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Intramolecular Cooperativity in a Protein Binding Site Assessed by Combinatorial Shotgun Scanning Mutagenesis

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Combinatorial shotgun alanine-scanning was used to assess intramolecular cooperativity in the high affinity site (site 1) of human growth hormone (hGH) for binding to its receptor. A total of 19 side-chains were analyzed and statistically significant data were obtained for 145 of the 171 side-chain pairs. The analysis revealed that 90% of the side-chain pairs exhibited no statistically significant pair interactions, and the remaining 10% of side-chain pairs exhibited only small interactions corresponding to cooperative interaction energies with magnitudes less than 0.4 kcal/mol. The statistical predictions were tested by measuring affinities for purified mutant proteins and were found to be accurate for five of six side-chain pairs tested. The results reveal that hGH site 1 behaves in a highly additive manner and suggest that shotgun scanning should be useful for assessing cooperative effects in other protein–protein interactions.

Keywords: cooperativity; additivity; protein engineering; phage display; growth hormone

Protein–protein interactions are central to most cellular processes, including metabolism, immune responses, signal transduction and gene regulation.[1–4] Accordingly, there is intense interest in an atomic-resolution exploration of these systems, because detailed knowledge of the physiochemical principles that govern protein–protein interactions would have immense impact on our understanding of biology. The structural database of molecular complexes has contributed tremendously to our understanding of protein–protein interactions, but three-dimensional structures do not address the energetics of binding interactions. Structural analyses can identify structural epitopes (residues in contact with a ligand), but mutagenesis approaches are necessary to define functional epitopes (contact residues that make energetic contributions to binding).

Alanine-scanning mutagenesis is the most popular method for mapping functional epitopes, as alanine substitutions remove side-chain atoms past the β-carbon without introducing additional conformational freedom.[5,6] However, the method is very laborious, as individual mutant proteins must be purified and analyzed separately. In addition, single mutations do not provide information about intramolecular cooperativity between protein residues.

Cooperativity can be detected by double-mutation cycles in which the free energy change caused by simultaneous mutation at two residue positions in a protein is compared with the sum of

Abbreviations used: ECD, extracellular domain; hGH, human growth hormone; hGHR, hGH receptor; wt, wild-type.

