Engineering Antibody Variable Domains for Improved Stability and Folding

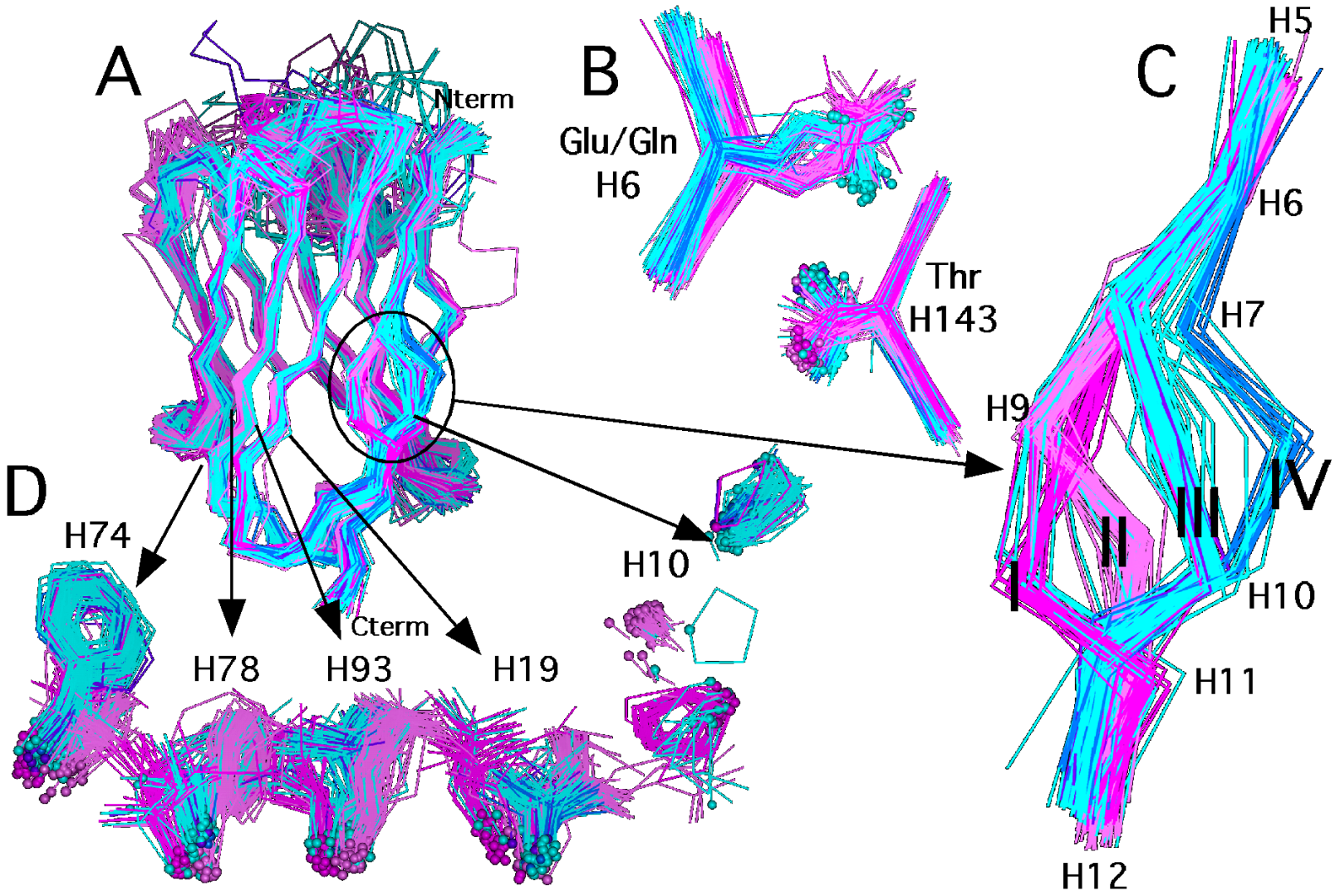
Effects on the biophysical Properties of scFv, Fab and whole Antibodies

Annemarie Honegger, Dept. of Biochemistry, Zürich University

It seems absurd,
but all good scFv
analyzed so far (+/- 1996)
seem to have an unpaired
negative charge (Glu) in
the core of V_H!

Why is hu4D5 such a good scFv?

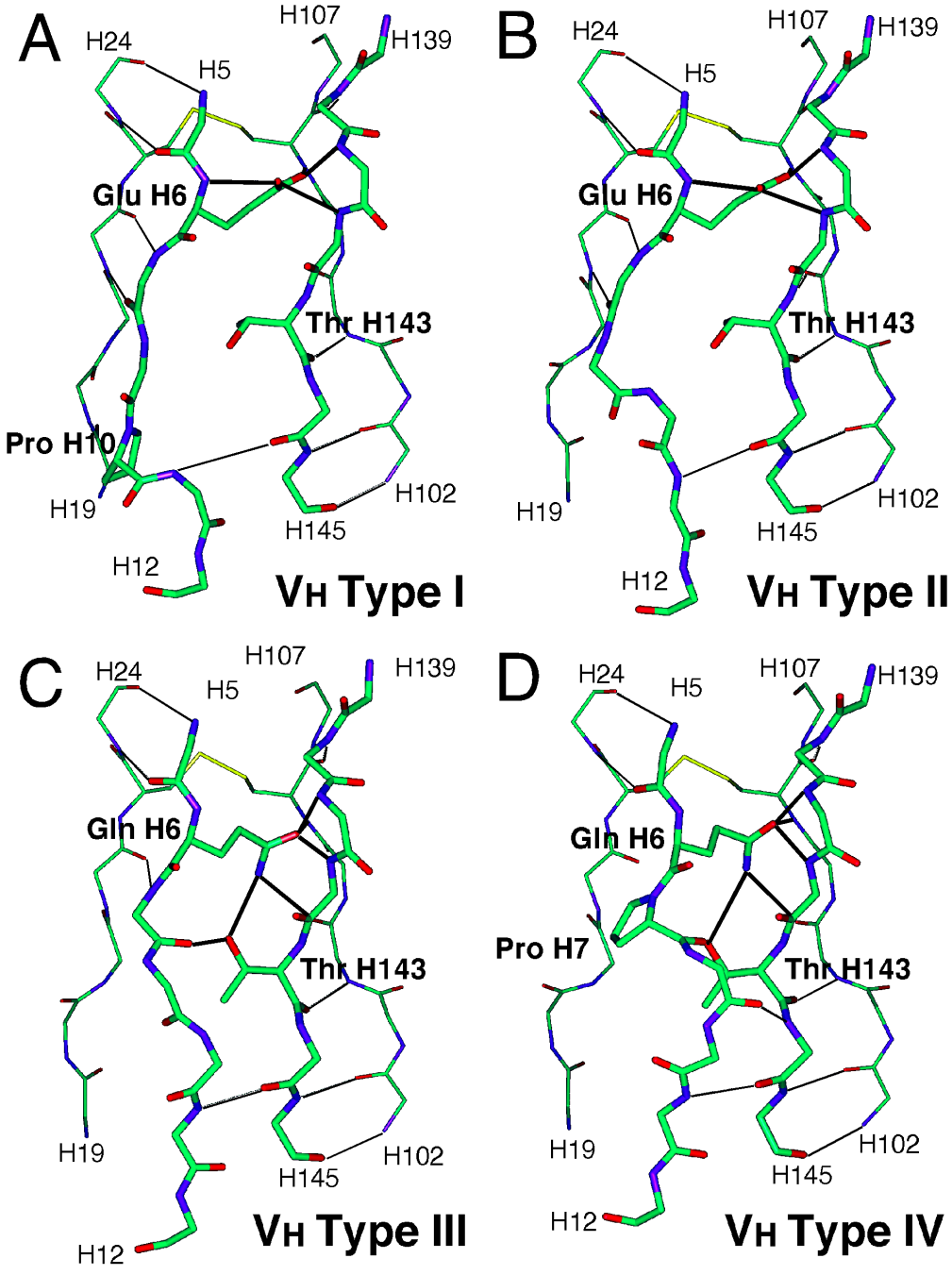
V_H Framework Structural Variability

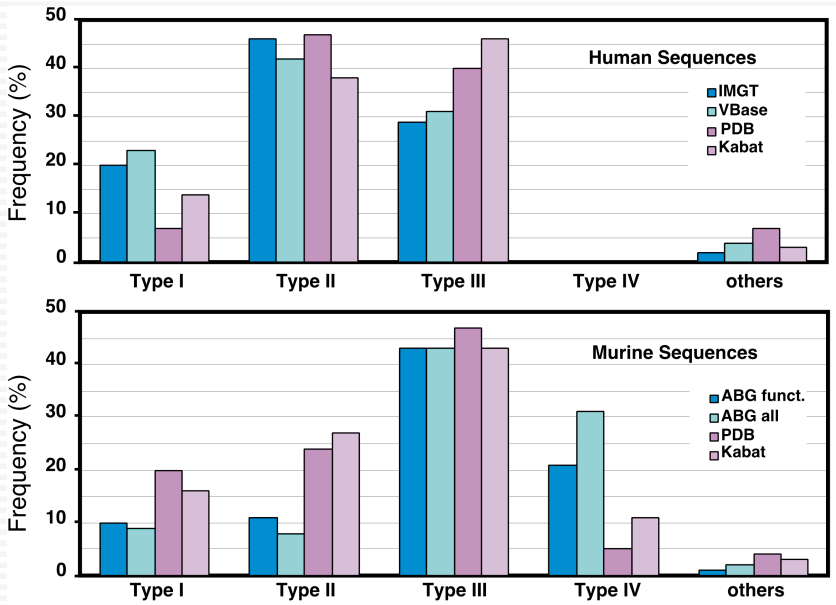


Human and murine V_H domains: 4 distinct structural subtypes

A.Honegger et al.: J. Mol. Biol. ***309*** *(2001) 687-699.*

V_H Diversity and the Role of H6





Type I: **E**SG**P**G

Type II: **ESGGG**

Type III: **Q**SG**A**E

Type IV: **Q**SG**P**G

*A.C.Langedijk et al.:J. Mol. Biol. **292** (1999)855-869*

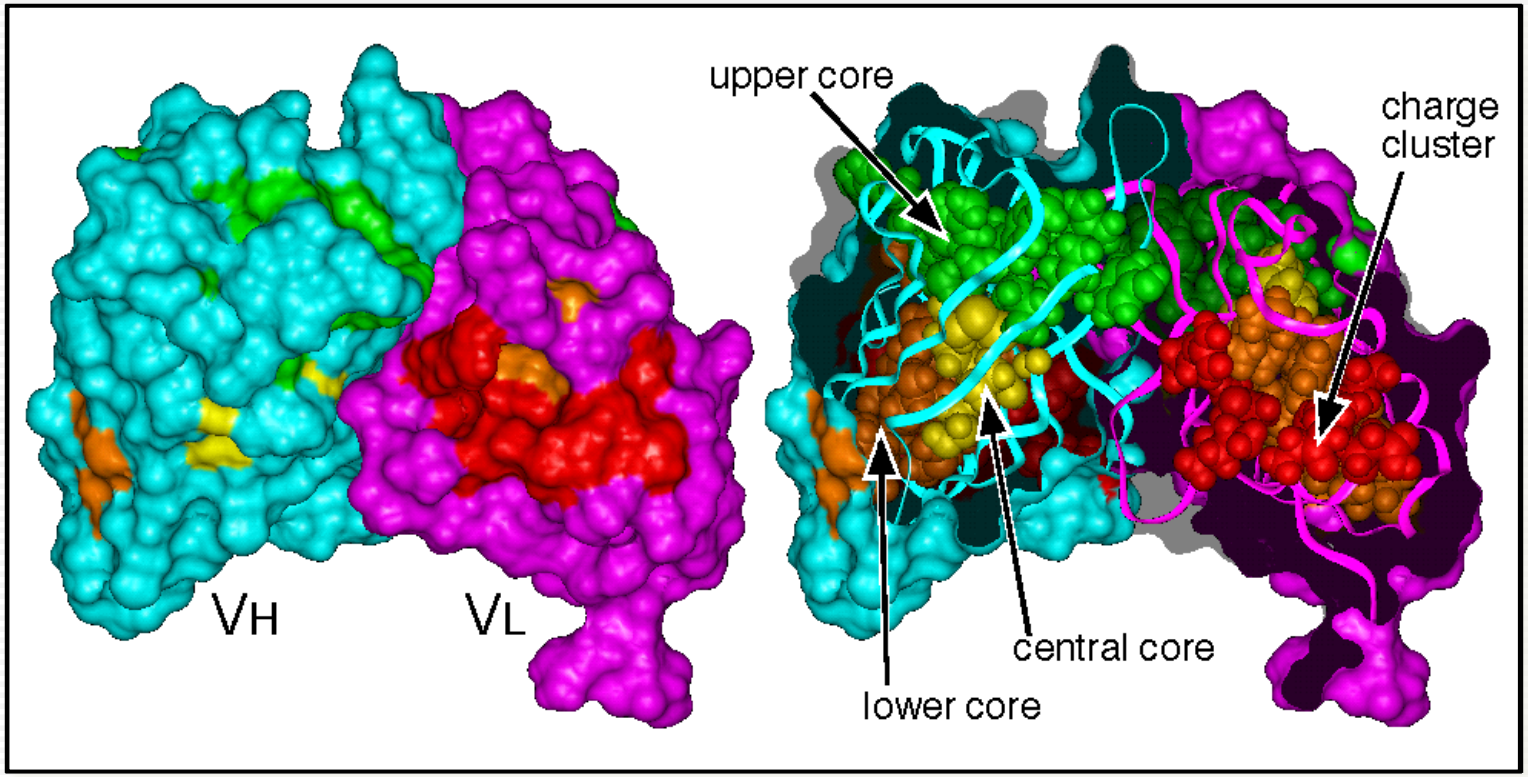
*S.Jung et al.: Mol. Biol. **309** (2001) 701-716*

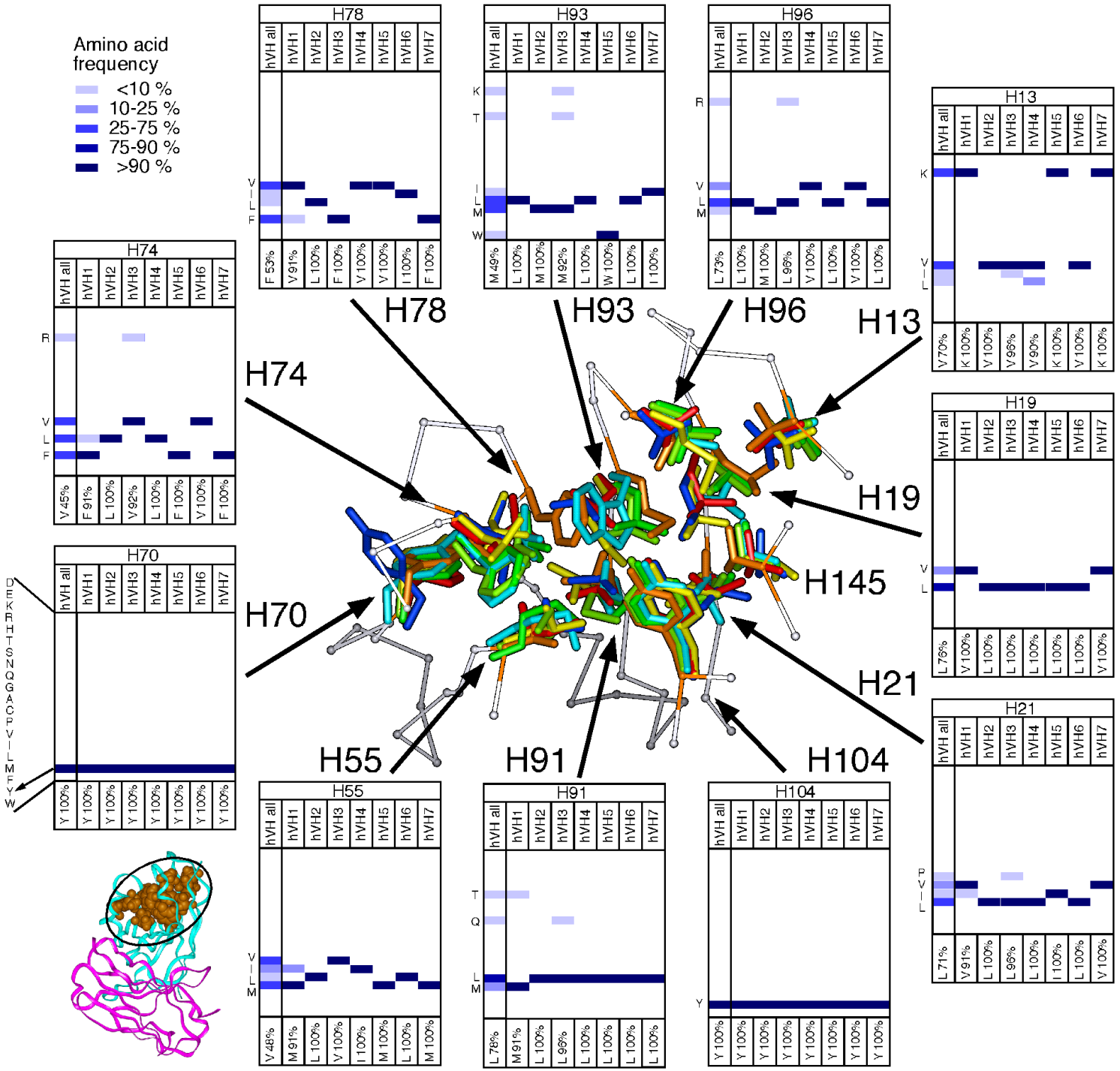
*A.Honegger et al.: J. Mol. Biol. **309** (2001) 687-699.*

