic and hydrophilic surface area, they proposed an alternative physical description of ΔC_p for protein-protein associations that are expressed through its linkage with ΔS to estimate the extent of conformational ordering that occurs on binding.

Application of this type of analysis has proven to be successful with regards to several protein systems (14), and in particular to ones where one of the binding partners is inherently disordered before binding (30, 31). These systems generally share the same thermodynamic fingerprint as is observed here for the hGH variants binding to hGHR- large unfavorable entropies of binding and large negative ΔC_p values (14). However, it appears that the similar correlations do not hold here with regard to using the Spolar-Record $\Delta C_p/\Delta S^\circ$ relationships as an indicator of conformational ordering on binding. This is demonstrated in our case by comparing the thermodynamic components of the *wt*-hGH and the high affinity variant hGHv in the context of the predicted conformational ordering. These relationships predict ordering of about 36 residues for *wt*-hGH binding and about 70 residues for hGHv binding. Although there might be somewhat more ordering in hGHv binding, the calculated values are a large overestimation of what can occur based on the knowledge of the structures of the unbound and bound forms of hGH and hGHv. Part of this overestimation may originate from the assumption in the method that ΔS_{sc} (the entropic component originating from side-chain restriction upon binding) is not explicitly accounted for in the calculations (14), but is believed to play a significant energetic role in folding and binding (47, 48).

Influence of SG Variants on Receptor Homodimerization

Biological signaling in the hGH system is triggered via a hormone-induced receptor homodimerization. The homodimerization proceeds in a controlled stepwise manner with the high affinity Site1 of hGH always being occupied first to form a stable 1:1 hormone-receptor intermediate. This 1:1 intermediate acts as a scaffold to bind a second receptor at Site2 through two spatially distinct binding contacts. One contact is on the opposite face of the hormone from Site1 and is called the Site2 contact; the other is through direct receptor-receptor contacts called the stem-stem contact region (9, 19, 25, 49).

The finding that the K_d for binding the second receptor is reasonably independent of the affinity of Site1 binding has several important biological implications that are noteworthy. First, it shows that the stability of the ternary hormone-receptor signaling complex is dictated by the binding contributions of Site2 and the stem-stem contact region. Weak Site1 binding produces a more transient 1:1 complex thus reducing the number of potential targets for the second receptor ECD, but once Site2 is productively occupied the persistence time for the stable 1:2 hormone receptor complex is not appreciably affected by the Site1 binding energetics, at least over 2 logs of binding. Second, this provides direct data showing that the dissociation of the 1:2 complex to a 1:1 intermediate always is initiated by loss of Site2 binding, a mechanism that was almost universally accepted, but not experimentally verified (3).

Allosteric Coupling of Binding Sites

In the studies characterizing the allosteric coupling between the Site1 and Site2 binding sites by Walsh *et al.* (28) it was found that hormone residue Asp-116, which is found in the middle of the Site2 interface, was a sensitive reporter group in the presence of the allosteric coupling phenomenon. The structural origin and energetic consequence of this allosteric effect have been characterized (25, 28). In *wt*-hGH, the D116A mutation has no effect on binding of the receptor at Site2. However, this mutation in the context of hGHv produces a 70-fold decrease in affinity. The D116A mutant was introduced into three SG variants (SG1–3), and the Site2 affinities were determined (Table II). Both SG1 and SG3 had similar reductions in binding affinities as the hGHv molecule. However, SG2 showed about an additional 3-fold decrease in binding. It is not clear whether this additional effect on binding represents a significant difference in relation to SG1 and SG3. Nevertheless, these data suggest that for these SG variants there exists an allosteric effect comparable to that seen for hGHv.

Although the analysis of the Site2 binding effects of the set of Site1 variants is not fully comprehensive, it is somewhat surprising that none of the SG variants in the absence of the D116A mutation appeared to have large negative effects on Site2 binding. This finding supports the observation that, although the allosteric coupling between Site1 and Site2 clearly exists and can be described unequivocally by both structural and functional analyses as a reorganization of the hormone and receptor residues at Site2, this reorganization produces only a minor overall effect on second receptor binding. This implies that the contribution of the stem-stem contact region to the binding of the second receptor may dominate and mask subtle changes in the energetics of the Site2 hormone-receptor contact.

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