Dissecting the Binding Energy Epitope of a High-Affinity Variant of Human Growth Hormone: Cooperative and Additive Effects from Combining Mutations from Independently Selected Phage Display Mutagenesis Libraries†

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ABSTRACT: Phage display mutagenesis is a widely used approach to engineering novel protein properties and is especially powerful in probing structure−function relationships in molecular recognition processes. The relative contributions of additive and cooperative binding forces and the influence of conformational diversity in producing a novel protein−protein interface is investigated using as a model an ultra-high-affinity receptor binding variant of human growth hormone (hGHv) that has been previously affinity matured. The modular aspect of how the mutations were grouped in the phage display libraries and combined allowed for a systematic probing of the inherent functional cross-talk between the different secondary structure elements that make up the remodeled hGHv binding surface. We performed an alanine scanning analyses of 35 hGHv residues and determined the kinetics of each variant by surface plasmon resonance (SPR). This analysis showed that there is a significant difference between the additive and cooperative binding forces existing among the selected residues in each library module, and the binding advantage of these residues is maximized over the original wild-type residue when in the context of the other mutations in the library. The degree to which residues in a particular mutagenesis library display binding cooperativity characteristics is generally correlated with the conformational plasticity of the polypeptide chain. Additionally, these cooperativity effects change when the mutations from one library are combined with the mutations from one or several of the other separate libraries. This supports the idea that significant functional cross-talk exists between the combined library modules that can affect the binding energetics of individual residues over a large distance.

The initiation and regulation of most essential biological activities are under the control of evolved molecular recognition processes. Structural studies have shown that the cytokine hormone receptor superfamily has a number of examples in which the primary binding determinants are not dominated by the amino acid sequence of the interacting peptide segments but also center on features relating to conformational variation (*1*−*4*). In this regard, human growth hormone (hGH)[1] binding to its cognate receptor (hGHR) and the related prolactin receptor (hPRLR) has been extensively characterized through mutagenesis and a number of biophysical methods (*5*−*9*). Taken together, these studies indicate that the hormone's biological functions are regulated through mechanisms involving extensive conformational and energetic adaptability (*10*, *11*).

A series of important molecular evolution studies using phage display mutagenesis have produced a number of variant hGH molecules with significantly altered binding properties (*7*, *12*). Pertinent to issues involving the coupling of sequence diversity and conformational plasticity are a set of variants derived from phage display selections aimed at producing higher-affinity receptor binding sites (*12*). The construction of these variants was carried out using a modular strategy. Four separate phage display libraries, each containing four or five residues and representing a different region of the binding site, were independently subjected to binding selections to isolate variants with improved affinity. The selected "best" sequences from each of the four libraries were then combined to produce final high-affinity variants. The high-affinity variant of hGH (hGHv) that has been best characterized contained 15 mutations in its high-affinity receptor binding site (Site1), and binds to the receptor ECD ∼400-fold more tightly than does wild-type (*wt*) hGH (*12*).

It is noteworthy that the production of this hGHv variant was not a case of fine-tuning the initial binding interface. Schiffer *et al.* (*13*) have shown that the new contact interface,

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[1] Abbreviations: hGH, human growth hormone; hGHR, human growth hormone receptor; hGHv, high-affinity variant of hGH; hPRL, human prolactin; hPRLR, human prolactin receptor; ECD, receptor extracellular domain; *wt*, wild-type; Xaa_R, hGHR residue; SPR, surface plasmon resonance; POH1, phage display-optimized helix 1; POMH, phage display-optimized mini-helix; PLOP, phage display-optimized loop; POH4, phage display-optimized helix 4; Ala-scan, alanine scanning mutagenesis; *wt*-scan, wild-type scanning mutagenesis; RU, SPR response units.

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the so-called structural epitope, has significantly more hydrophobic character and a rearranged H-bonding network and involved large conformational changes in both the hormone and receptor interfaces. Because the stereochemical character of these interfaces is so different, it is not surprising that the functional epitope, which describes the distribution of the binding energy in Site1 of high-affinity hGHv, has been shown to be fundamentally different from that of the *wt* hormone in several notable ways (*14*). In *wt* hGH, the functional epitope of Site1 is dominated by a small number of key residues that are spatially clustered into a so-called binding "hot spot" (*15*, *16*). In contrast, even though the binding of hGHv is much tighter, the binding contributions of the original hot spot residues are highly attenuated. This attenuation appears to be more than offset by the addition of a new set of contributing residues located at the periphery of the binding epitope, in effect resulting in an expanded and more diffuse hot spot (*14*).

Although high-resolution structures exist for the *wt* and hGHv complexes and the Site1 binding energy surfaces have been mapped, it has not been possible to identify the specific residues or the group of interactions that lead to the 400-fold improvement in binding. This suggests that there must be some level of cooperativity between residues, which may be a direct result of the nature of the phage display selection process. In this process, all possible combinations of amino acids at four or five randomized sites are tested in each independent library. It is probable that in some cases particular groupings of residue types will bind in a synergistic way, rather than additively. Further, although the libraries were screened separately, the close spatial relationship of the residues in the tertiary structure makes it almost a certainty that some functional cross-talk exists between the affinity-matured residues from different libraries when they are combined.

Because of these characteristics, we believe that the hGHv system offers an extremely powerful model system for deconvoluting the effects of additivity and cooperativity in a complicated protein−protein interface. Further, establishing the linkage between conformational plasticity and additive and cooperative effects is fundamental with respect to gaining a broader understanding of the structure−function relationships in play in protein−protein interactions. Unfortunately, dissecting energetic contributions due to these effects has proven to be a daunting challenge. However, in the case of hGHv, we believe that the effects from the four different phage display modules that were added together can likewise be systematically taken apart and studied to isolate different characteristics in the binding interface.