Checklist

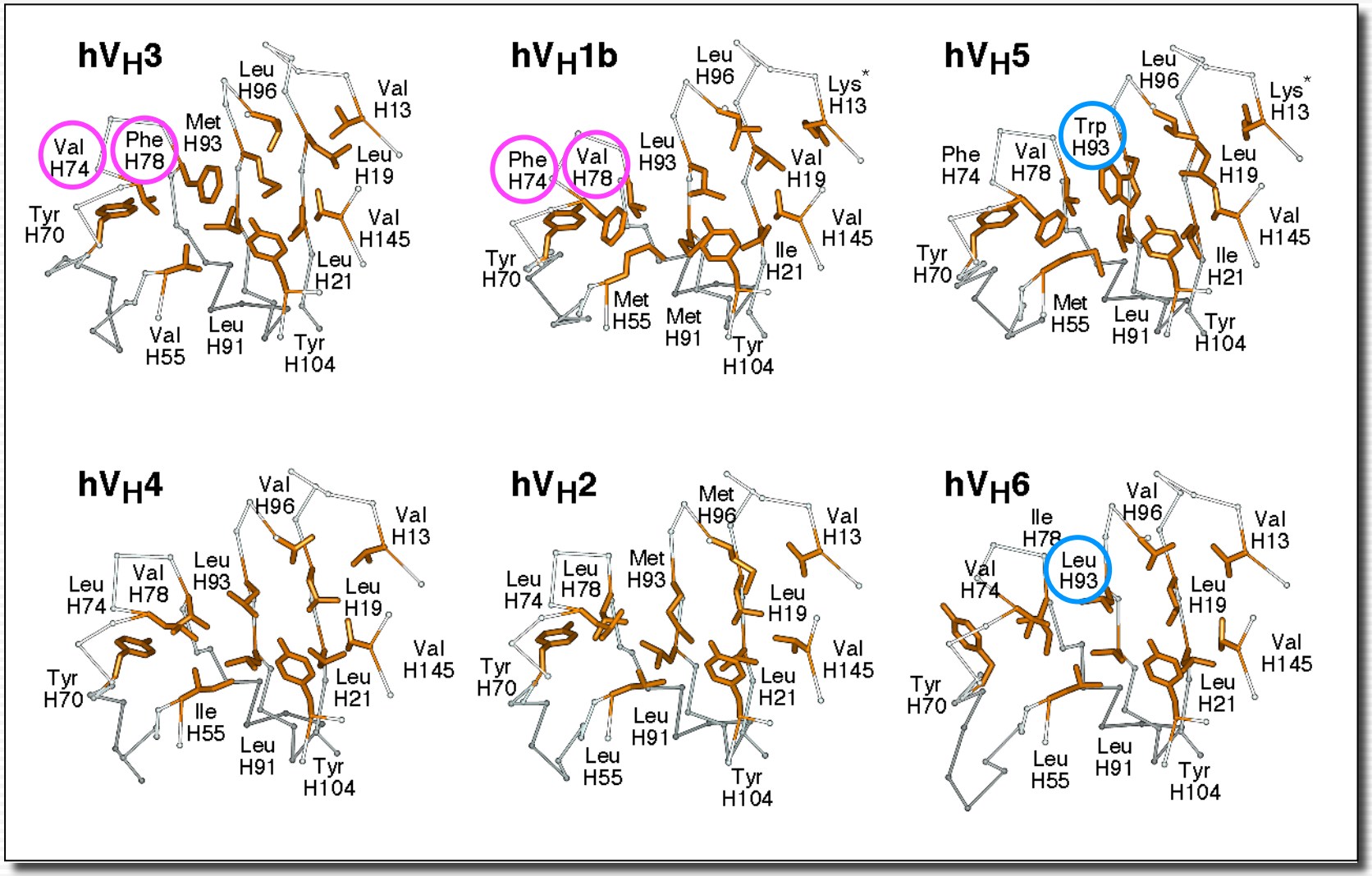
- **Hydrophobic core packing**
  Steric clashes and cavities destabilize the domain, as do buried hydrophilic side chains unable to satisfy their hydrogen bonding potential. Deviations from subtype-specific pattern!
- **Hydropathic contrast between core and surface**
  Hydrophobic surface residue can decrease folding efficiency
- **Conserved hydrogen bonding interactions in the core**
  Core hydrogen bonding network (E/Q 6, T 143, Y104, main chain)
- **Conserved charge interactions**
  Buried charge cluster (R 77, D 100, E 99, R/Q 45, E/R 53)
- **Conserved unusual main-chain torsion angles**
  Positions which enforce a positive Φ torsion angle, conserved Gly
- **Conserved Pro and Gly positions**
  cis-Pro L8 and and L136 of $V_L\kappa$, conserved *trans*-Pro in various positions
- **Secondary structure propensity and torsional preference**

Core packing



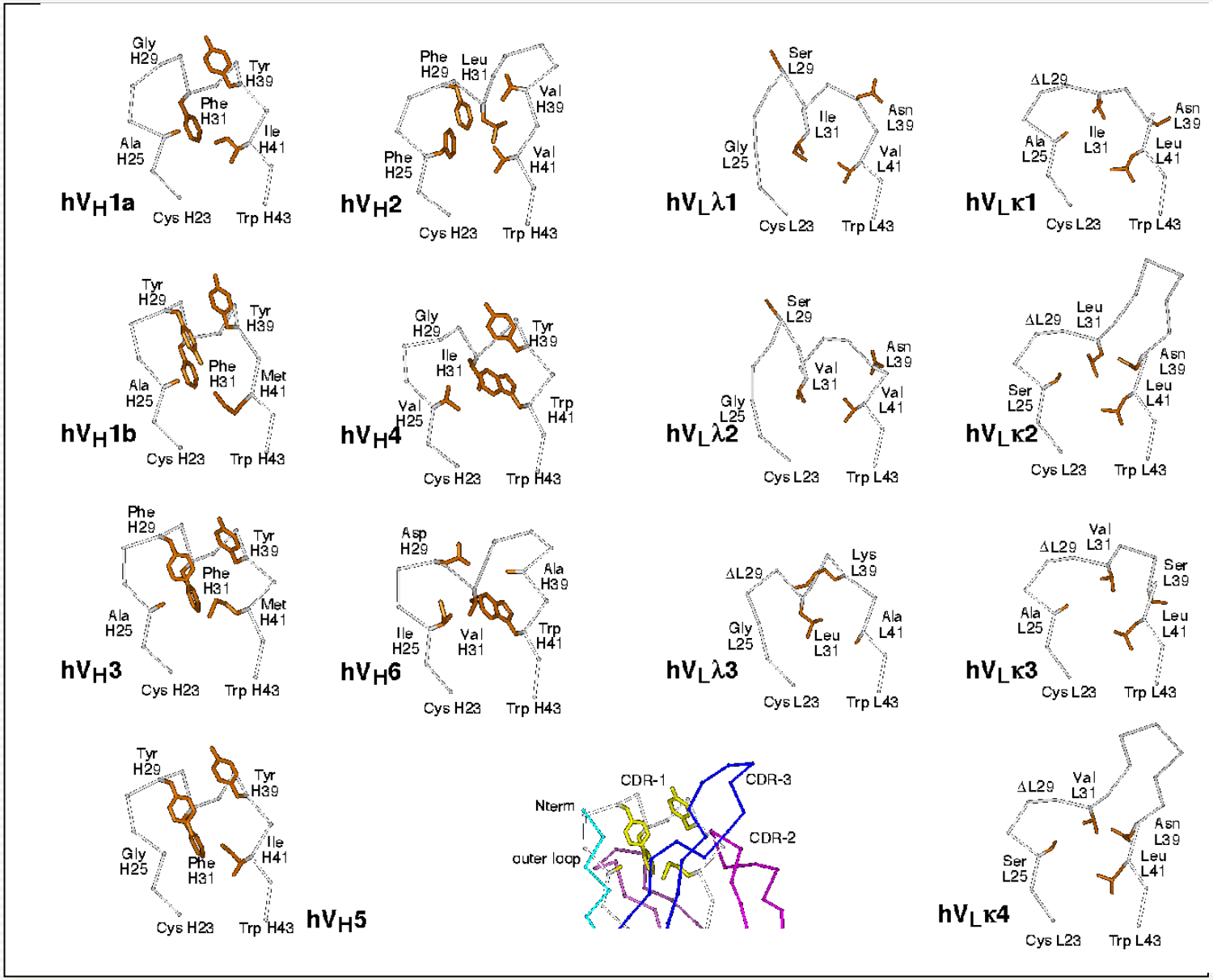

Amino acid frequency
<10 %
10-25 %
25-75 %
75-90 %
>90 %
H78
H93
H96
H74
H13
H19
H70
H145
H21
H55
H91
H104

Lower core packing



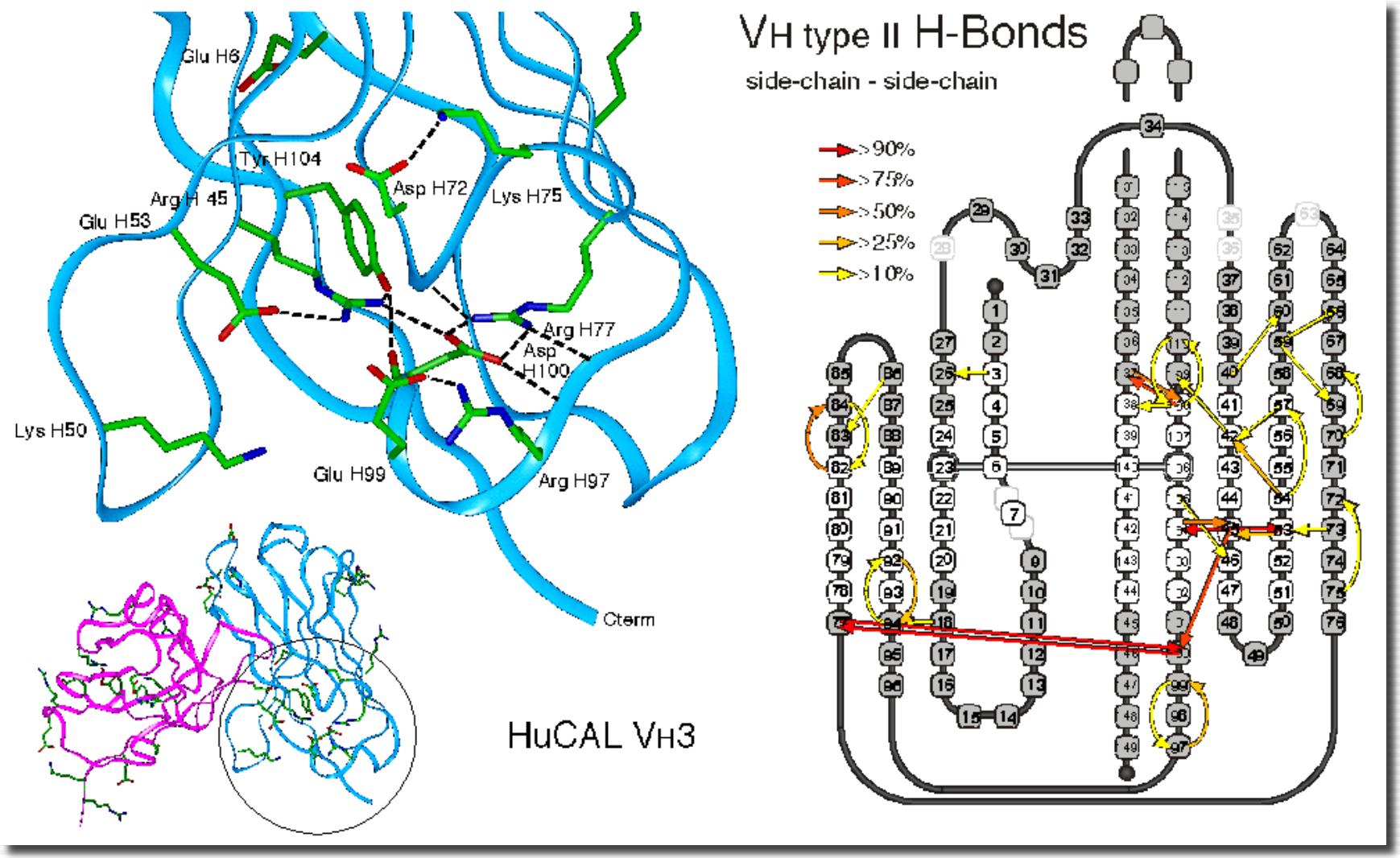
Differences in upper core packing:

CDR-1



Residue numbering: Honegger&Plückthun, J. Mol. Biol 309 (2001) 657-670

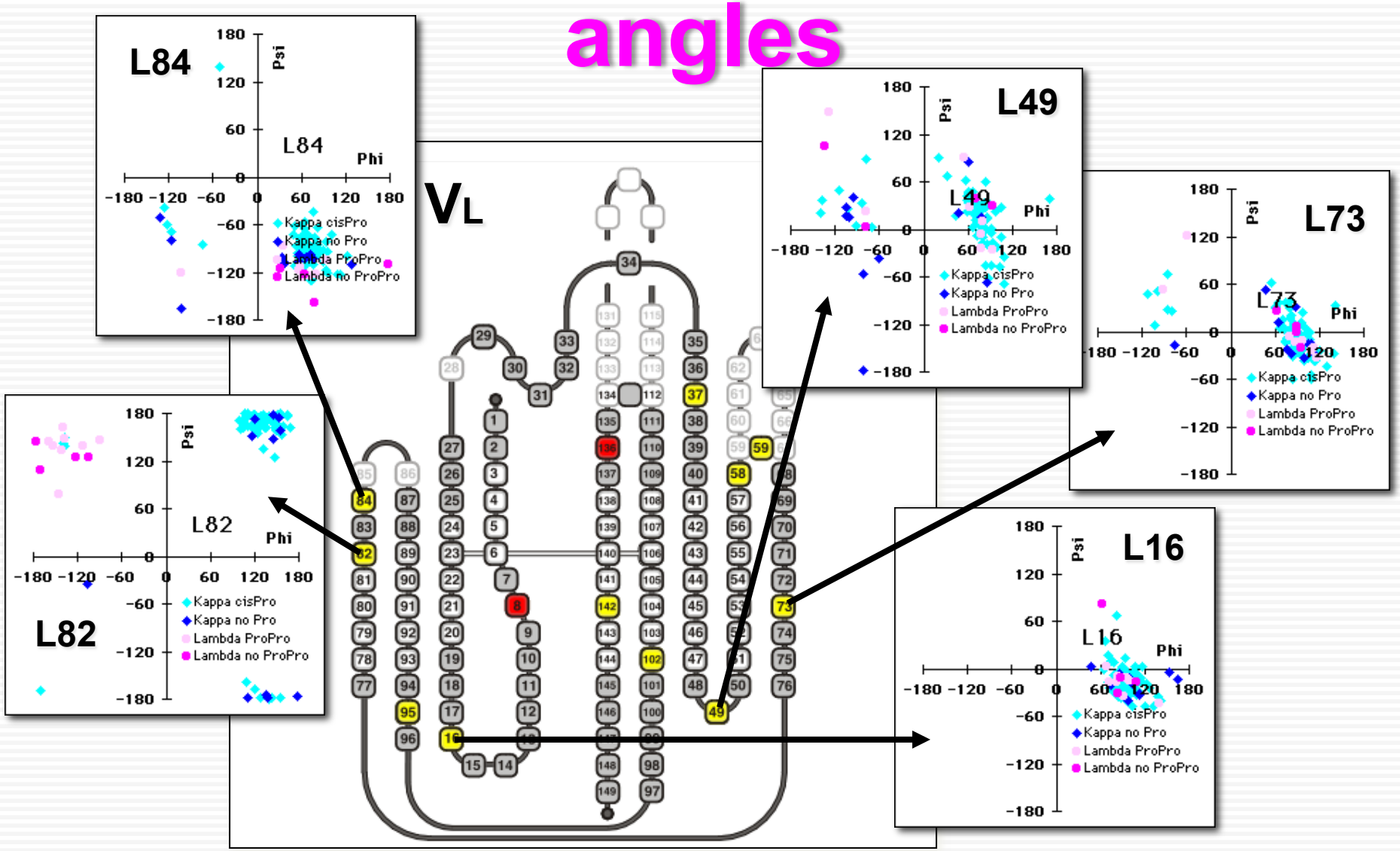
Charge cluster in hV_H3

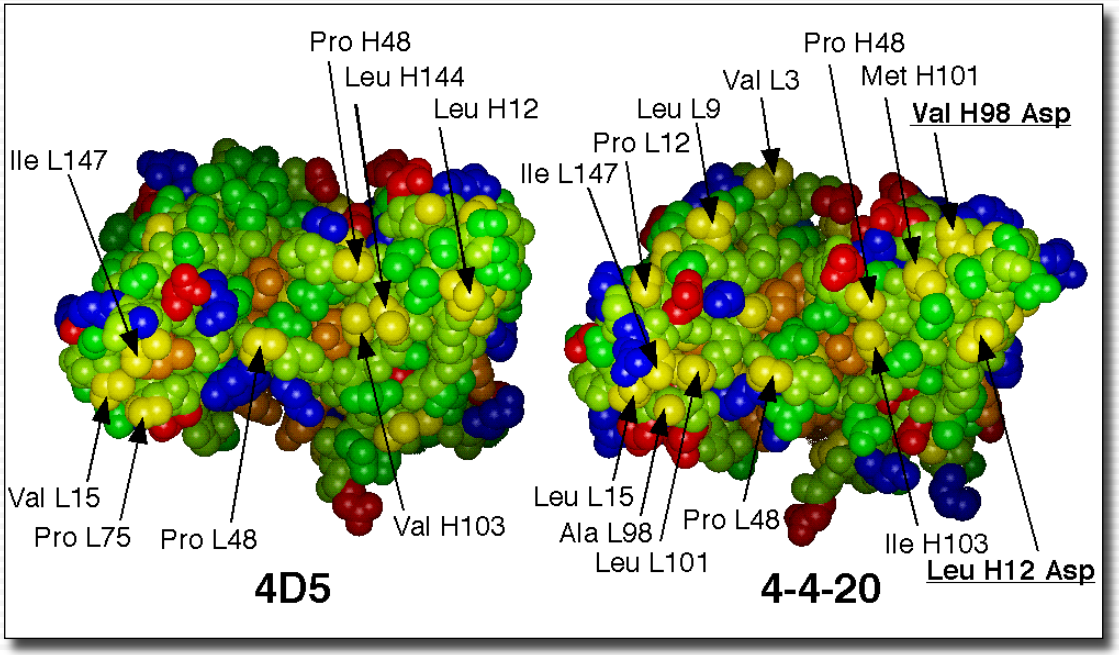


Lys H77 found 50% of murine Ab: replacement by Arg has a huge effect on stability

K.Proba et al.: *J. Mol. Biol.* **275** (1998) 245-253.

