Protein-Protein Interactions - II

-experimental dissection of protein-protein interactions.

-development of theory for understanding protein-protein interactions has occurred in parallel with refinement of methods for probing protein interactions through experiment.

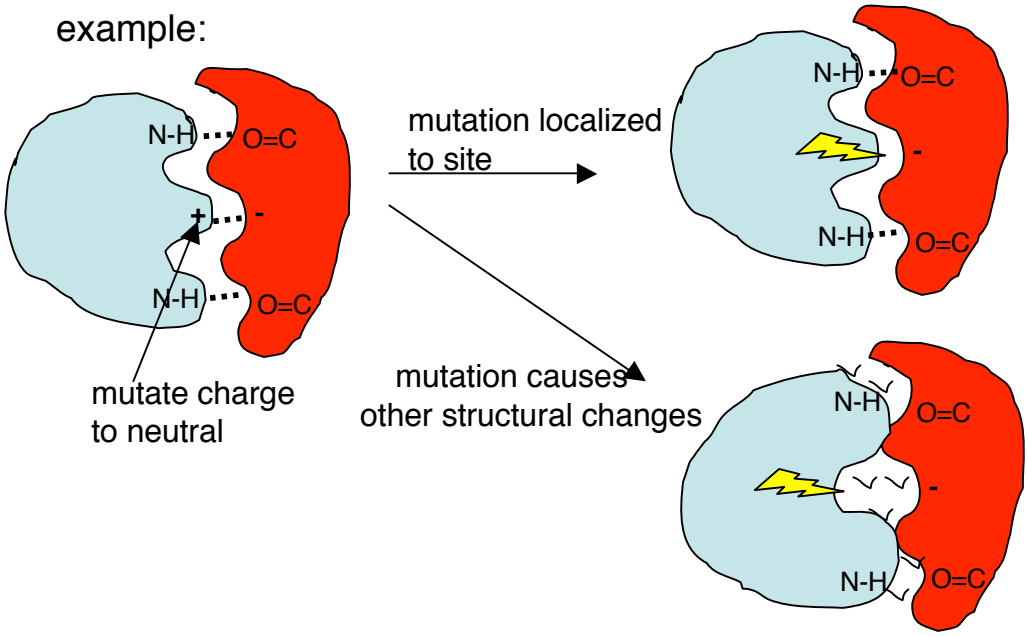
I. Site-directed Mutagenesis

$$\Delta\Delta G_{(X,Y)} = \Delta\Delta G_{(X)} + \Delta\Delta G_{(Y)}$$

1) Additivity principle.

- a critical - *BUT ERRONEOUS*- assumption in site-directed mutagenesis of a protein-protein interaction is that the mutation itself does not introduce structural perturbations in the protein that skew the functional readout of the mutation.

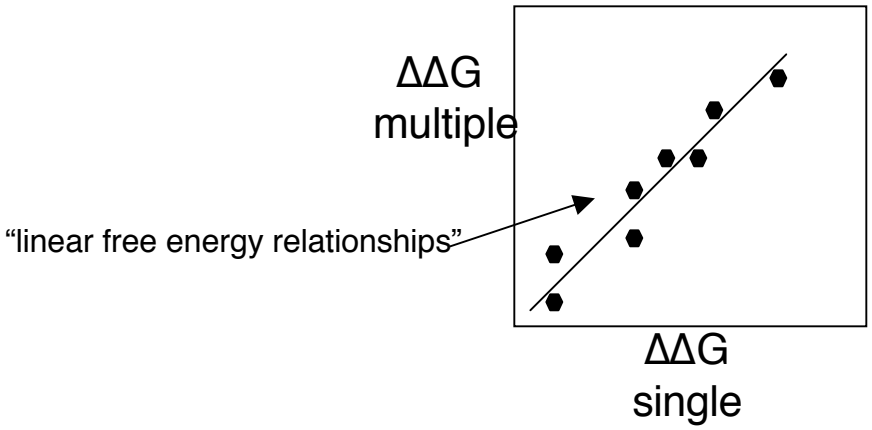
example:



-principle of additivity has been widely debated in the literature (Wells, Fersht, etc.)

$$\Delta\Delta G_{(X,Y)} = \Delta\Delta G_{(X)} + \Delta\Delta G_{(Y)} + \Delta\Delta G_{I}$$

$\Delta\Delta G_I$ is the "coupling energy" between residues X and Y
-normally $\Delta\Delta G_I$ is assumed to be zero.
-however, $\Delta\Delta G_I$ can be either positive or negative depending on whether the interactions between the sites enhances or reduces the functional property being measured.



-the additivity of protein mutational effects implies that proteins are modular and obey predictable rules of thermodynamics.
-also that mutational effects remain localized (evolution ?)
-divergence from additivity in cases of allostery, and conformational change,

2) Double-mutant cycles and additivity (Fersht, Winter).

-Fersht proposed that mutation of residue X to A is not the intrinsic binding free energy of residue X, but rather just the relative binding energy of X versus A.
-A better procedure (more work of course !) is to singly and doubly mutate pairs of residues X and Y.

$$\Delta\Delta G_{int} = \Delta\Delta G_{(X > A,\ Y > B)} - \Delta\Delta G_{(X > A)} - \Delta\Delta G_{(Y > B)}$$

$\Delta\Delta G_{(X > A, Y > B)}$ is the free energy change on the simultaneous mutation of X to A and Y to B

$\Delta\Delta G_{int}$ is then a measure of the cooperativity of interaction of two sites X and Y.

- if the effects are independent, then $\Delta\Delta G_{int}$ equals 0 and $\Delta\Delta G_{(X > A, Y > B)}$ is the sum of the free energy change of each of the single mutants.

- this method is not only useful for probing binding sites on one protein, but can also be used to identify interacting amino acids across a protein interface.

-Example: Barnase/Barstar (Schreiber & Fersht, 1995)

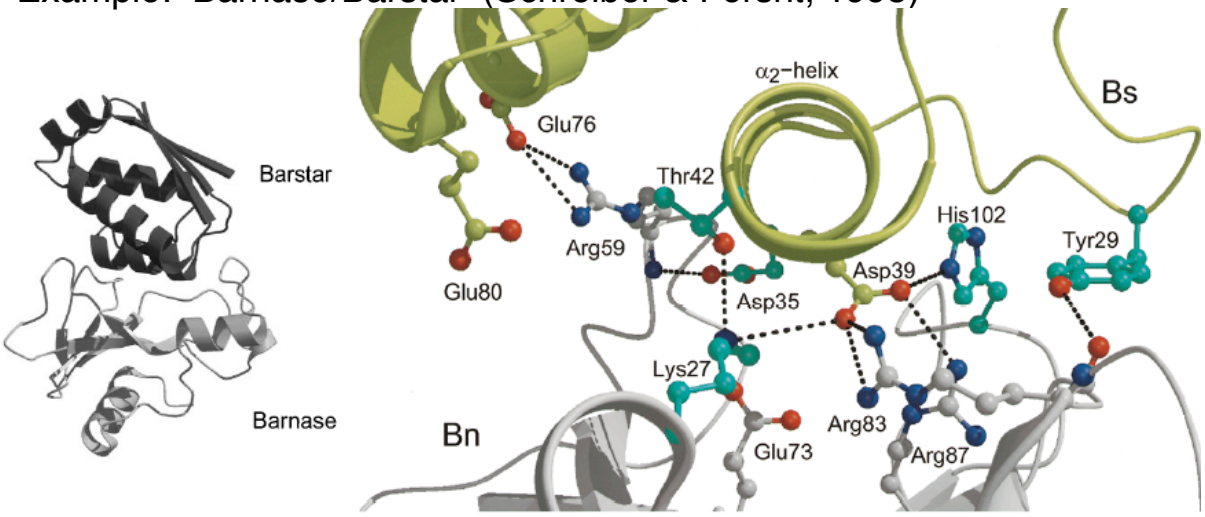
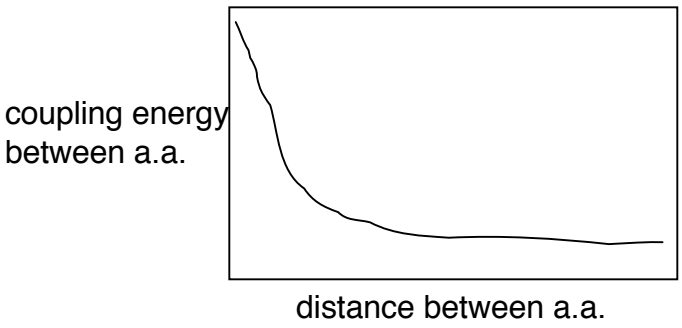


Table 3. Kinetics and equilibrium of association of barstar and barnase mutants

Barnase	Barstar	$k_1 \times 10^{-8}$ ($s^{-1}M^{-1}$)	$k_{-1} \times 10^3$ (s^{-1})	K_D (pM)	ΔG^a (kcal mol^{-1})	$\Delta\Delta G^b$ (kcal mol^{-1})	$\Delta\Delta G_{int}{}^c$ (kcal mol^{-1})
wt	wt	3.7	0.0037	0.01	19.0		
H102A	wt	4.0	129	320	12.9	6.1	
wt	Y29F	3.0	0.0024	0.008	19.1	−0.1	
H102A	Y29F	3.9	45	117	13.5	5.5	0.5 (0.36)
K27A	wt	0.51	4.5	88	13.6	5.4	
wt	D35A	1.9	3.8	20	14.5	4.5	
wt	T42A	3.2	0.072	0.23	17.2	1.8	
K27A	D35A	0.37	3600	1×10^5	9.5	9.5	0.4 (0.22)
K27A	T42A	0.43	6.8	157	13.3	5.7	1.5 (0.28)

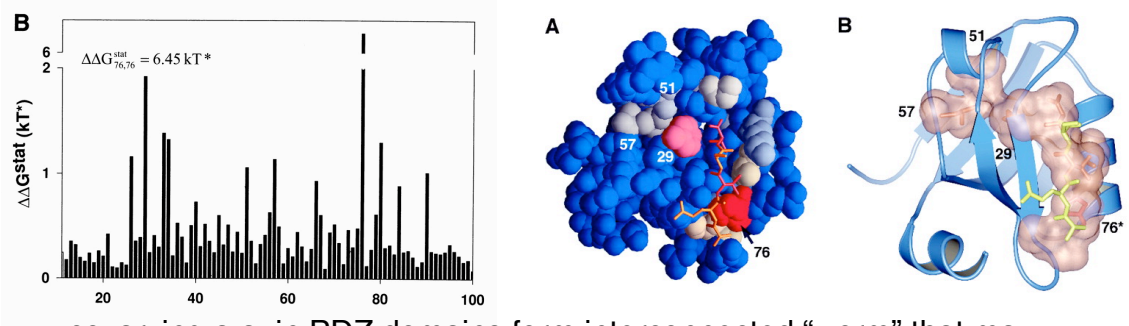
-in some case, coupling is observed between residues that do not interact.

-an important result of this work, and other related works, is that coupling energy is distance dependent.
-that is, coupling energies are observed for residue pairs up to 7Å apart.
-the range of amino acid interaction distances is more than 4Å even for uncharged interactions.
-energetics of a charged interaction cannot be assessed by only a distance dependence, is highly skewed to local environments.