We report here an extensive structure−function study using Ala-scan and wild-type (*wt*) scan mutagenesis with the aim of determining the interplay between additive and cooperative binding forces in a complex protein−protein interface. In particular, we aimed to determine the fidelity with which the properties derived from individual library phage display selections were retained when combined with the products of other libraries to produce the final high-affinity hGHv. Is the fact that there is no discrete and simple structural rationale that reflects the 400-fold increased affinity of hGHv for the receptor ECD due to cooperativity effects generated by how the four phage display libraries interact when combined? To gain some insights into the energetics of the molecular recognition process governing this protein−protein association, we hoped to correlate the binding kinetics with the changes in electrostatic and structural properties of the large panel of group and single-site mutations.

We find that the contributions of individual residues are highly context dependent. That is, the characteristics of the residues in the individual phage optimizations are many times not conserved when placed in the format of the hGHv molecule. This suggests that some form of functional cross-talk exists between the different groups contained in the phage display libraries when they are combined. We also find that binding effects of the phage display-derived mutations within a single library can act in cooperative fashion, and are generally correlated to the flexibility of the polypeptide chain. Interestingly, we find that the hGHv molecule binds the alanine mutants of the principal hot spot receptor residues, $W104_RA$ and $W169_RA$ (residue numbers with a subscript R are hGHR residues), more than 10^4-fold tighter than does *wt* hGH. We also find that in most cases the mutations that weaken the binding affinity have a negligible impact on the binding on-rates. This invariance implies that the level of stereospecificity existing in the encounter complex intermediate represented by the binding transition state is low. This suggests that the initial molecular recognition event, as defined by the transition state energy, is characterized by a hormone−receptor interface that is not highly formed.

MATERIALS AND METHODS

Materials. *N*-Ethyl *N*′-[3-(dimethylamino)propyl]carbodiimide hydrochloride, *N*-hydroxysuccinimide, and 2-(2-pyridinyldithio)ethaneamine hydrochloride were obtained from Biacore. Expression vectors for recombinant hGH (*16*) and hGHR ECD (*17*) were obtained from Genentech (South San Francisco, CA). For the hGHR ECD, a truncated version was used (residues 29−238) which has an unaltered activity and a superior expression level compared to the version with residues 1−245 (*18*). Oligonucleotides were from OPERON (Berkeley, CA) and Integrated DNA Technologies (Coralville, IA).

Mutagenesis. The Kunkel method was used for all mutagenesis (*19*), and the presence of the mutations was confirmed by using fluorescent dye terminator chemistry and analyzed on an Applied Biosystems 377 automated DNA sequencer.

Growth and Purification. hGH, hGH mutants, hGHR ECD, and hGHR ECD mutants were expressed in the periplasm of *Escherichia coli* essentially as described previously (*17*) except that BL21 cells from Novagen were used as a host, and the mutant hGHR ECD variants were expressed at 20 °C. The periplasmic protein fraction was prepared as described previously (*17*). All the hGH variants that were studied contained a Site2 mutation, G120R, which totally inhibits ECD binding at Site2 without affecting Site1 binding. The hormone variants were purified by using a Resource Q ion exchange column (Amersham Pharmacia) with some variants further purified on a Superdex 75 gel filtration column (Amersham Pharmacia). The hGHR ECD variants were purified over a hGH affinity column as described previously (*17*) except that 4.5 M $MgCl_2$ was used in the elution step. The defective $W104_RA$ and $W169_RA$ ECD

mutants were purified by ion exchange and gel filtration chromatography. All proteins were analyzed for purity by analytical HPLC, and the presence of the mutation was confirmed by electrospray mass spectrometry (PE Sciex API 150Ex instrument). The expression yield for hGH G120R was ~4 mg from 1 L of culture, while most hGH Site1 mutants were expressed at a level of ~1 mg/L. Typical yields for the hGHR ECD mutants were between 1 and 2 mg of protein purified from 1 L of culture. Protein concentrations were determined by using molar extinction coefficients at 280 nm of 16.2 and 55.0 mM^{-1} cm^{-1} for hGH and hGHR ECD, respectively, and these values were adjusted appropriately for mutations (*20*).

Surface Plasmon Resonance (SPR). All experiments were carried out on a Biacore 2000 instrument at 25 °C. hGHR ECDs were coupled to a Biacore Pioneer C1 sensor chip by disulfide bond formation through an engineered cysteine on the S237C mutant. This unpaired cysteine was typically modified in the bacteria by glutathione during expression. The glutathione modification was removed by treatment with 1 mM DTT for 30 min on ice. The DTT and glutathione were then removed from the sample by a desalting step on a PD-10 column (Amersham Pharmacia) equilibrated with 10 mM sodium phosphate buffer (pH 7.4). The ECD sample was then concentrated to 0.5−1 mg/mL for the subsequent immobilization step. The immobilizations and kinetic measurements were carried out by using HBS buffer [10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% Tween 20 (pH 7.4)] that had been filtered and degassed. The S237C hGHR ECD mutant was immobilized on the C1 sensor chip following the Biacore protocol. Briefly, the flow rate was set to 5 mL/min, and a 25 mL mixture of 100 mM *N*-hydroxysuccinimide (NHS) and 390 mM *N*-ethyl *N*′-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) was injected. It was followed by a 40 mL injection of 80 mM 2-(2-pyridinyldithio)ethaneamine hydrochloride (PDEA) in 50 mM sodium carbonate buffer (pH 9.5). A 50−100 mg/mL solution of the S237C hGHR ECD in 10 mM sodium acetate (pH 4.5) was then injected to achieve a level of 20−100 functional response units (RUs). The unreacted functional groups were blocked with a 30 mL injection of 50 mM reduced glutathione in 20 mM sodium acetate/1 M NaCl buffer (pH 4.5).