Positions with positive Φ torsion angles





Replacement of hydrophobic surface residues

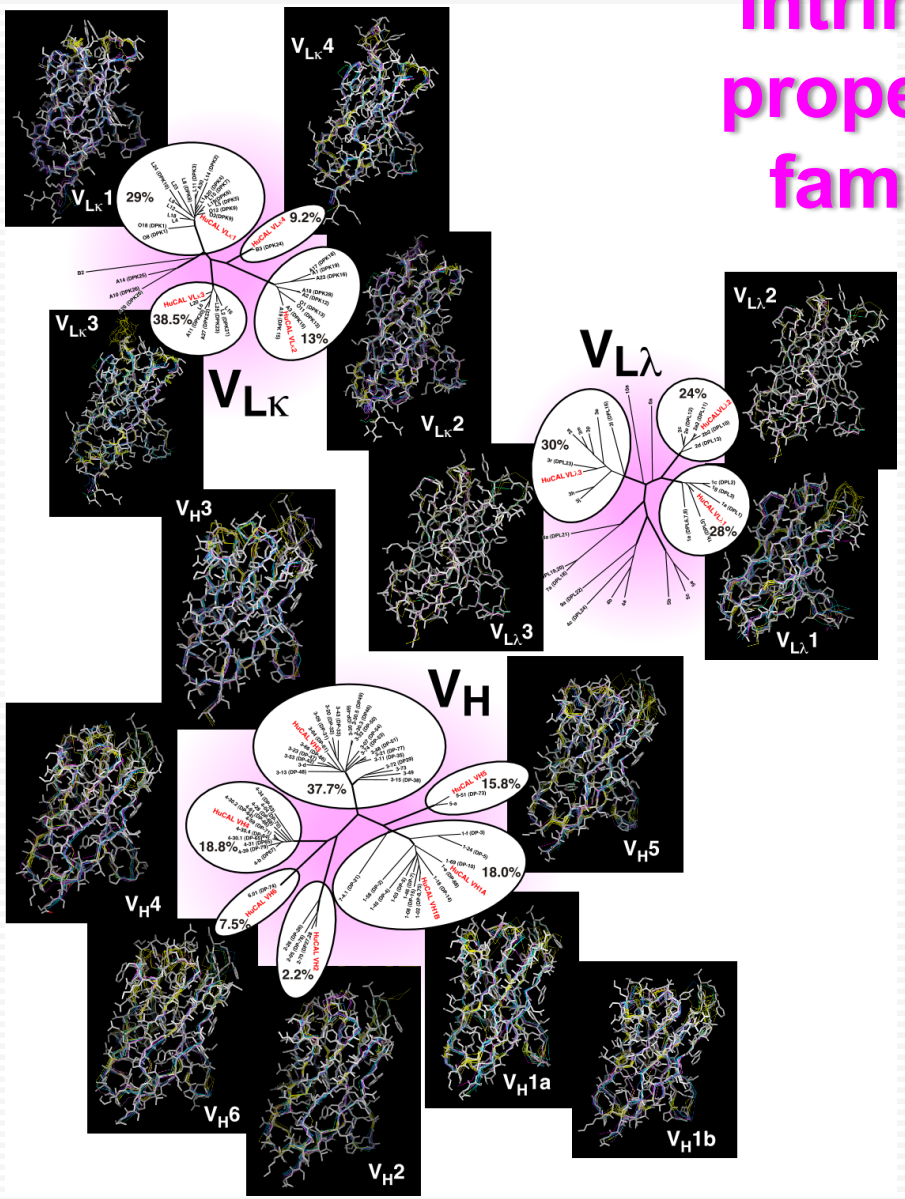
- Reduces rate of thermal aggregation
- Non-additive
- Highly context dependent
- No effect on the solubility of the native scFv

scFV	$Urea_{50}$	% soluble
4D5-8	4.3 M	>95%
4-4-20	4.1-4.3 M	<2%
4-4-20 Leu H12 Ser	4.1-4.3 M	<2%
4-4-20 Leu H12 Asp	4.1-4.3 M	25%
4-4-20 Val H98 Asp	4.1-4.3 M	50%
4D5Flu	6.4 M	50%

L. Niebe et al., Prot. Eng. 10 (1997) 435-444
S. Jung et al. J. Mol. Biol. ***294*** *(1999) 163-180*

What stabilities and folding efficiencies can we expect for average antibody domains?

Intrinsic stability and folding properties of human germline family consensus domains



HuCAL® germline family consensus frameworks
(A. Knappik et al. J. Mol. Biol. 296 (2000) 57-86)

CDR-1 and -2 derived from the germline family consensus,
CDR-L3κ from 4D5-8,
CDR-L3λ from consensus,
CDR-H3: in isolated domains, a long, stabilizing CDR-H3 allowing the production of isolated V_H domains, in scFv derived from 4D5-8

4 $V_L\kappa$ domains, 3 $V_L\lambda$ domains,
7 V_H domain and 14 out of 49 scFv tested

Stability of human consensus domains

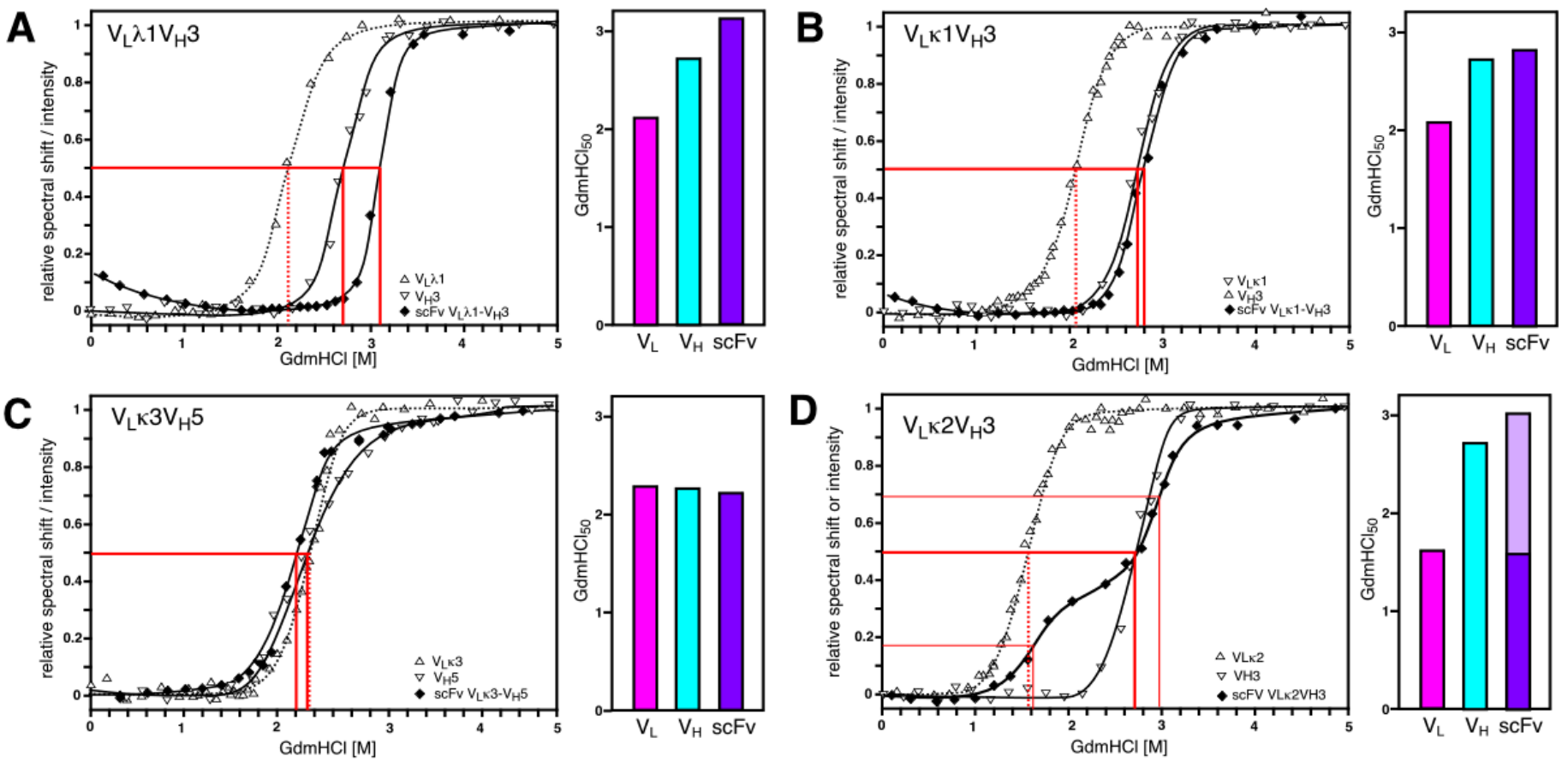
Domain	yield mg/L_{OD10}	oligomeric state	T_m °C	$[GdmHCl]_{50}$ M	$\Delta G(H_2O)$ kJ/mol	m kJ L/mol²
$hV_L\kappa1$	4.5	monomer	64	2.1	29	14
$hV_L\kappa2$	14.2	monomer	63	1.5	25	16
$hV_L\kappa3$	17.1	monomer	73	2.3	35	15
$hV_L\kappa4$	9.6	mono+dimer	58	1.5	*n.d.*	*n.d.*
$hV_L\lambda1$	0.3	monomer	64	2.1	24	11
$hV_L\lambda2$	1.9	monomer	50	1.0	16	16
$hV_L\lambda3$	0.8	mono+dimer	49	0.9	15	16
Domain	**yield mg/L_{OD10}**	**oligomeric state**	**T_a °C**	**$[GdmHCl]_{50}$ M**	**$\Delta G(H_2O)$ kJ/mol**	**m kJ L/mol²**
hV_H1a	1.0	monomer	41	1.5	14	10
hV_H1b	1.2	monomer	51	2.1	26	13
hV_H2	refolded	*n.d.*	45	1.4	*n.d.*	*n.d.*
hV_H3	2.4	monomer	65	3.0	53	18
hV_H4	refolded	*n.d.*	44	2.3	*n.d.*	*n.d.*
hV_H5	refolded	monomer	44	2.2	17	7
hV_H6	refolded	*n.d*	39	1.2	*n.d.*	*n.d.*

*S.Ewert et al.: J Mol Biol **325** (2003) 531-553.*

How does the stability of an scFv depend on the stabilities of the individual domains

Single chain fragments

V_H3 paired with any of the seven V_L fragments
$V_L\kappa3$ paired with any of the seven V_H fragments



From the domain to the scFv

scFv	yield mg/L_{OD10}	rel. yield %	% soluble	oligomeric state	[GdmHCl]$_{50}$ scFv V_L	[GdmHCl]$_{50}$ scFv V_H	[GdmHCl]$_{50}$ isol. domains V_L	[GdmHCl]$_{50}$ isol. domains V_H*
$V_L\kappa1$-V_H3	2.6	40	50	monomer	2.8		2.1	2.7
$V_L\kappa2$-V_H3	2.6	40	20	monomer	1.6	2.9	1.5	2.7
$V_L\kappa3$-V_H3	6.5	100	30	monomer	2.8		2.3	2.7
$V_L\kappa4$-V_H3	5.2	80	40	monomer	2.0	2.8	1.5	2.7
$V_L\lambda1$-V_H3	7.8	120	40	mono/dimer	3.0		2.1	2.7
$V_L\lambda2$-V_H3	5.9	90	10	mono/dimer	2.9		1.0	2.7
$V_L\lambda3$-V_H3	3.6	60	10	mono/dimer	2.8		0.9	2.7
$V_L\kappa3$-V_H1a	11.1	170	10	mono/dimer	2.8	1.8	2.3	1.2
$V_L\kappa3$-V_H1b	12.4	190	20	monomer	3.0	2.4	2.3	1.8
$V_L\kappa3$-V_H2	2.6	40	90	monomer	2.8	1.5	2.3	1.6
$V_L\kappa3$-V_H3	6.5	100	30	monomer	2.8		2.3	2.7
$V_L\kappa3$-V_H4	2.6	40	90	monomer	3.0	2.3	2.3	1.5
$V_L\kappa3$-V_H5	6.5	100	50	monomer	3.0	2.2	2.3	1.9
$V_L\kappa3$-V_H6	5.2	80	80	monomer	2.6	1.2	2.3	0.5

*S.Ewert et al.: J Mol Biol **325** (2003) 531-553.*

How many mutations are needed to improve the poorest consensus V_H domain to the level of the good ones?

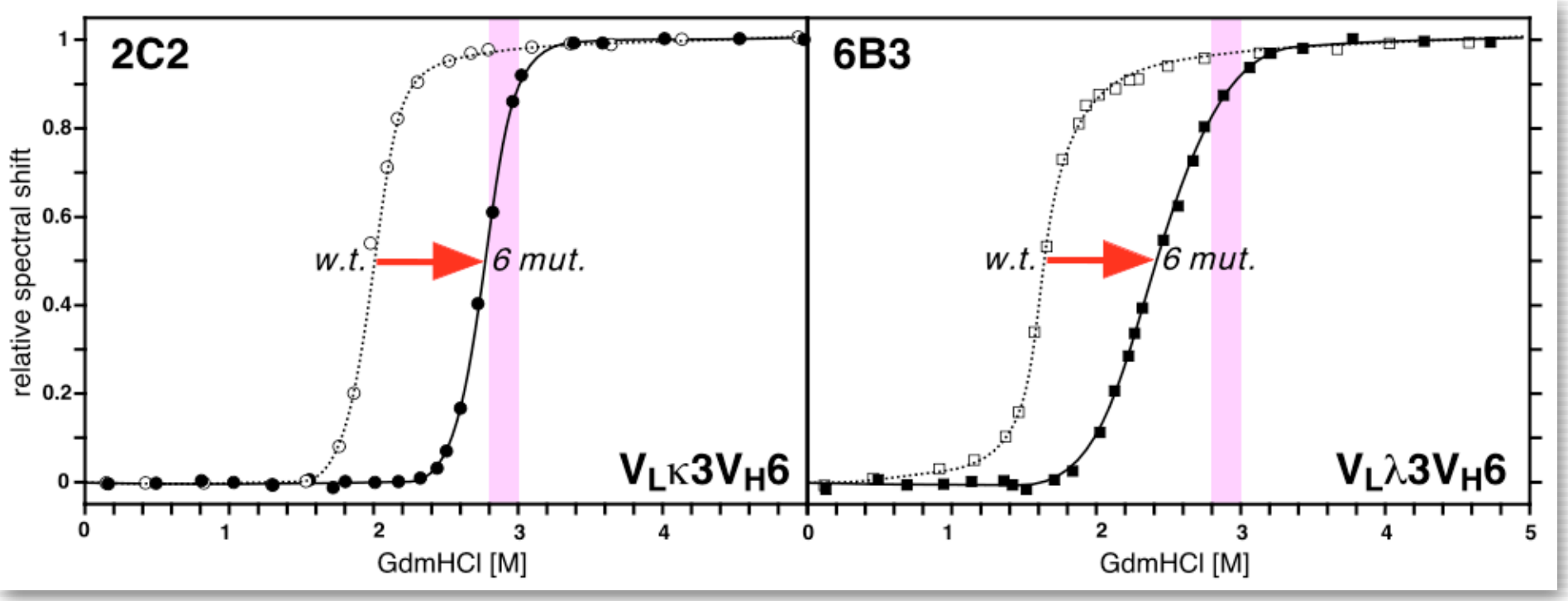
Improving the hV_H6 HuCAL framework

Six mutations were needed, five of them common to hV_H2, hV_H4 and hV_H6:

Three mutations improve both stability and yield

Two improve the folding yield, but have no measurable effect on thermodynamic stability

One significantly improves stability without affecting the yield

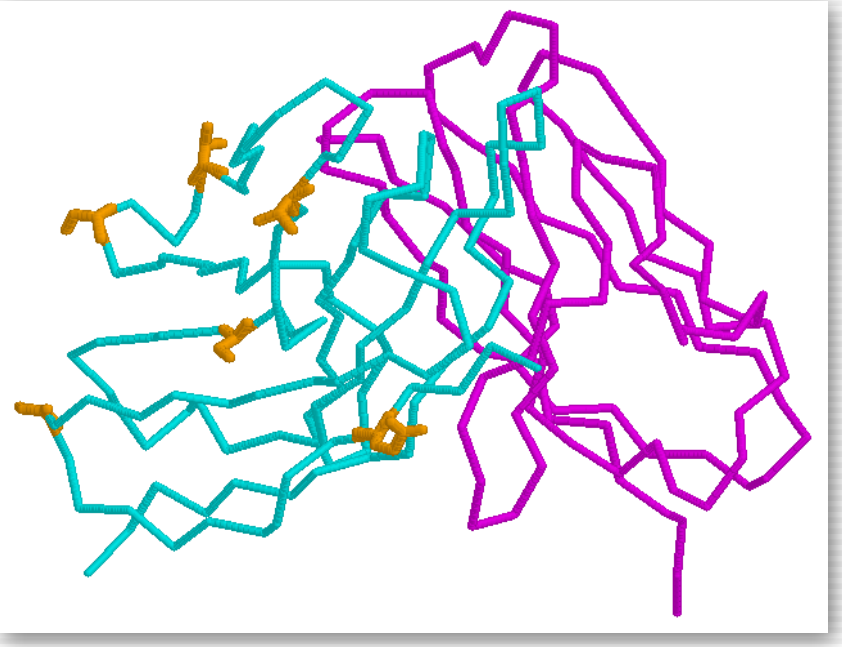


Mutations to huV$_H$6

Two different scFv: 2C2 (V$_\kappa$3-V$_H$6) and 6B3 (V$_\lambda$3-V$_H$6):

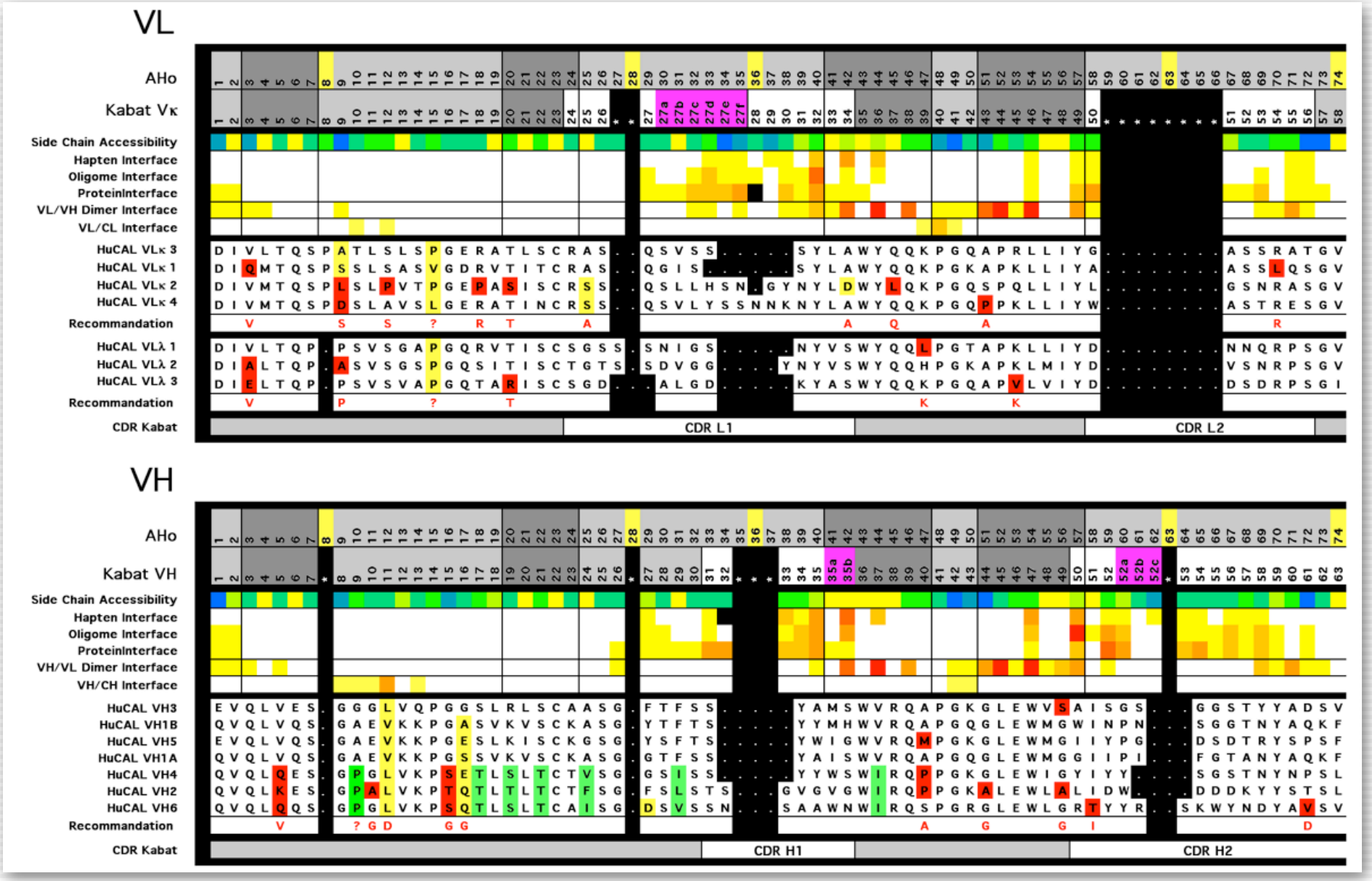
	yield	stability
Gln H5 Val (secondary structure propensity)	+	+
Ser H16 Gly (pos. Φ, conformational strain)	+	+
Thr H58 Ile (hydrophobic packing, to V$_H$ consensus)	0	+
Ser H76 Gly (pos. Φ, conformational strain)	+	+
Ser H90 Tyr (semiexposed hydrophobic, to V$_H$ cons.)	+	0
Val H72 Asp (exposed hydrophobic residue)	+	0

