Modeling Protein Degradation Processes and the Development of Rational Approaches to Stabilization

A New Strategy of Molecular QbD

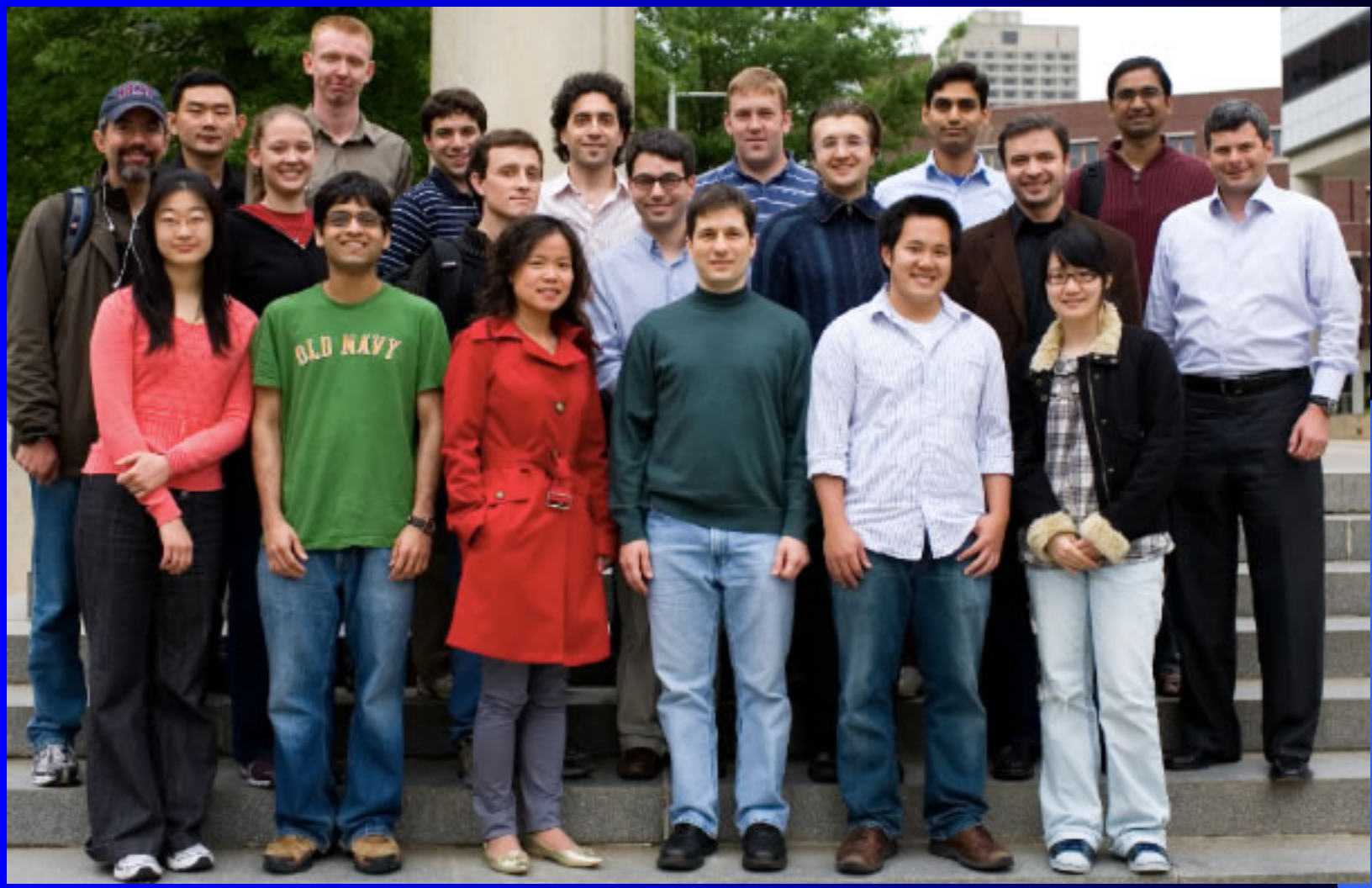
Naresh Chennamsetty[1],
Vladimir Voynov[1], Veysel Kayser[1], Curt Schneider[1],
Diwakar Shulka[1], Bernhard Helk[2] and Bernhardt L. Trout[1]

1) MIT 2) Novartis Pharma AG

New Strategic Approach

- Incorporate developability and manufacturability early.
- Incorporate QbD.
- Reduce overall time from discovery to market launch.
- Molecular QbD presents a new strategic option.

Trout Research Group



Research Areas in Trout Group Molecular QbD

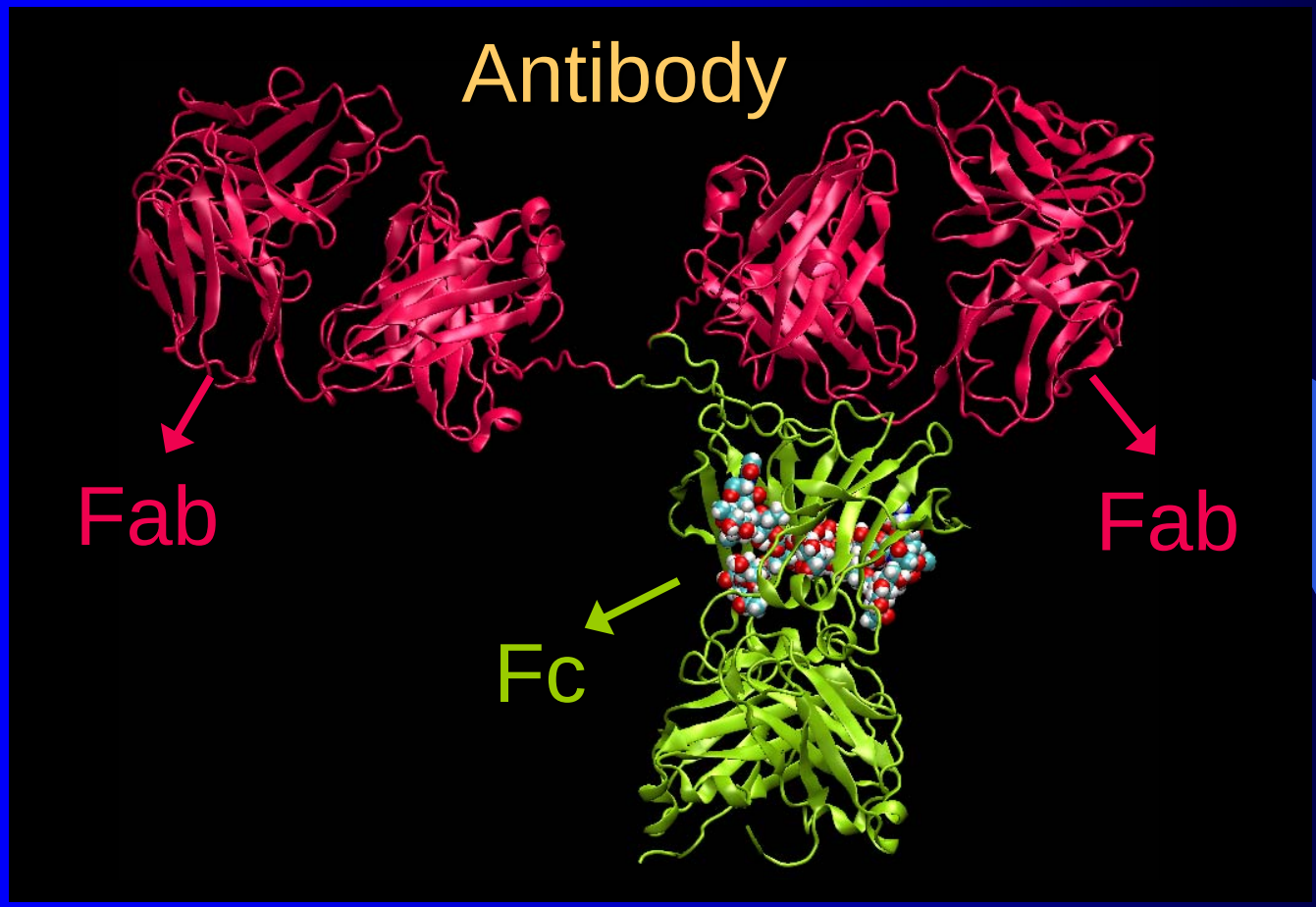
- Formulation and Stabilization of Biotherapeutics.
  - Aggregation
  - Oxidation
  - Deamidation
  - Hydrolysis
- Crystallization and New Technologies for the Manufacturing of Small Molecular Pharmaceuticals.
- Major Initiatives
  - Novartis-MIT Center for Continuous Manufacturing
  - Singapore-MIT Program on Chemical and Pharmaceutical Engineering

Objective for today:

Identify major problems that you face, and determine how we might be able to help.

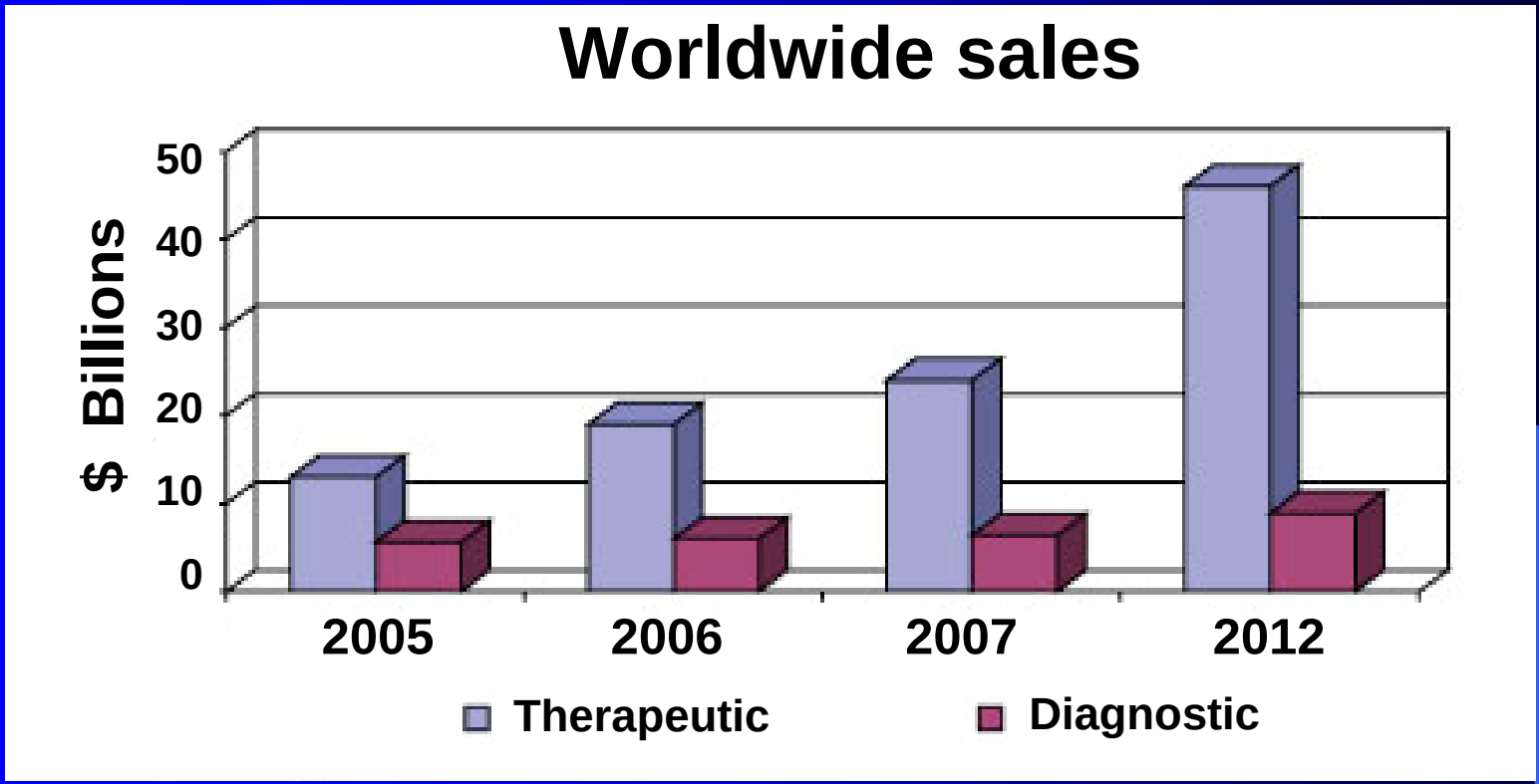
Molecular QbD for Therapeutic Antibody Stabilization

Aggregation of Therapeutic proteins: E.g. Therapeutic Antibodies



- Antibody is a large glyco-protein (~ 1300 residues,150kDa)
- Therapeutic antibodies are used in the treatment of cancer, Rheumatoid Arthritis, etc.

Therapeutic protein sales are growing fast



*Source: BCC Research

- Antibody sales are growing at a fast pace
- The sales could reach $56 billion by 2012, a compound annual growth rate (CAGR) of 13%

Problems: Antibody Aggregation

- Therapeutic antibodies aggregate during manufacture and storage

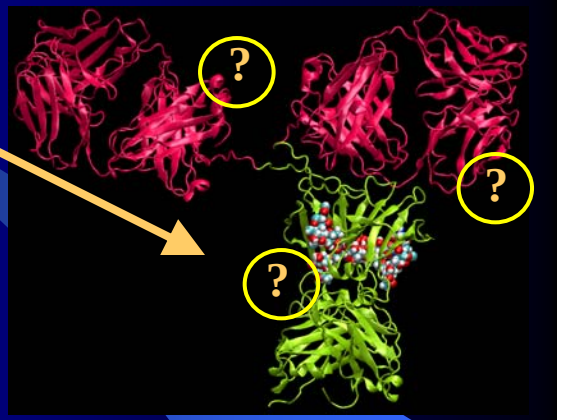
Antibodies

Aggregates are

- Inactive against disease
- Immunogenic
- Can have serious side effects

Why do antibodies aggregate ?

- What regions are aggregation prone?
- Can we modify these aggregation prone regions to enhance stability ?