For kinetic analysis, serial dilutions of hormone (typically, 40, 20, 10, 5, and 2.5 nM) were made in HBS at 25 °C. The flow rate was set to 50 μL/min, and 250 μL of hormone was injected for each concentration. Most of the mutants could be completely dissociated from the ECD surface by prolonged washing with buffer for 180 min. Increasing the flow rate to 100 μL/min had no effect on the rate constants, indicating that hGH binding is not limited by diffusion or a mass transport effect. The immobilized ECD on the C1 chip could be used for ~150 injections. The amount of functional response units (RUs) gradually decreased over time. The activated thiol surface was regenerated, allowing the C1 chip to be used several times. The data were fit by the CLAMP program using a simple $A + B \rightleftarrows AB$ binding model giving values for the k_{on} and k_{off} processes (*21*). The binding K_d was calculated from the k_{off}/k_{on} ratio. All binding experiments were repeated at least two times. Results were highly reproducible, and errors in K_d values were typically <20%.

Table 1: Binding of hGH and Phage-Optimized Libraries to hGHR1 (S237C) by SPR[a]

hormone	k_{on} ($\times 10^5$ M^{-1} s^{-1})	k_{off} ($\times 10^{-4}$ s^{-1})	K_d[b] (nM)	fold increase in binding ($K_d^{wild-type}/K_d^{mutant}$)
hGH	3.2	3.9	1.2	1
hGHv	5.0	≤0.05	≤0.01	>120
POH1	2.5	1.1	0.45	2.7
POMH	5.1	2.0	0.39	3.1
POH4	3.2	0.2	0.06	20
PLOP	6.4	1.9	3.0	0.4
POH1 and POMH	3.6	0.8	0.20	5.5
POMH and POH4	4.8	0.2	0.03	40
POMH1 and POH4	4.2	0.2	0.04	30
PLOP and POH4	1.4	0.25	0.02	60

[a] All mutants contain the G120R mutation. [b] The standard error for these measurements (K_d) does not exceed ±20%. hGHv, F10A, M14W, H18D, H21N, K41I, Y42H, L45W, Q46W, F54P, R64K, R167N, D171S, E174S, F176Y, I179T; POH1, F10A, M14W, H18D, H21N; POMH, K41I, Y42H, L45W, 46W; POH4, R167N, D171S, E174S, F176Y, I179T; PLOP, F54P, K64R.

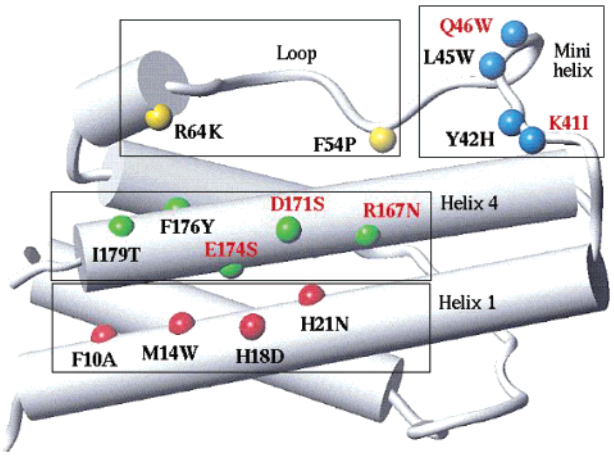

FIGURE 1: Residues contained in the individual phage display libraries: helix 1 (POH1) in red, mini-helix (POMH) in blue, loop (POLP) in yellow, and helix 4 (POH4) in green. The wild-type residue precedes the residue number, and the phage-optimized residue follows it. A residue with red lettering indicates that that residue was involved in either a salt bridge or H-bond interaction in the wild-type hGH−hGHR complex.

RESULTS

Additivity Effects between Mutations Combined from Different Phage Display-Derived Variants. The construction of the high-affinity hGH variant hGHv was based on the premise of additivity. To produce this variant, sets of residues from four independent phage-optimized variants were combined to give a molecule that contained 15 mutations in Site1 (*12*).

The values determined by surface plasmon resonance (SPR) describing the binding kinetics of the phage-optimized variants are given in Table 1. Wild-type (*wt*) hGH binds to Site1 of hGHR ECD with a K_d of 1.2 nM (*10*); the affinity of hGHv was too high for its kinetics to be measured accurately by SPR. On the basis of the experimental limits of measuring k_{off} rates on the instrument, we estimate that the K_d for hGHv is less than 10 pM, and it has been estimated by other methods to be ~2 pM (*12*).

Figure 1 shows the regions randomized in the four independent libraries used to optimize Site1 of hGH for

binding to the receptor ECD (hGHR) and the mutations accumulated in hGHv. The phage-optimized helix 1 library (POH1) contains mutations at residues 10, 14, 18, and 21. The mini-helix library (POMH) contained mutations at residues 41, 42, 45, and 46. The helix 4 (POH4) library had mutations at residues 167, 171, 174, 176, and 179. The fourth library, the phage-optimized loop (POLP), contained only two mutations, at positions 54 and 64, which are found in the polypeptide loop connecting helix 1 and helix 2.

Incorporating the four POH1 mutations into the *wt* hGH sequence produced a variant that bound to the hGHR ECD with an affinity of ~0.4 nM (Table 1). A similar binding affinity increase was observed for the set of POMH mutations. The ~3-fold improvement in binding for these two libraries determined here by SPR is close to the values reported previously using an RIA format (*12*). The hGH variant containing the mutations in the POH4 library shows a 20-fold increase in the level of binding. The two POLP mutations were reported to increase the level of binding by 2.5-fold in the study by Lowman and Wells (*12*); however, we find that these mutations have no measurable effect on binding using the methods described here.