[GdmHCl]$_{50}$ shifted from 2.0 to 2.8 M
and from 0.7 to 2.5 M *
Total stabilisation by 21 and 25 kJ/mol
from 51 to 72 kJ/mol
and from 42 to 67 kJ/mol *
Total increase in yield 4.3 and 4.2-fold,
from 1.2 mg/L to 5 mg/L
and from 0.4 mg/L to 1.7 mg/L

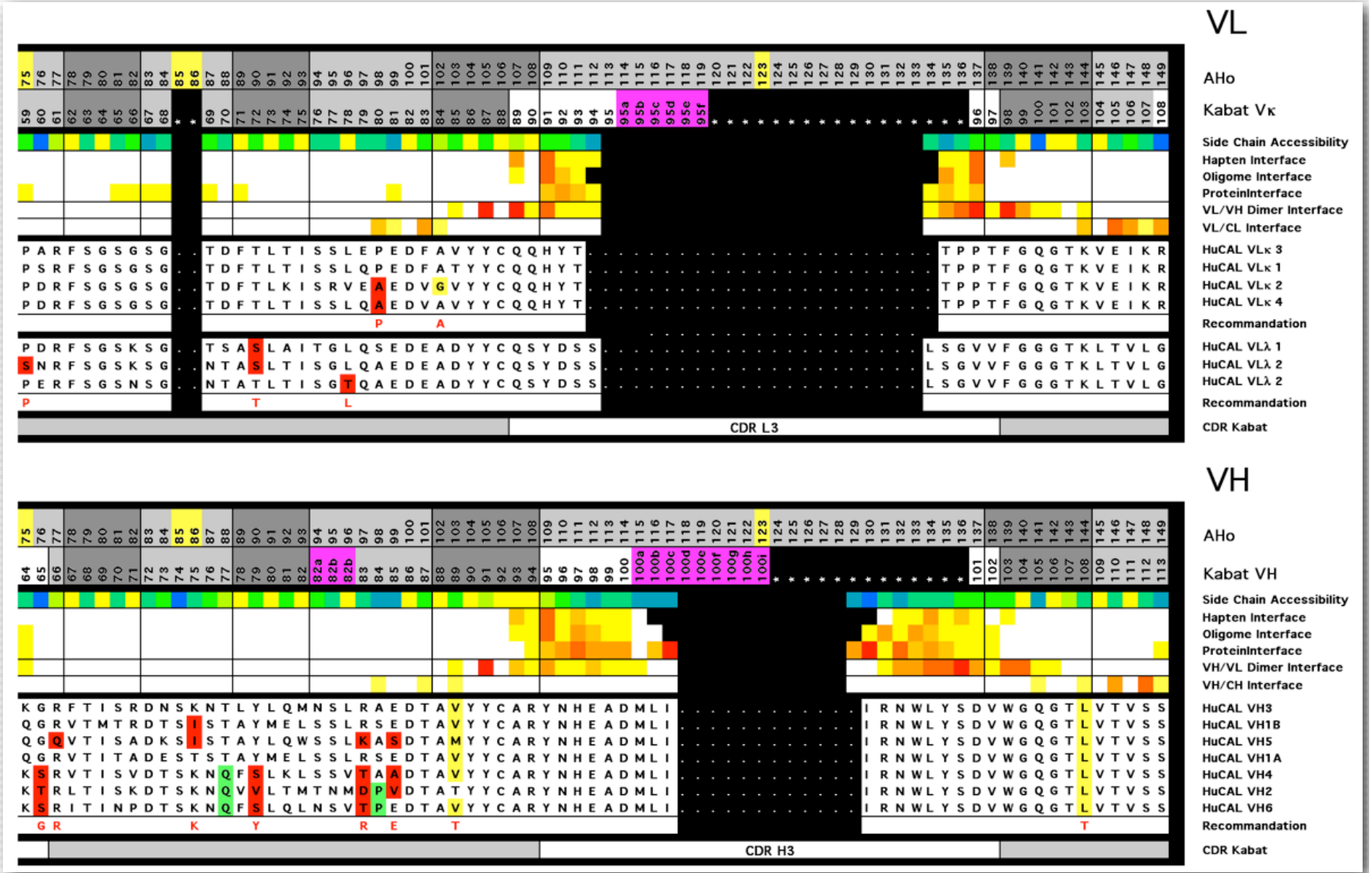


ΔΔG for scFv are highly suspect, see suppl. materials to
*Kügler et al.: Protein Eng Des Sel **22** (2009) 135-147*

How about the other Consensus Frameworks?

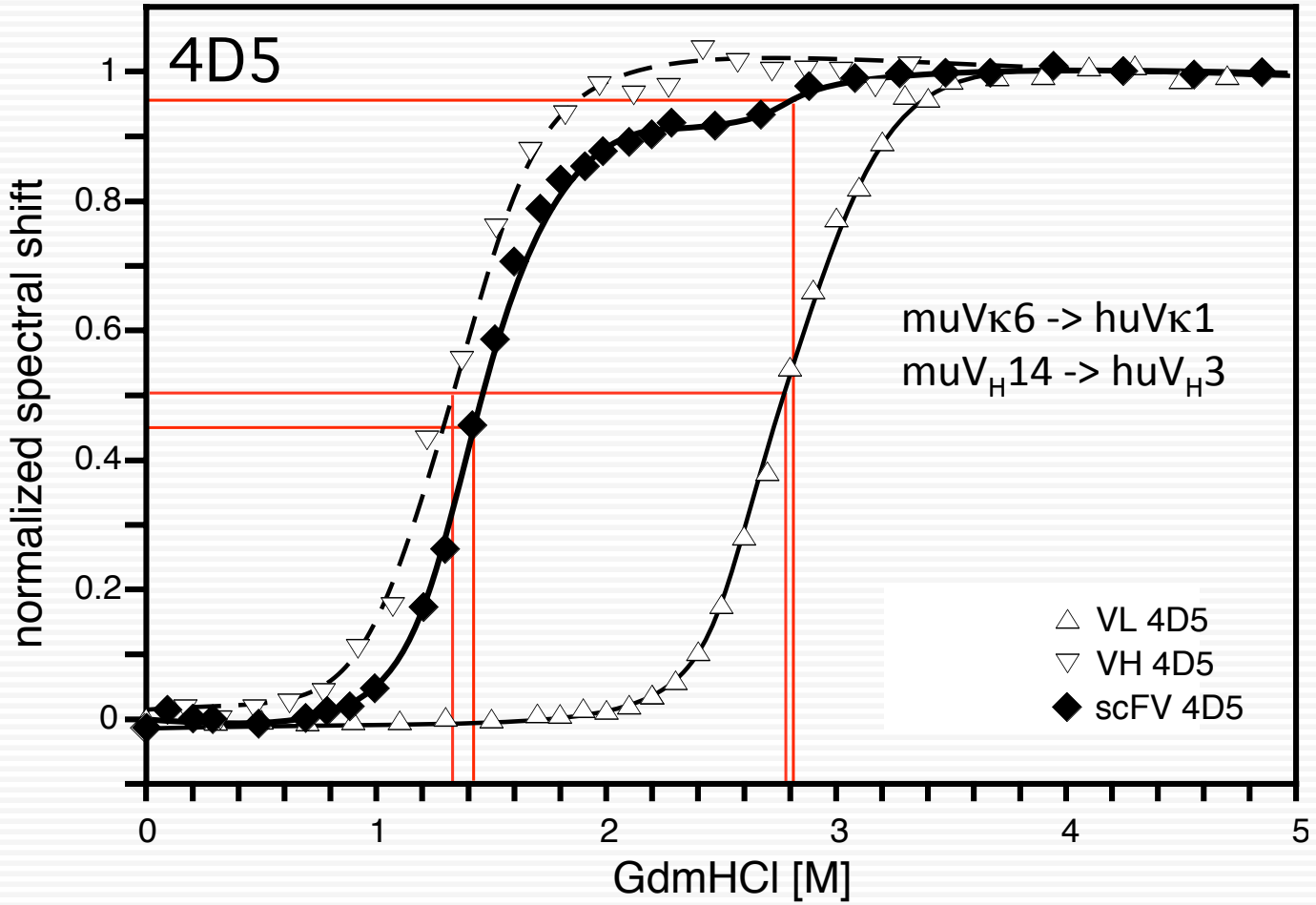


How about the other Consensus Frameworks?



Why is the equilibrium stability of hu4D5 so low?

The Gold Standard: hu4D5-V8 scFv



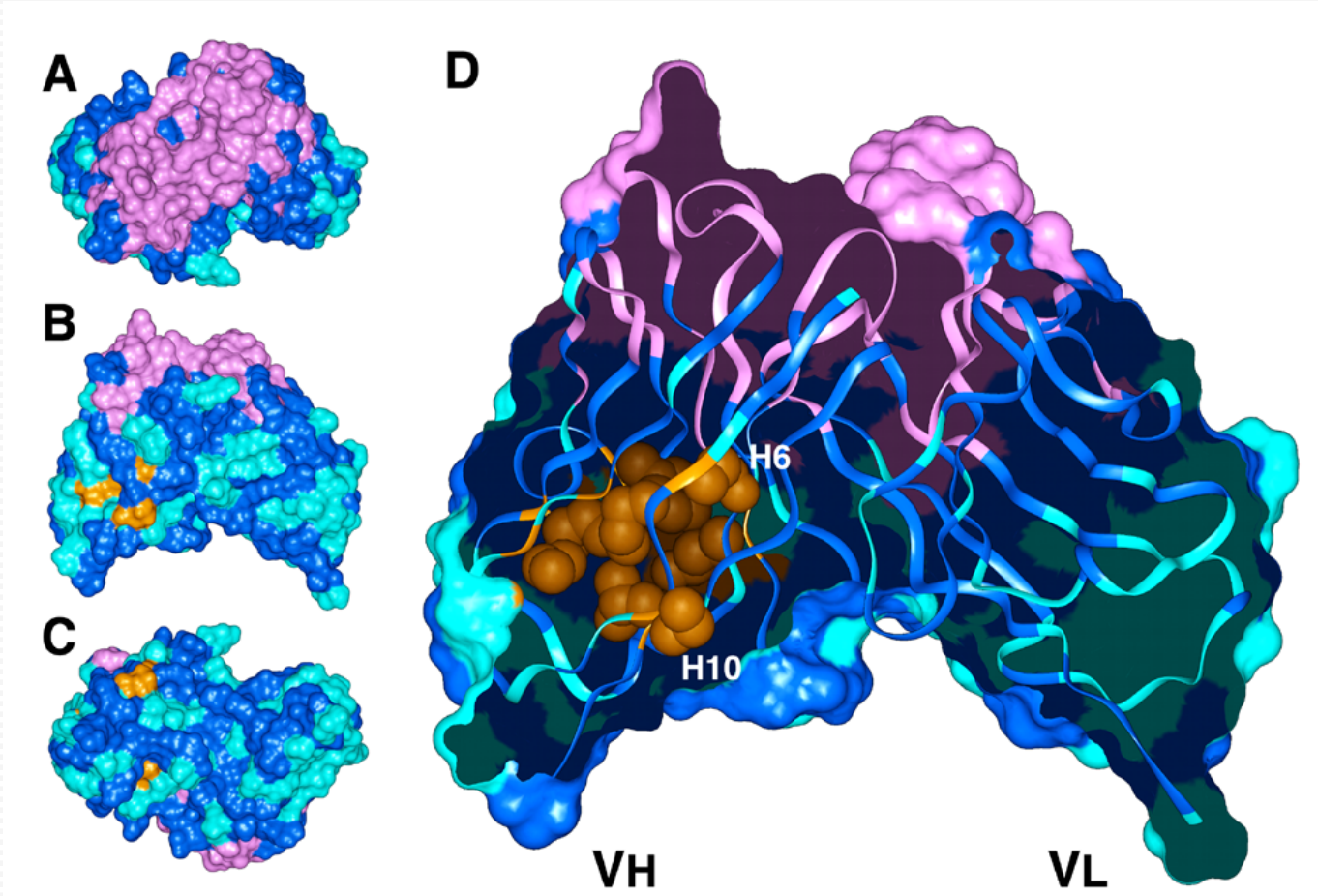
HuCAL Vκ1: [GdmCL]$_{50}$ = 2.3 M **4D5 Vκ1: [GdmCL]$_{50}$ = 2.8 M**

HuCAL V$_H$3: [GdmCL]$_{50}$ = 2.7 M[a] (3.0 M[b]) **4D5 V$_H$3: [GdmCL]$_{50}$ = 1.3 M**

[a] with CDR-H3 of 4D5, [b] with long, stabilizing CDR-H3

huVκ1/huV$_H$3 scFv: [GdmCL]$_{50}$ = 2.8 M **hu4D5-V8: [GdmCL]$_{50}$ = 1.4 M**

Sometimes a straight CDR grat is not enough

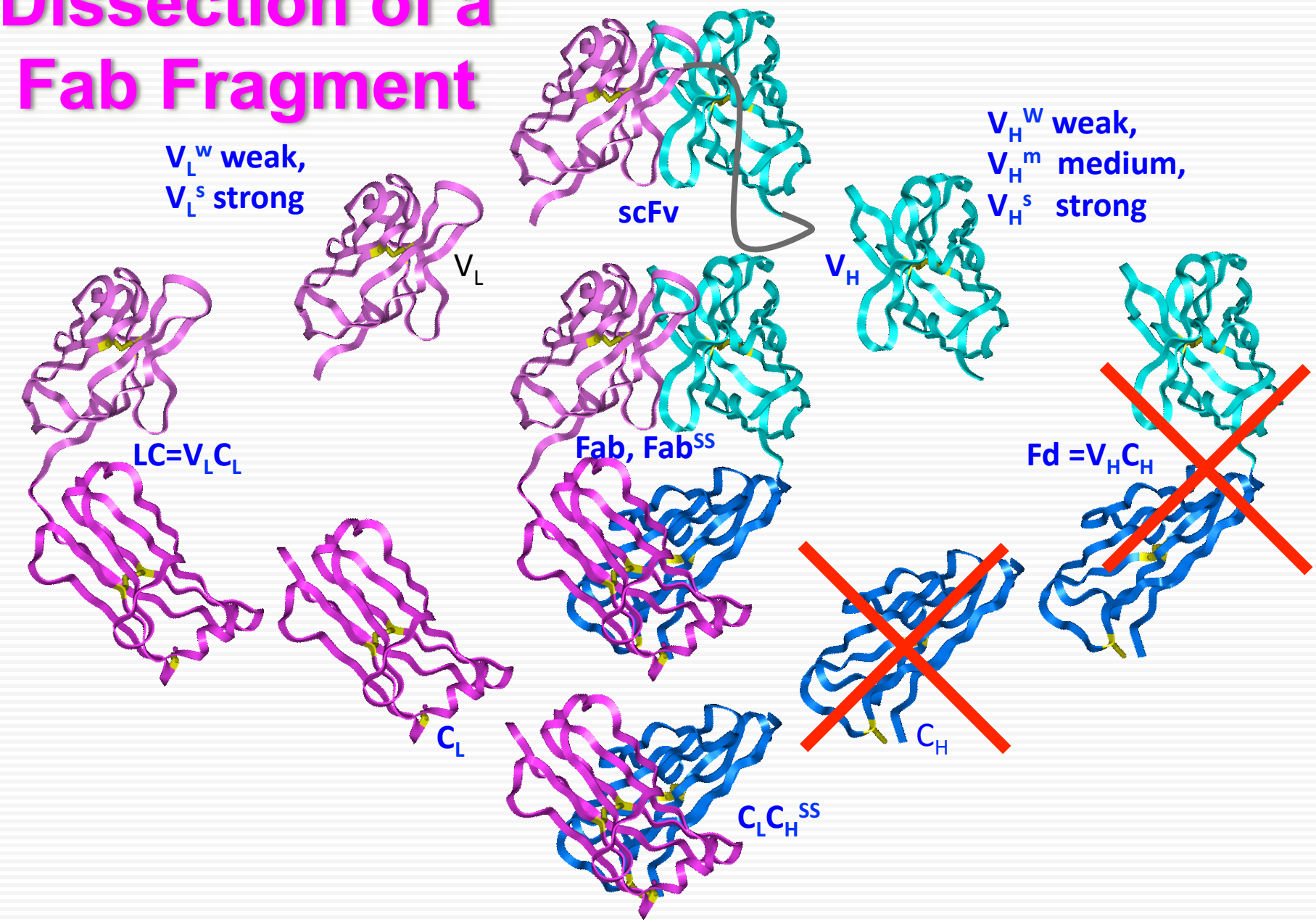


*Willuda et al.: Cancer Res. **59** (1999) 5758-5767: anti-EpCAM scFv 4D5moc, muVH9 to huVH3*
*Kügler et al.: Protein Eng Des Sel **22** (2009) 135-147: anti-CD19 4G7, muVH1 to huVH3*
*Honegger et al.: Protein Eng Des Sel **22** (20099121-134: Generic huVH1 and huVH5 to huVH3 graft*

Frequently Asked Question:

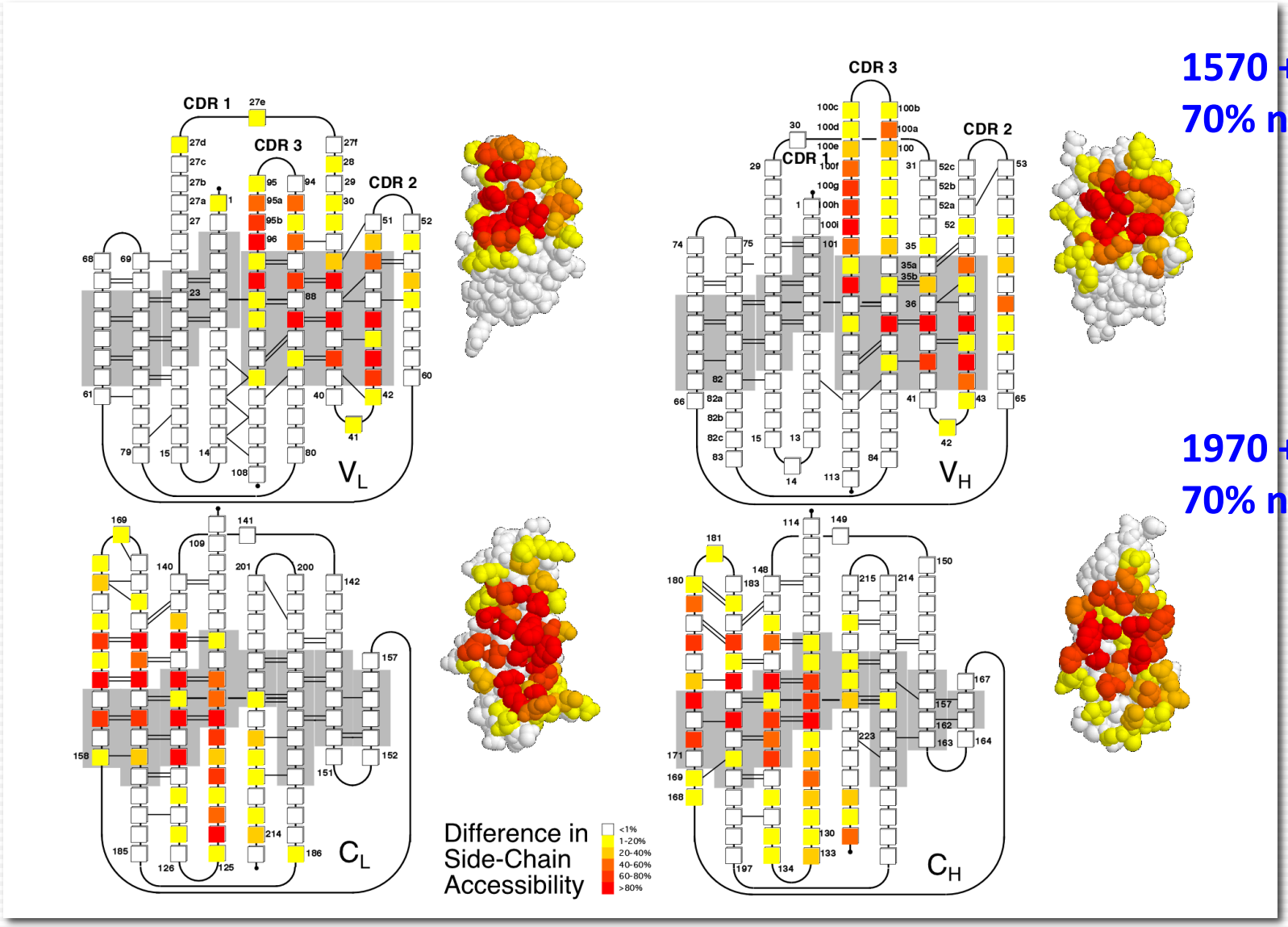
How much will a Fab fragment profit from improved variable domain stability?