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	K41	Y42	L45	P48	P61	S62	N63	R64	T67	Q68	Y164	R167	K168	D171	K172	T175	F176	R178	I179
K41		60 44.8	37 31.0	113 113	17 21.8	83 93.0	48 43.1	19 23.4	98 95.6	42 49.5	56 48.3	55 50.2	35 42.4	98 109	9 13.8	21 25.7	0 3.9	5 9.2	14 13.2
Y42	0.001		19 18.0	98 90.4	23 17.0	61 69.2	29 31.8	17 16.4	77 73.3	39 42.1	36 40.3	31 35.6	33 30.8	79 81.0	9 10.3	16 14.8	0 3.5	8 5.9	7 7.5
L45	0.15	0.25		78 66.4	10 8.8	47 55.9	28 25.3	11 12.2	44 56.0	21 29.4	33 25.5	33 28.2	21 24.2	62 60.3	4 6.1	11 17.0	0 1.6	2 4.9	5 8.0
P48	0.25	0.15	0.02		41 43.0	192 189	77 86.2	31 43.9	170 181	98 101	103 94.9	87 101	78 80.8	214 205	23 23.8	45 45.9	7 7.0	15 15.0	23 21.3
P61	0.2	0.1	0.25	0.25		31 33.8	8 12.9	7 8.0	41 32.6	15 19.7	14 16.8	21 20.5	12 13.2	39 37.2	8 4.2	5 8.2	1 1.2	5 2.6	1 3.7
S62	0.1	0.1	0.05	0.25	0.25		70 68.1	36 36.0	138 144	72 80.1	73 77.3	89 84.8	66 62.3	161 162	19 19.0	39 36.1	3 5.5	9 11.8	18 16.7
N63	0.25	0.25	0.25	0.1	0.1	0.25		27 19.6	80 66.9	34 34.8	36 35.2	35 38.9	27 30.3	67 77.8	7 9.2	10 15.0	2 1.7	5 5.5	7 7.7
R64	0.25	0.25	0.25	0.003	0.25	0.25	0.05		36 33.4	25 18.6	22 17.5	15 18.1	21 16.4	30 39.5	0 4.4	3 8.7	1 1.3	5 2.7	2 3.2
T67	0.25	0.25	0.01	0.1	0.05	0.25	0.01	0.25		88 79.4	68 69.4	77 76.4	61 59.4	150 156	23 18.0	31 34.8	7 5.2	6 11.6	16 20.3
Q68	0.15	0.25	0.025	0.25	0.2	0.15	0.25	0.1	0.15		38 38.5	47 45.6	36 35.3	80 89.5	8 10.8	13 19.7	3 3.0	6 6.8	13 9.1
Y164	0.1	0.25	0.05	0.15	0.25	0.25	0.25	0.2	0.25	0.25		44 35.4	34 33.0	89 81.3	7 9.1	11 19.6	3 2.4	3 6.8	6 10.6
R167	0.25	0.25	0.2	0.01	0.25	0.25	0.25	0.25	0.25	0.25	0.05		15 30.0	78 91.2	5 11.9	17 22.4	5 3.5	6 8.0	8 8.0
K168	0.1	0.25	0.25	0.25	0.25	0.25	0.25	0.15	0.25	0.25	0.25	0.001		69 57.6	11 9.0	13 15.1	1 1.8	6 5.9	8 7.5
D171	0.05	0.25	0.25	0.2	0.25	0.25	0.05	0.025	0.25	0.1	0.15	0.02	0.02		31 20.1	48 39.7	7 6.0	14 12.4	21 18.4
K172	0.1	0.25	0.25	0.25	-----	0.25	0.25	-----	0.15	0.25	0.25	0.02	0.25	0.001		2 4.5	0 0.6	2 1.5	0 1.6
T175	0.2	0.25	0.1	0.25	0.25	0.25	0.1	0.05	0.25	0.1	0.01	0.15	0.25	0.05	-----		1 1.3	4 2.6	4 1.5
F176	-----	-----	-----	0.25	-----	0.15	-----	-----	0.25	-----	-----	-----	-----	0.25	-----	-----		0 0.4	1 1.0
R178	0.1	0.25	-----	0.25	-----	0.25	0.25	-----	0.025	0.25	0.1	0.25	0.25	0.25	-----	-----	-----		1 0.7
I179	0.25	0.25	0.15	0.25	-----	0.25	0.25	-----	0.15	0.15	0.05	0.25	0.25	0.25	-----	-----	-----	-----	

Figure 1. Statistical analysis for the detection of cooperative interactions between side-chains in hGH site 1 for binding to the hGHR. The upper quadrant shows the observed (top number) and expected (bottom number) occurrence of double-alanine mutations for each pair of side-chains. The lower quadrant shows the *P*-value for the null hypothesis that the mutant effects should be additive. The grey squares denote pairs for which the expected double-alanine mutant count was insufficient for statistical analysis. The green squares denote pairs with no statistically significant deviation from additivity (*P*-value >0.05). The blue and red squares denote pairs predicted to exhibit positive or negative cooperativity, respectively (*P*-value <0.05). The construction and sorting of the phage-displayed library of hGH variants has been described.[12] Briefly, 19 codons chosen for mutagenesis were replaced simultaneously by shotgun alanine-scanning codons that ideally encoded for equal proportions of the wt and alanine, although two additional substitutions were permitted at some positions. Phage from the library were cycled through two rounds of binding selection with immobilized hGHR extracellular domain (hGHR-ECD) as the capture target. Following selection, individual clones were sequenced and filtered to remove any sequences that were identical with other sequences at all 19 mutagenized positions. The resulting data set of 709 unique hGH variants was used for the pairwise correlation analysis. The data set was analyzed by the program SGCOUNT, which aligned all 709 unique sequences and counted the occurrence of each natural amino acid at each position. For each (i);(j) pair of positions (where (i) and (j) are non-identical mutated positions), SGCOUNT extracted the subset of sequences which contained only alanine or wt at both positions; this was necessary because two additional non-alanine substitutions were permitted at some positions. SGCOUNT then tabulated the observed counts of each of the four possible classes: wt ($w_{(i)};w_{(j)}$), double-alanine mutant ($a_{(i)};a_{(j)}$), and the two single-alanine mutants ($a_{(i)};w_{(j)}$ and $w_{(i)};a_{(j)}$). The sum of the observed joint occurrences for the four classes ($O_{w(i);w(j)}$, $O_{a(i);a(j)}$, $O_{a(i);w(j)}$, and $O_{w(i);a(j)}$) equals the total number of clones ($N_{(i);(j)}$) with sequence containing either alanine or wt at positions (i) and (j). The number $N_{(i);(j)}$ depends on the particular pair of positions and varied between a minimum of 209 and a maximum of 709. The program also calculated the expected joint occurrence for each of the four classes ($E_{w(i);w(j)}$, $E_{a(i);a(j)}$, $E_{a(i);w(j)}$, and $E_{w(i);a(j)}$), assuming that mutations at the two positions (i) and (j) are independent. For example, the expected joint occurrence of the double-alanine mutant was calculated as the product of the individual counts of alanine at the two positions ($N_{a(i)}$ and $N_{a(j)}$), normalized by the total number of clones as follows:

$$E_{a(i);a(j)} = N_{(i);(j)} \frac{N_{a(i)}}{N_{(i);(j)}} \frac{N_{a(j)}}{N_{(i);(j)}} = \frac{N_{a(i)} N_{a(j)}}{N_{(i);(j)}}$$

Given the observed and expected counts, a standard chi-squared test was used to test the independence of the mutated positions.[15,16] The chi-squared statistic (CSS) with one degree of freedom was computed as follows:

$$\mathrm{CSS} = \frac{(O_{a(i);a(j)} - E_{a(i);a(j)})^2}{E_{a(i);a(j)}} + \frac{(O_{a(i);w(j)} - E_{a(i);w(j)})^2}{E_{a(i);w(j)}} + \frac{(O_{w(i);a(j)} - E_{w(i);a(j)})^2}{E_{w(i);a(j)}} + \frac{(O_{w(i);w(j)} - E_{w(i);w(j)})^2}{E_{w(i);w(j)}}$$

the free energy changes associated with single mutations at the two residue positions.[7–9] Deviations from additivity are indicative of cooperative interactions, and in this way, energetic coupling between protein residues can be quantified. Unfortunately, these analyses are even more laborious than single-mutation alanine-scanning, as a comprehensive assessment of cooperativity requires the analysis of not only all point mutations within a structural epitope, but also, the analysis of all possible double-mutation combinations.

We have previously described the use of phage-displayed protein libraries for the rapid mapping of functional epitopes.[10–12] Combinatorial shotgun alanine-scanning mutagenesis was used to study the high affinity site (site 1) of human growth hormone (hGH) for binding to the hGH receptor (hGHR).[12] A library of hGH variants was constructed such that 19 side-chains within site 1 were allowed to vary as the wild-type (wt) or alanine, although two additional substitutions were permitted at some positions. Following functional selections for binding to the hGHR, DNA sequencing was used to determine the wt/alanine ratios at the randomized positions, and these ratios were used to quantify the energetic contributions of each side-chain to the binding interaction.

It has been proposed that such a combinatorial method could also be used to detect intramolecular cooperativity by pairwise correlation analysis, as the occurrence of double-alanine mutations should be positively or negatively influenced by positive or negative cooperativity, respectively.[13] Herein, we describe the use of shotgun scanning mutagenesis to assess intramolecular cooperativity within hGH site 1.

Assessment of intramolecular cooperativity by shotgun scanning

Nineteen residues within the hGH site 1 for binding to the hGHR were randomized in a combinatorial, shotgun alanine-scanning library.[12] Following functional selection for binding to the hGHR, a data set of 709 unique sequences was used to detect cooperative interactions between side-chains. For each pair of positions, double-alanine mutants were counted. In addition, the expected occurrence of double-alanine mutants was calculated, assuming that the effects of the single-alanine mutations were independent of each other (Figure 1, top quadrant). Furthermore, the deviations from pure additivity were assessed by a statistical analysis, and probabilities that the effects of the two alanine mutations were purely additive were computed (Figure 1, bottom quadrant). We did not calculate probabilities for pairs at which the expected double-alanine count was less than five, as the statistics for such positions are likely to be unreliable.[14] Thus, we excluded 27 pairs from the analysis due to insufficient data (Figure 1, grey squares), but we were able to assess cooperativity between 144 of the 171 side-chain pairs.