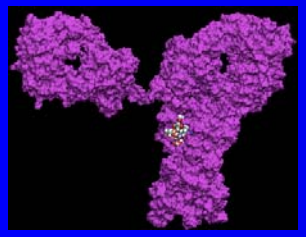


Molecular Simulations + Experiment

→ Molecular level detail on aggregating regions

→ Validation

Overview of methodology



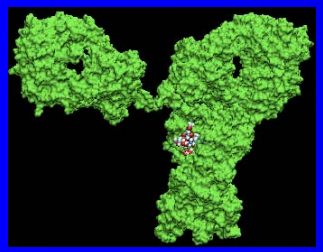
Unstable antibody

Molecular simulation of a single antibody

SAP Technology*

Identify aggregating regions

Mutate predicted aggregating regions

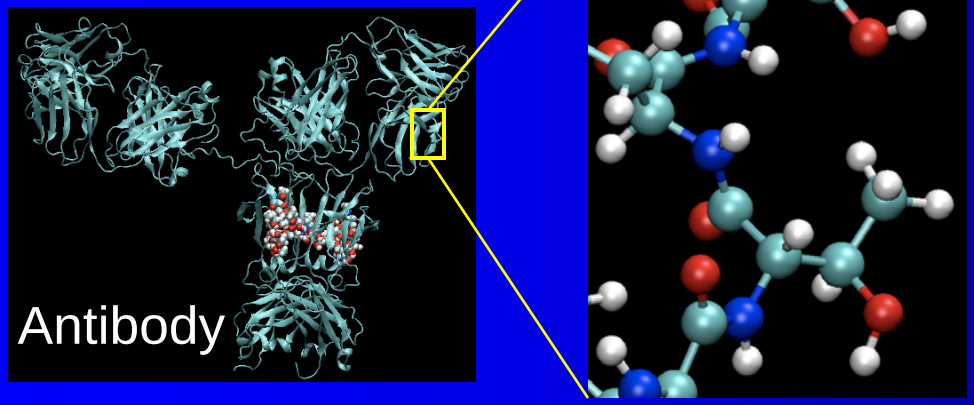


Stable antibody

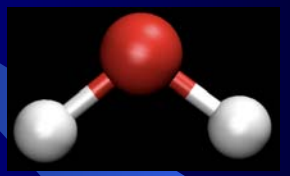
* SAP (Spatial Aggregation Propensity) technology developed in this project

Simulation methodology

- Detailed atomistic model for antibody



- Explicit atomistic model for water

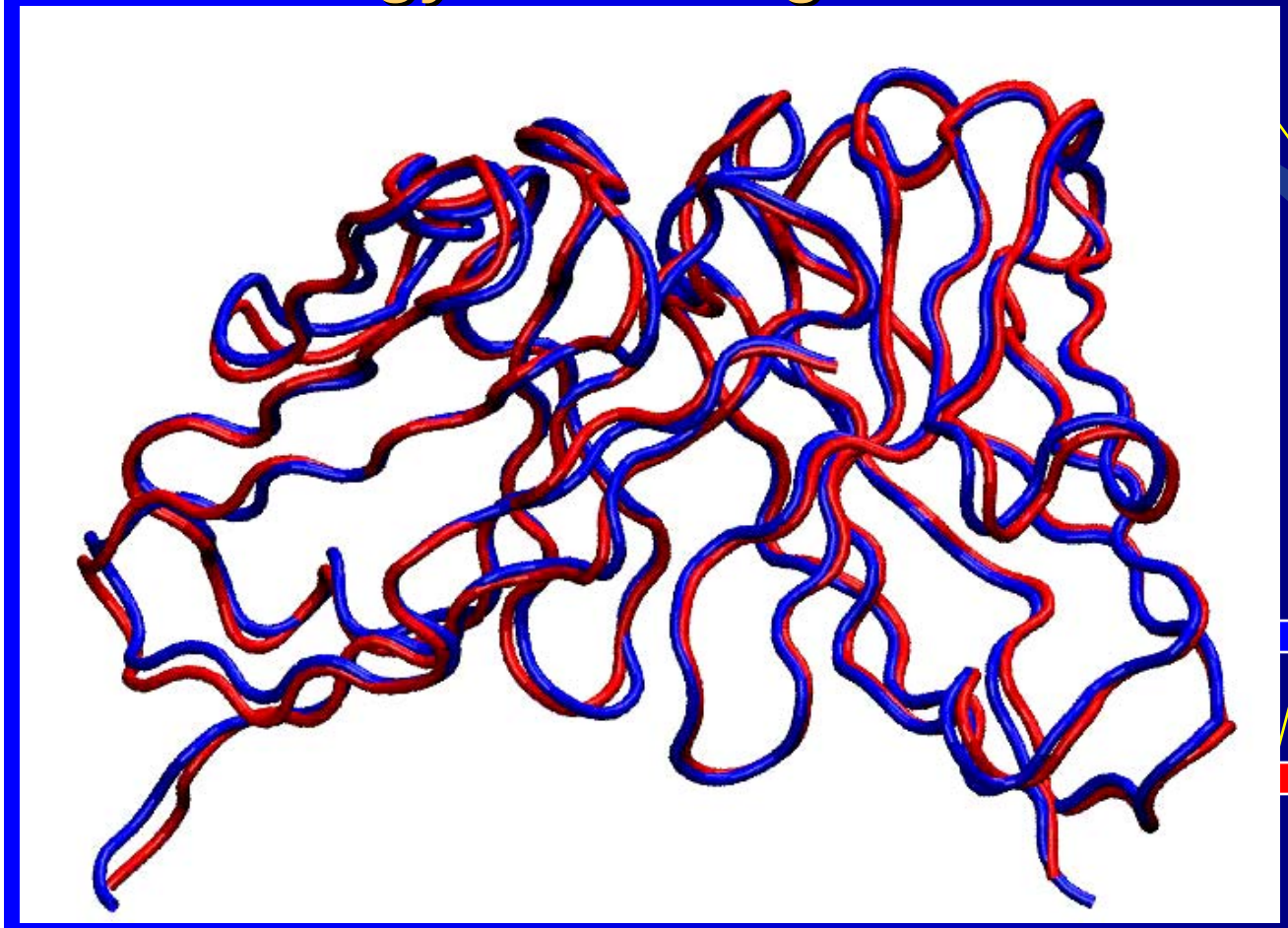


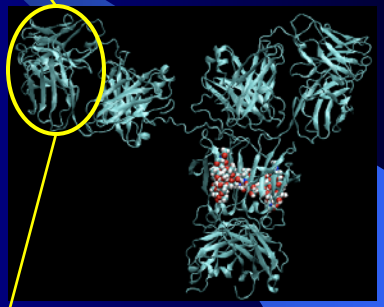
- CHARMM force field[1] for protein, TIP3P water model[2]
- CHARMM[3] and NAMD[4] simulation packages
- Simulations in the NPT ensemble at 300K and 1atm
- Ewald summation for electrostatics
- Supercomputer resources from NCSA

1) MacKerell, Jr., A. D *et. al.*, J. Phys Chem. B, 1998, **102**, 3586
2) Jorgensen, W. L. *et al.,* J. Chem. Phys., 1983, **79**, 926
3) Brooks, B. R. *et. al.*, J. Comput. Chem., 1983, **4**, 187
4) Phillips J. C., *et. al.*, J. Comput. Chem., 2005, **26,** 1781

For unknown X-ray structures:

Homology modeling with canonical structures[1-3]





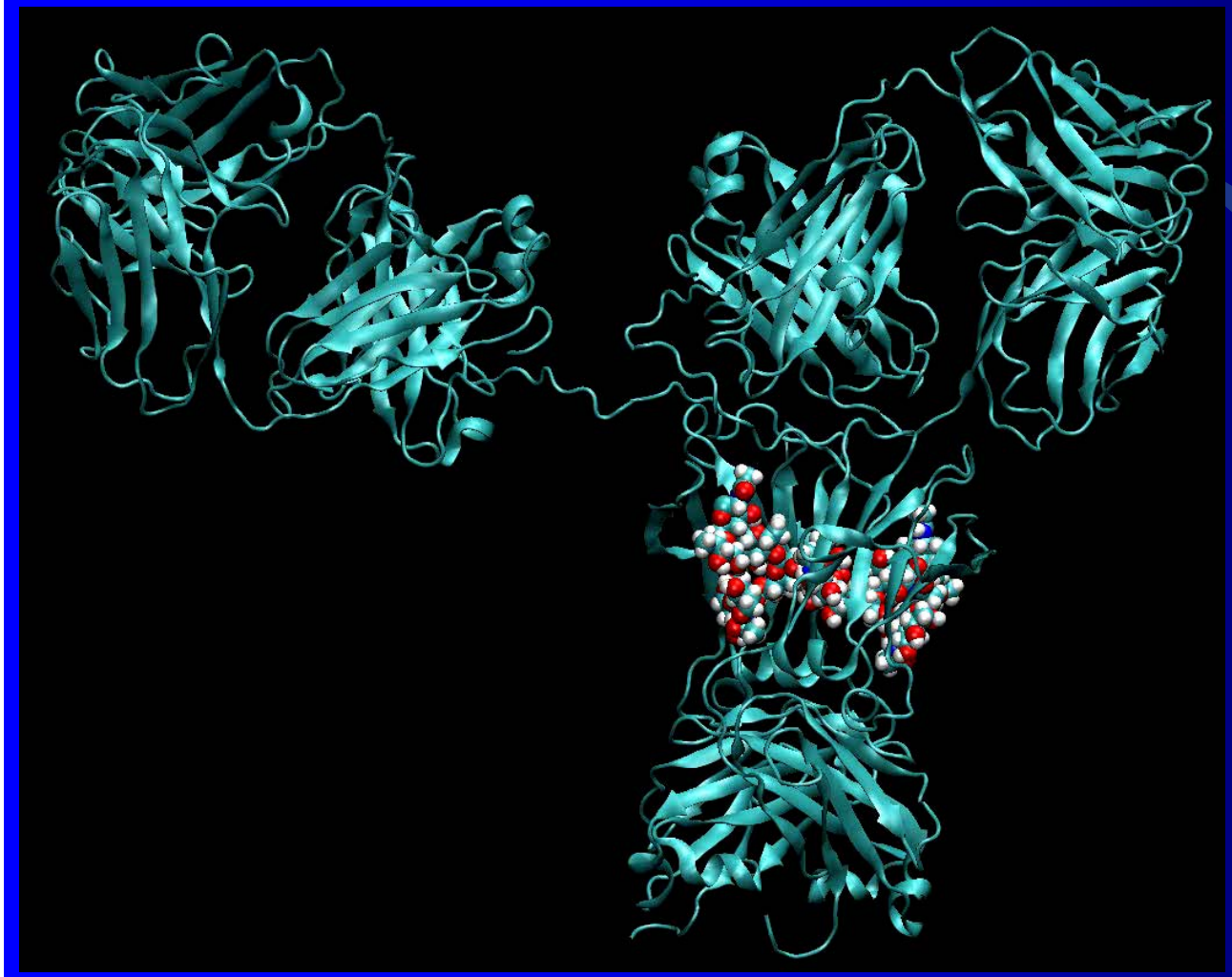
X-ray

Homology model

- Validation: Structure obtained by homology modeling matches very well with the X-ray structure

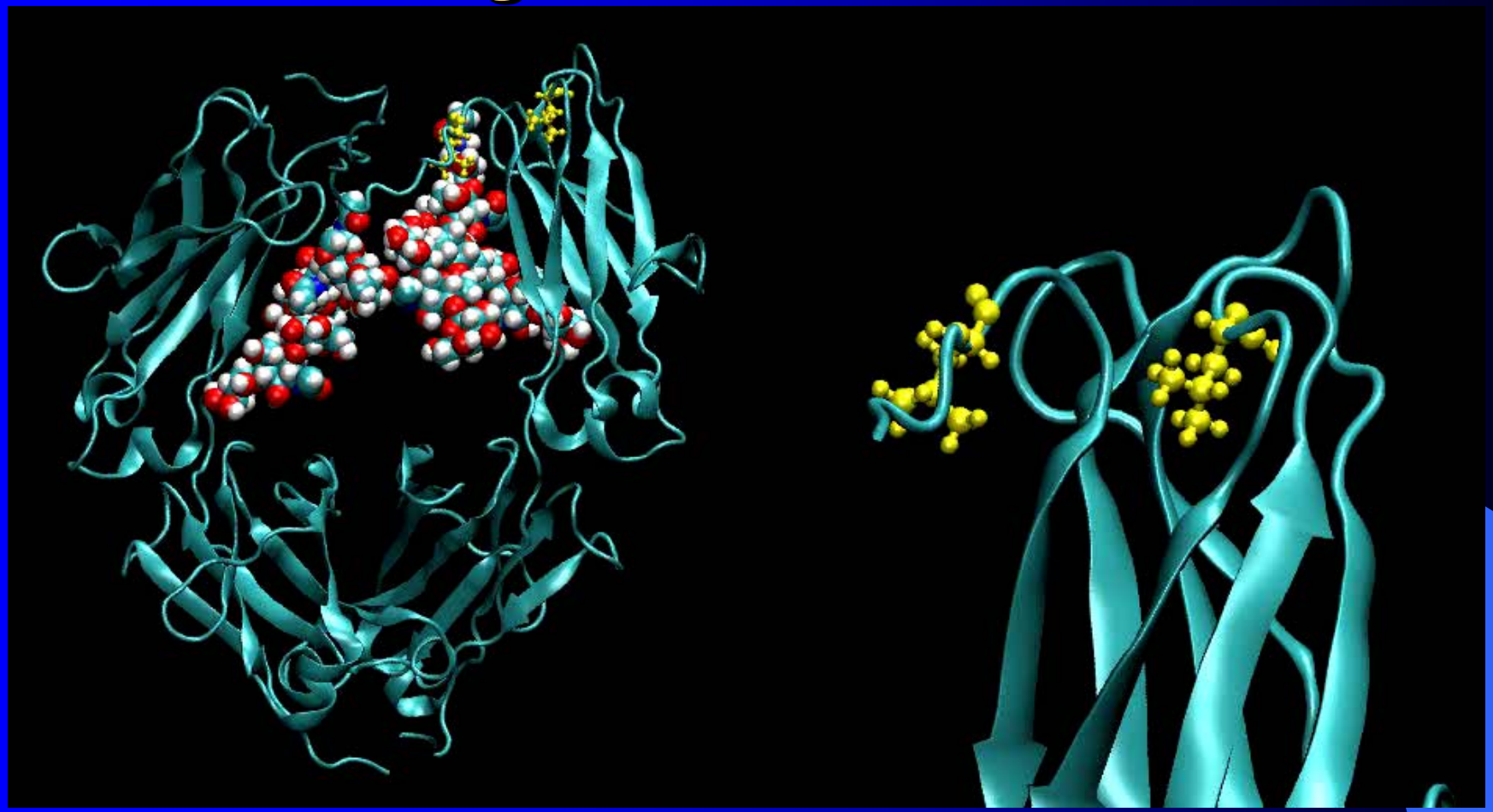
1) Chothia, C., and Lesk, A. J. Mol. Biol. (1987) 2) Chothia *et al* Nature (1989) 3) Al Lazikani et al, J. Mol. Biol (1997)