-the phenomenon of coupling energies may explain some of the deviations seen in correlations between buried surface area and free energy changes.

Digression - Interesting concept (Lockless & Ranganathan, 1999)



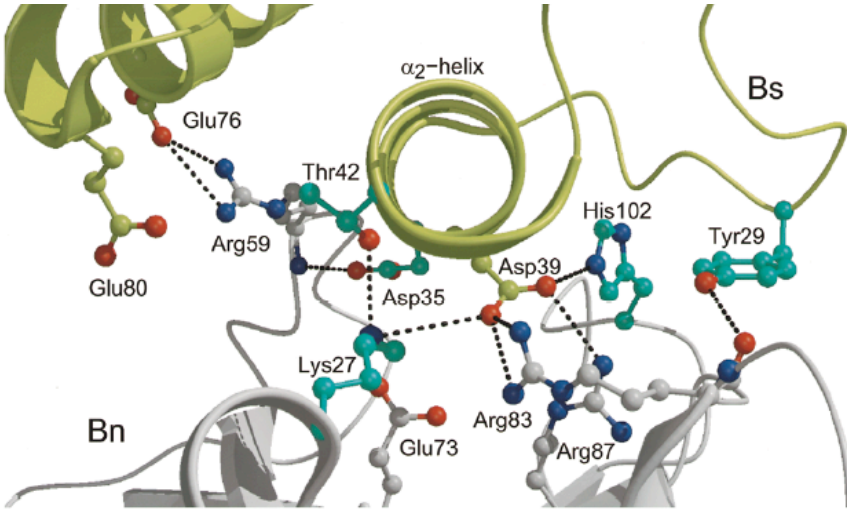
covarying a.a. in PDZ domains form interconnected “worm” that may indicate energetic coupling through the protein.

what about enthalpy-entropy compensation ?

(Frisch et al., 1997)

Table 2. Thermodynamic parameters of binding of mutants of both barnase and barstar

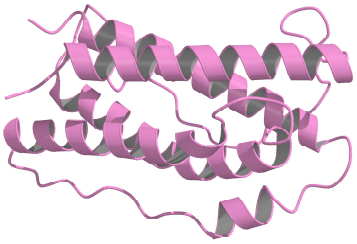
Barstar	Barnase	ΔH (kcal/mol)	$\Delta\Delta H$[a] (kcal/mol)	ΔG[b] (kcal/mol)	$\Delta\Delta G$[c] (kcal/mol)	K_a[d] (M^{-1})
wt	wt	−19.3		−19.0		1×10^{14}
wt	H102A	−10.9	−8.4	−12.9	−6.1	3.1×10^{9}
Y29A	wt	−14.2	−5.1	−15.6	−3.4	2.9×10^{11}
Y29A	H102A	−13.3	−6.0	−12.7	−6.3	2.4×10^{9}
Y29F	wt	−18.8	−0.5	−19.1	0.1	1.2×10^{14}
Y29F	H102A	−12.0	−7.3	−13.5	−5.5	8.5×10^{9}
D39A	wt	−7.4	−11.9	−11.3	−7.7	2.5×10^{8}
				−11.1[e]	−7.9[e]	1.3×10^{8}[e]
D39A	H102A	−2.4	−16.9	−10.1	−8.9	2.6×10^{7}
				−8.3[e]	−10.7[e]	1.3×10^{6}[e]
wt	R59A	−11.9	−7.5	−13.8	−5.2	1.4×10^{10}
wt	K27A	−13.1	−6.2	−13.6	−5.4	1.1×10^{10}
wt	R87A	−17.3	−2.0	−13.5	−5.5	8.3×10^{9}
D39A	R59A	−1.7	−17.6	−7.7[e]	−11.3[e]	4.7×10^{5}[e]
D39A	K27A	−10.6	−8.7	−10.8	−8.2	8.5×10^{7}
				−10.3[e]	−8.7[e]	3.7×10^{7}[e]
D39A	R87A	−13.4	−5.9	−11.9	−7.1	5.4×10^{8}



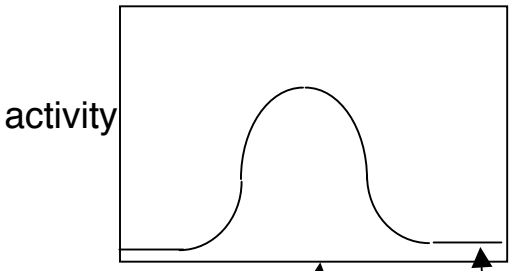
-mutations will, in general, lead to larger changes in enthalpy than is reflected in the overall binding free energy.
-hence, due to instability and poor estimation of energetics of polar interactions and hydration, overall free energy is much better correlated with mutation.
-a calculation of Cp based on the buried surface area gives a value of -85cal/K/mol, but a measurement of Cp=-235.

The Human Growth Hormone (hGH) System as a paradigm for protein-protein interactions and receptor dimerization:

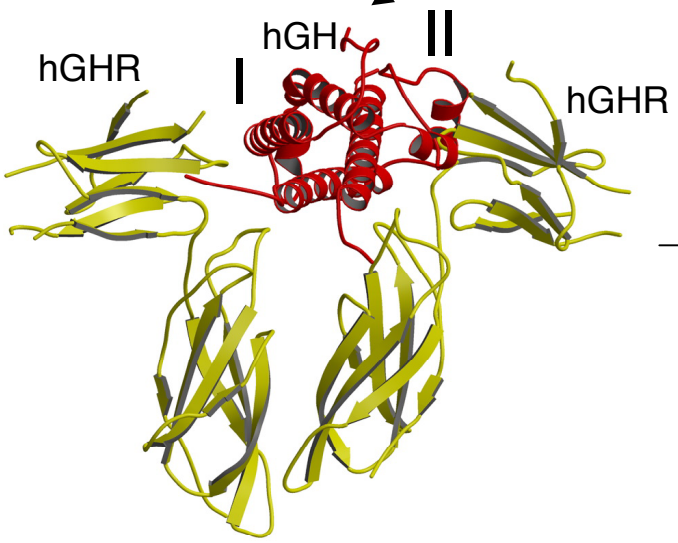
1) Jim Wells et al. have carried out a comprehensive structural and mutational analysis of hGH interaction with the hGH receptor (hGHR).
-bell-shaped activity curve for hGH activity on cells expressing hGHR.

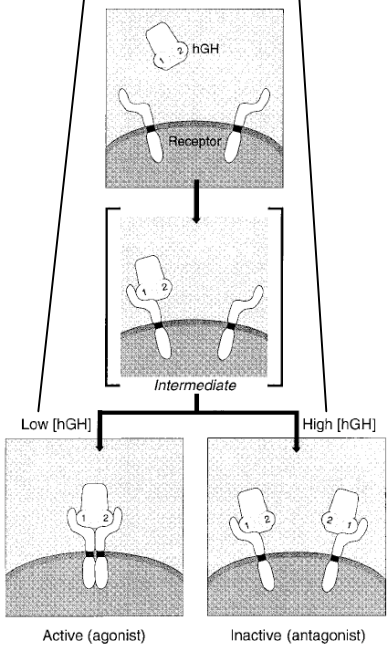


hGH



structure revealed why !





2:1 receptor/ligand stoichiometry

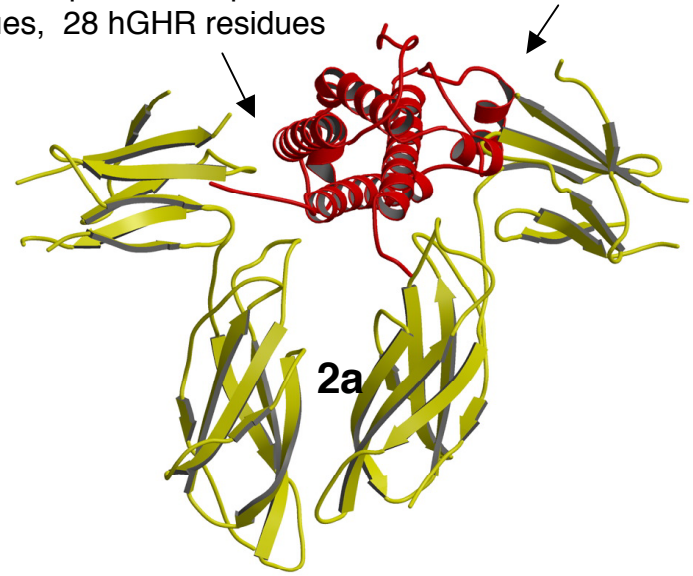
-simple dimerization alone not sufficient to activate signal, requires precise geometry.

2) Interface descriptions of the hGH/hGHR complex.

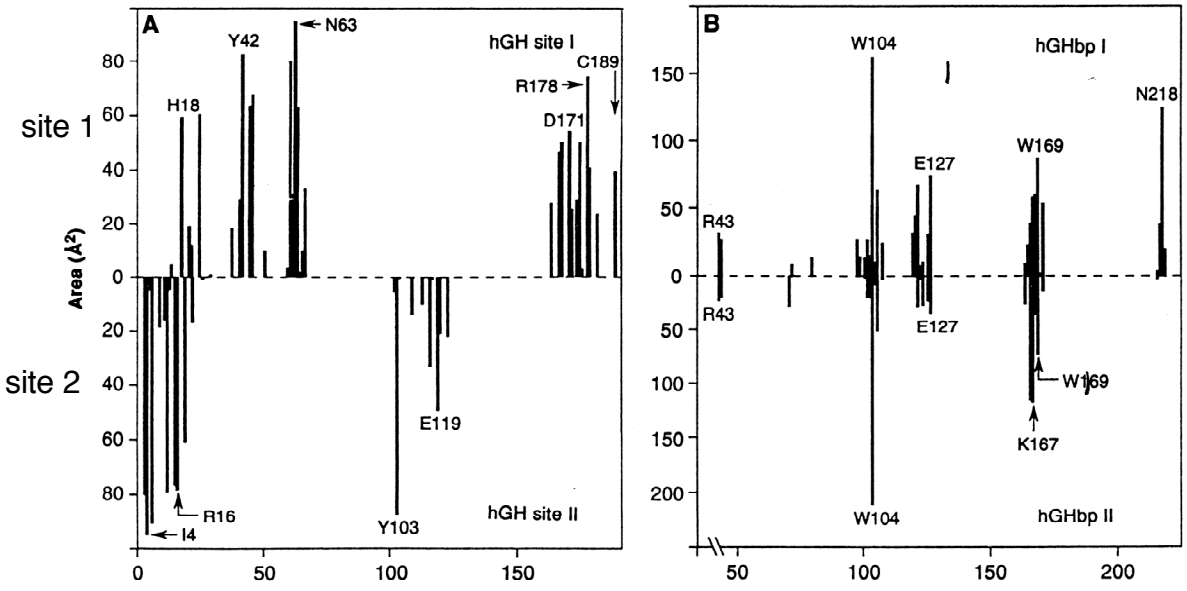
site 1: 1350Å^2, 52% non-polar, 48% polar,
31 hGH residues, 28 hGHR residues

site 2: 850Å^2, 52% non-polar, 48% polar,
20 hGH residues, 15 hGHR residues

"site 2a": 500Å^2, 17 residues

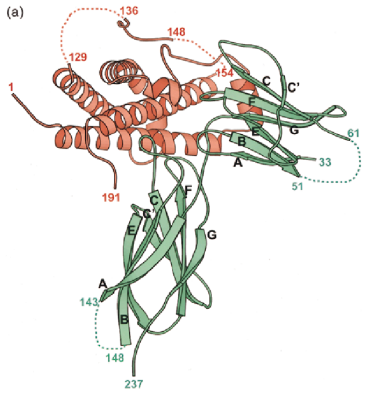


asymmetric hGH contacts, but roughly symmetric hGHR contacts.