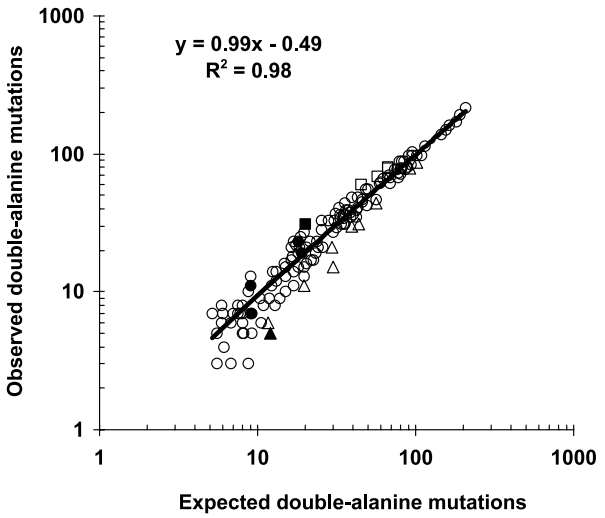

Figure 2. Correlation between the expected (x axis) and observed (y axis) counts of double alanine-mutations in hGH site 1 variants selected for binding to the hGHR. Each symbol represents one of the 144 side-chain pairs for which the expected double-alanine mutant count was sufficient for statistical analysis (Figure 1). Circles denote pairs with no statistically significant deviation from additivity. Squares and triangles denote pairs predicted to exhibit positive or negative cooperativity, respectively. The filled symbols denote pairs that were analyzed by double mutant cycle analysis (see Table 1). The least squares linear fit of the data is shown, with the corresponding equation and R^2 value given at the top left.

The expected *versus* observed double-alanine mutation counts were plotted to obtain an overall view of the pairwise interactions between the side-chains in hGH site 1 (Figure 2). A least squares linear fit of the data showed a strong correlation ($R^2 = 0.98$), with a slope close to unity (0.99) and a y intercept close to zero (-0.49). Deviations from the fitted line tend to be greater for pairs with low numbers of double-alanine counts, and this is likely due to the nature of stochastic sampling error, which is inversely proportional to the number of counts. Nonetheless, it is clear that, overall, the side-chains in hGH site 1 behave in a highly additive manner.

A P-value characterizing the statistical significance of deviation of observed and expected values was looked up from a table corresponding to the calculated CSS value at one degree of freedom. The P-value served as a test of the null hypothesis that the mutant effects should be additive.[13] A cutoff value ($P < 0.05$) was applied. P-values below the cut-off indicate residue pairs for which the probability that the calculated deviation is due only to stochastic sampling error is less than 5%, and therefore, there is a greater than 95% probability that the two positions are not independent.

Of the 144 pairwise combinations for which the data was sufficient to provide statistically reliable predictions, a chi-squared test for independence[15,16] predicted that only 15 pairs (10%) behave in a non-additive manner at a confidence level greater than 95% (P-value < 0.05). Five of these pairs were predicted to interact with positive cooperativity (blue squares in Figure 1) and ten were predicted to interact with negative cooperativity (red squares in Figure 1). However, it should be noted that, while the deviations from the expected counts were statistically significant for these 15 pairs, even the largest deviations between the expected and observed double-alanine counts were only about twofold. Again, this analysis emphasizes that hGH site 1 is highly additive, as 90% of the analyzed pairs are predicted to be purely additive, and even the largest deviations from additivity are only predicted to cause twofold deviations in binding affinities of double-alanine mutants compared with predictions derived from the binding affinities of the component single-alanine mutants.

Of the 19 side-chains analyzed, eight are predicted to have cooperative interactions with two or more of the other side-chains (Figure 3). It is noteworthy that five of these side-chains are charged, suggesting that many of the cooperative effects arise from electrostatic interactions. Of particular interest is a cluster of three basic side-chains (Arg167, Lys168 and Lys172) and one acidic side-chain (Asp171) at the center of the binding site, as these side-chains exhibit significant cooperativity with each other.