Full antibody simulation



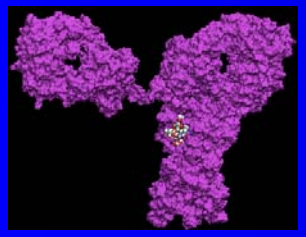
- Full antibody constructed from fragments using another antibody, 1HZH, as template
- Simulated using supercomputer
- First full MAb simulation in the literature

Fc fragment simulation



- Significant fluctuations in protein and sugar groups
- These fluctuations could dynamically expose buried hydrophobic residues

SAP tool applied after simulation



Unstable antibody

Molecular simulation of a single antibody

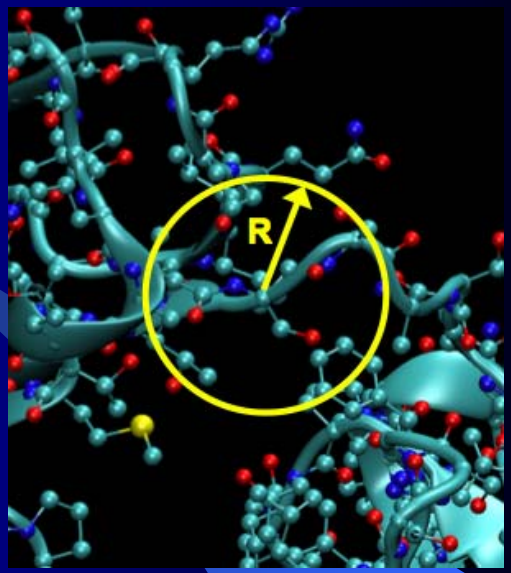
SAP Technology

SAP to Identify aggregating regions

Spatial–Aggregation–Propensity (SAP)

SAP finds the dynamically exposed hydrophobic patches on the protein surface

SAP = Residue fraction exposed X Residue Hydrophobicity



$$SAP = \sum_{\text{Simulation Average}} \left\{ \sum_{\text{Residues within R}} \left(\frac{\text{SAA of side chain atoms within radius R}}{\text{SAA of side chain atoms of fully exposed residue}} \times \text{Residue Hydrophobicity} \right) \right\}$$

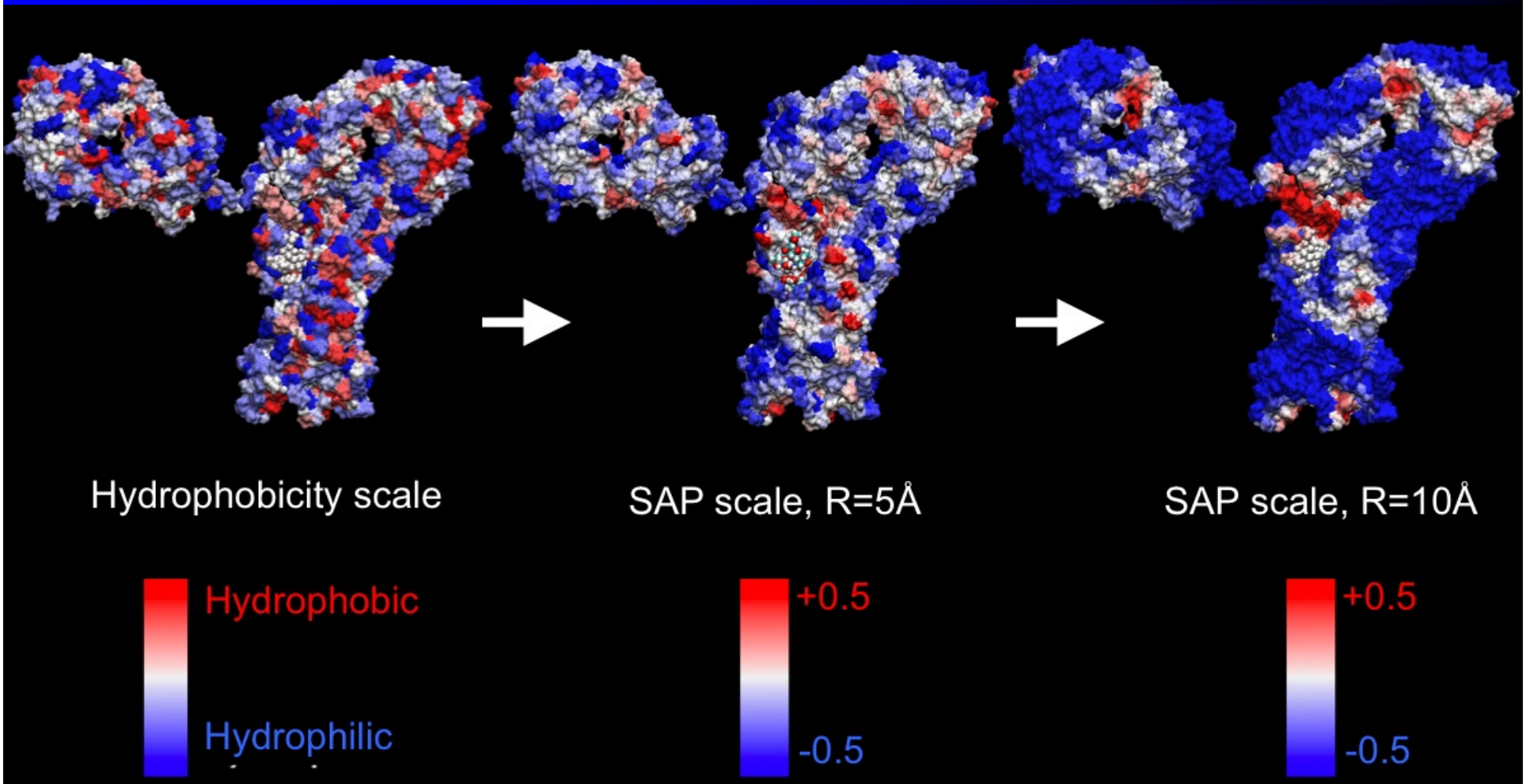
SAA of middle residue in extended tripeptide 'Ala -X- Ala'

SAA = Solvent Accessible Area

*N. Chennamsetty, et al., PNAS, 106, 11937 (2009).

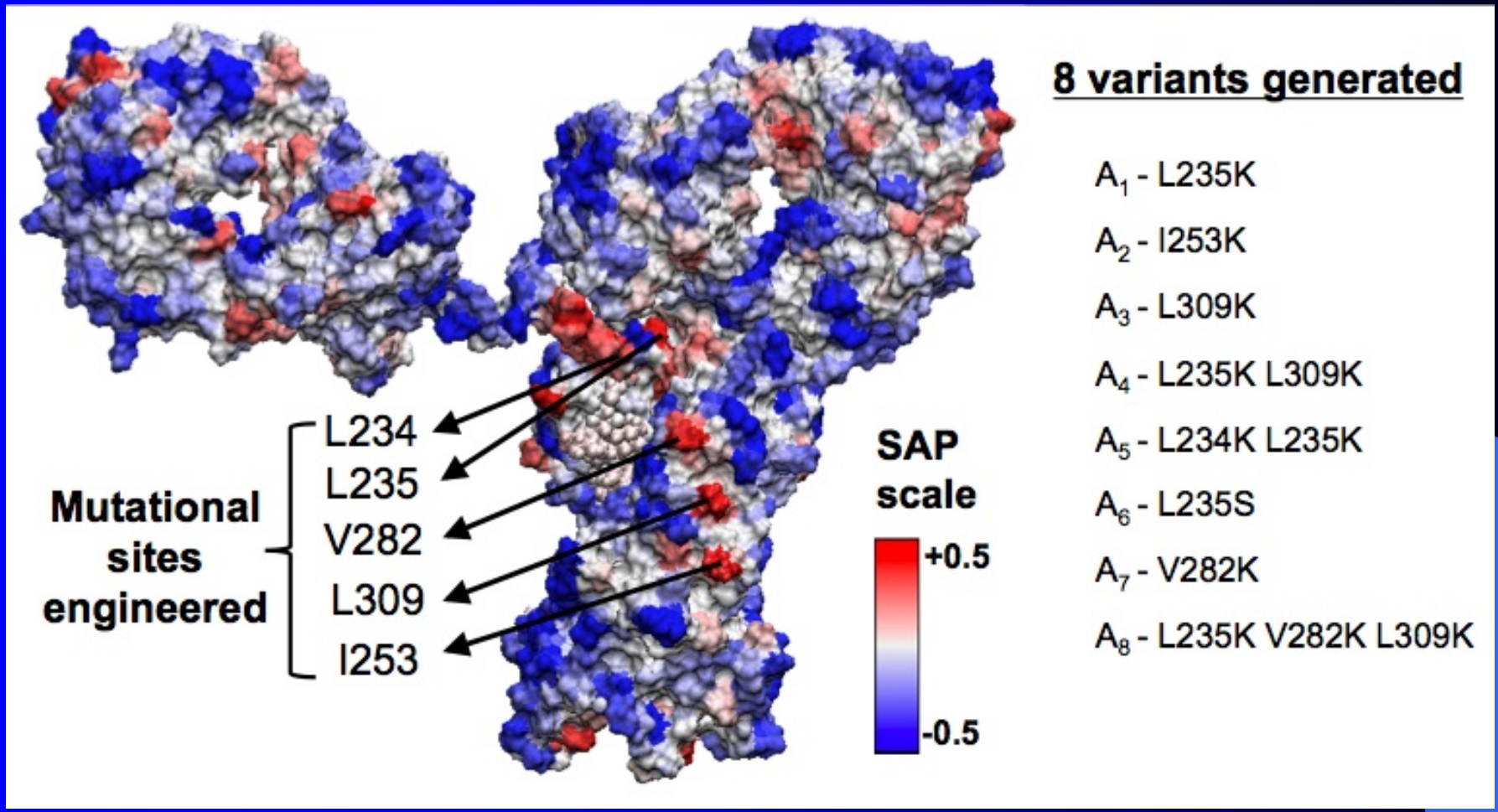
17

SAP mapped onto antibody structure



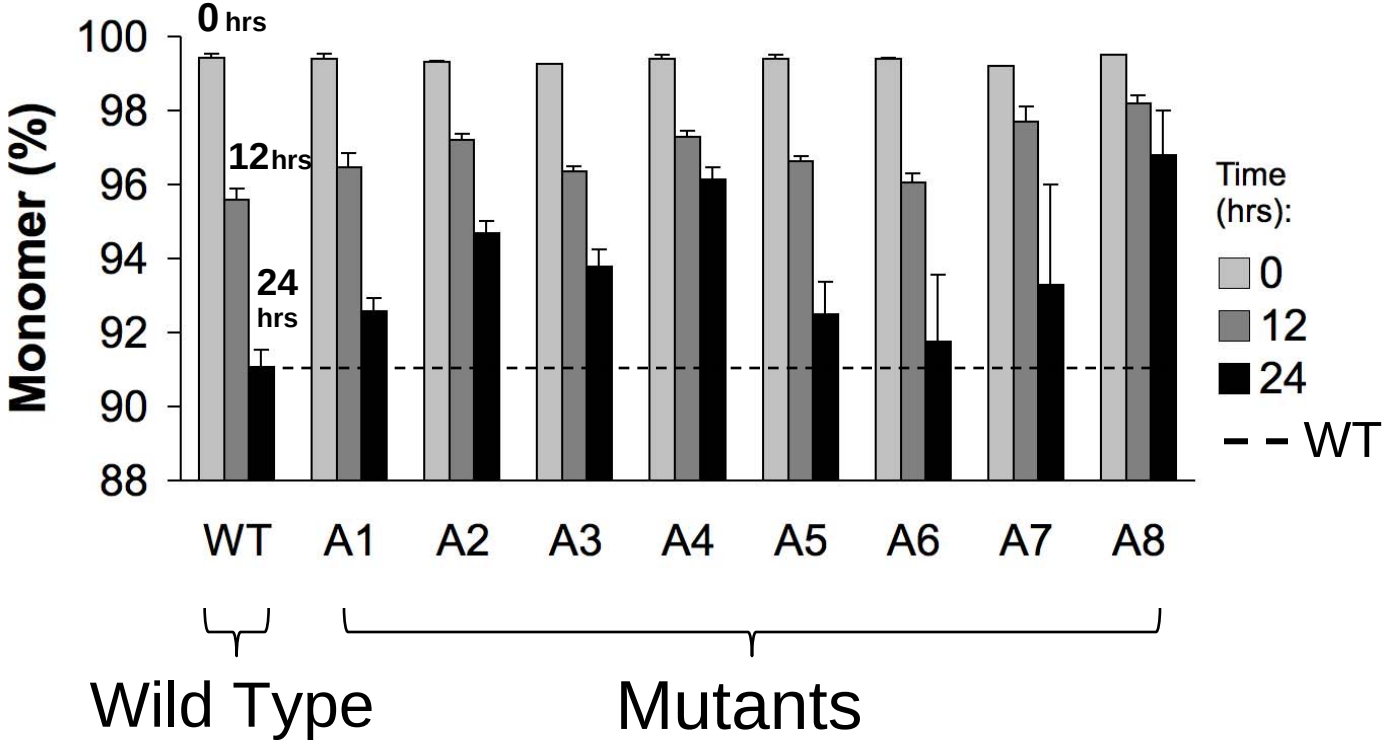
- RED regions are highly hydrophobic dynamically exposed patches
- BLUE regions are highly hydrophilic dynamically exposed patches

Mutation of SAP predicted aggregation prone regions



- 5 sites with high SAP values selected for mutations
- These sites are mutated to more hydrophilic residues