3) Non-equivalence between “structural” and “functional” epitopes.

-the site 1 interface is the highest affinity.
-an hGH antagonist can be engineered by mutating site 2 contact residues on hGH. This “1:1” complex has been the target of most of the mutational studies.

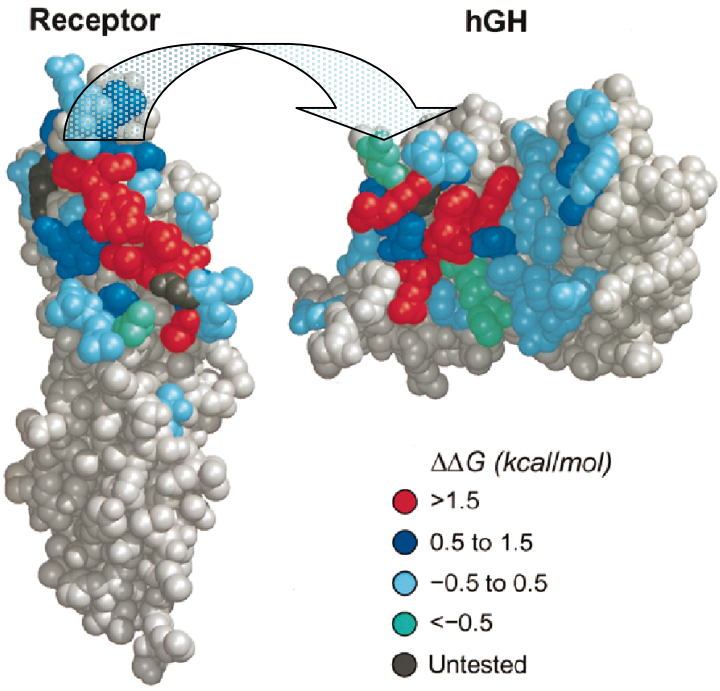


-all side chains in both hGH and receptor interface have been “**Ala scanned**” to yield a surprising result - only a few of the many amino acids are energetically important. These have been termed the “energetic hotspot.”

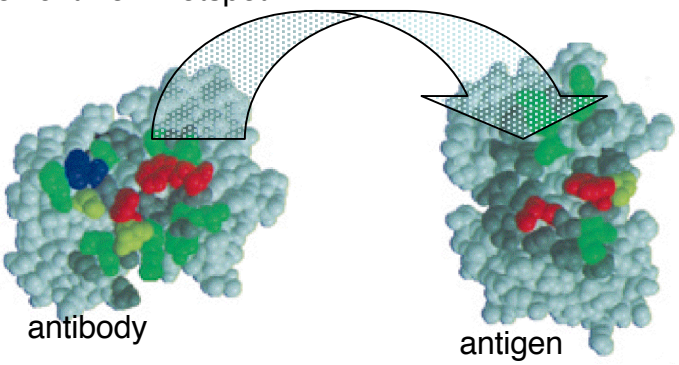
	Loss in side-chain solvent accessibility[a] (Å^2)	K_d (nM)	Relative K_d (mut/wt)	$\Delta\Delta G_{(mut\text{-}wt)}$ (kcal/mol)[b]
Alanine scan of structural epitope:[d]				
Wild-type	–	0.34 ± 0.07	(1)	–
R43A	30.9	12.1 ± 3.0	35.7	2.12
E44A	34.0	5.92 ± 0.80	17.4	1.69
R70A	1.1	1.09 ± 0.14	3.20	0.69
R71A	80.7	0.84 ± 0.10	2.47	0.54
T73A	0.0[e]	0.41 ± 0.08	1.20	0.11
Q74A	8.9	0.34 ± 0.04	1.00	0
T75A	22.8	0.29 ± 0.05	0.84	−0.10
W76A	57.6	0.81 ± 0.15	2.38	0.51
W80A	4.2	0.33 ± 0.07	0.97	−0.02
S98A	15.3	0.31 ± 0.07	0.92	−0.05
S102A	22.3	0.25 ± 0.05	0.72	−0.2
I103A	8.1	5.13 ± 0.5	15.1	1.61
W104A	135.3	>1000	>2500	>4.5
I105A	13.0	9.01 ± 2.44	26.5	1.94
P106A	36.2	90.1 ± 6.0	265	3.31
E120A	34.6	0.25 ± 0.02	0.73	−0.19
K121A	12.0	0.39 ± 0.03	1.15	0.08
S124A	12.7	0.54 ± 0.08	1.60	0.28
D126A	16.6	1.82 ± 0.15	5.35	0.99
E127A	65.9	1.75 ± 0.33	5.15	0.97
D164A	16.7	4.18 ± 0.74	12.3	1.49
I165A	18.7	12.4 ± 2.9	36.5	2.13
Q166A	6.6	0.35 ± 0.06	1.03	0.02
K167A	37.5	0.33 ± 0.05	0.96	−0.02
W169A	88.7	>1000	>2500	>4.5
V171A	43.2	0.12 ± 0.02	0.34	−0.64
T195A	7.0	0.29 ± 0.06	0.86	−0.09

-in fact, a plot of ΔΔG versus loss of ASA reveals NO correlation.
-one reason for the divergence in the system may be structural plasticity, which is difficult to predict and even more difficult to predict the energetic cost.

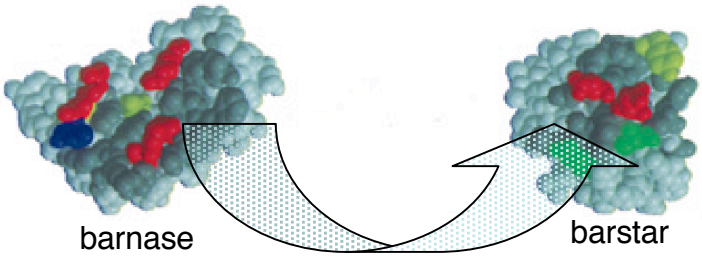
“energetic hotspot” concept:



-appears as if there is a central core hotspot surrounded by a halo of functionally inert residues responsible for “presenting” the hotspot.
-hotspot residues tend to be large (Trp, Tyr and Arg), while halo residues tend to be small and polar. Halo residues likely “steer” proper docking orientations by presenting anchor points at periphery, as well as serve as a “seal” to occlude bulk solvent from hotspot.

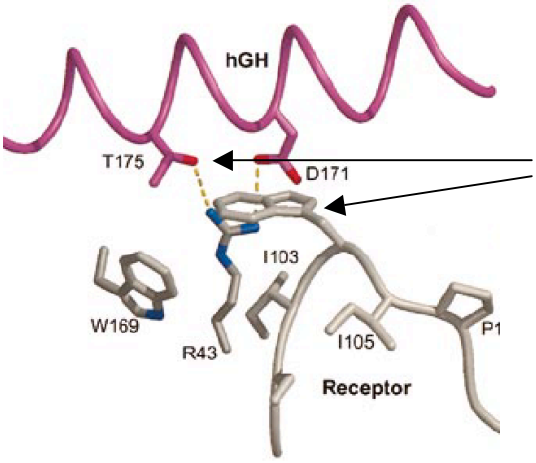


-double mutant cycles in an Ab/Ag interface revealed that only 2 out of 14 direct interatomic contacts were energetically significant (Dall’Acqua et al., 1999)



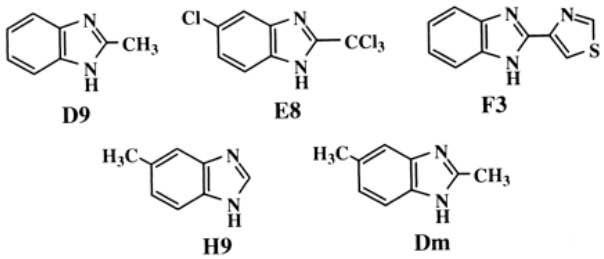
-”hotspot” concept is highly prevalent across many, but not all protein-protein interactions.

-the hotspot concept has major implication for the pharmaceutical industry.
-suggests that protein-protein interactions can be modulated by a small molecule that does not have to encompass a surface area the size of a protein interface.
-in comparison to the deep clefts of enzyme active sites, protein-protein interfaces present a much more difficult challenge for drug design. why ?
-in fact, Peter Schultz group did a very clever experiment (Guo et al., 2001):

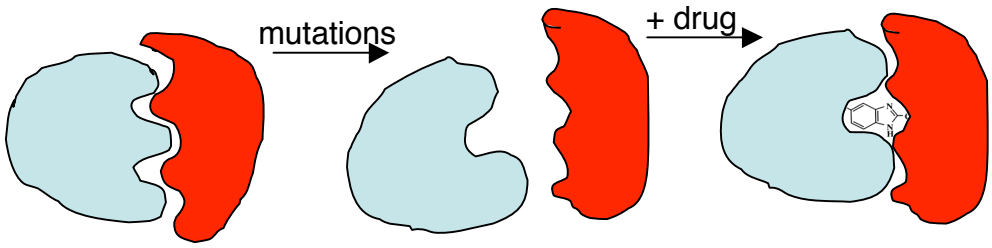


mutated both to Glycine to create a "hole" in the interface. These mutants had negligible affinity for each other (K_d > 1mM).

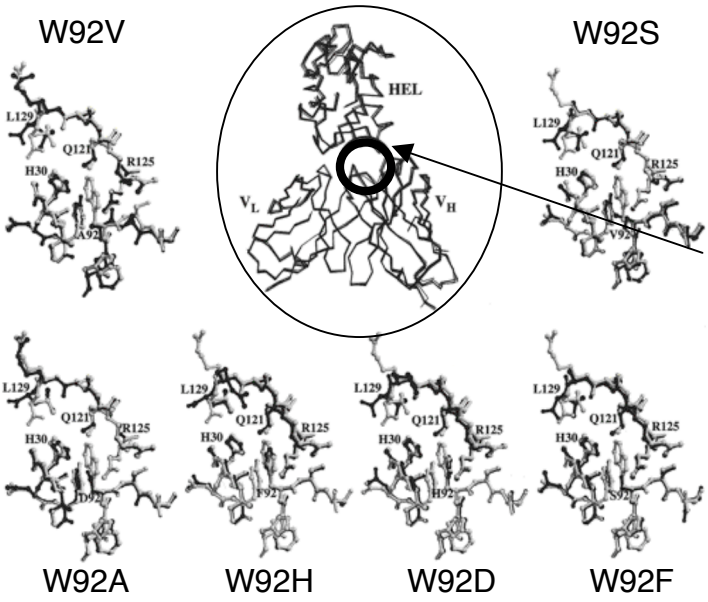
-screened for activation of the mutant hGH receptor by the mutant hGH in the
-presence of small molecule chemical libraries based on these structures.