Pairwise combinations of mutations from three of the libraries result in variants that exhibit binding additivity (ca. ±0.2 kcal/mol). For instance, combining the eight mutations derived from the POH1 and POMH libraries, each of which individually exhibited ~2.7- and 3.1-fold affinity increases, results in a variant that binds ~6-fold tighter than the *wt*. When either POH1 or POMH is combined with the tighter binding variant from the POH4 library, they give rise to variants that bind ~40-fold (POMH and POH4) and ~30-fold (POH1 and POH4) more tightly than the *wt* molecule. In contrast, combining the mutations derived from libraries POH4 and POLP should, by additivity, not show any increase in the level of binding over the library POH4 variant itself. However, this pairwise combination results in a K_d of ~18 pM, an increase in the level of binding of ~60-fold compared to the *wt*. This suggests some form of functional linkage between groups in these libraries (see below).

Alanine Scanning of Variants Derived from Individual Libraries. Each mutant residue from the four library variants was independently analyzed by alanine (Ala-scan) and wild-type (*wt*-scan) substitution. The Ala- and *wt*-scans provide different but complementary binding information. The Ala-scan gives a measure of the binding energy contributed by each side chain, while the *wt*-scan provides information about the degree to which a mutation produces a binding advantage over the original *wt* residue when placed in the context of the other mutations within the particular hGH variant. Therefore, if additivity applies, the sum of the binding advantages determined from the *wt*-scan data would equal the overall improvement of the binding affinity of the variant over the *wt* hormone. The degree to which the *wt*-scan data do not reflect the binding affinity produced by the mutations in a library as a group is an indication that the selections involved some form of cooperativity among these groups as a whole.

Helix 1 Library (POH1). For the POH1 library-derived variant, three of the four sites that were mutated were Ala-scanned: W14, D18, and N21. Position 10 was not Ala-scanned because the variant contained an Ala mutation (F10A) at this site. Although the helix 1 mutations produced a modest 3-fold improvement in binding relative to the *wt*, the individual Ala substitutions produced substantial decreases in the level of binding (Table 1). The W14A mutant produced a 14-fold reduction in the level of binding, while D18A and N21A resulted in 8- and 6-fold reductions in the level of binding, respectively.

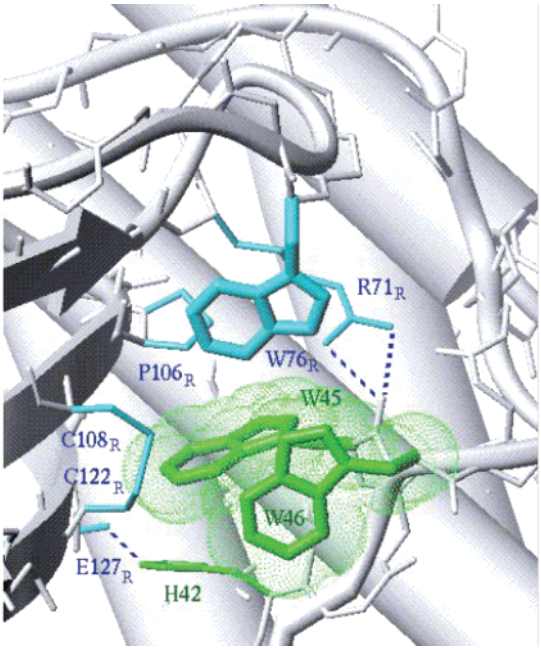

FIGURE 2: Packing interactions between the POMH residues (green) and the receptor residues (blue). W45 and W46 of POMH form a "Trp" sandwich with $W76_R$ of the receptor. This orientation is set up by the H-bond between the backbone carbonyl of residue 45 and $R71_R$.

The *wt*-scan of POH1 produced a set of effects different from that of the counterpart Ala substitutions. The A10F mutation increases the level of binding by ~2-fold, indicating that, surprisingly, the *wt* residue is slightly better than the selected alanine in the context of the other three selected mutations. At position 14, while introduction of the *wt* Met improves the binding relative to the binding with Ala, it is ~3-fold worse than the binding with the selected Trp. This contrasts with the *wt* substitution at position 18 where the *wt* His has a considerably more deleterious effect than Ala. In the case of the N21H substitution, introduction of the *wt* His results in a 3-fold decrease in the level of binding, which is similar to the effect of the Ala mutation.

Mini-Helix Library (POMH). The recently published structure of the hGHv−hGHR complex shows that the POMH residues form a number of interactions with Site1 of hGHR (*13*) (Figure 2). Thus, it is not surprising that in the Ala-scan of POMH significant decreases in the level of binding at all of the phage-optimized positions were observed. The I41A mutation produced a 6-fold decrease, and H42A, which eliminates at least one H-bond to the receptor, produces an 11-fold decrease. The structure of the complex reveals that W45 and W46 form a large hydrophobic cleft that makes extensive interaction with the receptor, and presumably, the finding that the W45A (15-fold) and W46A (13-fold) mutations have a significant negative effect on binding reflects some disruption of this interaction.

The *wt* substitution at position 41 (I41K) does not appreciably affect binding compared to the Ala substitution.