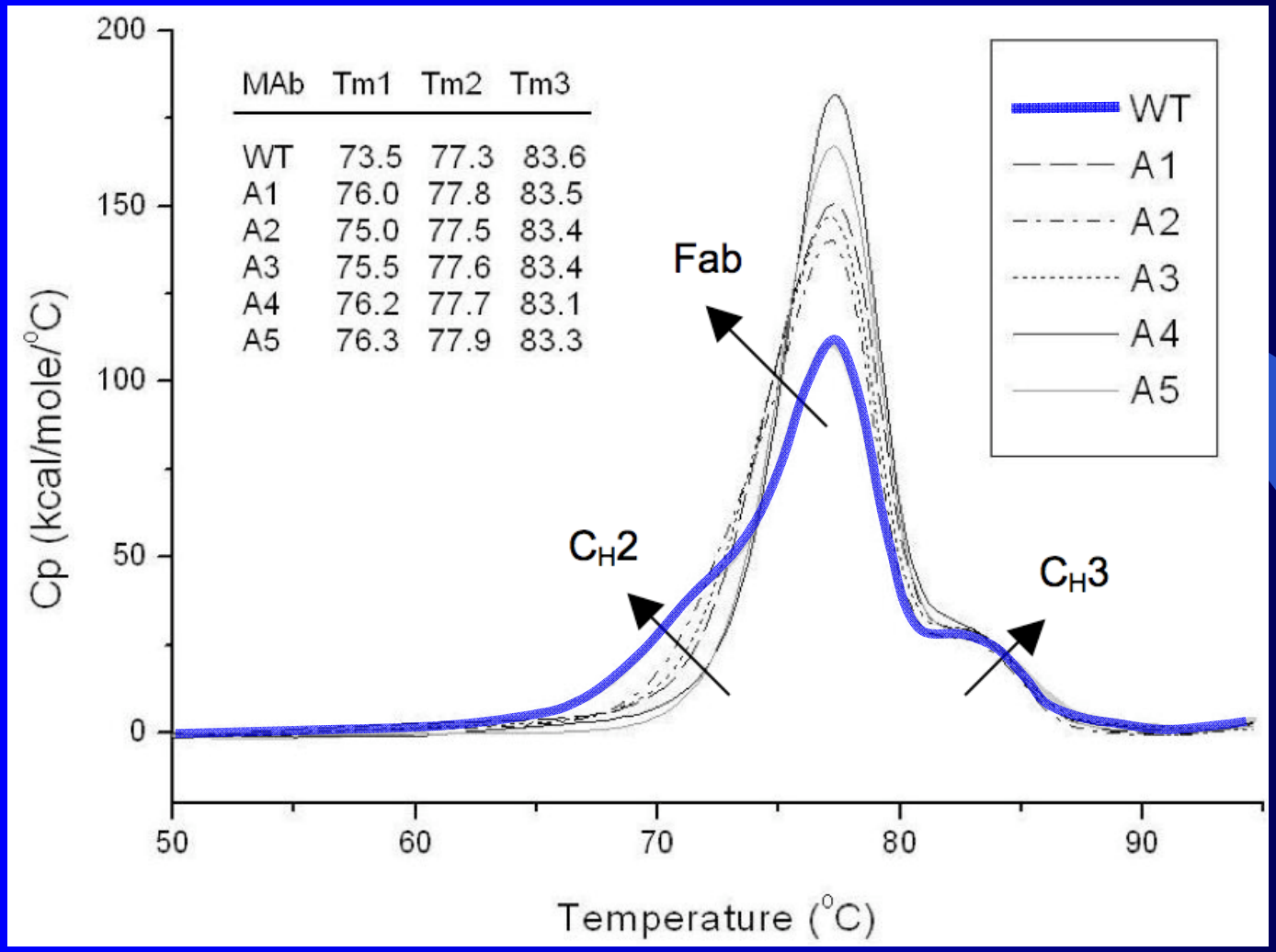
Stability analysis of mutants by SEC-HPLC



Heat stress at 58°C

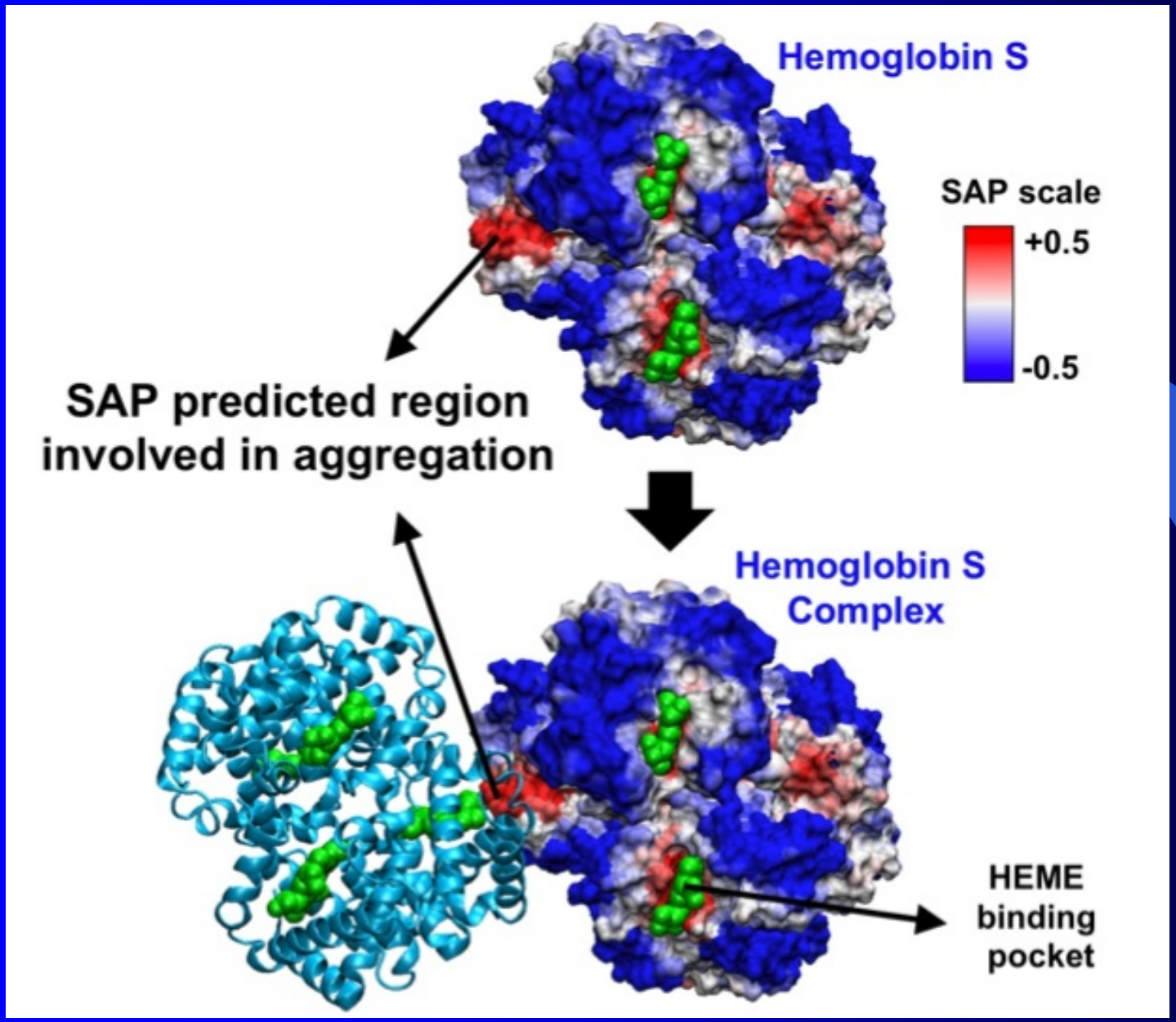
- All 8 mutants lead to increase in monomers (decrease in aggregates)
- This validates SAP predictions

DSC analysis of mutants



- The mutants have higher melting transition for the C_H2 domain
- This indicates increased stability of the mutants

SAP predicts the aggregation prone region of Hemoglobin S



* PNAS (2009)

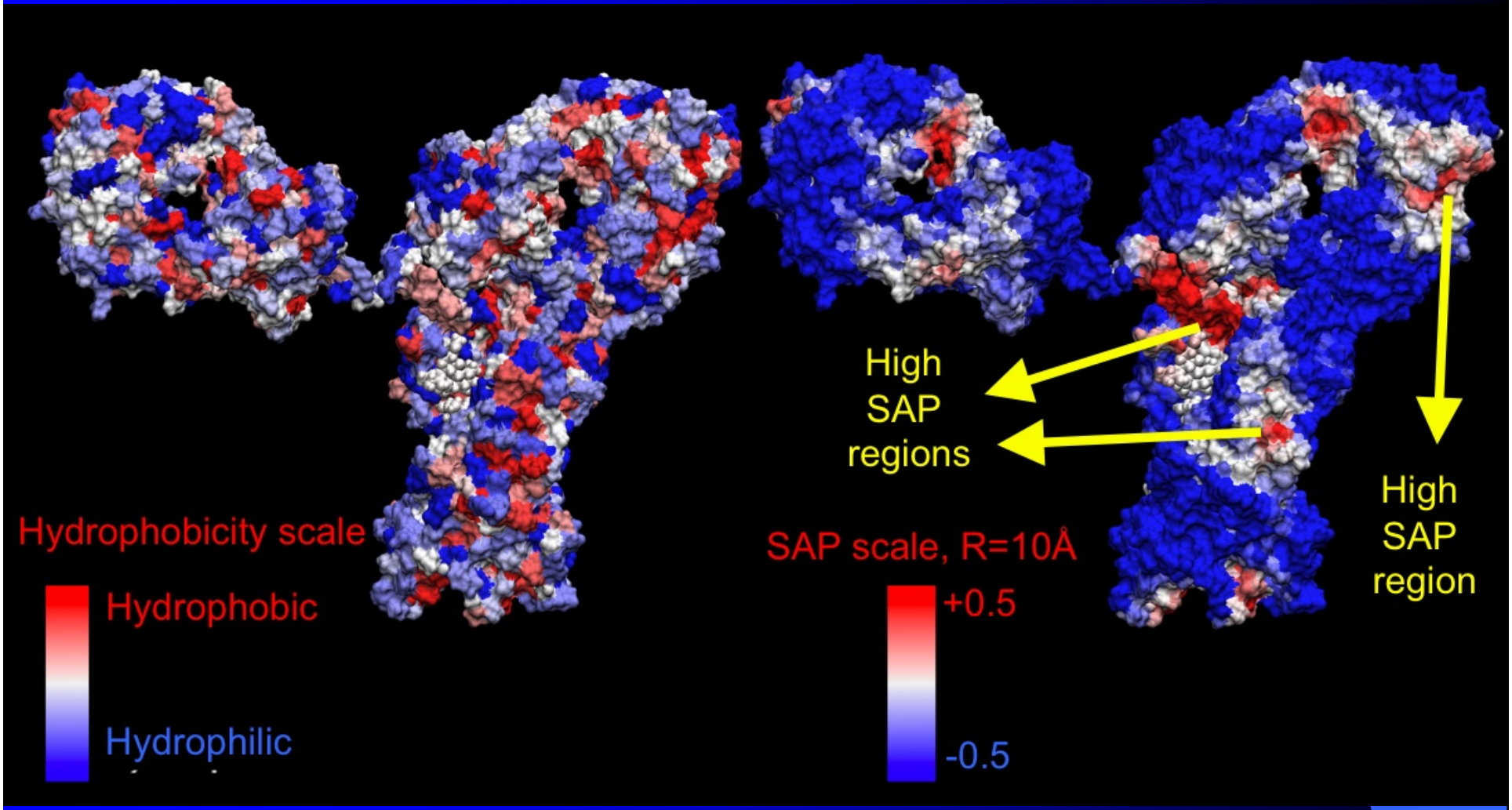
Can SAP predict protein binding regions?

Predicts aggregating regions

Predicts binding regions ?