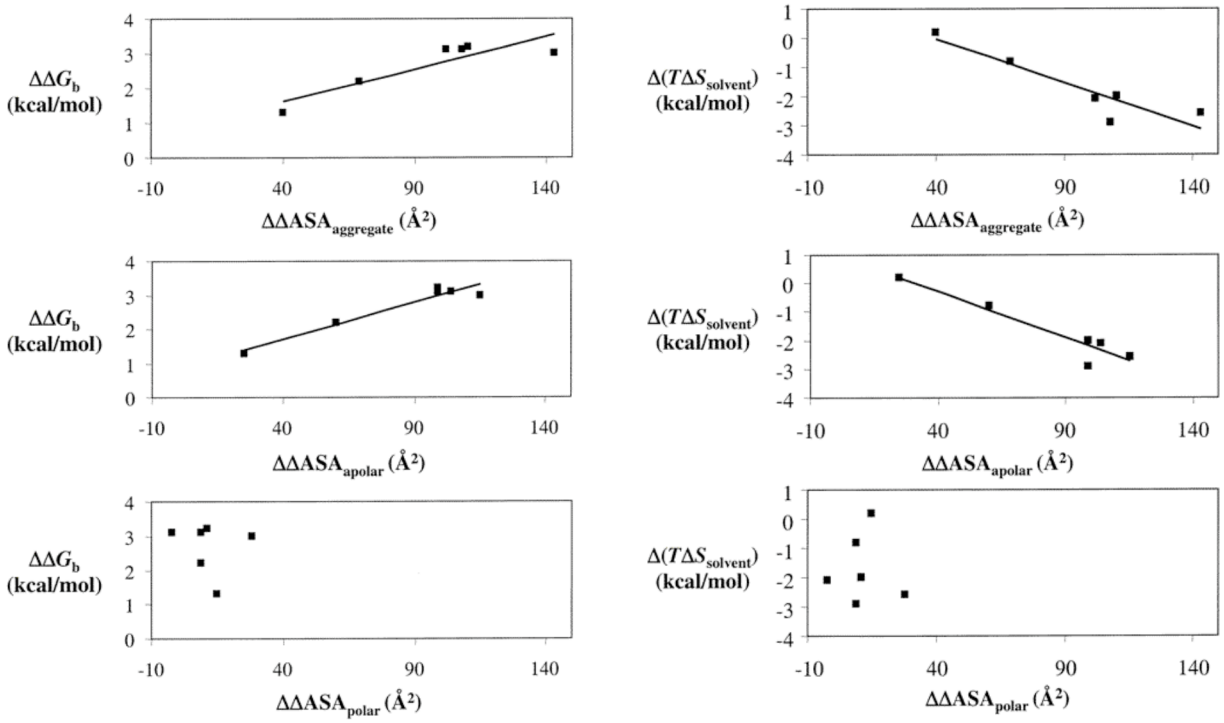
-identified a variety of these small molecules which can complement the mutations and lead to high affinity interactions (K_d ~nM). The small molecules will "switch on" signaling.



-with the hotspot idea in mind we can revisit idea of ASA and free energy (Sundberg et al., 2000)

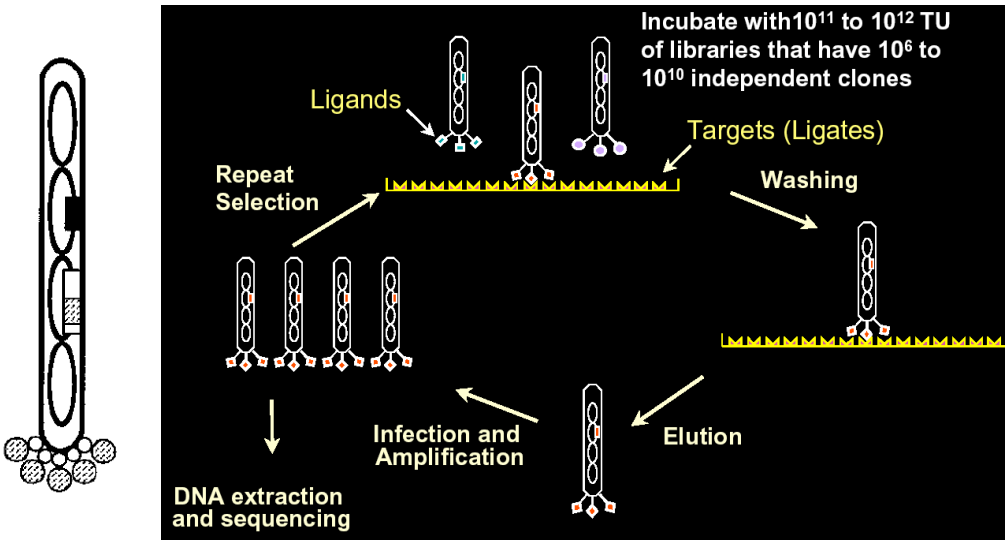


1- buried Trp in antibody/lysozyme interface mutated to a series of side chains varying in size of apolar groups.
2- ddG measured for each mutant, as well as enthalpy and entropy.
3- plots of free energy and entropy against change in BSA are linear.
4- Calculated value of 21 cal mol-1 $Å^{-2}$

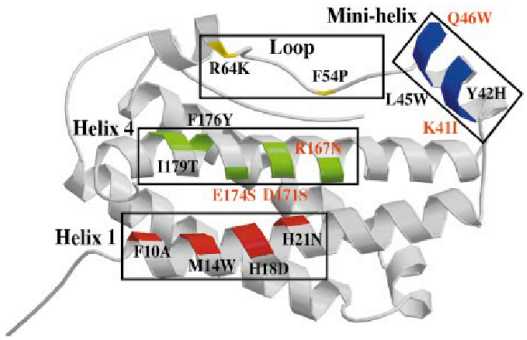


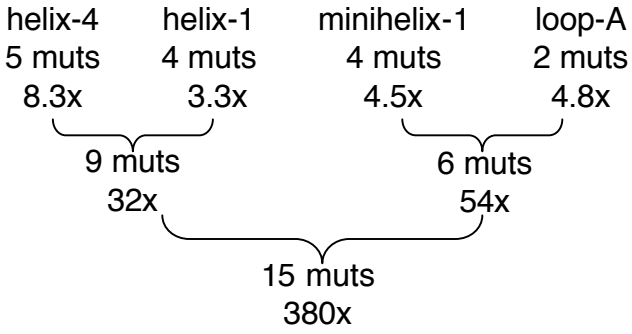
4) Hotspot & additivity concepts in protein engineering - phage display.

-design of a high affinity “supermutant” of hGH by phage display (Lohman & Wells, 1995).
-phage display is a technique based on the fact that protein-protein interactions are dictated by a relatively small number of amino acids.

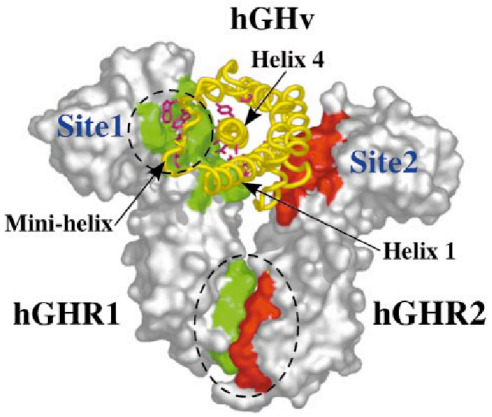


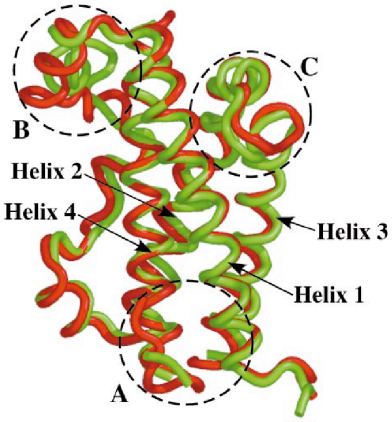
-hGH is displayed on phage.
-regions of the helices which contact the hGHR are converted into cassettes.
-random oligonucleotide cassettes inserted in hGH-phage and high affinity variants are selected.
-using additivity principle, each optimized helix cassette is sequentially combined.
-result is that final combined variant exhibits additive increase in binding free energy resulting from sum of individual variants.



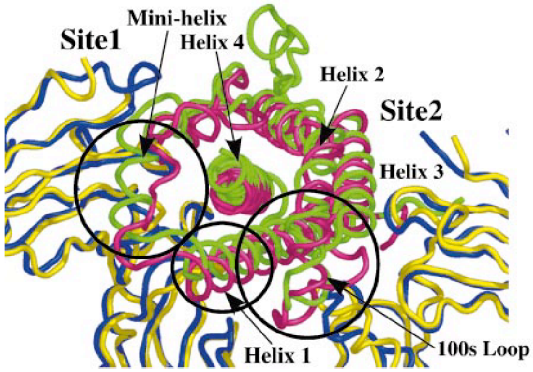


-is the explanation additivity ? Initial assumption was yes!
-but x-ray structure of supermutant complex shows large, unexpected structural rearrangements in the hGH molecule upon binding - up to 9Å in some contact sites.



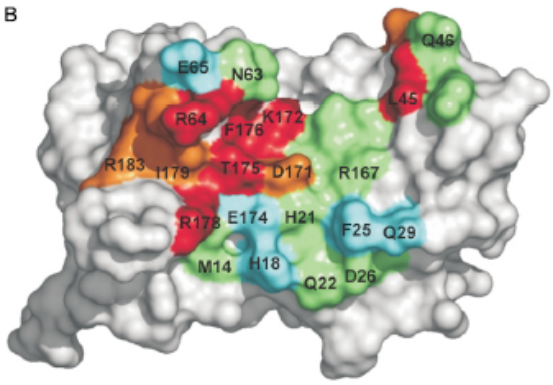


-also significant global structural rearrangements are propagated to site 2.
-since only site 1 was mutated, contribution of site 2 to overall affinity increase is purely through coupling - positive cooperativity.

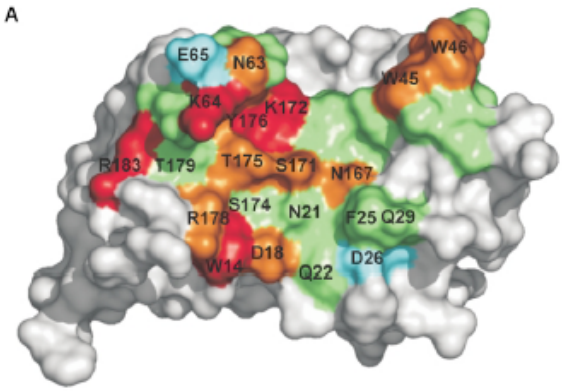


conclusion: additivity assumption from phage display was too simplistic.

-recently, “shotgun Ala scan” of supermutant hGH revealed that energetic hotspot was now spread over a broader surface. But most interesting, basis of affinity enhancement was the REMOVAL of negative bystander residues in interface, rather than insertion of hotspot residues.