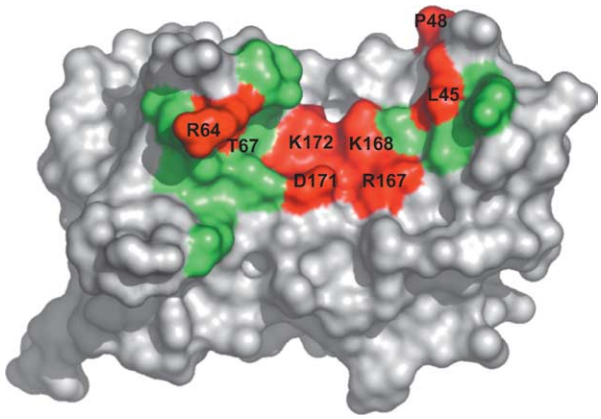

Figure 3. Predicted cooperative interactions in hGH site 1 for binding to the hGHR. The three-dimensional structure of hGH[20] is shown as a molecular surface. The 19 residues analyzed by shotgun scanning are colored red or green. Red color indicates residues for which two or more statistically significant pair interactions were detected (see Figure 1). The structure was rendered in Pymol (DeLano Scientific, San Carlos, USA).

Double mutant cycle analysis

To test the validity of the statistical shotgun scanning predictions, we selected a set of double-alanine mutants for analysis by surface plasmon resonance. We focused on double-alanine mutants that contained a common alanine substitution for Lys172, because the Lys172 side-chain is an energetically important component of the functional binding epitope,[5,6,12] and it was predicted to participate in both positively and negatively cooperative interactions (Figure 1). We purified wt hGH and a panel of 13 hGH mutants to measure cooperativity between Lys172 and six other side-chains. The shotgun scan analysis predicted that Lys172 should exhibit positive cooperativity with one of these side-chains (Asp171), negative cooperativity with another (Arg167), and additivity with the remaining four (Ser62, Asn63, Thr67 and Lys168) (Figures 1 and 2).

In the classical double mutant cycle analysis, cooperativity can be detected and quantified by comparing the observed dissociation constant for a double mutant ($K_{d,obs}$) to the dissociation constant calculated from affinity measurements for the two corresponding single mutants ($K_{d,calc}$).[7,9] $K_{d,obs}$ values that are greater or less than $K_{d,calc}$ are indicative of negative or positive cooperativity, respectively, and the ratio of the two dissociation constants can be used to derive an interaction energy ($\Delta G_{int} = RT \ln[K_{d,obs}/K_{d,calc}]$) that provides a quantitative measure of the degree of cooperativity.[7,9]

The results of the kinetic analysis of hGH mutants are presented in Table 1. The double mutant cycle analysis confirmed the predicted positive or negative cooperativity between Lys172 and Asp171 or Arg167, respectively. The degree of cooperativity was very slight, as the deviations between $K_{d,obs}$ and $K_{d,calc}$ were only about twofold ($|\Delta G_{int}| < 0.4$ kcal/mol), and again, this was in agreement with the statistical predictions. The statistical predictions were also confirmed for three of the four side-chains (Ser62, Thr67 and Lys167), which were predicted to exhibit additivity with Lys172; however, the fourth side-chain (Asn63) exhibited slight negative cooperativity ($\Delta G_{int} = 0.41$ kcal/mol). These results indicate that the statistical method is highly accurate and sensitive in detecting cooperativity, but the discrepancy for the Asn63/Lys172 double-alanine mutant suggests that interaction energies with magnitudes less than 0.4 kcal/mol represent the limit of detection.

Differences in folding efficiency and expression level amongst variants could lead to biases in the sequences of the library pools. The effects of such biases have been addressed previously and were found to be minor, at least for the 19 solvent accessible residues analyzed herein.[12] Nonetheless, these biases could be significant enough to account for the observed limits of detection for interaction energies. However, we note that the observed subtle differences in interaction energy also approach the limits of accuracy for surface plasmon resonance, and thus it is possible that, at least in part, the discrepancy may be due to limitations in the