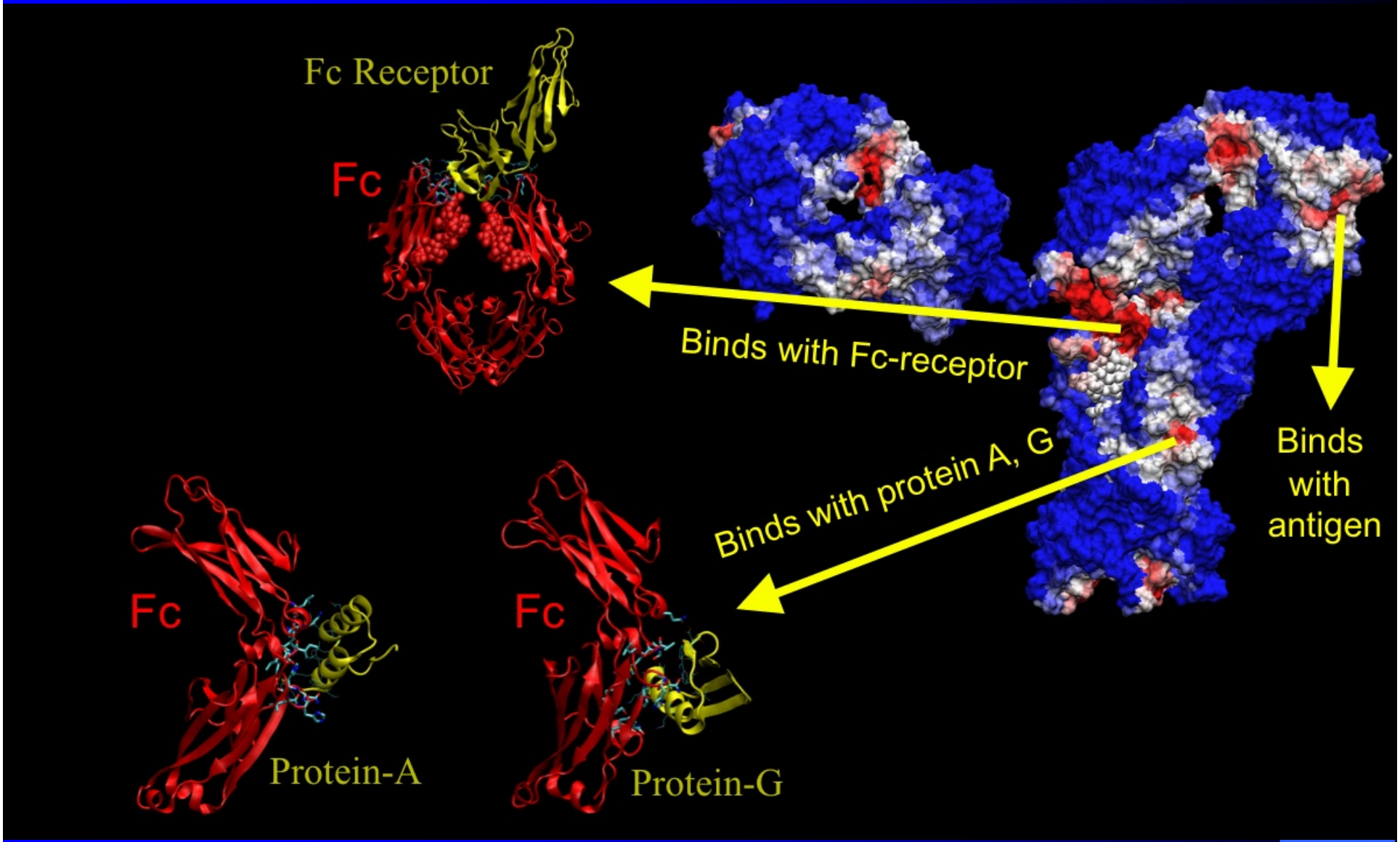
23

SAP predicts protein binding regions as well



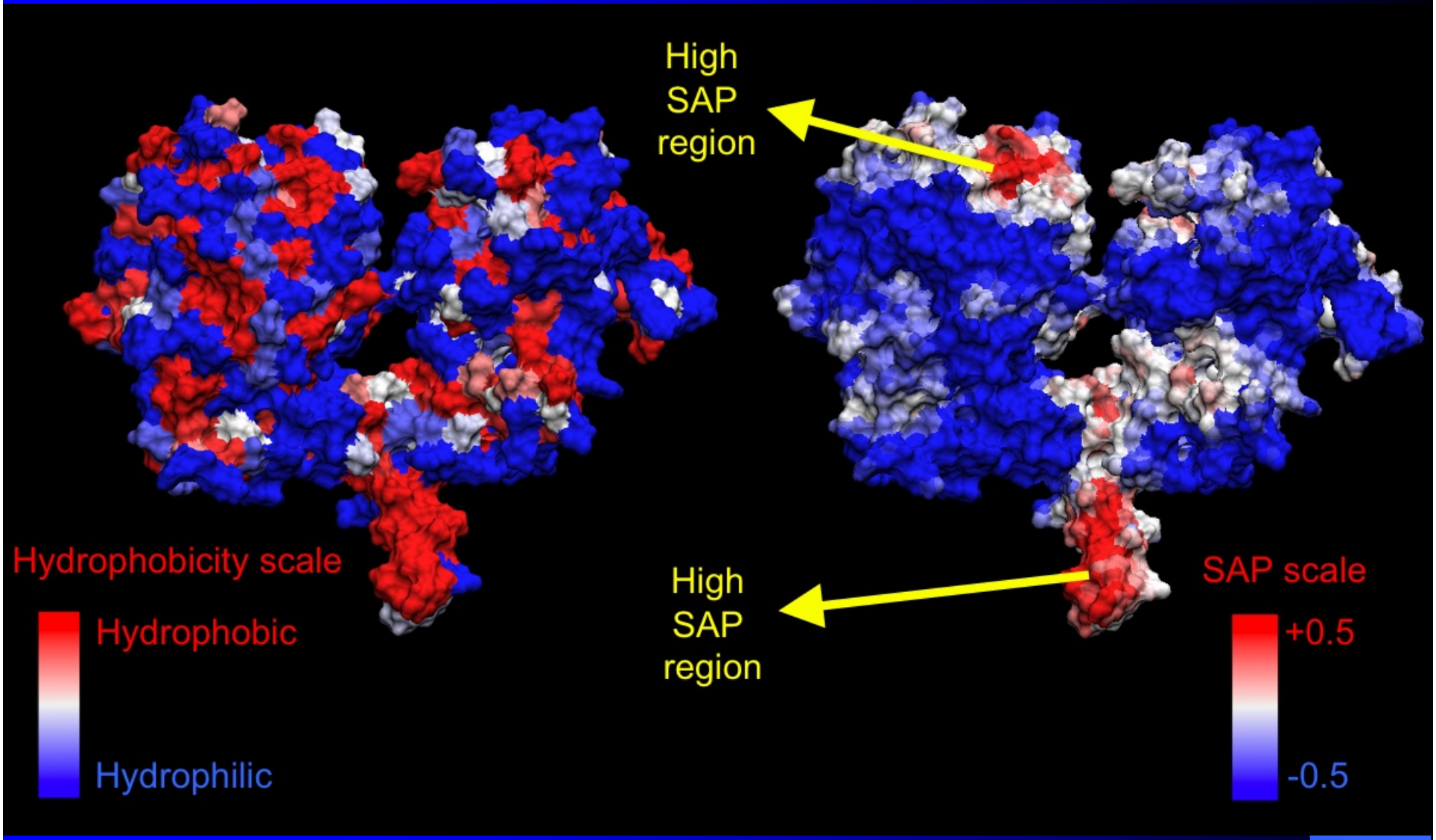
- Using simple hydrophobicity would be difficult to predict binding regions
- High SAP regions correlate well with protein binding regions

SAP predicts protein binding regions of antibody



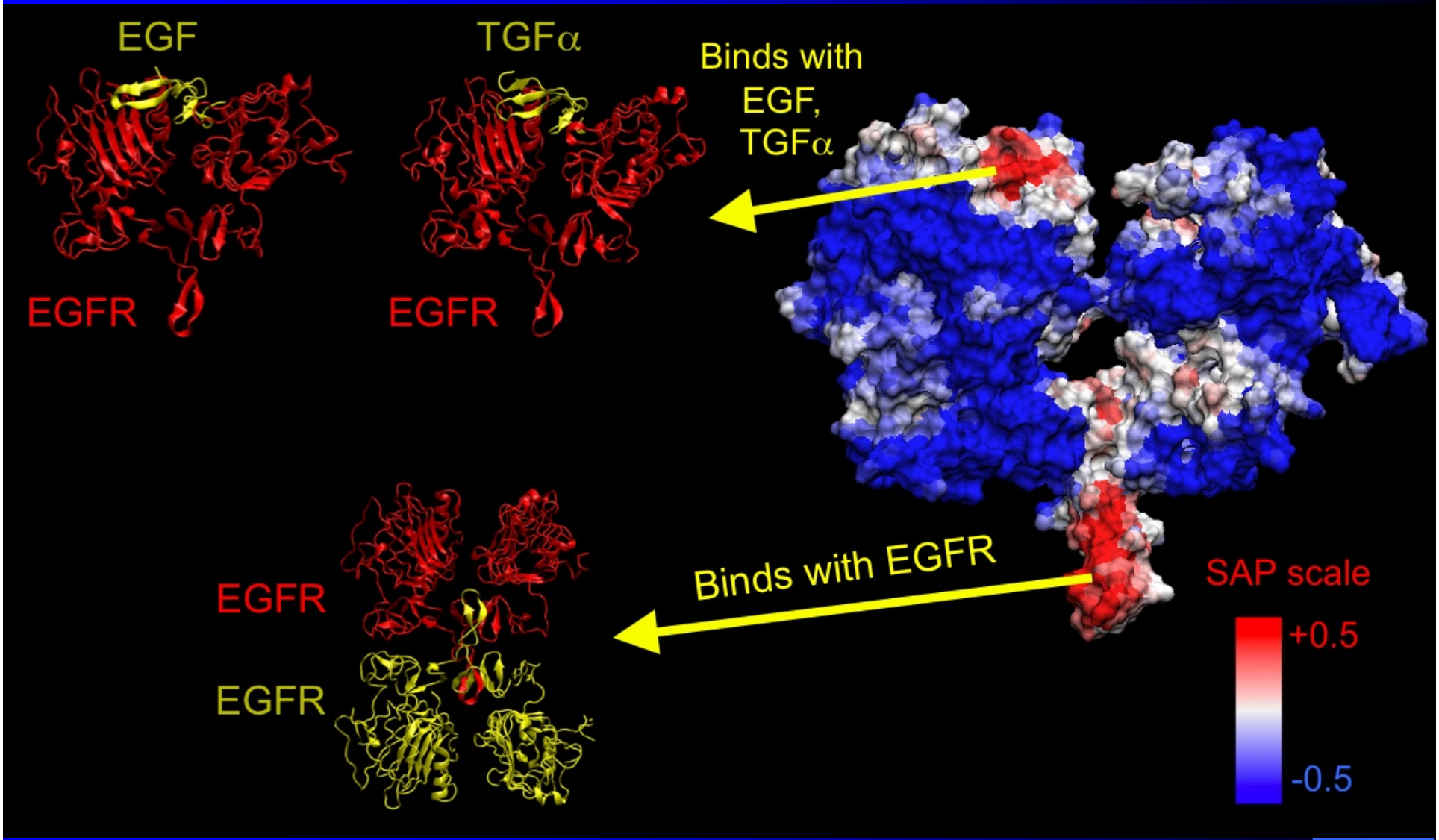
- High SAP regions correlate well with protein binding regions

SAP predicts binding regions of EGFR



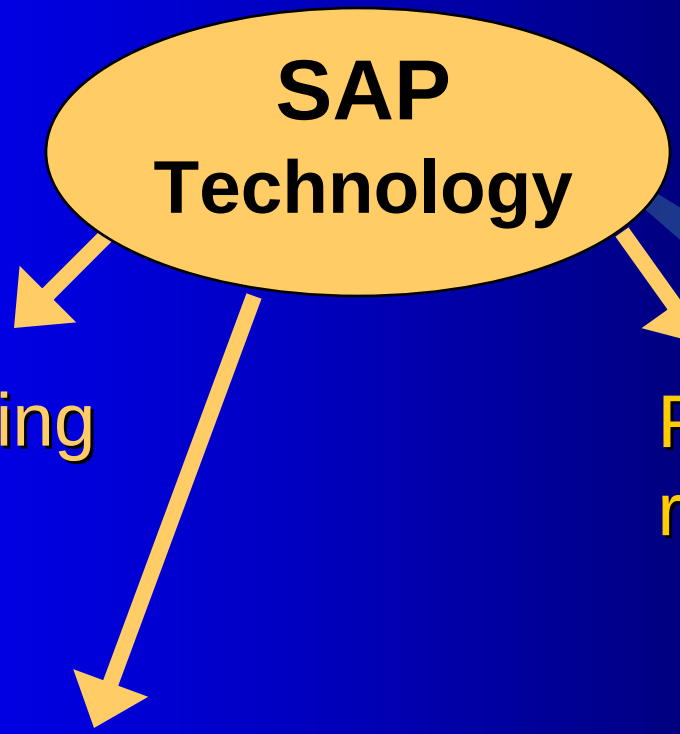
- Using simple hydrophobicity would be difficult to predict binding regions
- High SAP regions correlate well with protein binding regions

SAP predicts binding regions of EGFR



- High SAP regions correlate well with protein binding regions

More SAP applications



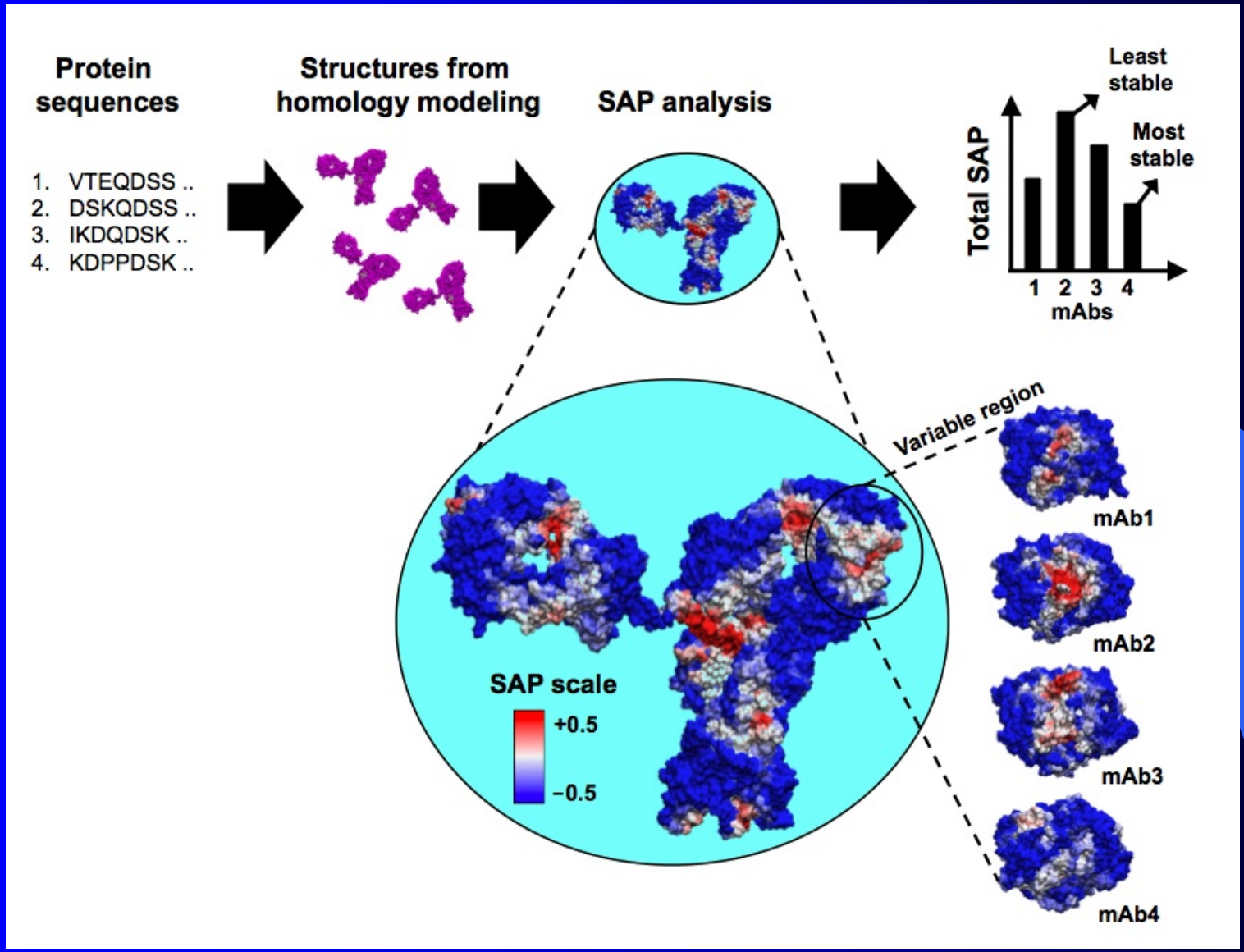
Predicts aggregating regions

Predicts binding regions

Developability ranking ?
(Aggregation propensity ranking)