Table 2: Binding of Alanine and Wild-Type Mutants in Phage-Optimized Libraries to hGHR1 (S237C) by SPR[a]

hormone	k_{on} ($\times 10^5$ M^{-1} s^{-1})	k_{off} ($\times 10^{-4}$ s^{-1})	K_d[b] (nM)	fold decrease in binding ($K_d^{mut.}/K_d^{ind.lib.}$)
POH1	2.5	1.1	0.44	1
A10F[c]	3.8	0.6	0.16	0.4
W14A	2.0	13	6.5	15
W14M	2.4	3.9	1.6	3.6
D18A	3.1	1.1	3.5	8.0
D18H	2.0	11	5.5	13
N21A	2.8	6.9	2.5	5.7
N21H	2.8	3.8	1.4	3.2
POMH	5.1	2.0	0.39	1
I41A	2.2	5.3	2.4	6.2
I41K	3.2	5.3	1.7	4.4
H42A[d]	1.2	5.0	4.2	11
W45A	2.9	18	6.2	16
W45L	3.4	5.3	1.6	4.1
W46A	2.2	12	5.5	14
W46Q	1.9	13	6.8	17
PLOP	0.6	1.3	3	1
P54A	0.9	8.0	9	3
F54A	1.4	2.9	2	0.7
R64A	0.3	54	171	57
R64K	2.2	1.1	0.5	0.2
POH4	3.2	0.20	0.06	1
N167A	3.0	0.42	0.14	2.3
N167R	4.0	0.65	0.16	2.7
S171A	2.7	0.55	0.20	3.3
S171D	2.2	0.33	0.15	2.5
K172A	3.6	0.63	0.18	3.0
S174A	1.4	0.03	0.02	0.33
S174E	2.3	0.44	0.19	3.2
T175A	1.6	0.38	0.24	4.0
Y176A	2.1	0.68	0.32	5.3
Y176F	2.8	0.03	0.01	0.2
R178A	2.6	1.0	0.38	6.3
T179A	3.8	0.26	0.07	1.2
T179I	4.0	0.27	0.07	1.2

[a] All mutants contain the G120R mutation. Mutations are listed in the context of each phage-optimized library (i.e., 45L contains all of the phage-optimized residues for the mini-helix (41I, 42H, and 46W in addition to the 45L mutation). [b] The standard error for these measurements (K_d) does not exceed ±20%. [c] POH1 contains alanine at position 10; therefore, this substitution could not be tested. [d] The 42Y mutant did not express in *E. coli*.

However, substituting the *wt* Leu residue back at position 45 increases the level of binding by ~3-fold over that with the Ala substitution, although it is still an ~4-fold poorer binder than the Trp residue in the context of the other phage-optimized substitutions. In contrast, the *wt* substitution of a Gln at position 46 produces a decrease that is similar to the effect of the W46A mutation. These results suggest that there is a measurable cooperativity effect between the adjacent Trp residues selected in the phage optimization procedure, which is also reflected in the structural organization of the residues at the hormone−receptor interface (Figure 2).

Loop Library (POLP). Although the two residues in the POLP library as a group have little measurable effect on binding, the R64K mutation alone produces a 5-fold increase in affinity (Table 2). The POLP variant is noteworthy in that it has a measurably lower (5-fold) on-rate than the *wt* hormone, while a majority of the other variants have on-rates that are virtually identical (within 2-fold) to that of the *wt* hormone. The lower on-rate is surprising on the basis of conventional electrostatic assumptions because the Lys side chain in the R64K mutant makes two salt bridge interactions, whereas the Arg side chain in the *wt* complex makes none. The F54P mutation alone has no effect on binding, and the Ala mutation (P54A) produces a modest decrease in affinity. This contrasts with the effect of the K64A mutation, which produces an ~60-fold reduction in affinity. This is somewhat larger than that observed in the Ala-scan of *wt* hGH, which results in a 16-fold decrease in affinity (*22*). The absence of any cooperativity between residues 54 and 64 is indicated by the fact that the P54A/K64A double mutation results in a variant with a binding affinity almost identical to that of the variant containing the single K64A mutation.

Helix 4 Library (POH4). While the original helix 4 library (POH4) was randomized at seven sites, two positions (175 and 178) showed a strong preference for their *wt* residue type. Thus, this library yielded an optimized variant that contained only five mutations. While this variant exhibited a much larger increase in affinity in comparison to variants obtained from the other libraries (20-fold compared to ~3-fold for the POH1 and POMH variants), both the Ala- and *wt*-scans of the seven sites randomized in the library indicated that none of the Ala or *wt* substitutions alone resulted in an effect as large as that seen for the Ala and *wt* substitutions in the other two libraries. This is noteworthy because the helix 4 residues were implicated as being part of the binding hot spot in the *wt* hGH−receptor contact surface (*15*). Overall, most of the individual Ala mutations resulted in modest decreases in the level of binding on the order of ~2−4-fold. The largest change was seen at position 178 where the R178A mutant produced an ~7-fold decrease. Interestingly, at position 175 where the phage selection showed an absolute preference for Thr, the Ala mutation caused only a modest 4-fold decrease in affinity.

The effects observed upon individually substituting the *wt* residues back into the helix 4 variant were on the same order of magnitude observed for the Ala substitutions. Interestingly, while the *wt*-scans of the other libraries suggested larger affinity improvements than those that were observed, the improvements in the binding of the individual POH4 mutations suggest a smaller than 20-fold increase in binding affinity based on additivity. Only two positions (174 and 176) showed significant differences when reverted back to the *wt* sequences. The Y176F mutation increased affinity by ~5-fold, indicating that the selection at this site was not fully optimized. At position 174, substitution of the *wt* Glu for Ser reduced the binding affinity by a factor of 3. Interestingly, the S174A mutation increased the binding affinity by 4-fold. This is not surprising because an Ala residue at position 174 was found in a number of clones in the original binding selections (*12*).