Dissection of a Fab Fragment



Röthlisberger et al., J Mol Biol (2005) **347**, 773-789.

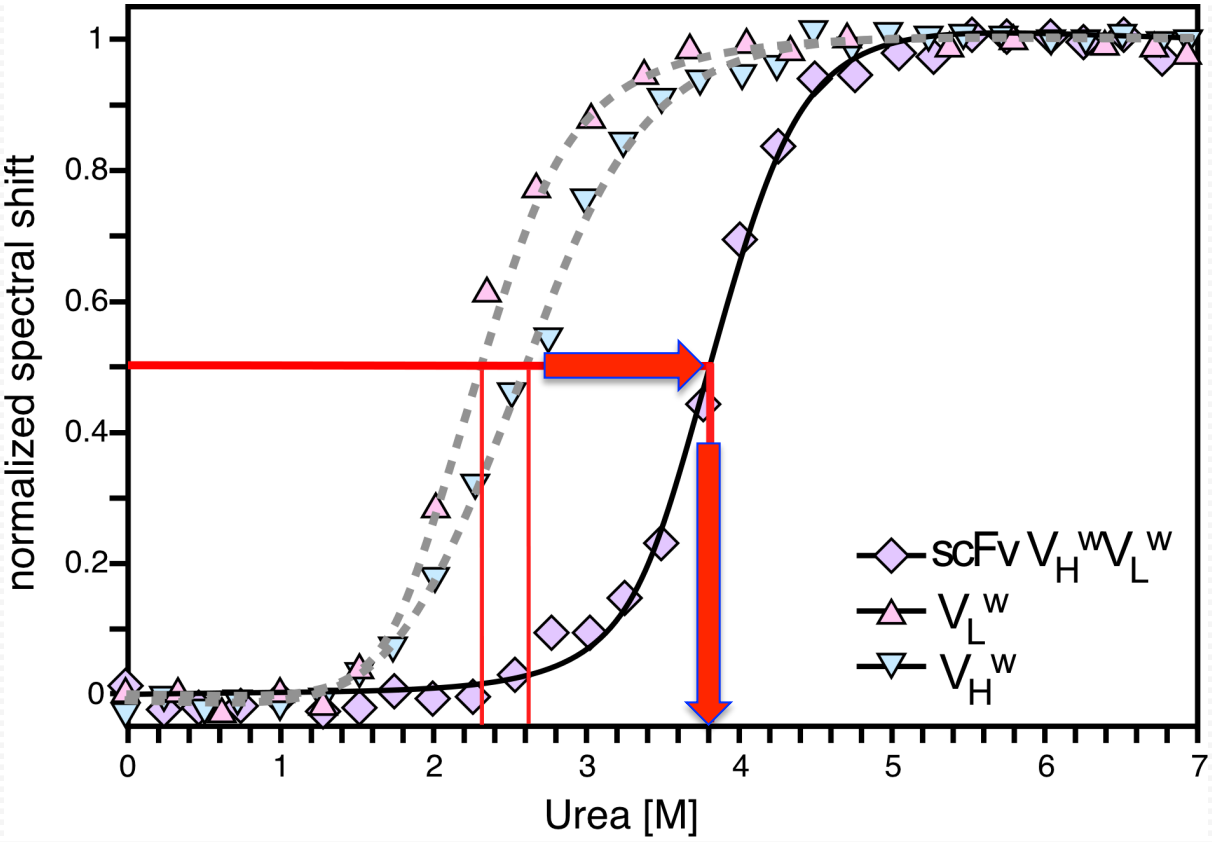
V_L/V_H and C_L/C_H Interface

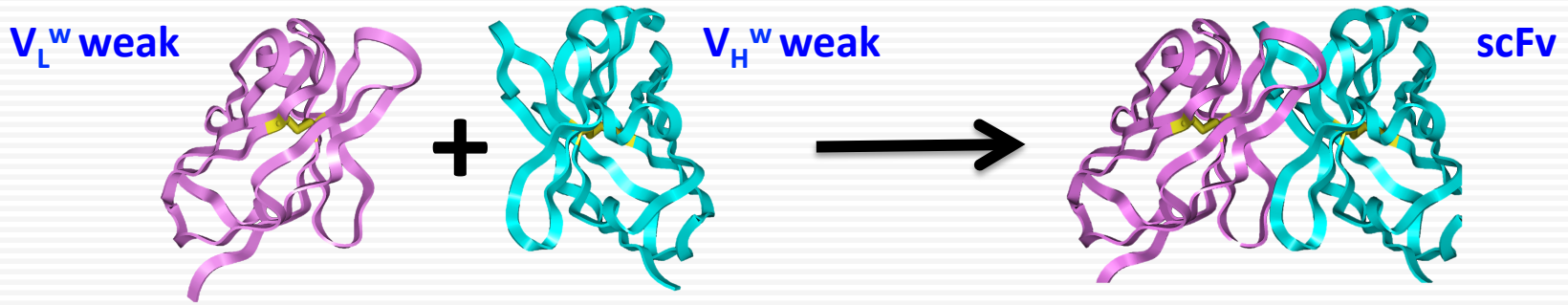


1570 +/- 160 Å^2
70% non-polar

1970 +/- 160 Å^2
70% non-polar

Mutual Stabilization of Domains in scFV





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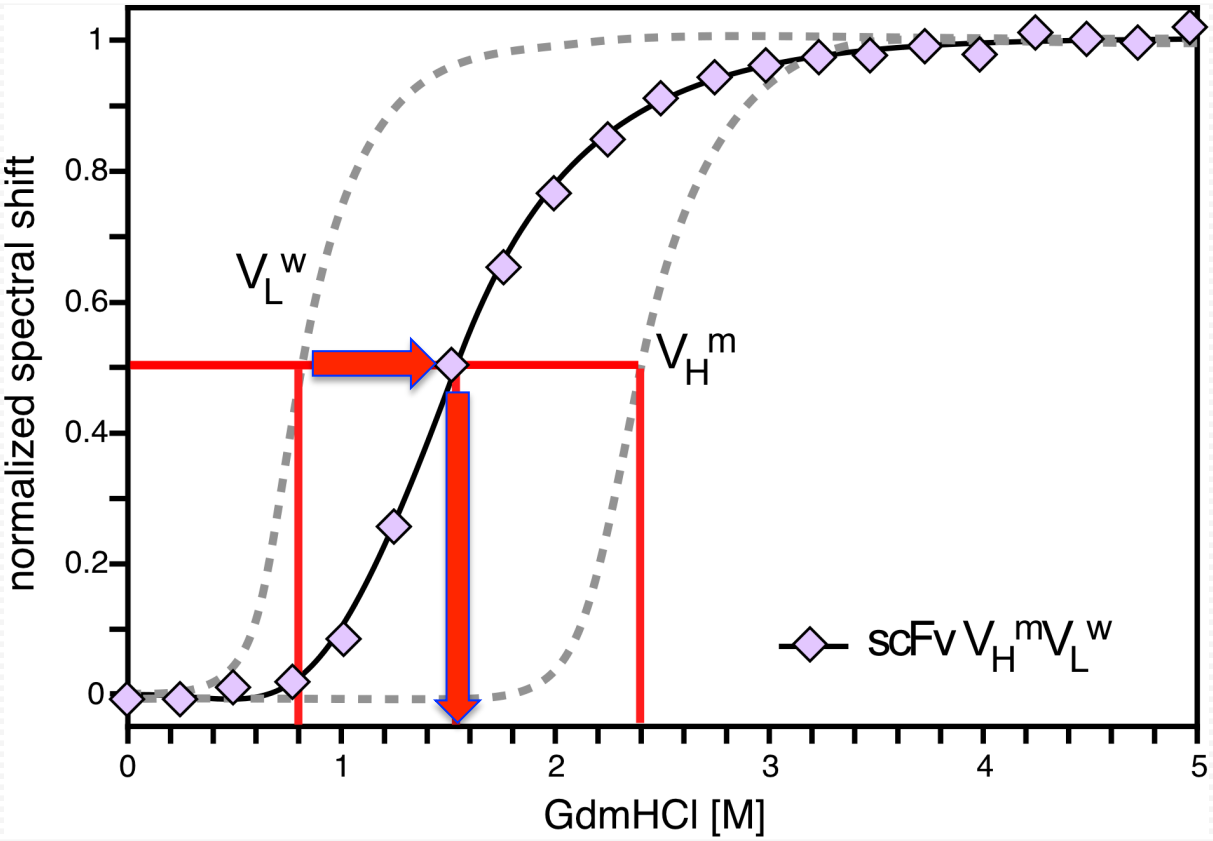
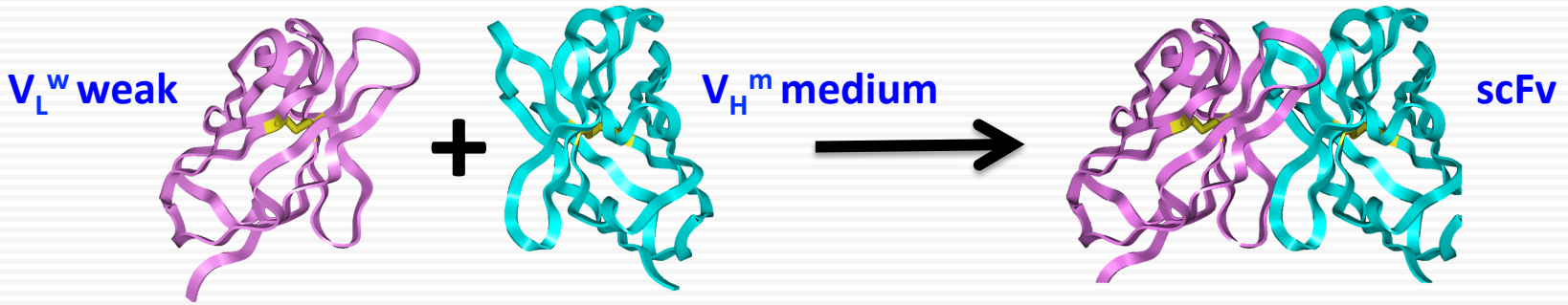
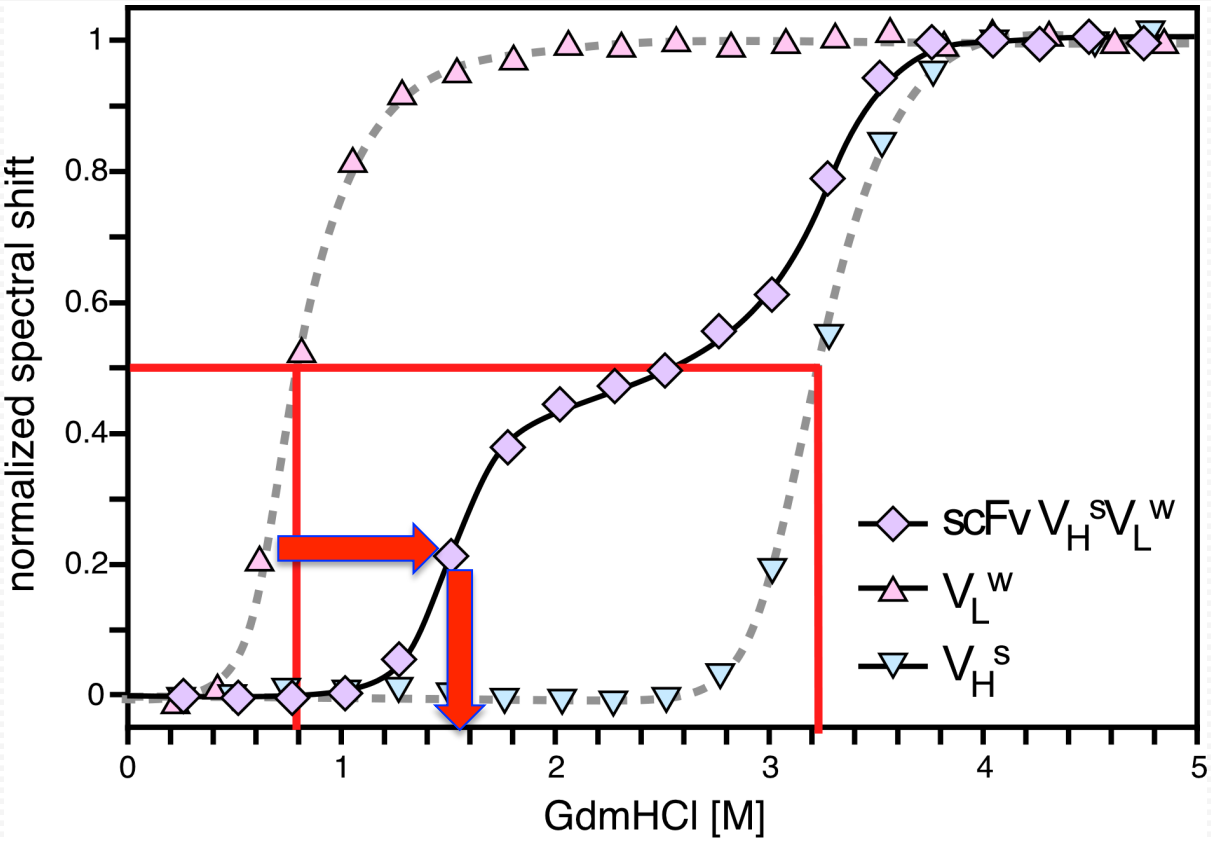
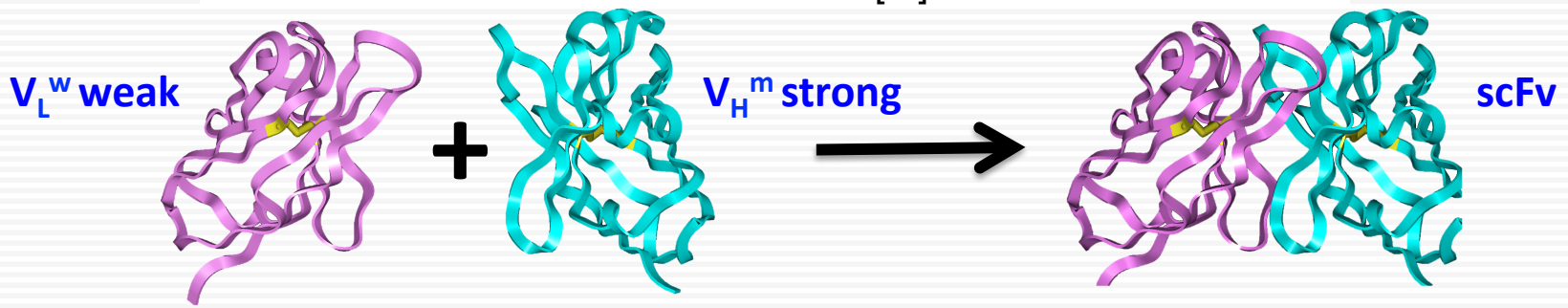