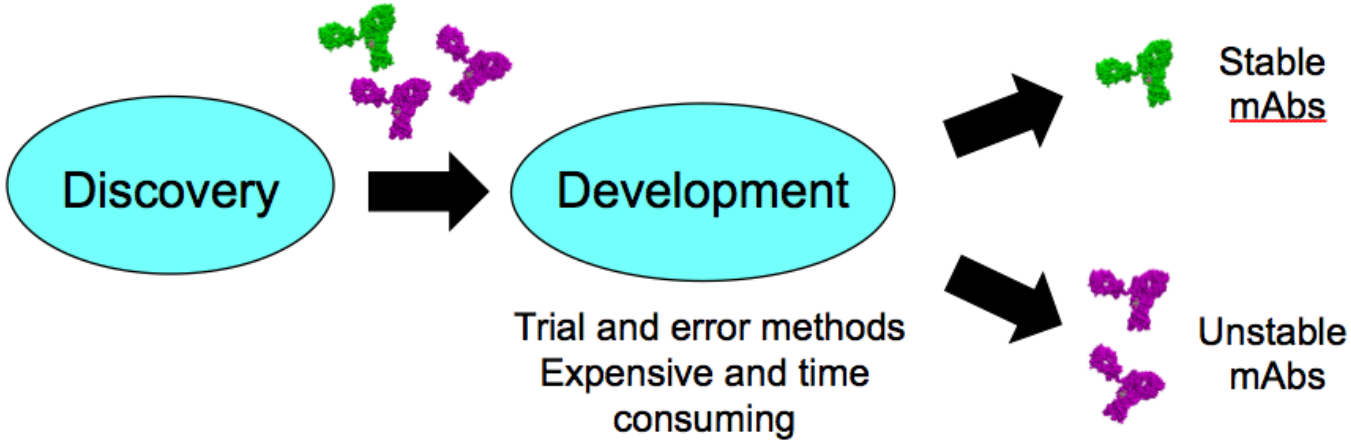
28

SAP for developability ranking

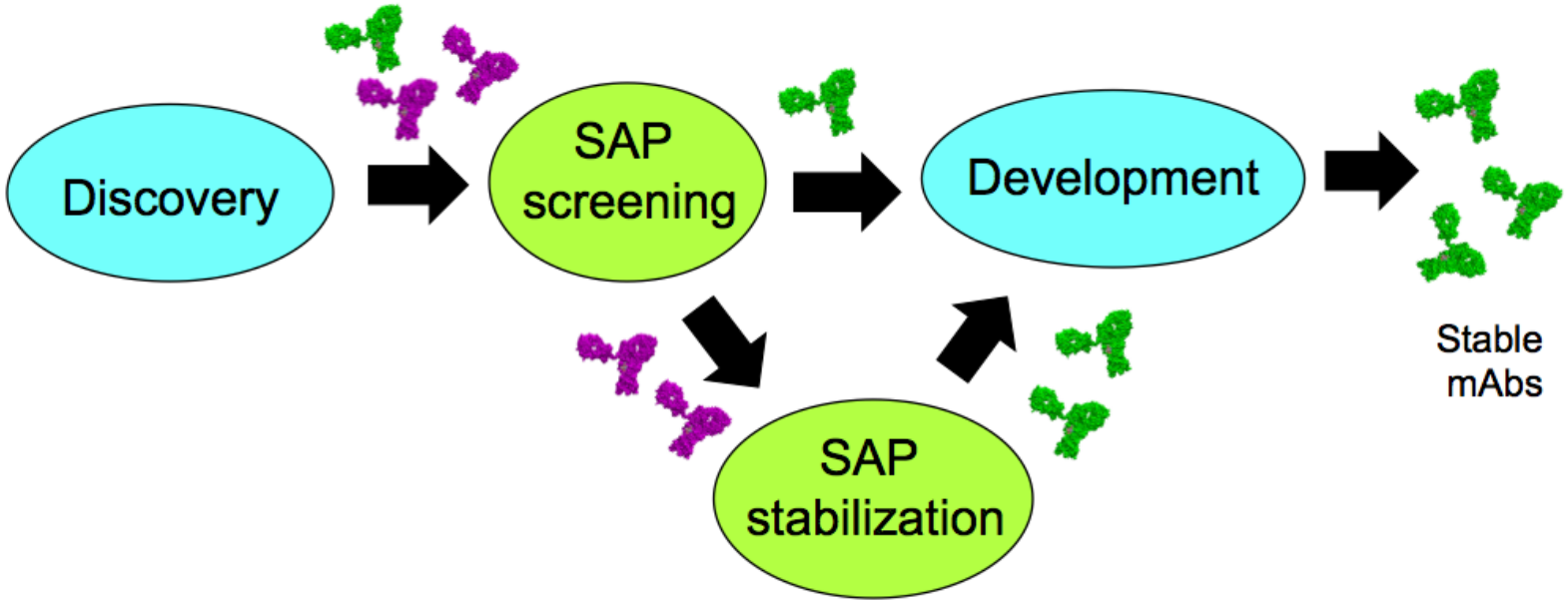


SAP will be optimized for developability ranking

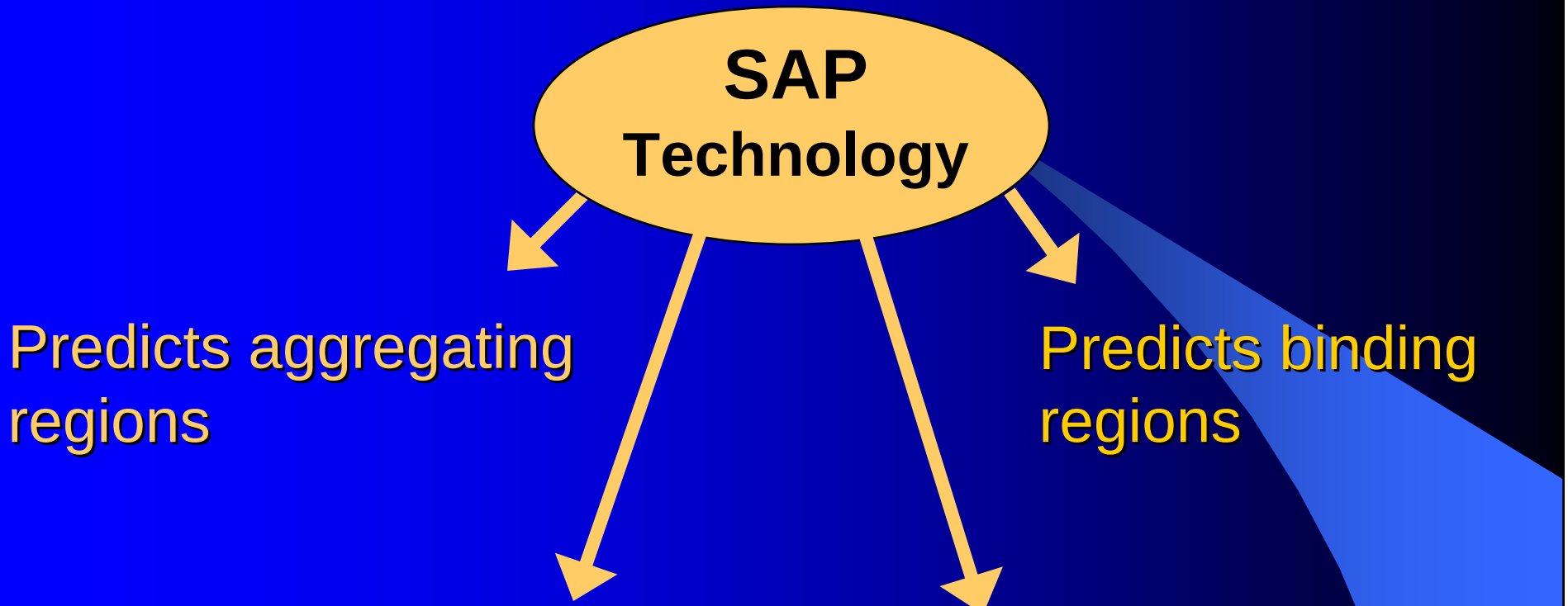
Current drug discovery process



Improved drug discovery process using SAP



More SAP applications



Predicts aggregating regions

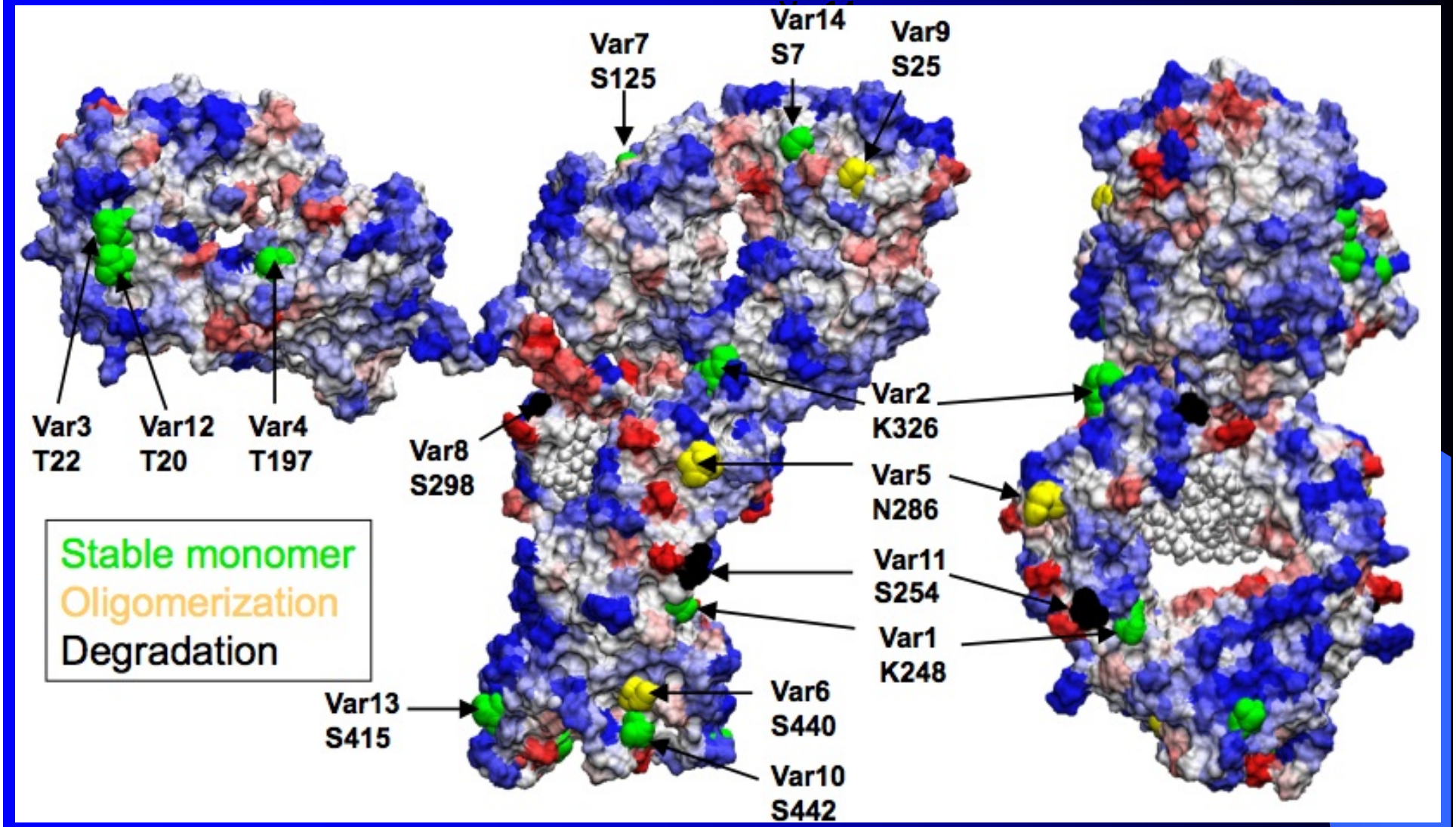
Predicts binding regions

Developability ranking

Find stable payload conjugation sites ?