Table 1. Kinetic analysis of hGH mutants binding to the hGHR

hGH mutant	k_{on} ($M^{-1}s^{-1}\times10^{-5}$)	k_{off} ($s^{-1}\times10^{4}$)	$K_{d,obs}$ (nM)	$K_{d,calc}$ (nM)	$K_{d,obs}/K_{d,calc}$	ΔG_{int} (kcal/mol)
Wt	3.38±0.14	4.54±0.33	1.35±0.12			
S62A	3.23±0.20	7.40±0.28	2.29±0.17			
N63A	2.62±0.20	5.91±0.01	2.26±0.18			
T67A	2.18±0.13	3.42±0.31	1.57±0.17			
R167A	2.51±0.15	2.06±0.15	0.82±0.08			
K168A	3.40±0.16	2.76±0.33	0.81±0.10			
D171A	3.95±0.46	29.7±1.5	7.57±0.96			
K172A	2.55±0.13	140±5	55.0±3.5			
K172A/S62A	1.83±0.75	231±5	126±6	93±18	1.35±0.27	0.18±0.04
K172A/N63A	1.77±0.75	327±26	184±16	92±18	2.00±0.43	0.41±0.09
K172A/T67A	1.71±0.17	96.1±6.7	56.9±6.9	64±13	0.89±0.22	−0.07±0.02
K172A/R167A	1.90±0.14	116±8	60.8±6.3	34±7	1.81±0.41	0.35±0.08
K172A/K168A	1.73±0.14	79.2±3.0	45.8±4.1	33±7	1.38±0.33	0.19±0.05
K172A/D171A	2.27±0.16	381±14	168±14	308±67	0.55±0.13	−0.35±0.09

Kinetic analysis was performed by surface plasmon resonance, and a 1 : 1 Languir model was used with simultaneous fitting of rate constant values k_{on} and k_{off}. The observed dissociation constant ($K_{d,obs}$) was determined from the k_{off}/k_{on} ratio. For each double-alanine mutant at two sites *X* and *Y*, the calculated K_d ($K_{d,calc}$) was determined from the $K_{d,obs}$ values for the component single-alanine mutants by the following equation: $K_{d,calc,XY}=(K_{d,obs,X})(K_{d,obs,Y})/K_{d,obs,wt}$. The interaction energy (ΔG_{int}) for each double-alanine mutant was determined by the following equation: $\Delta G_{int}=RT\ln[K_{d,obs}/K_{d,calc}]$. Mutations were generated by site-directed mutagenesis using the method of Kunkel *et al.*[21] Mutants of hGH were made in the background of the G120R mutation which ensures monovalent binding to hGHR,[22] and proteins were expressed and purified as described.[23] A truncated form of the hGHR-ECD (residues 29–238) with a S237C mutation was expressed and purified as described.[24,25] The purities of all protein samples exceeded 95% as judged by RP-HPLC and SDS-PAGE, and the identities of all mutants were confirmed by mass spectrometry. Protein concentrations were determined by amino acid analysis performed in triplicate. Protein affinities were measured at 25 °C by surface plasmon resonance on a Biacore 2000 instrument (Biacore Inc., Piscataway, NJ). The hGHR-ECD was immobilized through the engineered cysteine residue on a Pioneer C1 sensor chip at ~50 response units, and unreacted functional groups were blocked with glutathione, as described.[19] For kinetic analysis, twofold serial dilutions of hGH variants in HBS buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20, pH 7.4) were injected, and binding responses to the hGHR-ECD were corrected by subtracting responses from running buffer injections (no hGH injected) and by subtracting responses on a blank flow cell (no hGHR-ECD immobilized). High affinity ($K_d<40$ nM) or low affinity ($K_d>40$ nM) proteins were injected at concentrations of 2–32 nM or 8–128 nM, respectively.

accuracy of the kinetic measurements. Overall, the double mutant cycle analysis confirms the predictions of the shotgun alanine-scanning analysis.

Conclusions

Shotgun alanine scanning demonstrates that the high affinity hGH site 1 behaves in a highly additive manner. The largest cooperative interactions detected by the statistical analysis only caused approximately twofold deviations between the calculated and observed K_d values, and only 10% of the analyzed side-chain pairs were predicted to exhibit even these slight cooperative effects. These results are in good agreement with the results of a classical alanine-scan of hGH site 1 which revealed that the cumulative energetic effects of single-alanine mutants summed to a value close to the binding energy of the hGH:hGHR interaction, an observation that suggested that cooperative effects play only a minor role in the binding interaction.[5]

These results, and the results of previous studies, suggest that additivity of mutational effects is likely the general rule for protein–protein interactions.[7,8,13,17] Nonetheless, many exceptions to the additivity rule have been observed, and a complete understanding of molecular recognition will require accurate accounting of cooperative interactions.[18,19] The rapidity and general applicability of the shotgun scanning strategy should expedite the analysis of many other protein–protein interactions, and it will be interesting to compare and contrast the findings for different systems.

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