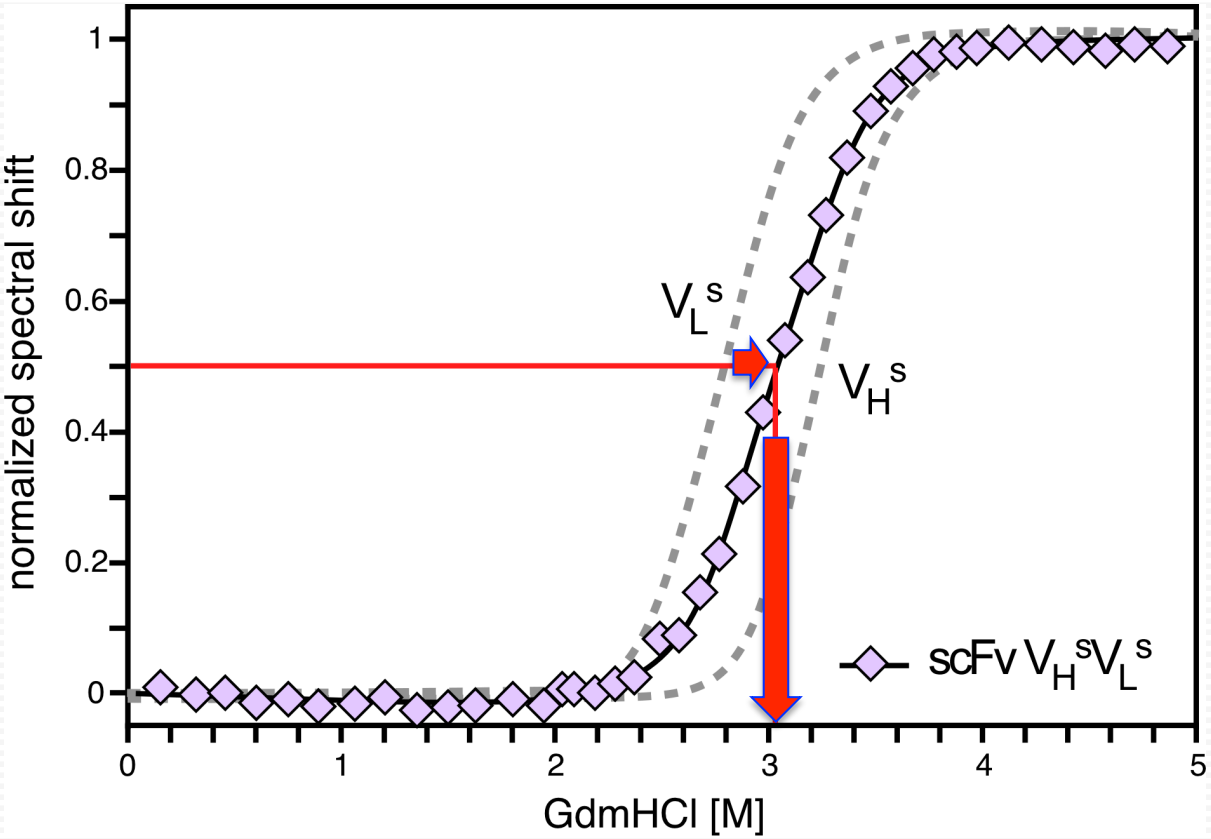


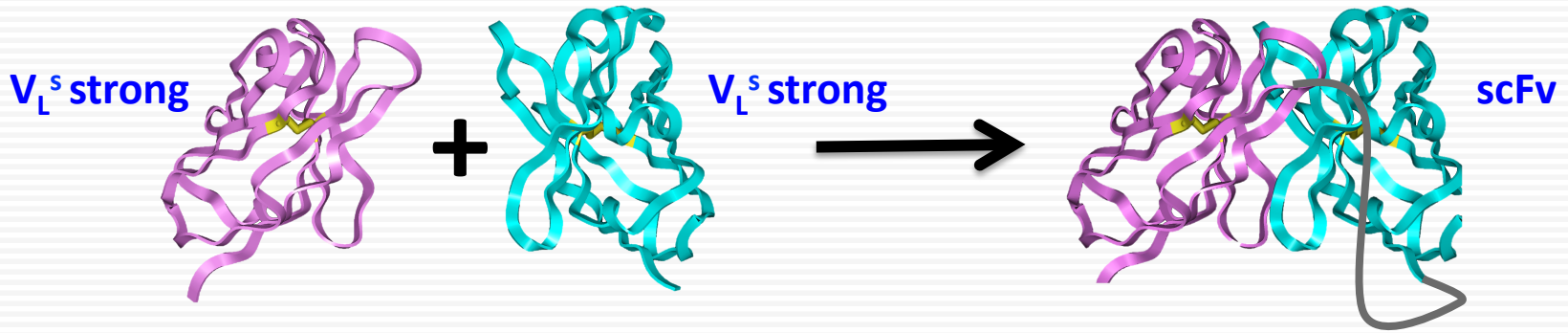
Mutual Stabilization of Domains in scFV



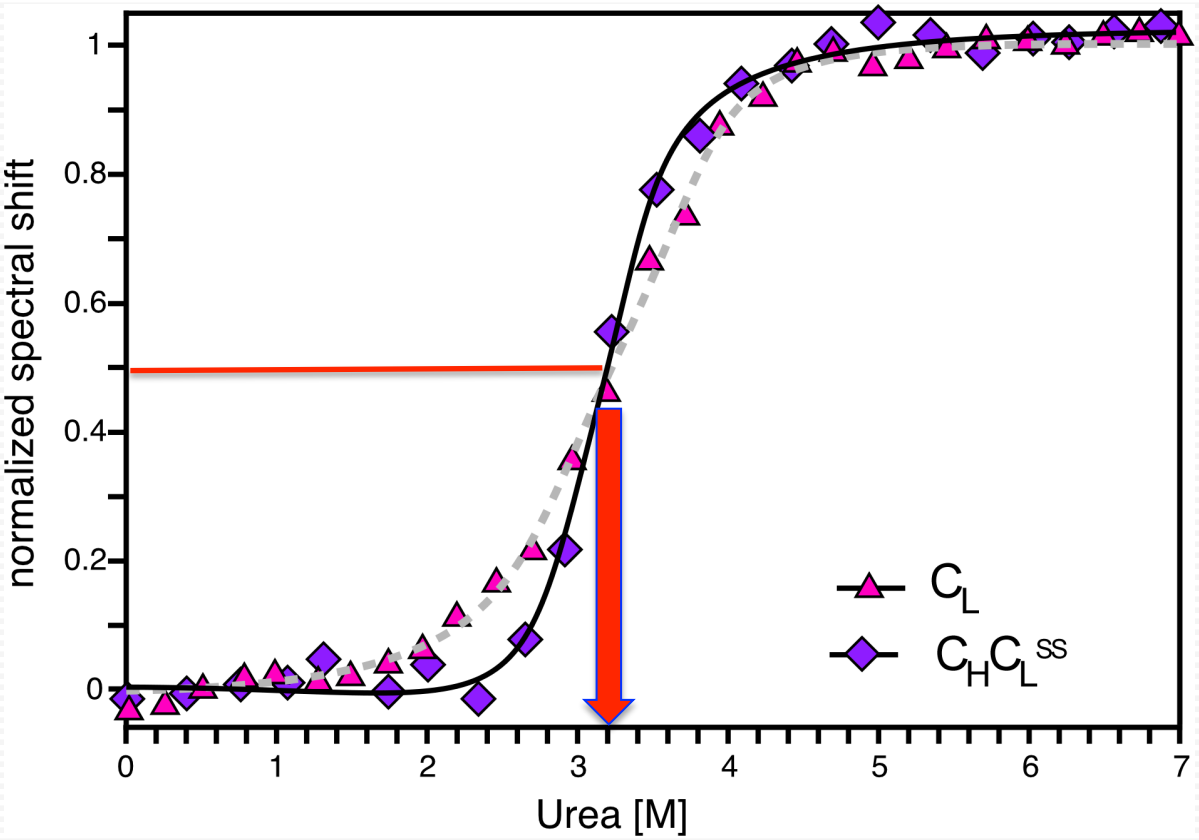


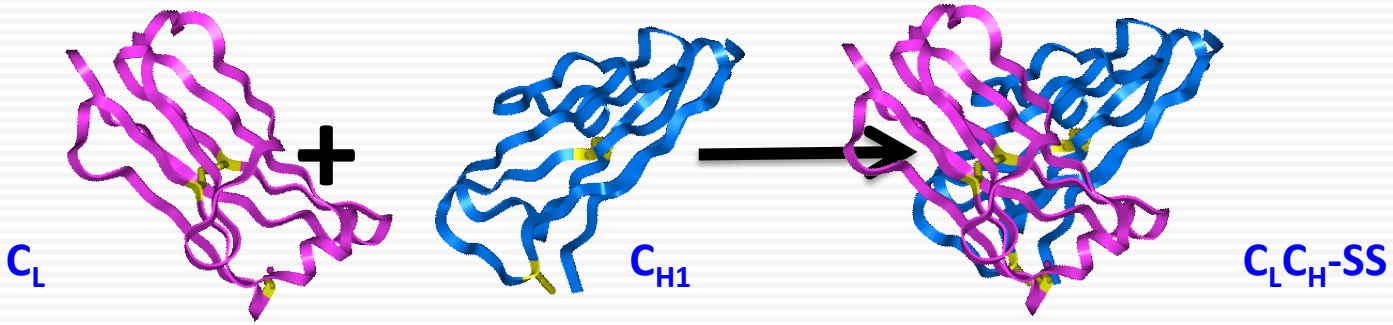
Mutual Stabilization of Domains in scFV



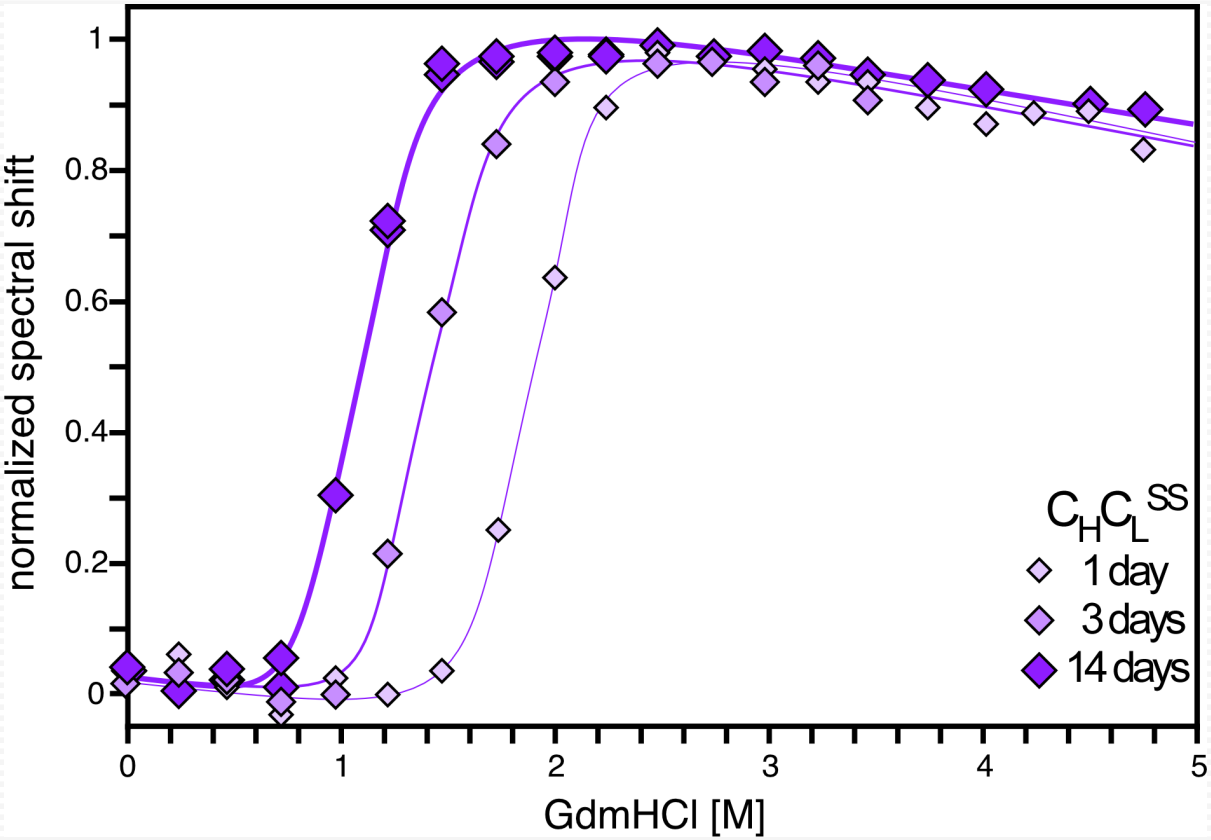


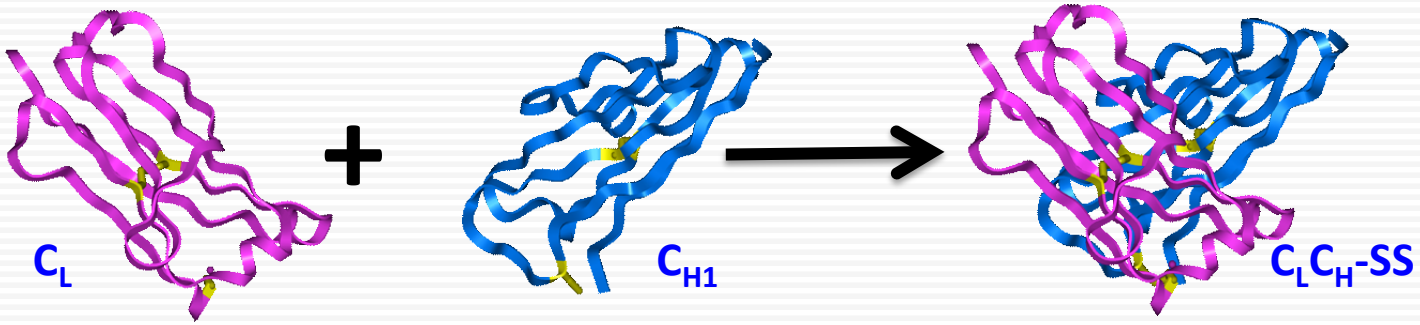
Mutual Stabilization of C_L and C_{H1}



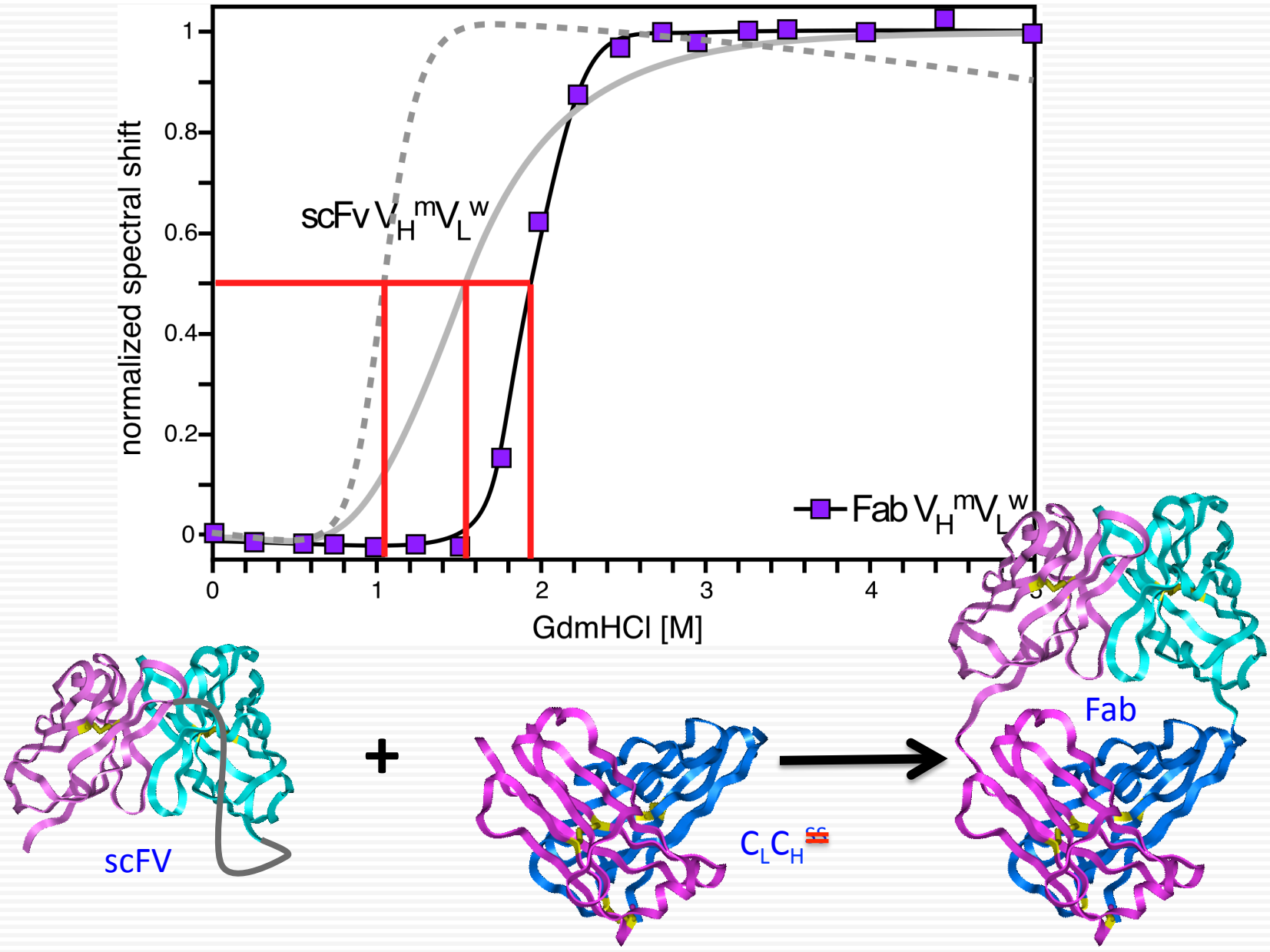


Mutual Stabilization of C_L and C_{H1}

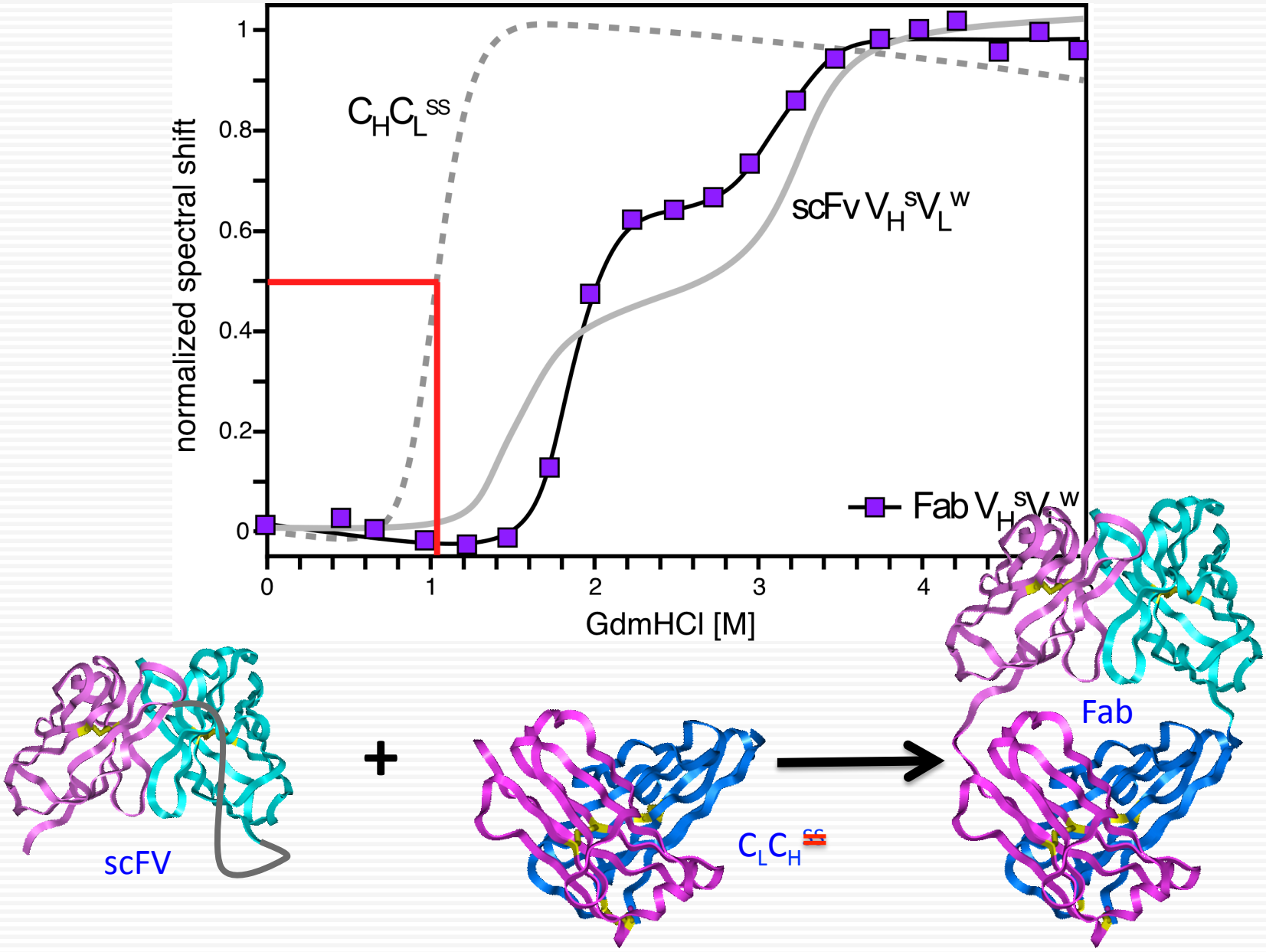




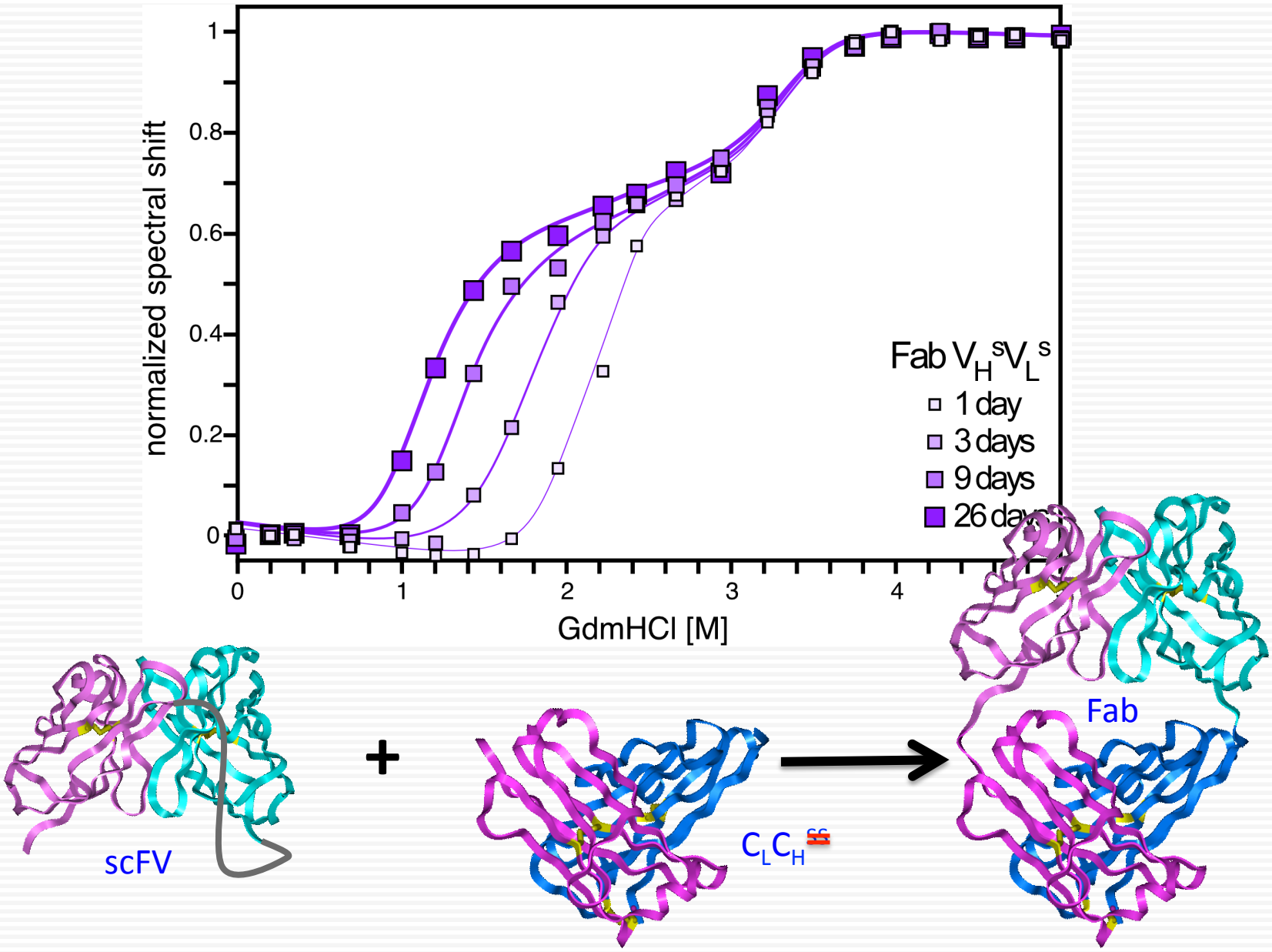
from scFv to Fab



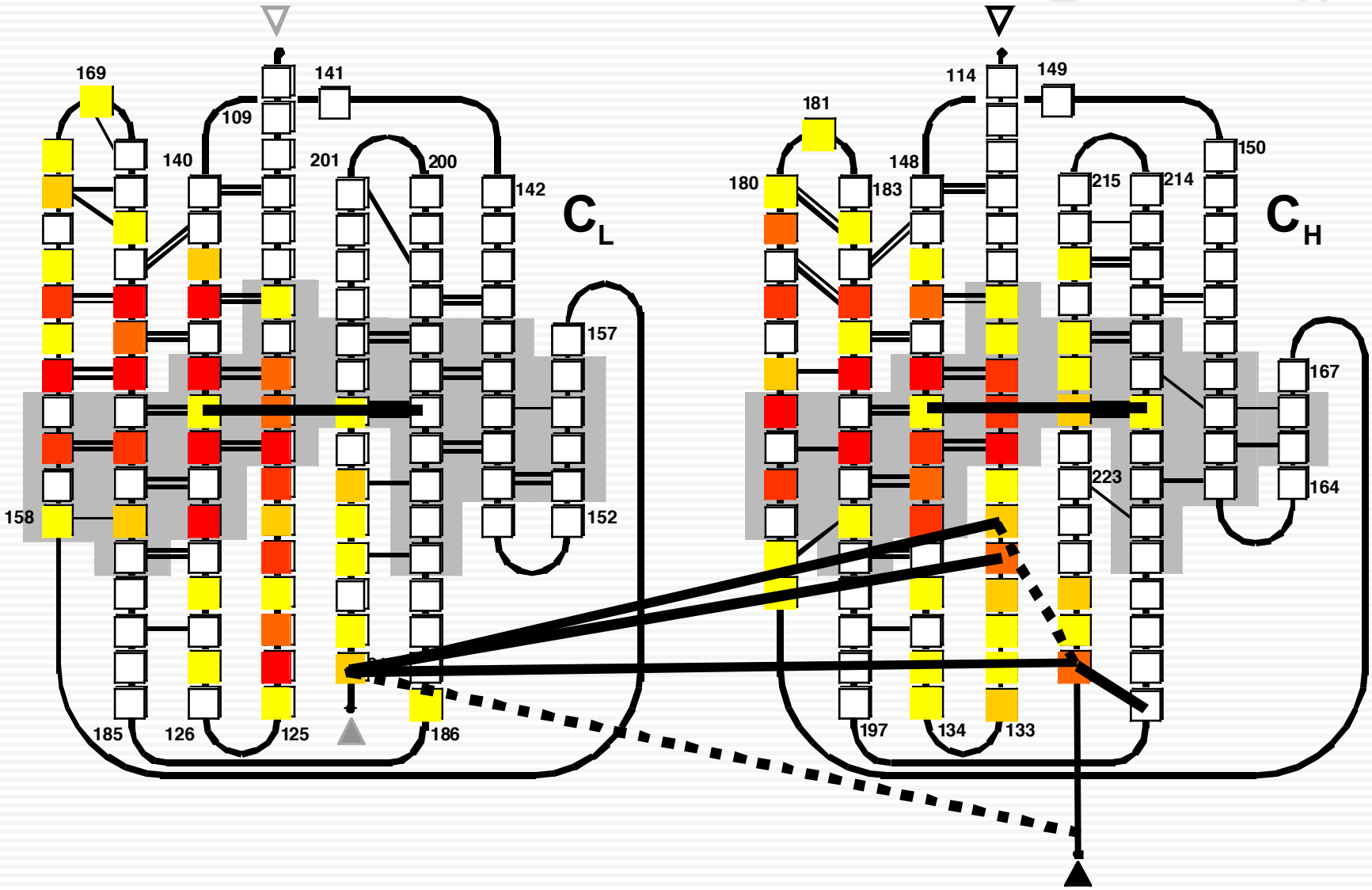
from scFv to Fab



from scFv to Fab, without L-H SS-Bond

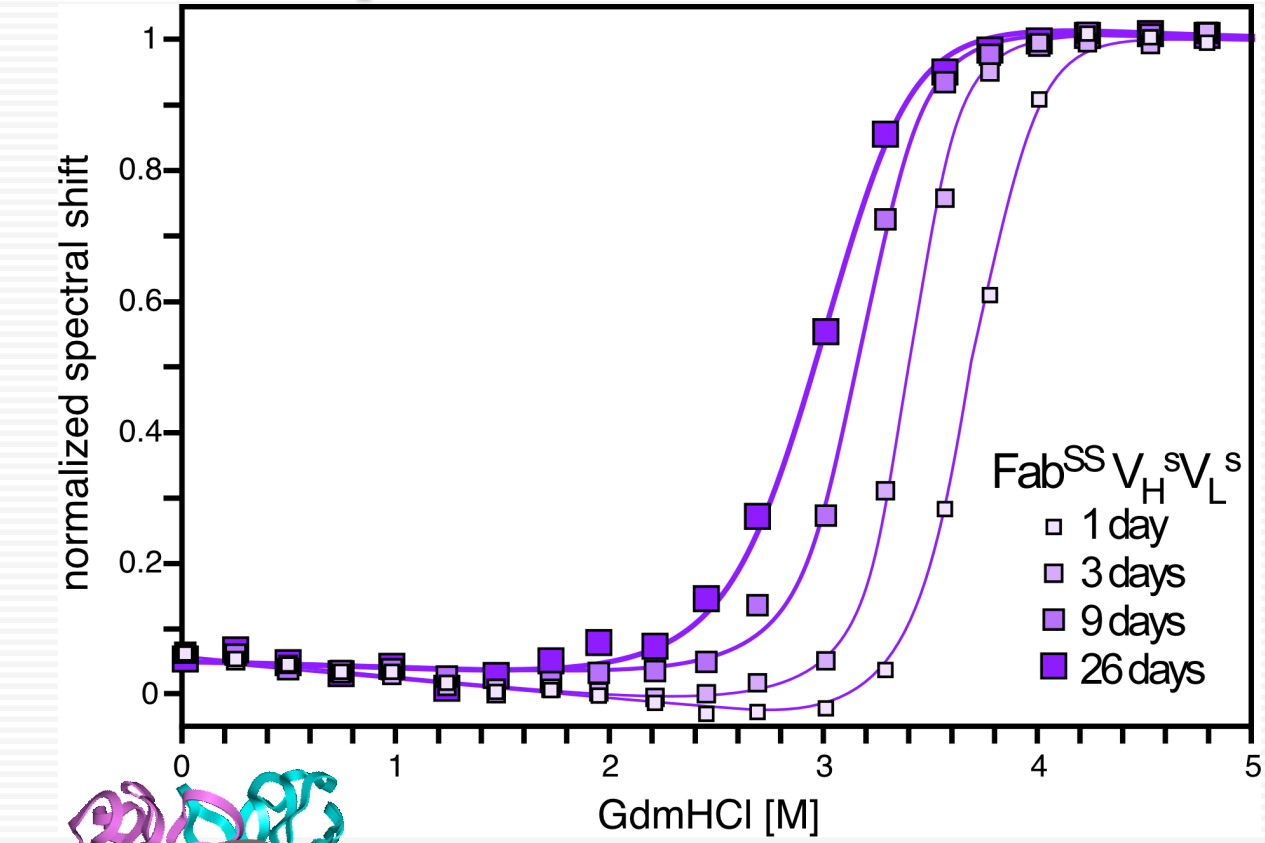


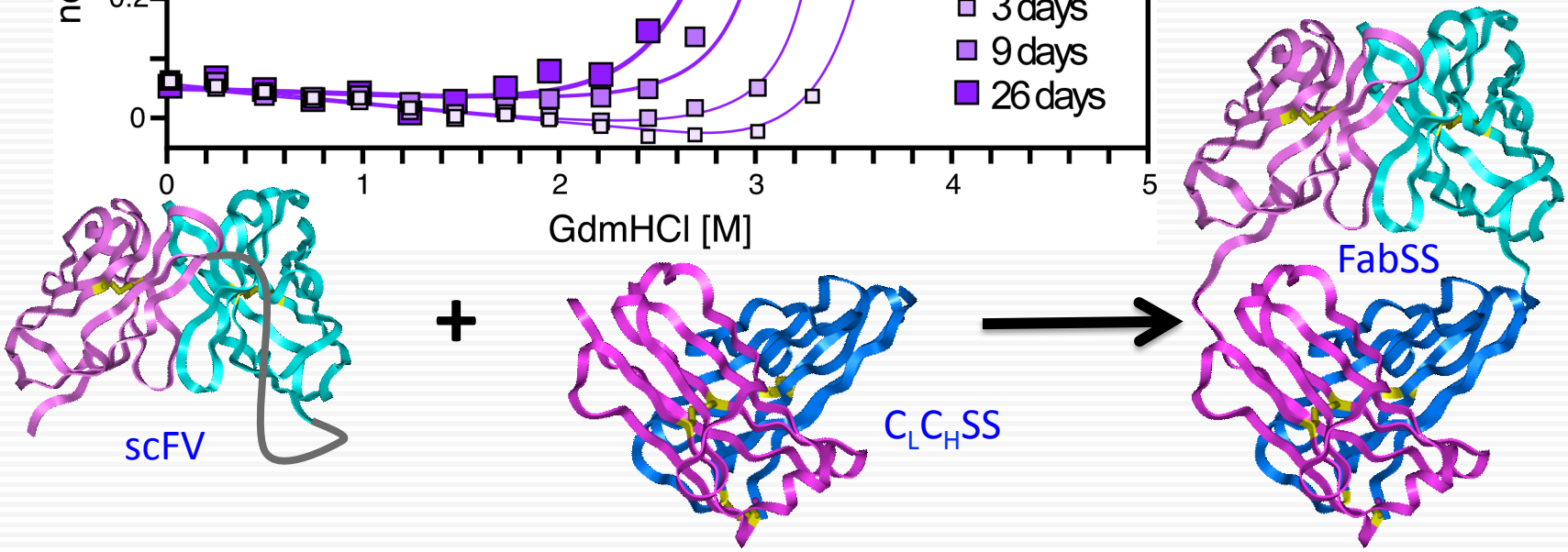
Disulfide Bridge between C_L and C_H



IgG1: L214 to first Cys in hinge, **IgG2**: L214 to H128**,**
IgA: L214 to H128 or intradomain H198 to H223B, **IgM**: L214 to H127

from scFv to Fab:
The importance of the L-H SS-Bond





Summary

- The extent of mutual stabilization depends on the individual sequences due to the strong contribution of CDR-3s to the domain interface, and is mainly relevant for weak domains.
- There is no significant stabilization between V_L and C_L in the isolated light chain, nor between V_H and C_H in the Fd fragment.
- The $C_L C_H$ heterodimer dissociates in the absence of an interdomain disulfide bond.
- $[GdmHCl]_{50}$ of $C_L C_H^{SS}$ equals $[GdmHCl]_{50}$ of the isolated C_L domain.
- Kinetic stabilization of the disulfide linked $C_L C_H^{SS}$ heterodimer.
- Above a $[GdmHCl]_{50}$ of the scFv of 1.5 - 2 M, the stability of the constant domains becomes limiting for the stability of the non-disulfide-linked Fab
- **In the disulfide-linked Fab, even strong variable domains profit from the kinetic stabilization of the $C_L C_H^{SS}$ heterodimer, while the $C_L C_H^{SS}$ is significantly stabilized by ist interaction with the $V_L V_H$ heterodimer.**

Frequently Asked Question:

Does Variable Domain Stability matter for a whole IgG expressed in mammalian Cells?