31

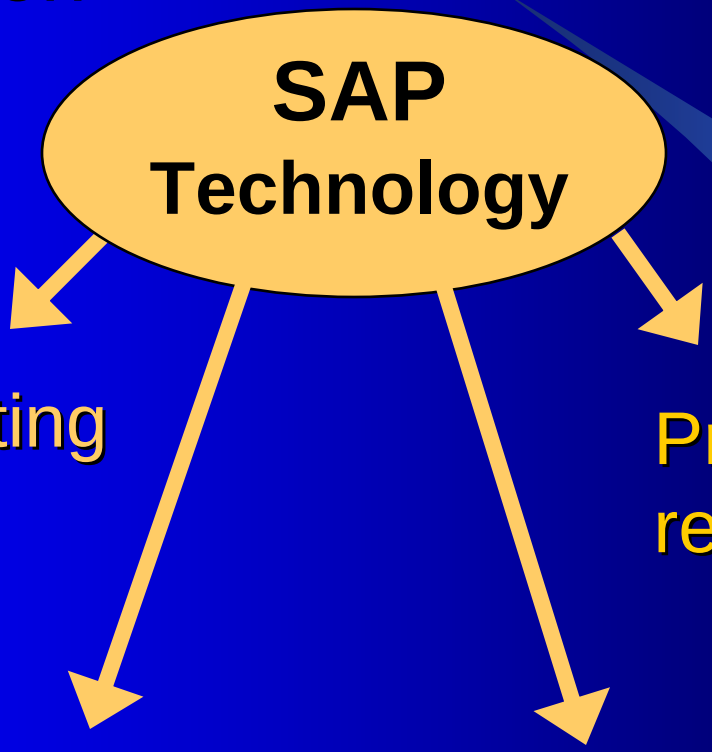
SAP improves the determination of sites for payload conjugation



To obtain conjugation sites that yield stable monomers, these sites should be partially exposed and away from high-SAP regions

Summary:

Developed the SAP tool to aid in discovery-commercialization



Predicts aggregating regions

Predicts binding regions

Developability ranking

Find stable payload conjugation sites

33

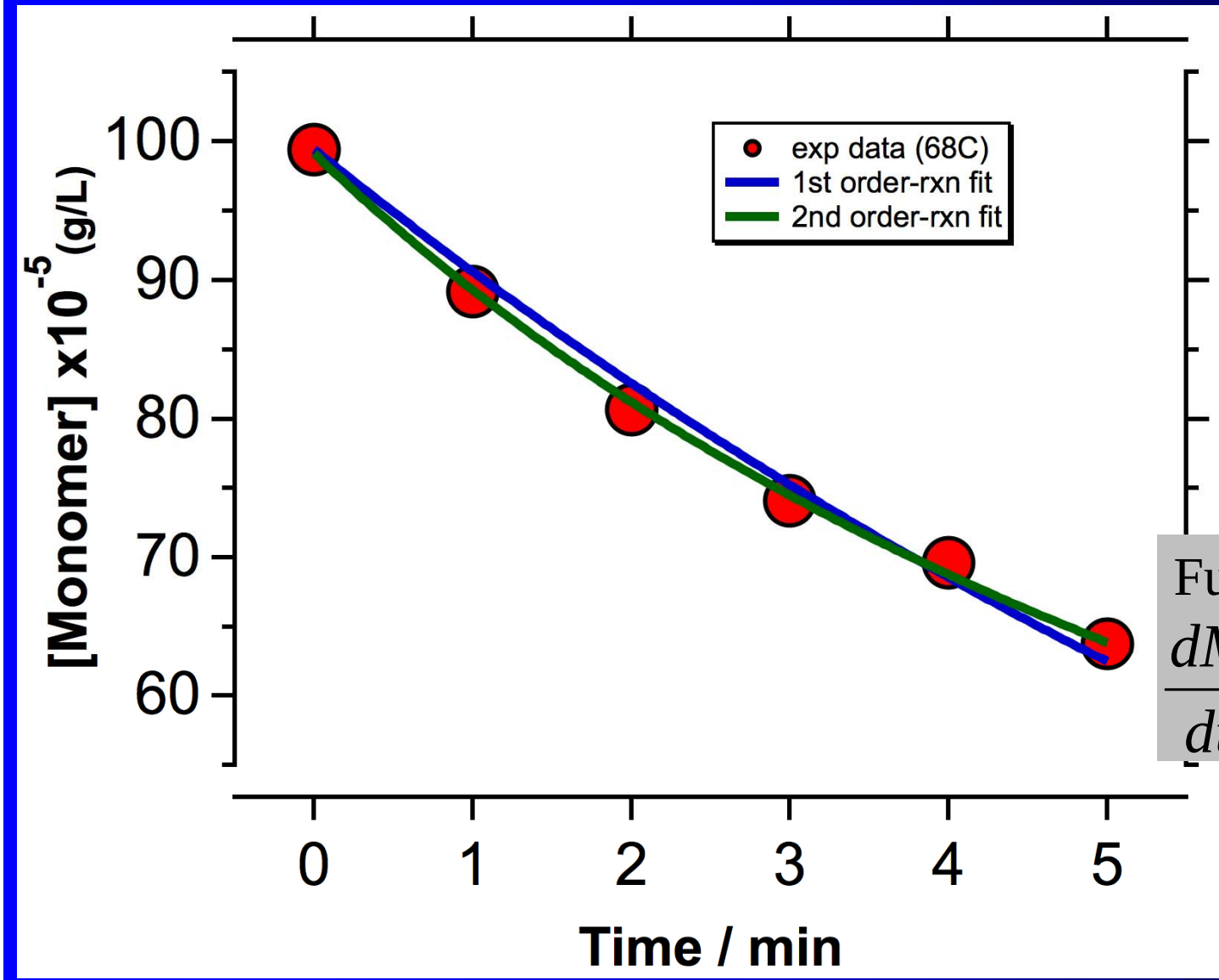
Macroscopic modelling

Macroscopic modeling and mathematical connection between long-term and short-term stability tests

Need model of aggregation for a given temperature and the temperature dependence of the rate constants

34

Monomer loss kinetics: Examples of 1st -, 2nd-order fits



1st order:

$$\frac{dM}{dt} = -k_{obs}M$$

2nd order:

$$\frac{dM}{dt} = -k_{obs}M^2$$

Full function:

$$\frac{dM}{dt} = -nk_nM^n - k_gMC$$

35

Temperature dependence: Kinetics are Non-Arrhenius

$$k = A\exp(-E_a / RT)$$

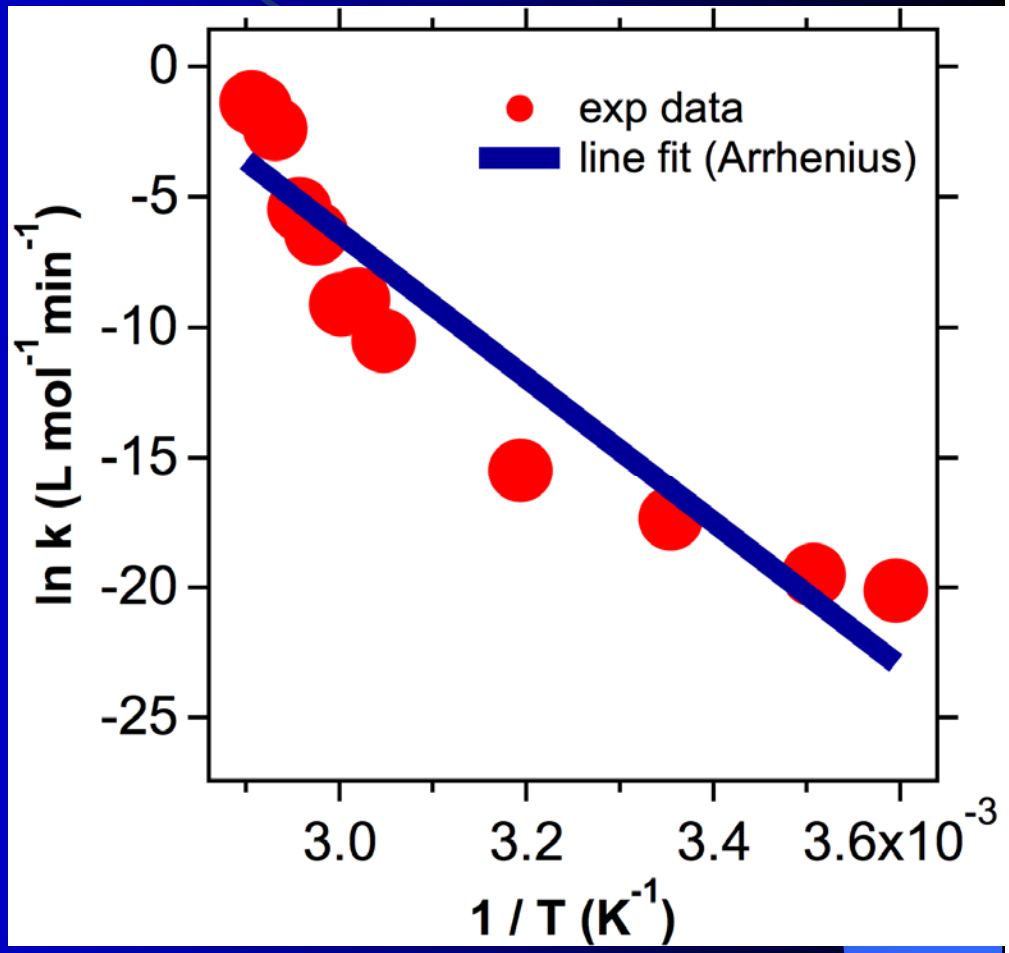
$$\ln k = \ln A - E_a / RT$$

A: pre-exponential coef.

E_a: activation energy

R: gas constant

T: temperature



Need Non-Arrhenius model

VFT method

(Vogel, Fulcher, Tammann)

$$Arrhenius:$$

$$k = A\exp(-E_a / RT)$$

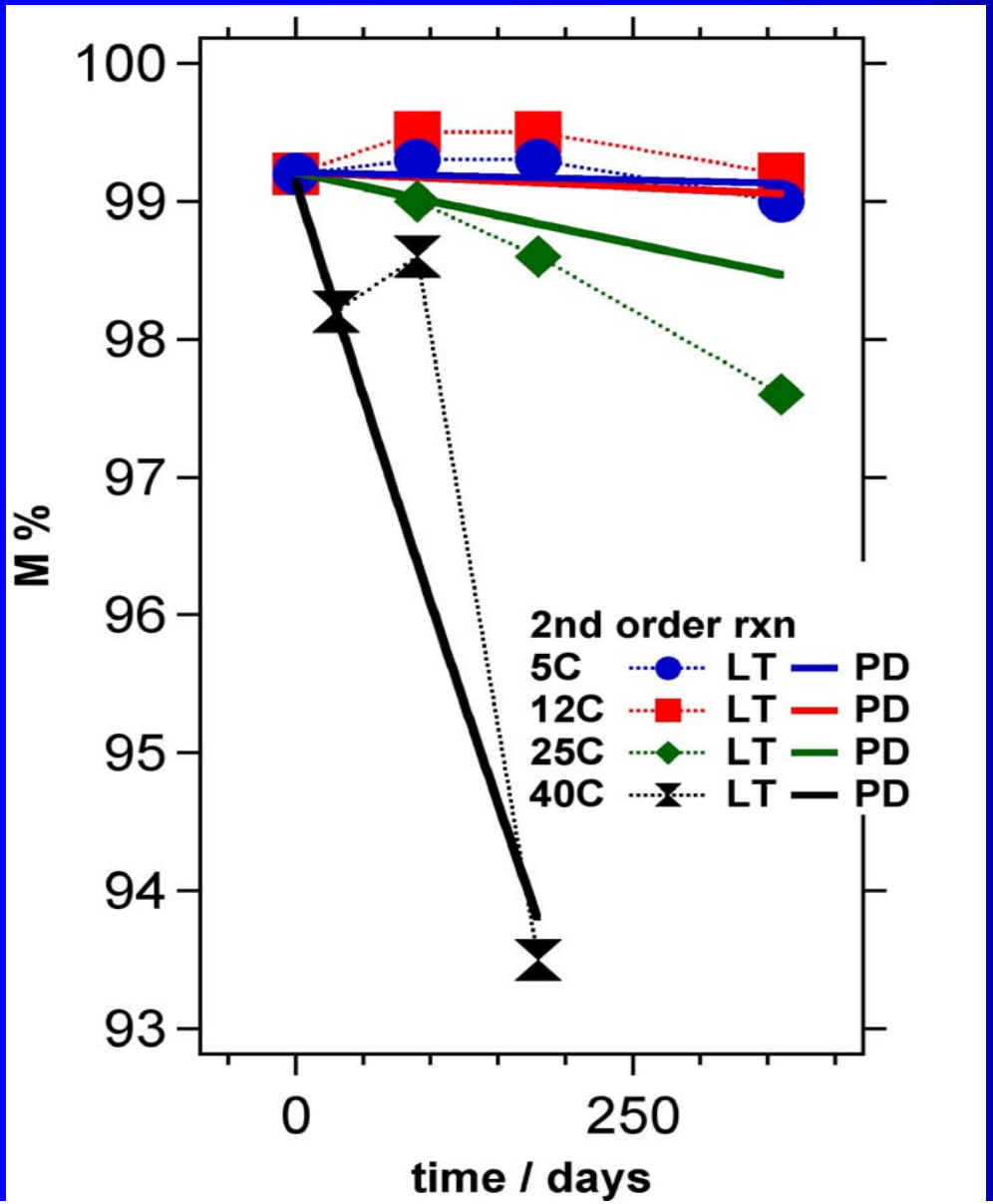
$$VFT:$$

$$k = A\exp(B/(T - T_o))$$

- Where T_o is a reference temperature at which the relaxation time relevant to molecular displacements becomes infinite, i.e. where the entropy changes suddenly
- Liu et al. found that $T_o = T_m$ for H exchange rates (DNA melting T)
- Can we also use VTF for highly non-Arrhenius behaving aqueous protein samples?
- We have found a similar trend for MAB2 but a higher T for MAB1

H. Levine (ed.), Amorphous Food and Pharmaceutical Systems, 2002, p131
Angell *et al.*, J. Appl. Phys., Vol. 88, No. 6, 15 September 2000
Liu *et al.*, Physics Letters A 361 (2007) 248-251