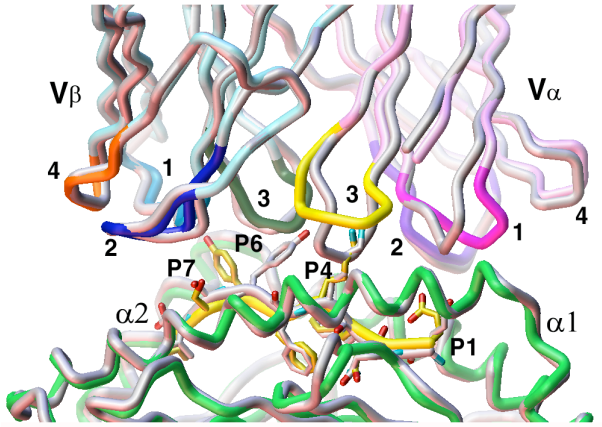
Wild-type hGH site I



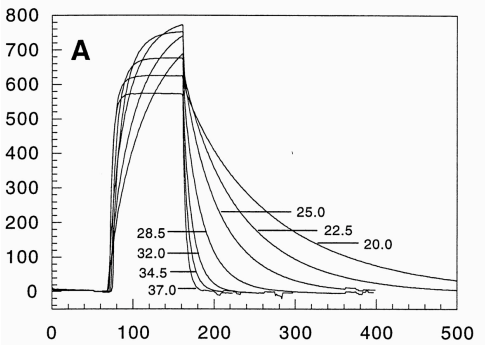
supermutant hGH site I

Conformational rearrangements in protein-ligand interactions.

Example #1. Large structural changes in T cell receptor upon binding its pMHC ligand.

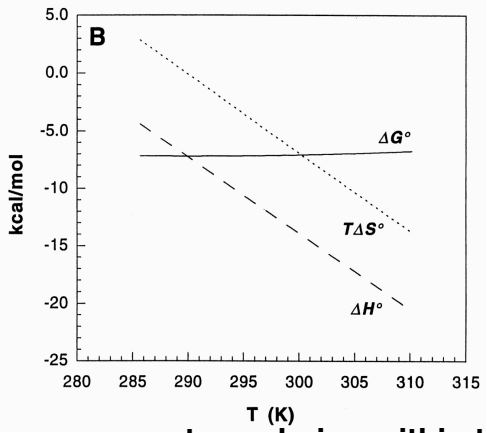


-temperature dependent on- and off-rates are a signature of a process requiring energy for binding. Why ?



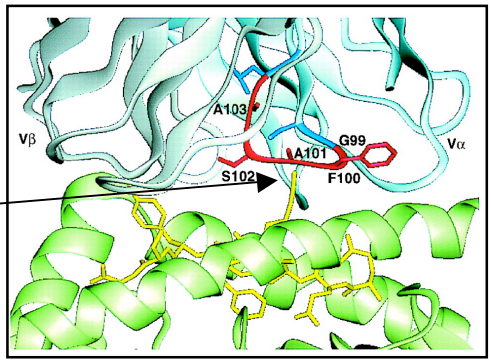
-both calorimetric and van't hoff analysis revealed significant unfavorable entropy upon binding. Binding exhibits entropy-enthalpy compensation.

ΔG=-7.0kcal/mol,
ΔH=-13.0kcal/mol
ΔS=-18.7cal/mol-deg
ΔCp=-600cal/mol-deg (anomalously large Cp) what does it mean ?



-is unfavorable entropy due to conformational change, or water ordering within the interface ?

-In the absence of direct structural proof, an intriguing result emerged from phage display.
-*in vitro* evolution of the TCR loop undergoing the conformational change identified variants with 3 orders of magnitude higher affinity !!



High affinity sequences are polyproline !

-reduction of conf. entropy appears to enhance binding since these residues do not contact ligand.

wildtype= SSYGNYL
"supermutants"= SLPPPLL

Are structural changes" induced" by ligand, or does ligand "select" from a population of structures ?

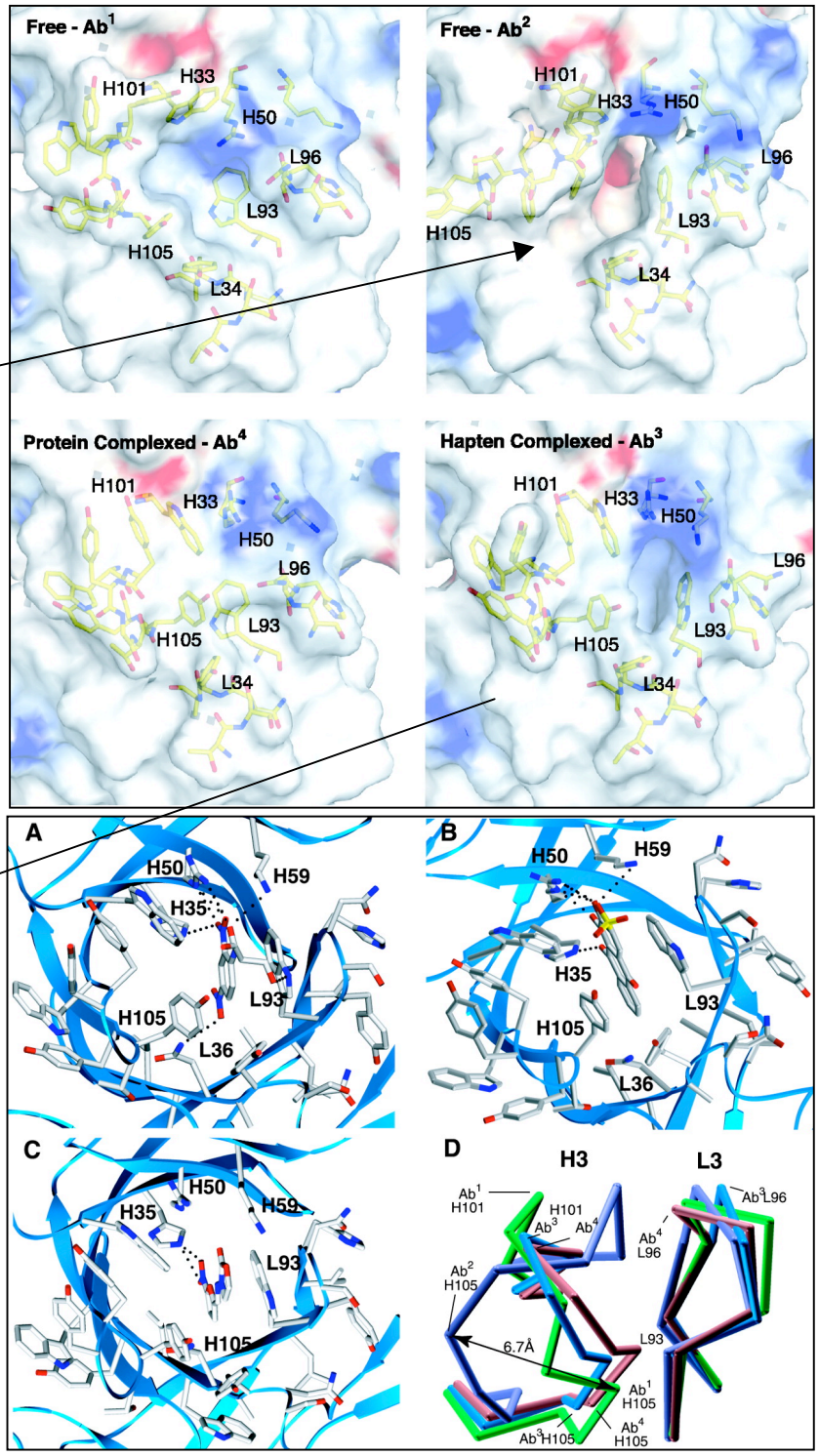
-general assumption has been ligand induces specific conformations within a flexible binding site.

Example #2. Conformational isomers in antibody binding site - "multispecificity"

-an antibody binding site exists as interchanging structural isomers.

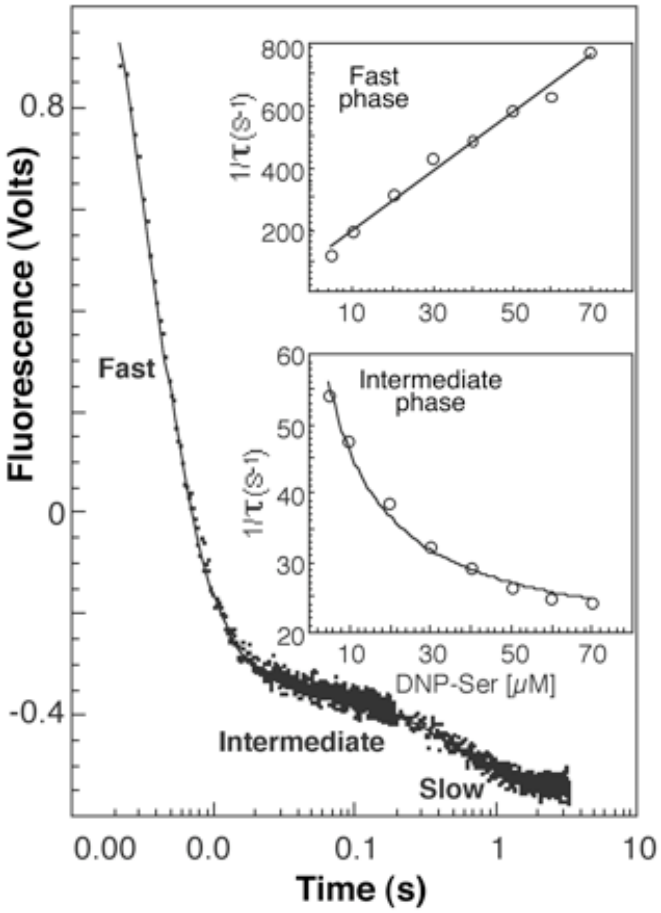
Promiscuous isomer

-each isomer binds ligand in unique way



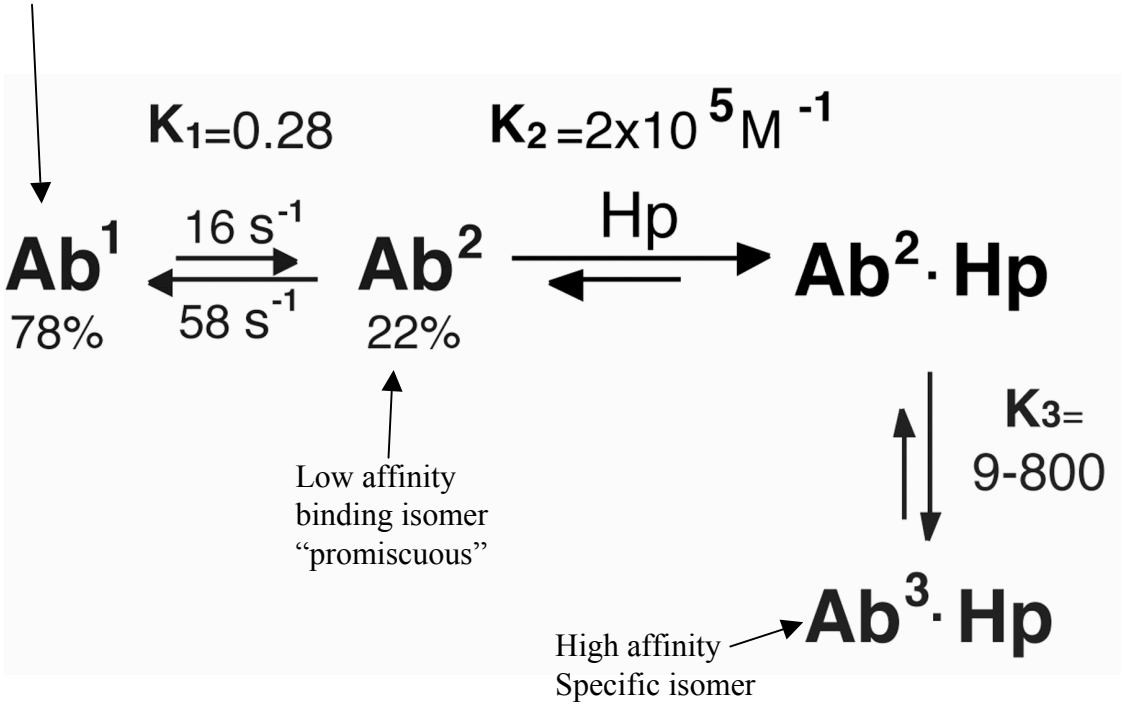
-what is the mechanistic explanation for this observation ?