Effects on the Binding Contributions of hGHR Hot Spot Residues $W104_R$ and $W169_R$. Substitution of Ala for either $W104_R$ or $W169_R$ of the receptor ECD essentially eliminates binding to the *wt* hormone. In contrast, hGH variants derived by combining mutations selected from two or more Site1 libraries retain relatively high-affinity binding to these Ala-substituted receptors (Table 3). The variant hGHv, which contains mutations derived from all four libraries, binds to either Ala-substituted receptor with an affinity comparable to that of *wt* hGH for the *wt* hGHR, although these affinities are at least 100-fold lower than that observed for binding of hGHv to the unmodified *wt* hGHR.

Table 3: Binding of hGH and hGH Mutants to W104A hGHR1 (S237C) and W169A hGHR1 (S237C) by SPR[a]

hormone	k_{on} ($\times 10^5$ M^{-1} s^{-1})	k_{off} ($\times 10^{-4}$ s^{-1})	K_d (nM)	fold increase in binding (K_d^{hGH}/K_d^{mutant})
	W104A hGHR1			
hGH[b]	0.0024	170	72000	1
hGHv	1.5	3.5	2.3	31000
POH1 and POMH	0.5	25	50	1400
POMH and POH4	1.8	140	80	920
POMH1 and POH4	1.5	14	9.1	7800
	W169A hGHR1			
hGH[b]	0.01	200	20000	1
hGHv	2.1	19	9.2	22000
POH1 and POMH	2.0	110	55	360
POMH and POH4			≥500	40
POMH1 and POH4	1.3	480	370	54

[a] All mutants contain the G120R mutation. [b] Measured with the steady state affinity model.

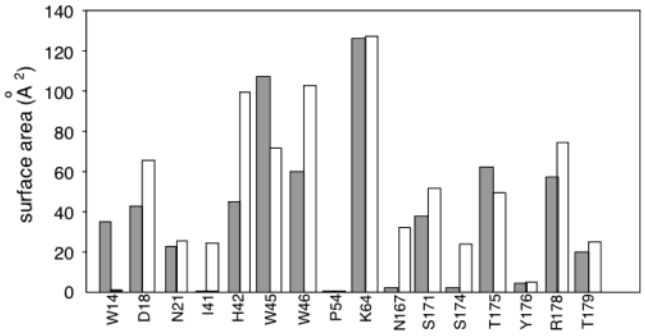

FIGURE 3: Comparison of the buried surface area (square angstroms) of phage-optimized residues (gray bar) and *wt* hGH (white bar) in Site1.

As expected, the combination of mutations derived from only two sets of libraries produced variants with affinities somewhat lower than those of hGHv. The combination of mutations from libraries POH1 and POMH produced an hGH variant that binds each Ala-substituted receptor with a K_d of ~50 nM. It is noteworthy that the residues in these two libraries are located some distance from the contact regions of the two tryptophans in the complex. Interestingly, the pattern of grouping of the two phage-optimized libraries that produces the most competent binding hormone variant is mutant-dependent. For instance, for the W104 $_R$A mutant, the tightest binding grouping is POMH and POH4 (K_d = 9 nM), whereas for W169 $_R$A, the tightest binding is generated with the POMH and POH1 grouping (K_d = 50 nM).

DISCUSSION

The overall character of the 15 mutations introduced into hGH to produce the high-affinity hGHv molecule leads to a somewhat smaller Site1 binding interface with a significantly increased hydrophobic character (*13*). This trend toward a more hydrophobic interface is exemplified by the fact that a number of H-bonding interactions were "mutated" out of the interface and replaced with nonpolar groups (*13*). Figure 3 compares the amount of surface area of burial on binding of the mutated phage-optimized residues to that of the *wt* residue counterparts. There are considerable differences in distribution within the library groupings. For instance, in the POH1 variant, W14 buries ~35 Å^2 compared to M14 of the *wt* hGH, which remains solvent-exposed on receptor binding. For POMH, the *wt* residues in positions 41, 42, and 46 bury more surface area than the phage-optimized mutations. Of the phage-optimized residues, only W45 buries more than its *wt* counterpart. The trend of the larger surface area burial for hGH in the *wt* complex extends to helix 4. Interestingly, although the helix 4 residues in hGHv bury 30% less surface area and make only two H-bonds to the receptor compared to six H-bonds in the *wt* complex, introducing the POH4 mutations into hGH produces a molecule that binds 20-fold tighter than *wt* hGH.

Ala-Scan Data and Identification of Binding Hot Spots. A common feature of protein−protein associations is that their interfaces contain binding energy hot spots (*1*, *15*, *22*, *23*). These hot spots generally make up a minor portion of the total binding surface yet contain the set of residues that contribute a disproportionally large amount of binding energy to the interaction. For the *wt* hGH system, the Site1 interactions between the hormone and receptor have been extensively characterized, showing that the majority of important residues are organized in a focused contiguous patch on the binding interfaces (*15*, *22*).

The recent analysis of the high-affinity hGHv binding surface contrasts with the *wt* hGH hot spot picture (*14*). In hGHv, the influence of the residues identified to be the main contributors in *wt* hGH was highly attenuated, with the binding energy being distributed more equally throughout the interface. However, it was not apparent whether the above characteristics were a function of the phage display selections or were partly the product of negative cooperativity via the combination of the mutations from the four libraries into the single hGHv molecule.

The extent to which the pattern of the energetics changes between *wt* hGH and hGHv is exemplified by the altered functional significance of the two residues that showed a strong preference for their *wt* sequence in the POH4 selection (T175 and R178). Together, they constitute a major component of the binding hot spot in the *wt* interaction (*22*); Ala substitutions in the *wt* hormone decreased the level of binding by 35- and 67-fold for T175A and R178A, respectively. In contrast, their respective contributions in hGHv are significantly diminished. In hGHv, the T175A mutation decreases the level of binding by only 4-fold, while R178A shows an only 6-fold effect (*14*), the essentially same values observed for the Ala-scan of the mutations in the POH4 variant (Table 1). On the basis of the differences with the *wt* scan, it is surprising that the crystal structure of the hGHv variant complexed to two copies of the hGHR ECD showed that the local interactions involving these two residues were virtually identical to those observed for the *wt* structure (*13*).