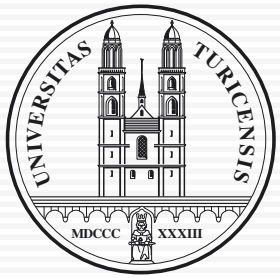
=> Jonas Schaefer

J.Schaefer et al.: J. Mol. Biol. **417**, 309-335

Andreas Plückthun

many generations of PhD students,
diploma- and masters- students

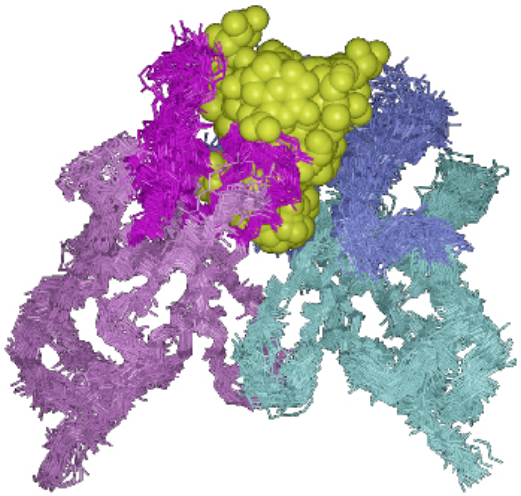
morphosys



Zürich University

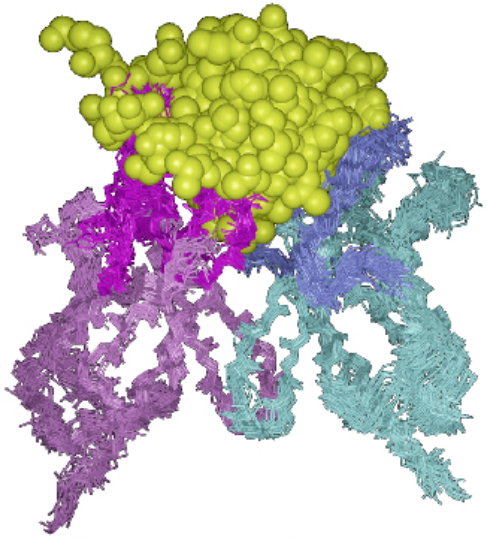
All Plückthun Group Publications by topic:

http://www.bioc.uzh.ch/plueckthun/index.php?pid=3-2-0



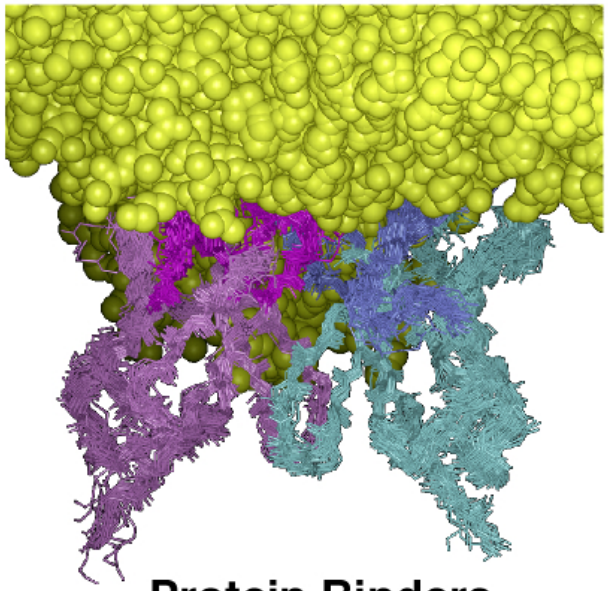
Hapten Binders

(52 structures)



Oligomer Binders

(30 structures)

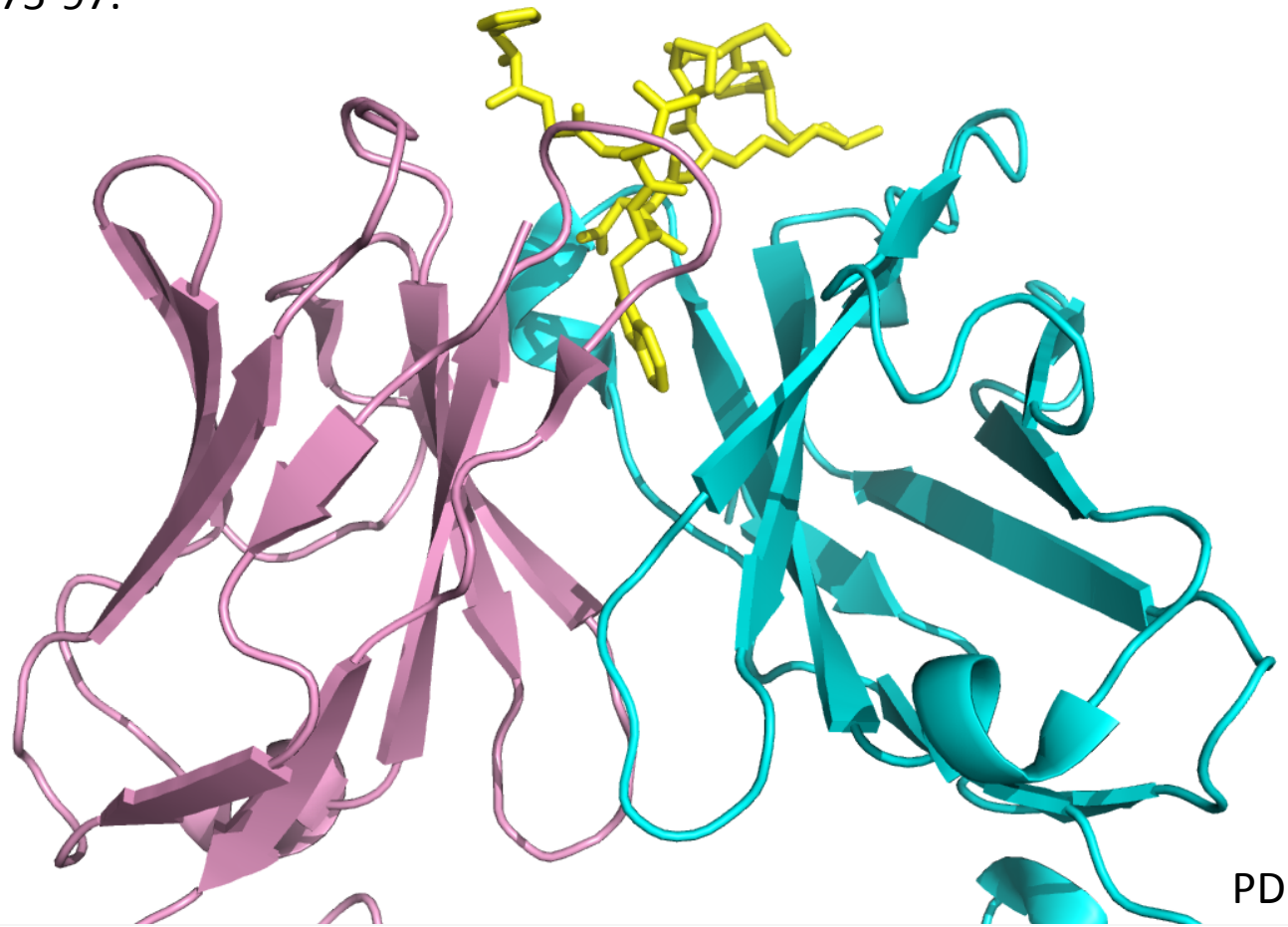


Protein Binders

(45 structures)

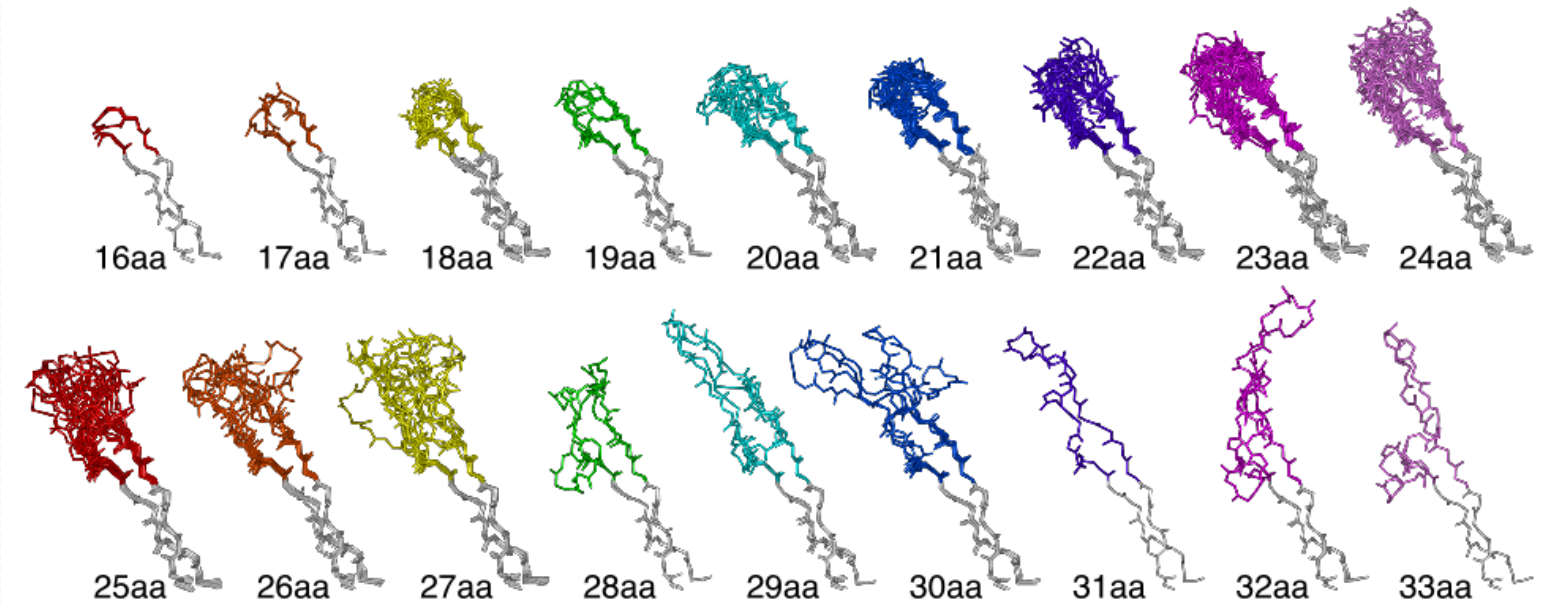
A typical "Hapten" Binder

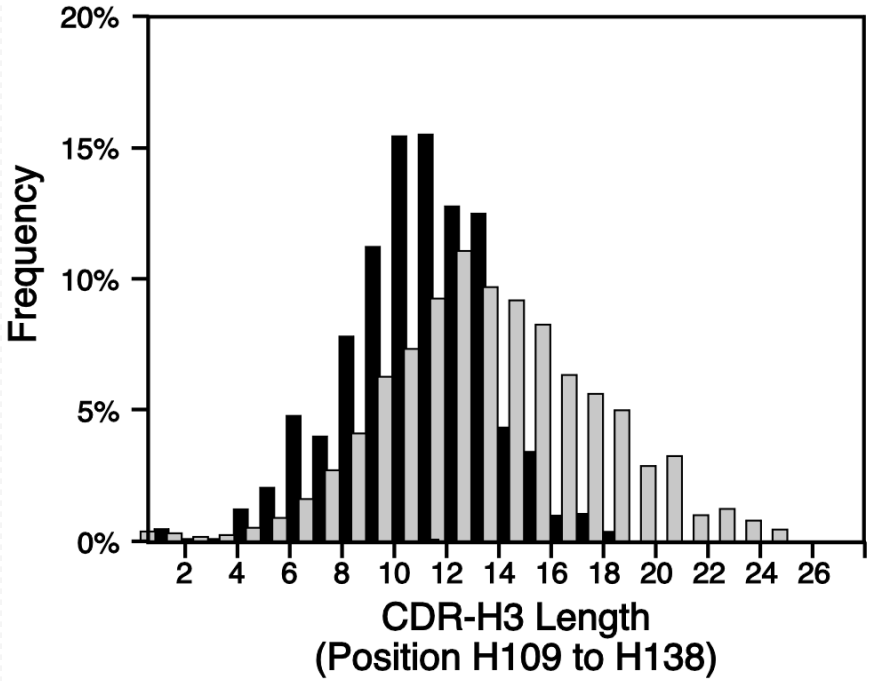
Luginbühl, B., Kanyo, Z., Jones, R. M., Fletterick, R. J., Prusiner, S. B., Cohen, F. E., Williamson, R. A., Burton, D. R., and Plückthun, A. (2006). **Directed evolution of an anti-prion protein scFv fragment to an affinity of 1 pM and its structural interpretation**. *J Mol Biol* **363**, 75-97.



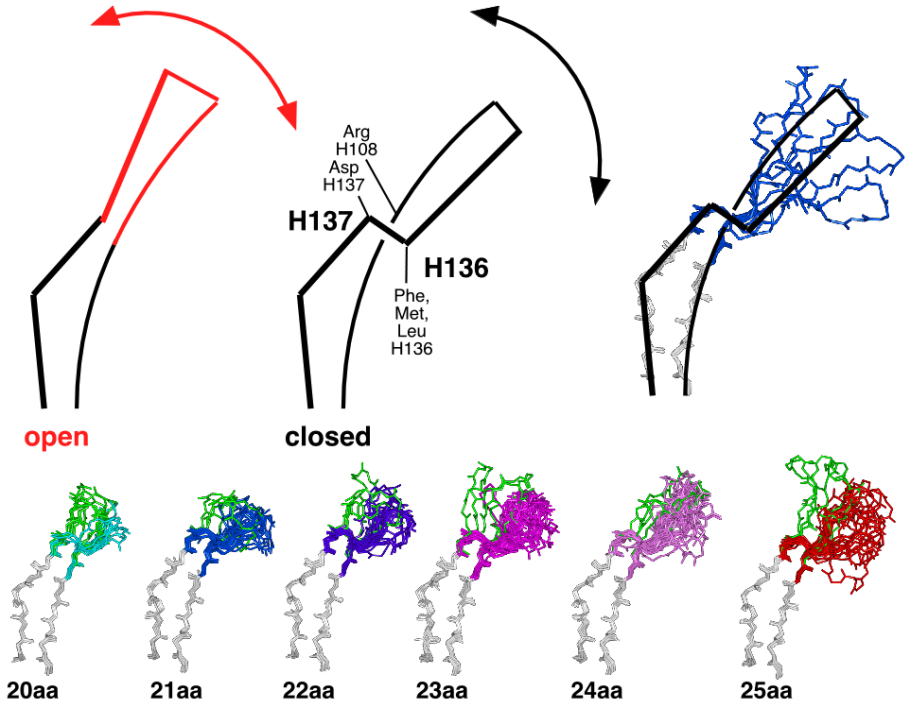
PDB entry 2HHO

= low pM binder to a protruding loop of a protein

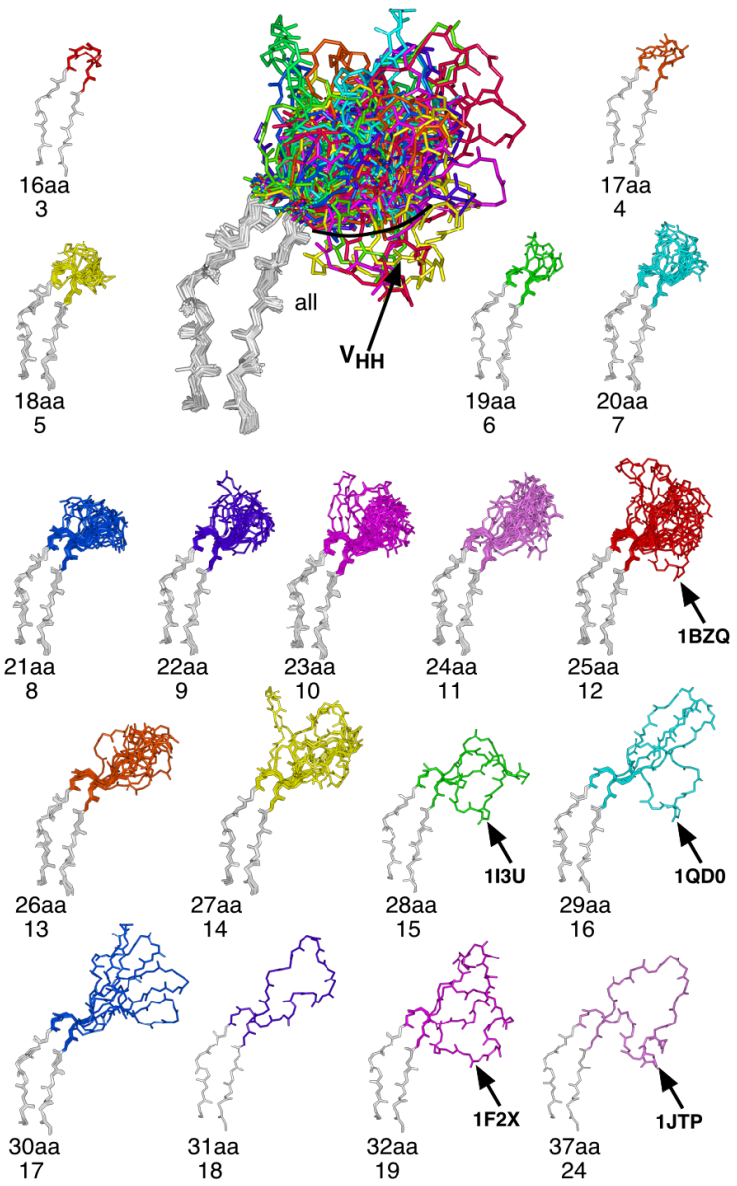


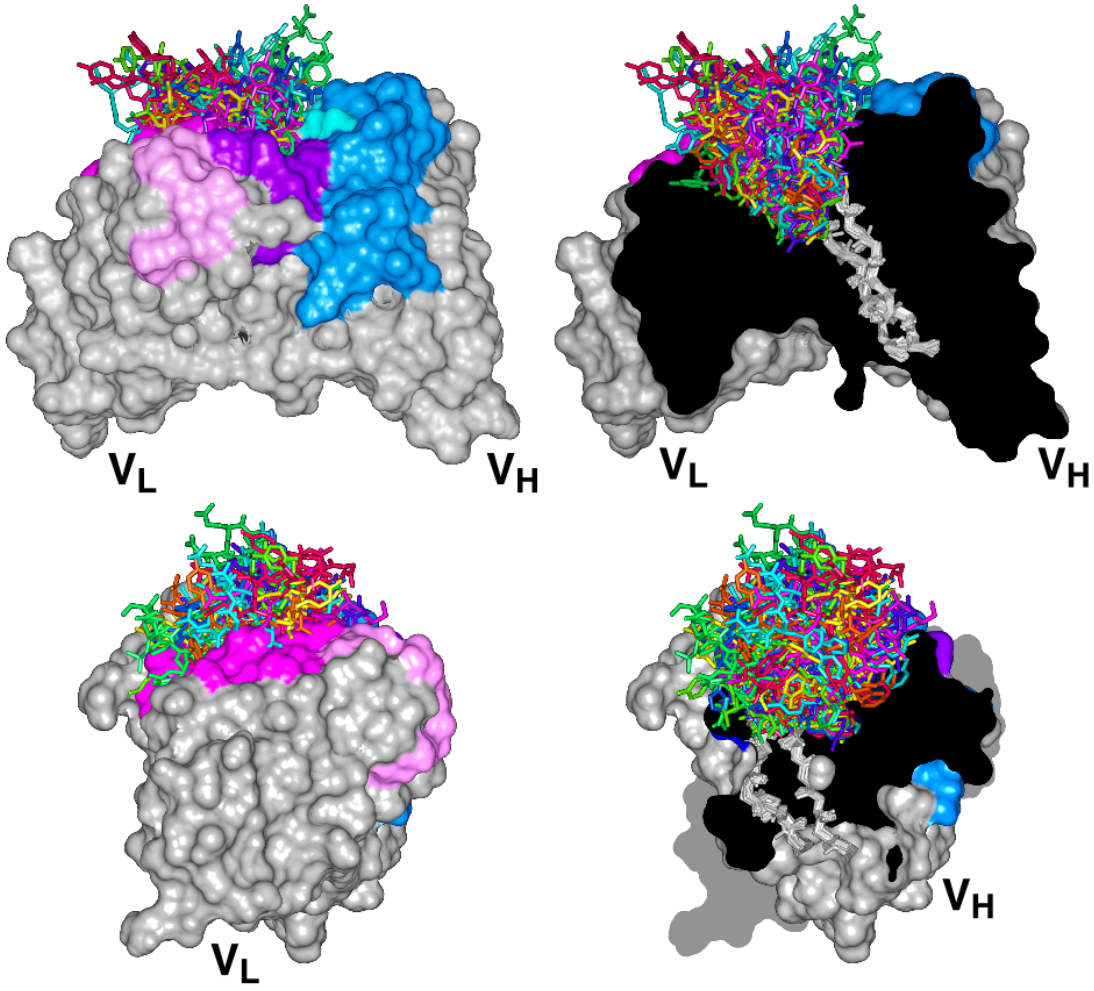


CDR-H3-length = segment length-13



CDR H3


16aa
3
all
V_{HH}
17aa
4
18aa
5
19aa
6
20aa
7
21aa
8
22aa
9
23aa
10
24aa
11
25aa
12
1BZQ
26aa
13
27aa
14
28aa
15
1I3U
29aa
16
1QD0
30aa
17
31aa
18
32aa
19
1F2X
37aa
24
1JTP


V_L
V_H
V_L
V_H
V_L
V_H