-pre-steady state flourescence quenching of the Ab upon addition of ligand (DNP). Curve can be fitted to three exponentials.



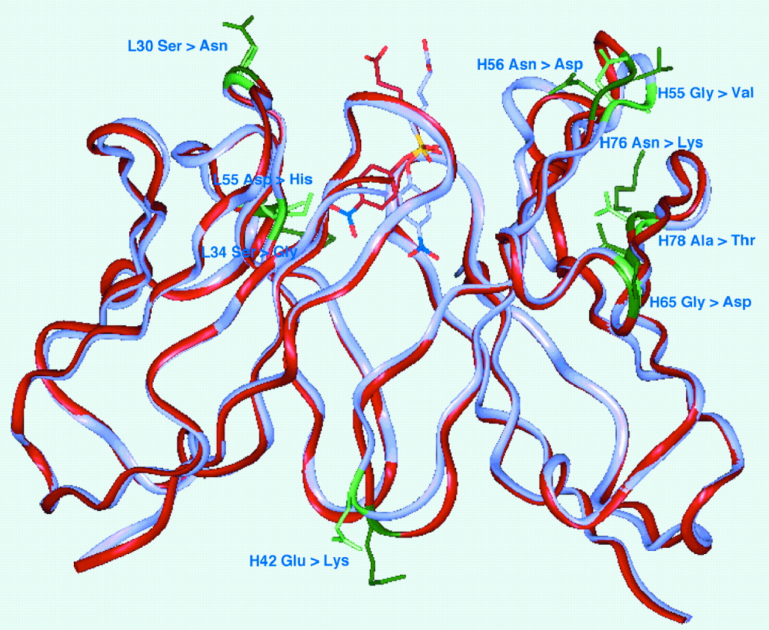
-in fast phase, DNP binds to preformed Ab2.
-in intermediate phase, DNP encounters a non-binding Ab1 which isomerizes to Ab2.
-in the slow phase, the AB2-DNP complex further isomerizes to a higher affinity state.

Non-binding isomer



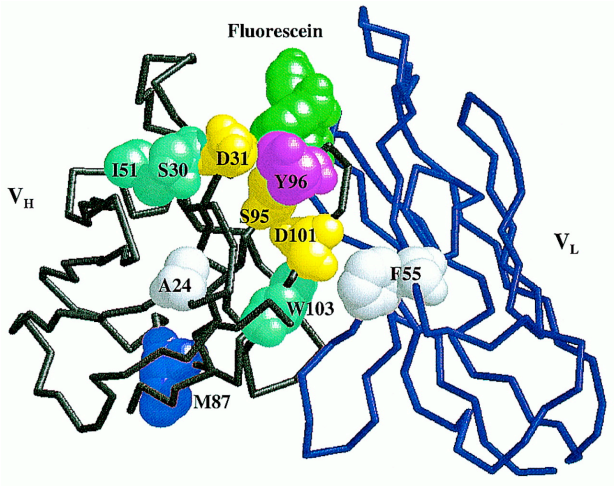
Example #3: Conformational changes away from binding interface can impact upon binding interactions in unexpected ways.

A. "Affinity maturation" of antibodies can increase affinity for ligand by as much as 6-orders of magnitude. An important result from recent structural studies is that many of these "affinity-enhancing mutations" occur far away from binding site (<20Å).



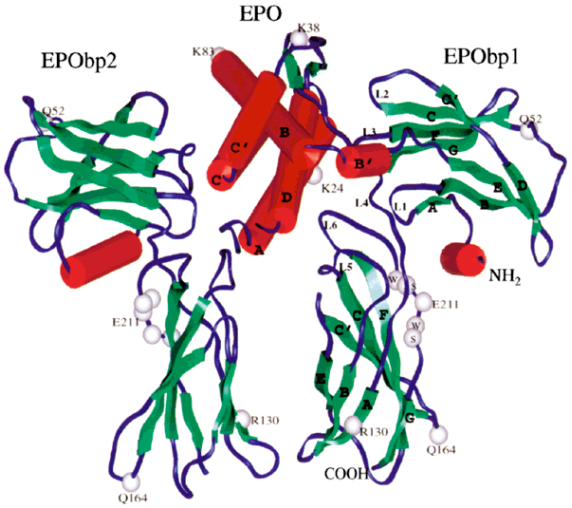
Wedemayer GJ, et al. Structural insights into the evolution of an antibody combining site. Science. 1997 Jun 13;276(5319):1665-9

B. *In vitro* evolution of an anti-fluorescein antibody (D. Wittrup, *PNAS*, 2000).
-evolved an antibody to have >1000-fold higher affinity for ligand (K_d ~ femtomolar).
-only 1 out of the 14 mutations in direct contact with ligand.



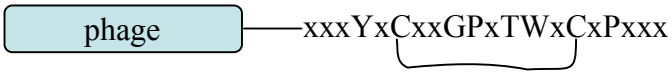
Concept of structural "mimicry" in protein interactions

3) Erythropoietin story: (Wilson & Jolliffe, 1999)
- cytokine receptor complex highly similar to growth hormone.
- dimerization leads to activation of intracellular jak/stat cascade.
- EPO receptor complex interface also has a hotspot, as in hGH.

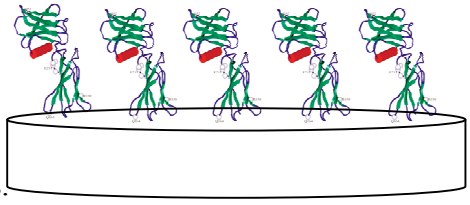


-disulfide-constrained phage peptide libraries were screened against the EPO receptor.
-constrained library strategy to reduce conformational entropy cost of binding.
-peptides capable of activating the EPO receptor were discovered.

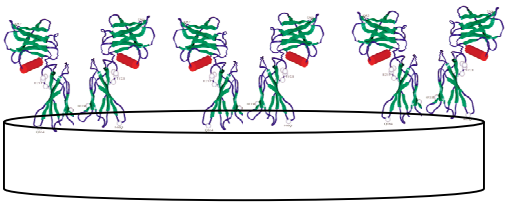
-important strategy was to first screen using POLYVALENT phage, followed by lead optimization with MONOVALENT. why ?



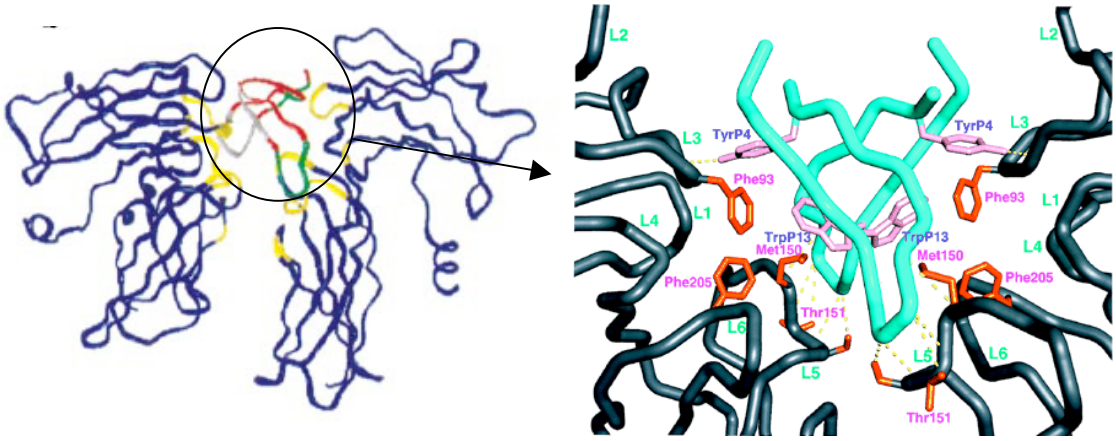
-coupling density of the receptor on plastic was critical.
-low coupling density gave antagonists.



-high coupling density gave agonists.