Additive and Cooperative Effects in the Libraries. As evidenced above, the procedure of combining the mutations from the four independently optimized libraries to construct the hGHv variant leads to a complicated set of functional relationships. The possibility of functional interplay among mutations in the four library groupings is particularly relevant with regard to the residue sets contained in helix 1 and helix 4, which are structurally coupled through extensive packing interactions (Figure 1). This property has broader implications outside the growth hormone system because these two helices form the high-affinity receptor binding sites in a number of four-helix bundle cytokine hormones (*2*, *24*).

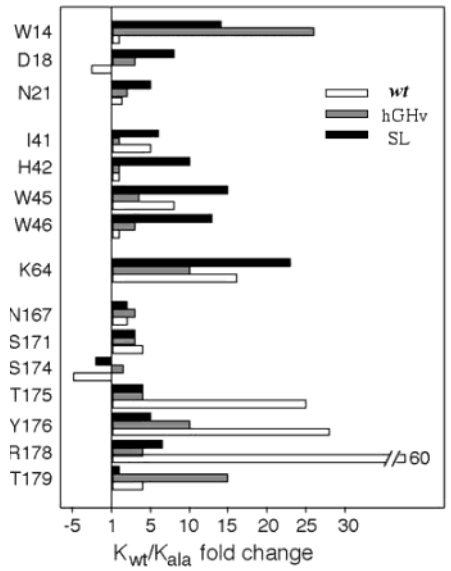

FIGURE 4: Comparison of the Ala-scan data for Site1 resides in wild-type hGH (*wt*, empty bars) and in the context of the single libraries (sl) (black) and in hGHv (gray). P54 is not included because it was not measured in *wt* hGH or hGHv. The wild-type data were taken from ref *22* and the hGHv data from ref *14*. SL data are taken from Table 2.

The existence and extent of the structural coupling between helix 1 and helix 4 were shown though a comparison of the *wt* hGH and hGHv structures (*25*). On the basis of the structural changes, it is not surprising that some of them appear to have functional consequences. Comparing Ala-scan data among *wt* hGH, hGHv, and four individual library groupings suggests that the effects of individual mutations are highly context dependent. The Ala-scans of the POH1 and POMH libraries generally show larger effects for the individual residues than when the same residues are scanned in the context of all the other 14 mutations that make up the hGHv variant (Figure 4). Additionally, the data from the *wt* hGH Ala-scan do not track with either the individual library scans or the hGHv scan. For instance, the POH4 mutations increase the Site1 binding affinity by ~20-fold compared to that of the *wt* hormone. These residues in the context of the *wt* molecule make up the majority of the binding hot spot and add ~6 kcal/mol to the binding based on the Ala-scan data (*22*). It is noteworthy, however, that in the context of the POH4 mutations alone, the contribution to binding is only 2.5 kcal/mol, even though they were the only residues mutated and that together they produced a 20-fold increase in affinity over that of the *wt*.

An opposite trend is observed for the POH1 residues. In this case, residues 14, 18, and 21 have much larger effects in the context of the individual library than in the *wt* molecule. Both D18 and N21 make new specific interactions in POH1. However, residue 14 is a "framework" residue that does not directly interact with the receptor, but rather supports the conformation of other important residues through local packing effects. On the basis of protein expression data (*14*), it is possible that the effects seen in POH1 and hGHv are due to protein stability. This appears to be a case in which the M14W mutation picked up in the phage display selection increases binding affinity, not by improving a specific interaction forming in the bound state but rather by destabilizing the unbound state. The additional free energy of stabilization toward binding is provided from a raised ground state, rather than a lowered final state.

In the case of the four residues comprising the POMH library (residues 41, 42, 45, and 46), it was determined that in the *wt* hormone only residue 45 (L45A) produced an effect of more than 10-fold (*22*). In the hGHv molecule, no single residue had an effect as great as 5-fold, suggesting little positive binding energy is generated by the mutations in the context of the other hGHv mutations. In contrast, when the same mutations are scanned in the context of the single POMH library (Figure 4), alanine substitutions at any of the four residues produce a decrease in affinity of at least 5-fold, with H42A, W45A, and W46A producing decreases in affinity of >10-fold. Thus, the POMH residues alone, with no other mutations in the molecule, appear to form a distinctive hot spot binding interaction.

We note, however, that the POMH substitutions produce an only 3-fold increase in the level of binding overall (Table 1), which is quantitatively inconsistent with the Ala-scan data, even after normalization to the *wt* hGH Ala-scan effects where only L45A produced a significant effect (*22*). The pattern of these data suggests that there are cooperativity effects among the mini-helix residues. This assumption is supported by structural data that show that the mini-helix undergoes a large conformational rearrangement of up to 8 Å on binding to hGHR. In the bound form, there is a distinctive packing arrangement among the side chains of H42, W45, and W46, which could be altered if any one of the three side chain groups was removed (Figure 2). Thus, it is probable that the binding effects that are observed in the Ala-scan of the POMH variant are due in large part to synergistic conformational factors related to the packing of the three side chains together, rather than the influence of the removal of any of the side chains alone.

Mechanism of Binding. Hormone−receptor binding involving large interfaces proceeds through three distinct stages: the unbound state, the encounter complex (a state closely associated with the transition state intermediate, TS), and the bound complex. The encounter complex is inherently unstable and can be considered a loosely organized ensemble of dynamic structures that might ultimately evolve into productive sets of specific interactions. In a sense, it represents the initial molecular recognition event, but its formation does not necessarily guarantee a biological response. The biologically relevant molecular recognition event involves considerably more structural organization and occurs somewhere later along the binding pathway.