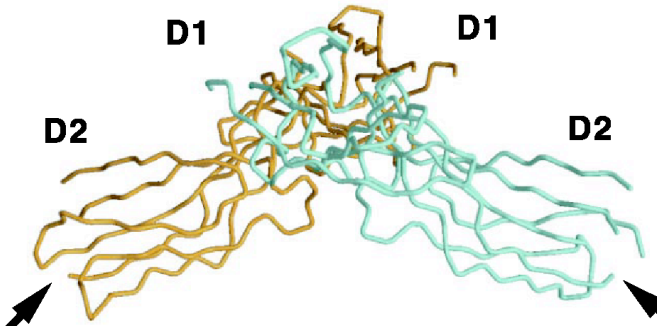


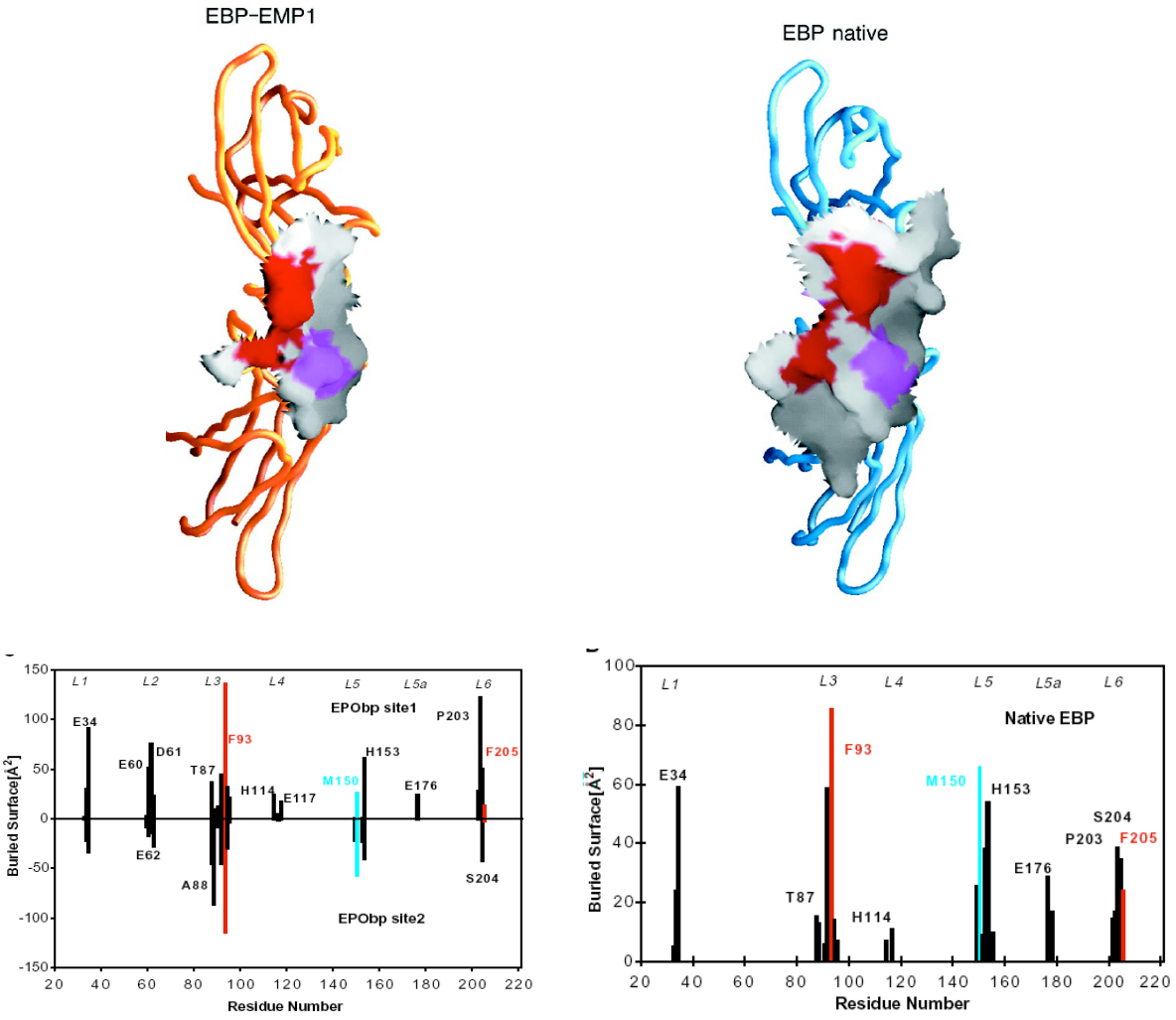
crystal structure of epo agonist peptide complexed to epo receptor (Livnah et al., 1996)



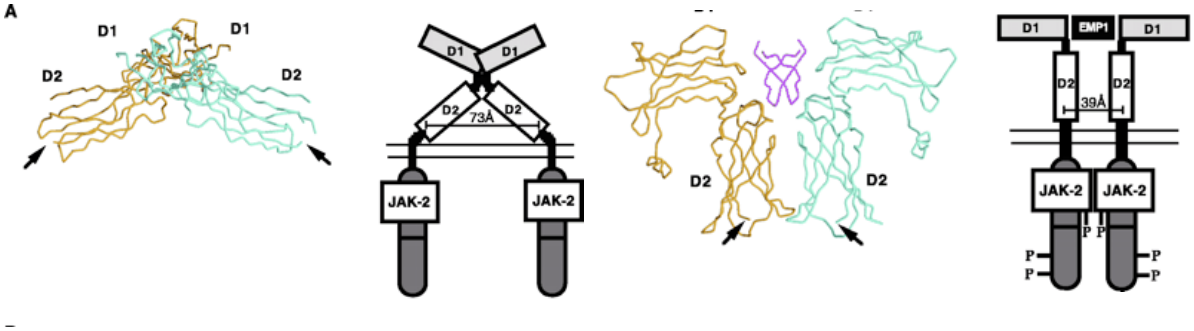
-really wild surprise, a 13 amino acid peptide dimerizes the receptor.
-the peptides are unrelated in sequence to EPO, but yet use the same EPO receptor hotspot contact residues.
-an important take home message from this experiment is that from a library of over 10^{12} different peptide sequences, all the binders target the normal functional binding site of the receptor - there were no "hits" on other surfaces of the receptor.
-this has implications for the idea of targeting the non-natural binding sites of proteins for drug design - they just may not be evolved to serve in a binding capacity.
-efforts to engineer "artificial" binding sites in proteins have been largely unsuccessful.

-in the absence of EPO, the receptor appears to exist as a homodimer mediated through receptor-receptor contacts almost identical to those used for ligand recognition.



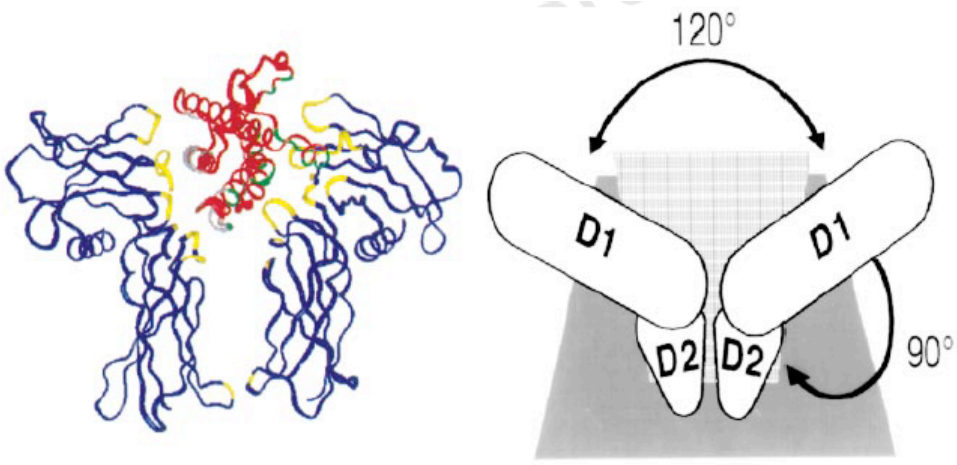


-the receptor uses the same hotspot for engaging EPO, the EPO peptide, and itself.
-clearly, this is an interaction site that is drawing interacting ligands away from other sites on the protein.
- hostpot-mediated homodimerization may prevent unwanted signaling.

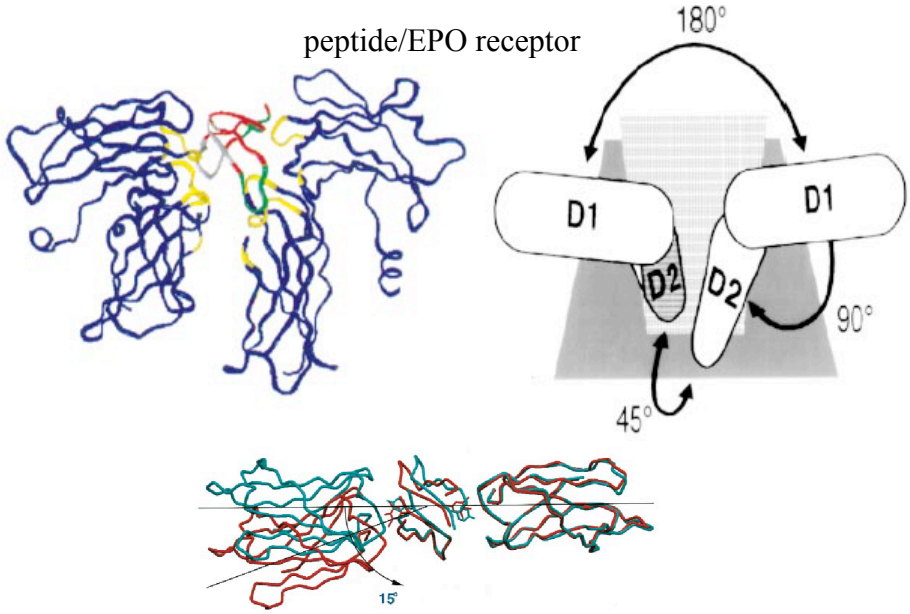


-the interactions of the EPO ligands with the receptor are not functionally inert.
-the nature of the receptor/ligand contact determines the signaling outcome by dictating the "orientation & proximity" (Crabtree) of the extracellular domains.

EPO/EPO receptor



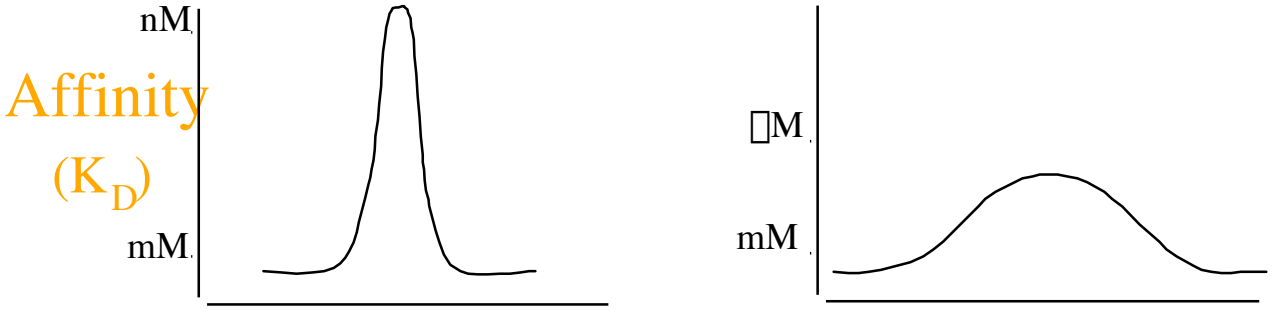
peptide/EPO receptor



-the two-fold orientations differ, as do the biological activities of the agonists.
-EPO is much more potent at activating the receptor than the peptides.
-main message is that for receptor-mediated signaling, not just any dimer will do.

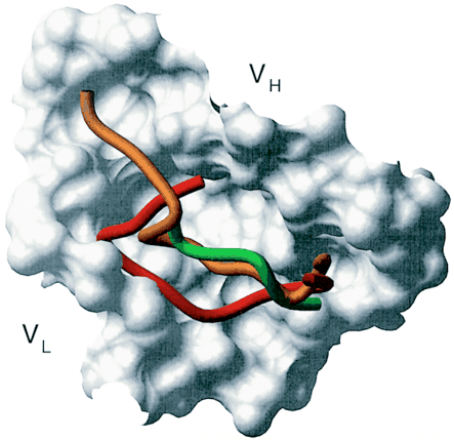
4) Addressing the issue of cross-reactivity with peptide libraries (Kramer et al. 1997).

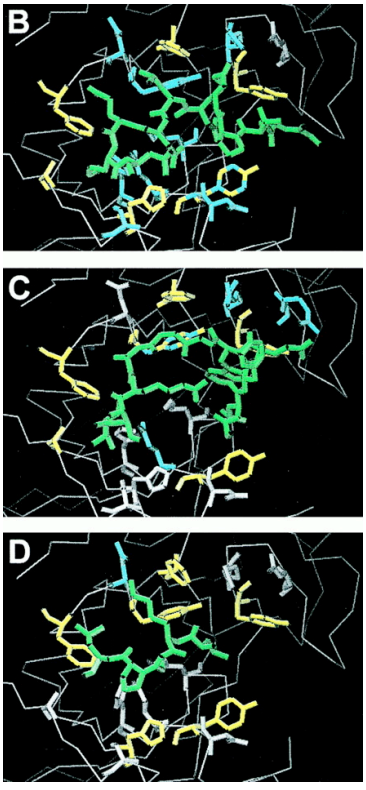
-in general, protein hotspots will select a very narrow set of binding solutions, either chemically and/or structurally. Protein binding sites are inherently "specific"to prevent spurious interactions with other proteins (e.g. hotspot "seal")
-however, some proteins such as those shared by numerous ligands, exhibit an astounding degree of cross-reactivity.
-antibody/antigen have so far revealed the most information about this phenomenon.



example: A monoclonal antibody is screened against a synthetic peptide library. Completely divergent sequences are identified.