Much of our conceptual view of molecular recognition focuses on this second molecular recognition event since it more closely resembles the structural state observed in crystallographic analyses, and is directly linked to biological activity. In contrast, we note that although there is an ever-expanding database of structural information for unbound and bound proteins, information about the structural state of the encounter complex−TS intermediate is much less plentiful (*26*, *27*). In this regard, we believe that the combination of kinetic and structural information developed from this study provides some important insight into the nature of the TS intermediate and how it is affected by conformational and electrostatic effects.

Even though the extensive set of variants generated by this study is comprised of mutations contained in very

different secondary structure types with different degrees of electrostatic complementarity and which undergo varying degrees of conformational change on binding, their k_{on} rates are remarkably constant (factor of ~2). This is true whether they are assayed as single mutants, in the context of a library, or as groups of libraries (Table 1). This indicates that the large changes seen in these K_d values do not involve processes that affect the structure of the TS. That is, changes in binding energy do not involve a reduction or increase in the TS energy but are manifested in structural phenomena directly related to factors that increase or decrease k_{off}. This has been shown to be a characteristic of binding events that are strongly influenced by van der Waals, not electrostatic, effects (*27*−*29*). These observations suggest that the TS encounter intermediate has little inherent specificity and the structure of the interface is not optimally formed. Thus, the formation of the encounter complex intermediate is characterized by a rather loose association between the hormone and receptor that apparently retains flexibility to make adjustments during formation of the complex without having any additional high-energy barriers to overcome.

We note that an important aspect of modeling the interactions involved in the TS encounter intermediate structure is the role of hydration. This is especially pertinent in this case, because the hydrophobic part of the interface is the major component in the binding hot spot. It is not clear whether upon reaching the TS structure most waters have been expelled from the interface, or whether what we are seeing is the energies used for conformational adjustments involved in forming the detailed atomic interactions being masked by the entropic gain upon the release of bound waters at the interface to the bulk. Additional biophysical studies are underway to address these issues (Horn and A. A. Kossiakoff, unpublished results).

Conclusions and Implications. Phage display mutagenesis in a sense is "evolution in a test tube". Although the extraordinary diversity that is available through phage display can generate an array of novel binding solutions, it is important to discriminate between the molecular strategies used in this method from those in play in the natural evolutionary process. A single-site mutation in a protein that is under evolutionary control occurs through a single-base change phenomenon; the probability of any mutation requiring the simultaneous change of two bases in a codon is extremely small. Additionally, sets of changes involving several residues occurring simultaneously are equally rare, except during events such as recombination (or in systems with inherently error prone polymerases). Phage display mutagenesis is not encumbered by these types of limitations; the process encourages multiple mutations, none of which is limited by only a single-base change route.

The *wt* hGH residues involved in the Site1 interaction act in a highly additive fashion (*22*), which is consistent with the co-evolution of the hormone and receptor interaction interface under the control of single-base change evolution (*30*, *31*). What is observed in the phage display-generated interactions is quite different. Since the diversity in the library is generally large enough to include most possible combinations of the potentially encoded amino acid types, it is reasonable to assume that, since the residues were selected as a group, some of the binding solutions will act as a group; that is, they will act in a cooperative fashion.

For an interaction that exhibits cooperativity effects, the impact on binding of each mutated residue is a function of the other mutated residues in that particular phage display library. Generally, we found that when a wild-type residue was substituted for a mutated residue, it had a more deleterious effect on binding than an alanine substitution. This indicates a strong context dependence within the libraries. In cases of cooperativity, Ala-scan mutagenesis does not accurately measure the energetic contributions of the individual residues. From our data, it appears that the mini-helix library selection (POMH) involved some level of cooperativity, especially between W45 and W46. The limitations of the Ala-scan in providing a semiquantitative assessment of the energies contributed by individual residues are readily seen in the data for helix 4 (Table 1).

The high-affinity variant does not contain a hot spot as highly focused as that observed for *wt* hGH, but rather has a more even distribution of contributions to binding among the residues in the contact interface (*14*). This feature raises some important questions about the mechanism of how such a phage display bio-panning process selects high-affinity mutations. Since phage display selections in this instance are directed to binding only, it is possible that the most efficient solutions will result from selecting sets of residues that individually are more or less equipotent; that is, each mutation produced a small positive improvement in the whole.

An interesting observation is that merging mutations from the individual libraries to make hGHv was a reasonably additive process; that is, the binding advantage generated by each of the phage display selections was conserved when put in combination with any of the other libraries. However, the apparent contribution of an individual residue based on Ala scanning differed significantly whether it was assayed in the context of the individual library from which it was derived or in the context of the final hGHv high-affinity variant. This suggests that residues in a library act together in additive fashion, not individually, but as a unit, through some subtle cooperative effects.

The binding kinetics of the extensive set of hGH mutants provide some important insights into the mechanism of the molecular recognition process for binding of hGH to its receptor. A pertinent question is whether there might be some inherent feature of the phage display bio-panning procedure, with its long ligand target incubation times, that preferentially favors sequences that have strong selectable on-rate traits. The members of a set of phage display-generated variants, as well as many tens of other hGH variants containing both single and multiple mutations (*10*, *11*, *15*, *22*), vary in a broad range of biophysical features of the hormone, changing charge, hydrophobicity, and conformational characteristics. Nevertheless, the binding kinetics of all the mutants show highly similar k_{on} values, suggesting that the phage display-generated variants are not selection artifacts and that the binding kinetics of these and most other hGH (and hGHR) variants are governed by the thermodynamics of the dissociation of the 1:1 complex, rather than by its formation.

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