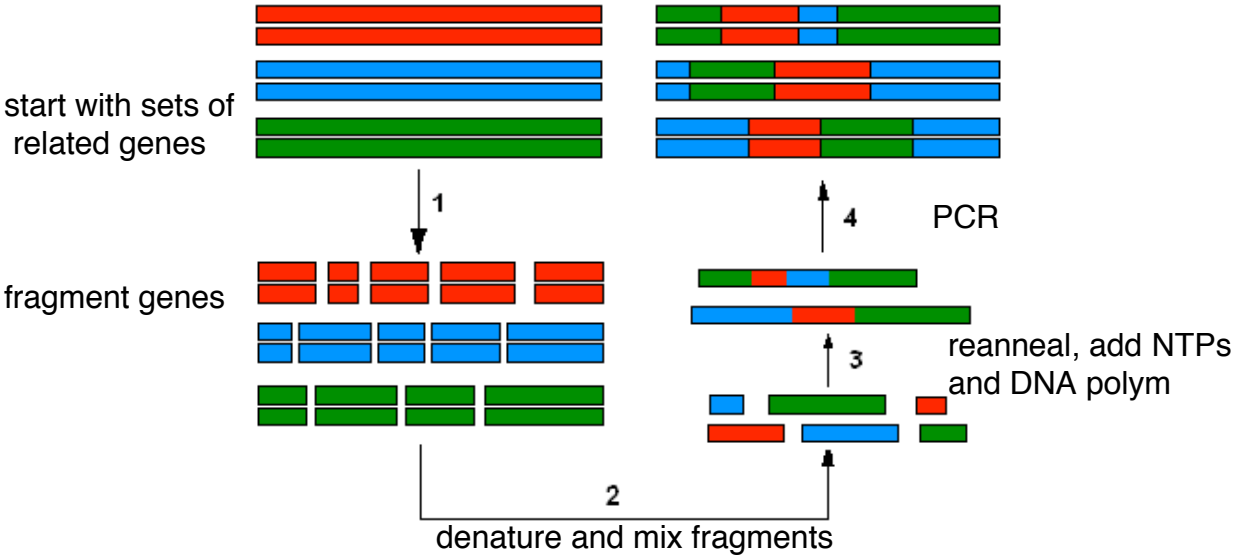
first screening step	deconvoluted peptides	K_I [ELISA] (M)	K_D [ET] (M)
XXXXX**LN**XX**L**XXXX	(1) GATPEDLNQKLAGN	1.2×10^{-9}	1.4×10^{-9}
XXXX**WXG**XX**I**XXXX	(2) GLYEWGGARITNTD	2.0×10^{-7}	6.1×10^{-8}
XXXXX**E**X**N**X**I**XXXX	(3) RDFDKEWNLIEQNS	2.7×10^{-6}	1.9×10^{-6}
XXXXX**PXRW**XXXXX	(4) FDEDSQPRRWQRLS	2.2×10^{-4}	n.d.†
xxxxx**Gp**x**l**xxxxx	(5) efslkGpllqwrsG	1.9×10^{-5}	n.d.†



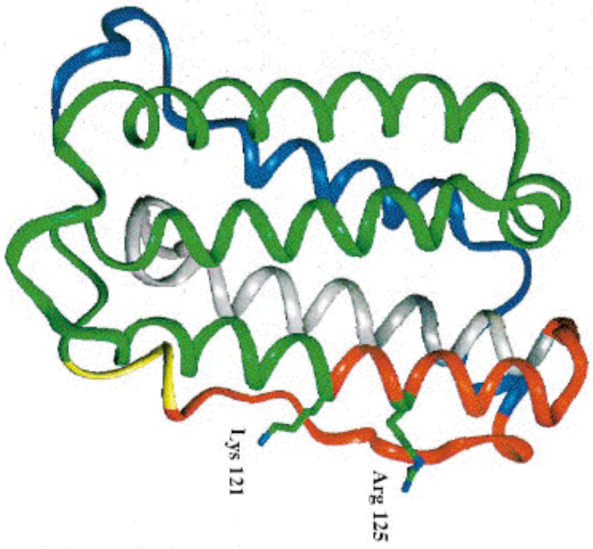


5) Additivity in protein engineering - gene shuffling (Stemmer et al., 1994).

-phage display does not really take full advantage of the additivity principle.
-limited to small peptides and small regions of proteins.
-gene shuffling relies on the concept that domains of proteins appear to be modular, and can be recombined in a fashion similar to the hGH supermutant



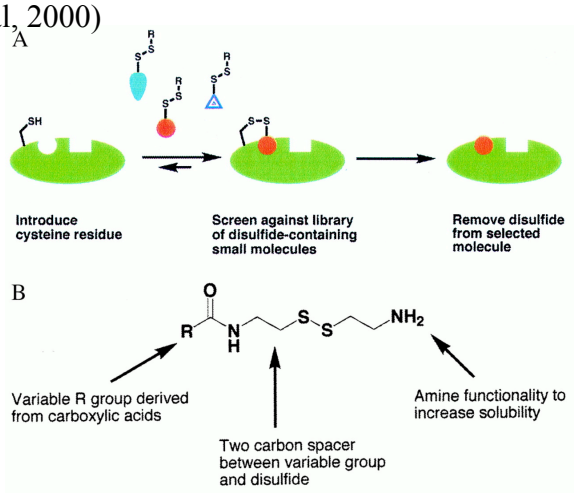
Experiment: evolve a four-helix cytokine by gene shuffling
-interferon is an anti-viral protein with a 4-helix bundle topology.
-each helix can be shuffled among related four-helix bundle proteins to generate large numbers of chimeric cyokines.



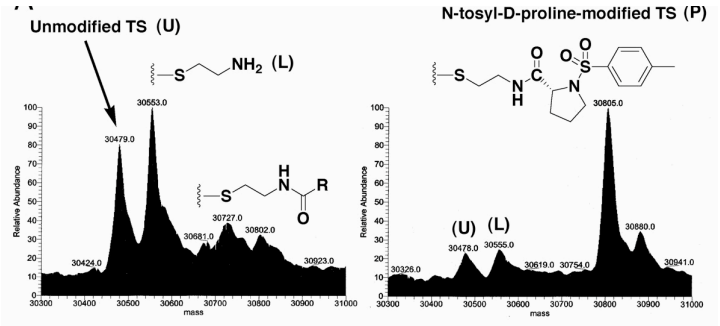
6) Question: How do we target non-hotspot regions of protein ? Will "any" part of protein surface bind a ligand, or are we restricted, for physical/chemical reasons to the hotspot ?

A) Cysteine fishing (Erlanson et al, 2000)

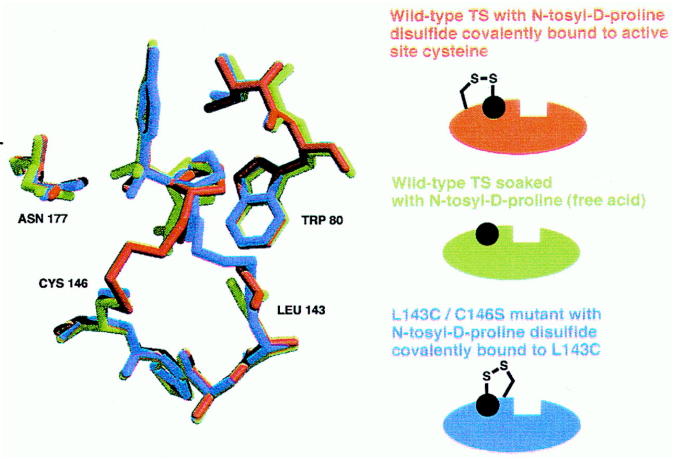
introduce free thiol into region of interest in the protein



screen large library of small molecules which also have free thiol



capture "leads" which appear to cross-link to the free thiol at a greater frequency than background.
-release cross-link in optimization



References & Reviews:

Frisch C, Schreiber G, Johnson CM, Fersht AR.
Thermodynamics of the interaction of barnase and barstar: changes in free energy versus changes in enthalpy on mutation.
J Mol Biol. 1997 Apr 4;267(3):696-706.

Wells JA.
Additivity of mutational effects in proteins.
Biochemistry. 1990 Sep 18;29(37):8509-17

Schreiber G, Fersht AR.
Energetics of protein-protein interactions: analysis of the barnase-barstar interface b y single mutations and double mutant cycles.
J Mol Biol. 1995 Apr 28;248(2):478-86.

Clackson T, Ultsch MH, Wells JA, de Vos AM.
Structural and functional analysis of the 1:1 growth hormone:receptor complex reveals the molecular basis for receptor affinity.
J Mol Biol. 1998 Apr 17;277(5):1111-28.

Wells JA.
Binding in the growth hormone receptor complex.
Proc Natl Acad Sci U S A. 1996 Jan 9;93(1):1-6.

Clackson T, Wells JA.
A hot spot of binding energy in a hormone-receptor interface.
Science. 1995 Jan 20;267(5196):383-6.

Lowman HB, Wells JA.
Affinity maturation of human growth hormone by monovalent phage display.
J Mol Biol. 1993 Dec 5;234(3):564-78.

Wilson IA, Jolliffe LK.
The structure, organization, activation and plasticity of the erythropoietin receptor.
Curr Opin Struct Biol. 1999 Dec;9(6):696-704.

Middleton SA, Barbone FP, Johnson DL, Thurmond RL, You Y, McMahon FJ, Jin R, Livnah O, Tullai J, Farrell FX, Goldsmith MA, Wilson IA, Jolliffe LK.
Shared and unique determinants of the erythropoietin (EPO) receptor are important for binding EPO and EPO mimetic peptide.
J Biol Chem. 1999 May 14;274(20):14163-9.

Chang CC, Chen TT, Cox BW, Dawes GN, Stemmer WP, Punnonen J, Patten PA.
Evolution of a cytokine using DNA family shuffling.
Nat Biotechnol. 1999 Aug;17(8):793-7.

References & Reviews (cont'd):

James LC, Roversi P, Tawfik DS.
Antibody multispecificity mediated by conformational diversity.
Science. 2003 Feb 28;299(5611):1362-7.

Pal G, Kossiakoff AA, Sidhu SS.
The functional binding epitope of a high affinity variant of human growth hormone mapped by shotgun alanine-scanning mutagenesis: insights into the mechanisms responsible for improved affinity.
J Mol Biol. 2003 Sep 5;332(1):195-204.

Schiffer C, Ultsch M, Walsh S, Somers W, de Vos AM, Kossiakoff A.
Structure of a phage display-derived variant of human growth hormone complexed to two copies of the extracellular domain of its receptor: evidence for strong structural coupling between receptor binding sites.
J Mol Biol. 2002 Feb 15;316(2):277-89.