Prediction of MAB1 aggregation with the model fitted to short term data

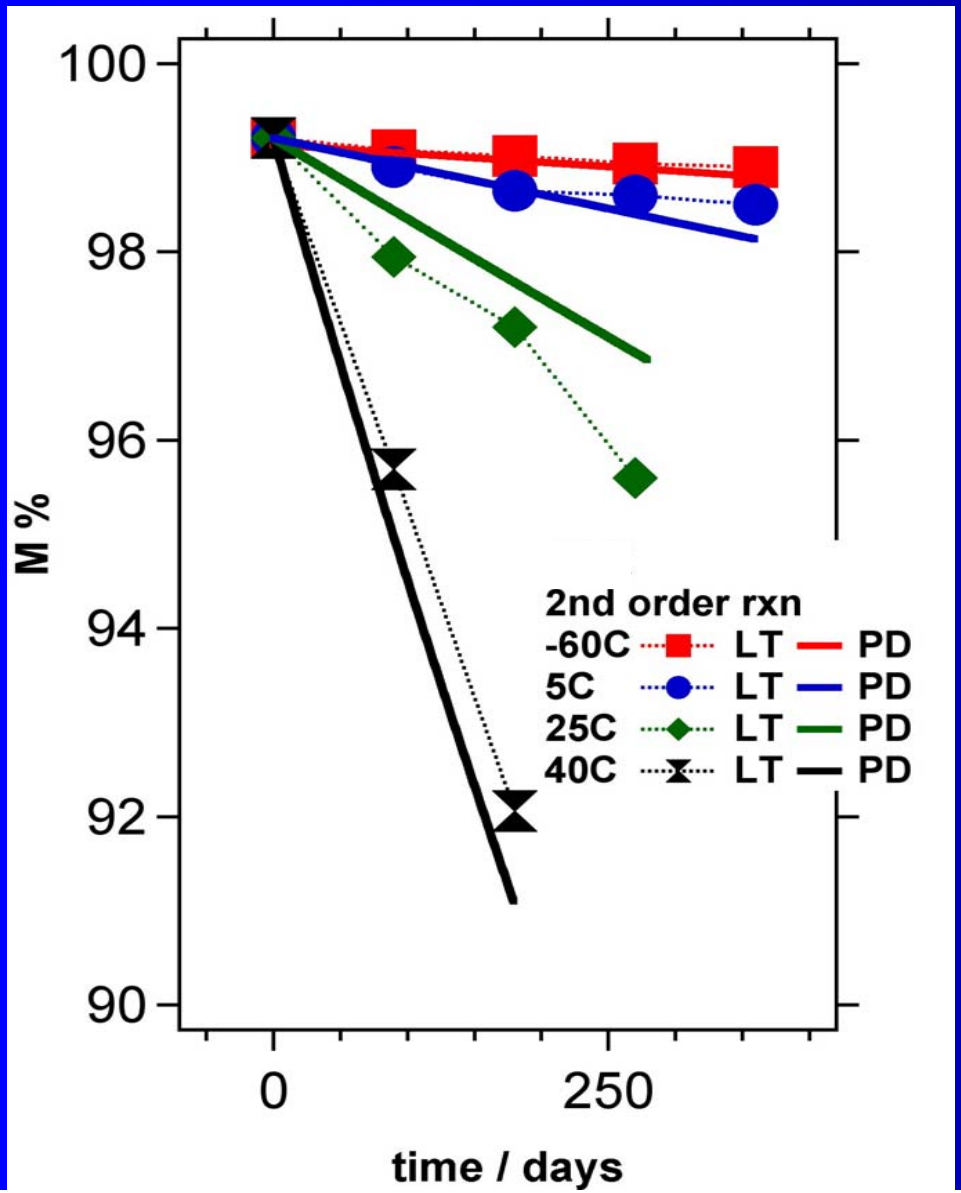


2nd order reaction

LT: long-term data
PD: predicted kinetics

38

Prediction of MAB2 aggregation with the model fitted to short term data



2nd order reaction

LT: long-term data
PD: predicted kinetics

39

Long-Term vs Predicted

- Time in months
- LT: long-term data
- 2^{nd} order reaction fit

MAB1

5C

time	2^{nd}	LT
0	99.2	**99.2**
3	99.19	**99.3**
6	99.17	**99.3**
12	99.13	**99**

12C

time	2^{nd}	LT
0	99.2	**99.2**
3	99.17	**99.5**
6	99.13	**99.5**
12	99.05	**99.2**

25C

time	2^{nd}	LT
0	99.2	**99.2**
3	99.02	**99**
6	98.84	**98.6**
12	98.47	**97.6**

40C

time	2^{nd}	LT
0	99.2	**99.2**
1	98.2	**98.2**
3	96.4	**98.6**
6	93.81	**93.5**

MAB2

m60C

time	2^{nd}	LT
0	99.21	**99.21**
3	99.06	**99.08**
6	98.97	**99.02**
9	98.89	**98.94**
12	98.8	**98.9**

5C

time	2^{nd}	LT
0	99.21	**99.21**
3	98.94	**98.9**
6	98.67	**98.65**
9	98.4	**98.6**
12	98.14	**98.5**

25C

time	2^{nd}	LT
0	99.21	**99.21**
3	98.45	**97.95**
6	97.69	**97.2**
9	96.86	**95.6**

40C

time	2^{nd}	LT
0	99.21	**99.21**
3	95.01	**95.69**
6	91.1	**92.06**

Molecular QbD for the Design of Protein-Cosolute Interactions

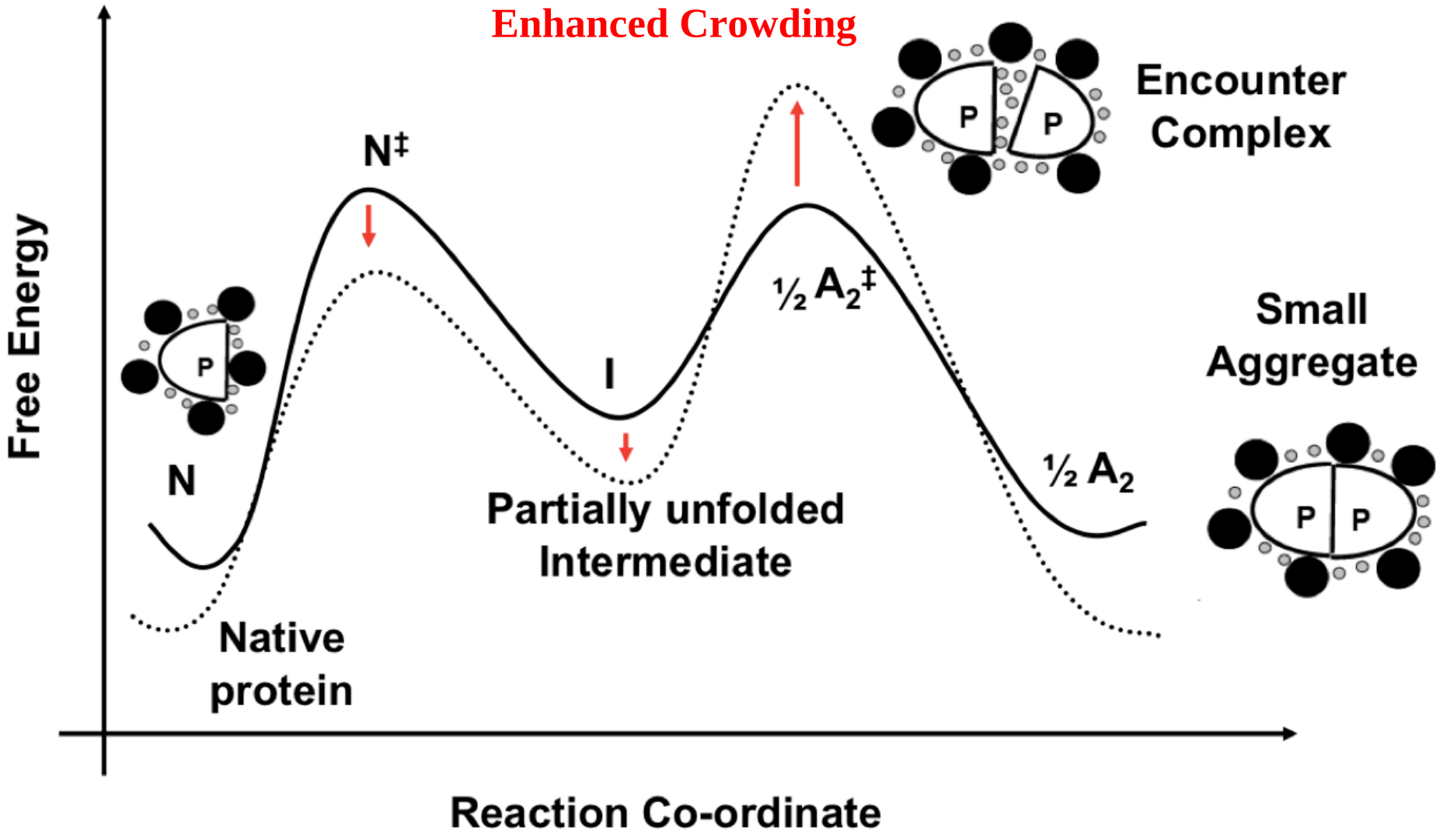
41

Starting Point: Arginine

- Well known stabilizer.
- Action of stabilization unknown.
- Seems to interact net neutrally with biomolecules.
- Can we better understand arginine and develop better additives?

42

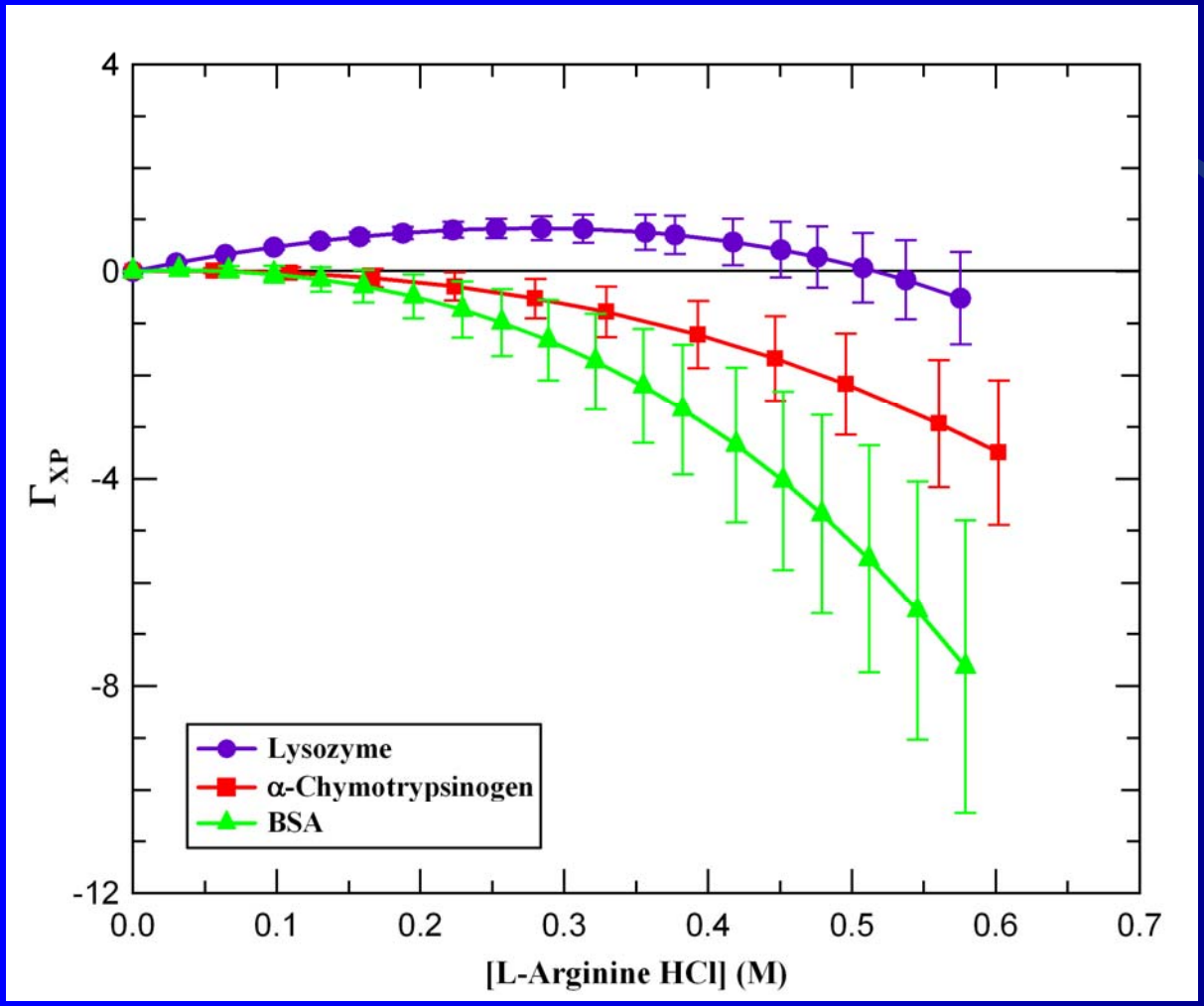
Proposed mechanism by which arginine inhibits aggregation



Preferential Interaction Coefficients

- Measures the degree of attraction or repulsion of cosolutes to proteins.
- Positive values means that cosolutes are attracted to the protein. (e.g. Gnd, urea)
- Negative values mean that cosolutes are repelled from the protein. (e.g. sucrose, mannitol)
- Indicates the degree to which an additive stabilizes the folded state of a protein.

Arginine Preferential Interactions



$$\Gamma_{XP} \equiv \left(\frac{\partial m_x}{\partial m_p}\right)_{T,P,\mu_x} = -\left(\frac{\partial \mu_p}{\partial \mu_x}\right)_{T,P,m_p}$$

VPO Technique:

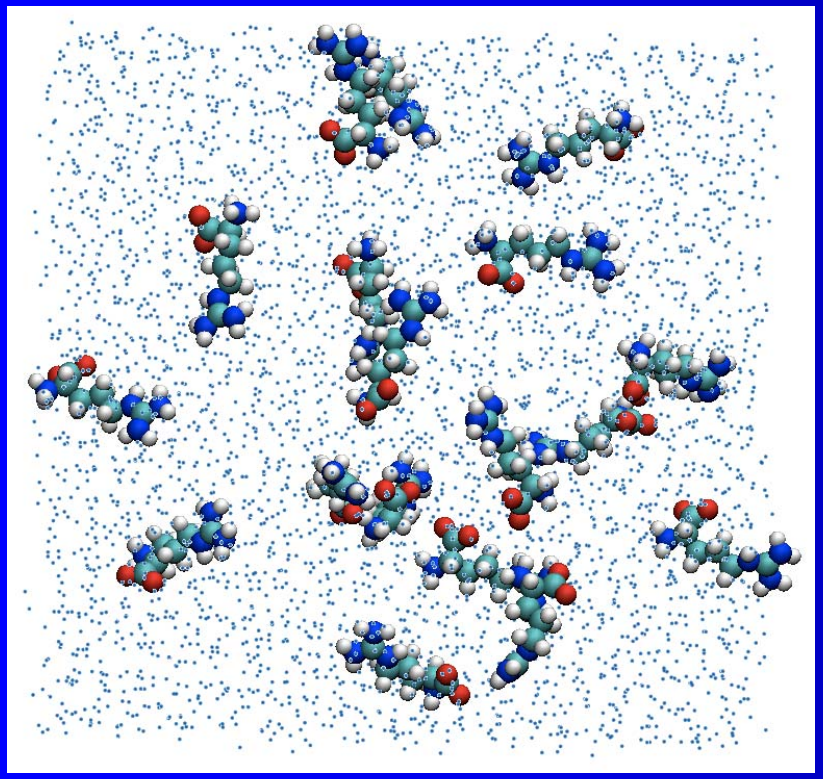
$$\Gamma_{XP} \cong \frac{m_x}{m_p}\left(1 - \frac{\left(\partial Osm/\partial m_x\right)_{m_p}}{\left(\partial Osm/\partial m_x\right)_{m_p=0}}\right)$$

- Arginine has a concentration dependent preferential interaction.

Schneider, C.P. and B.L. Trout, J. Phys. Chem. B, 2009. **113**(7): p. 2050-2058.

45

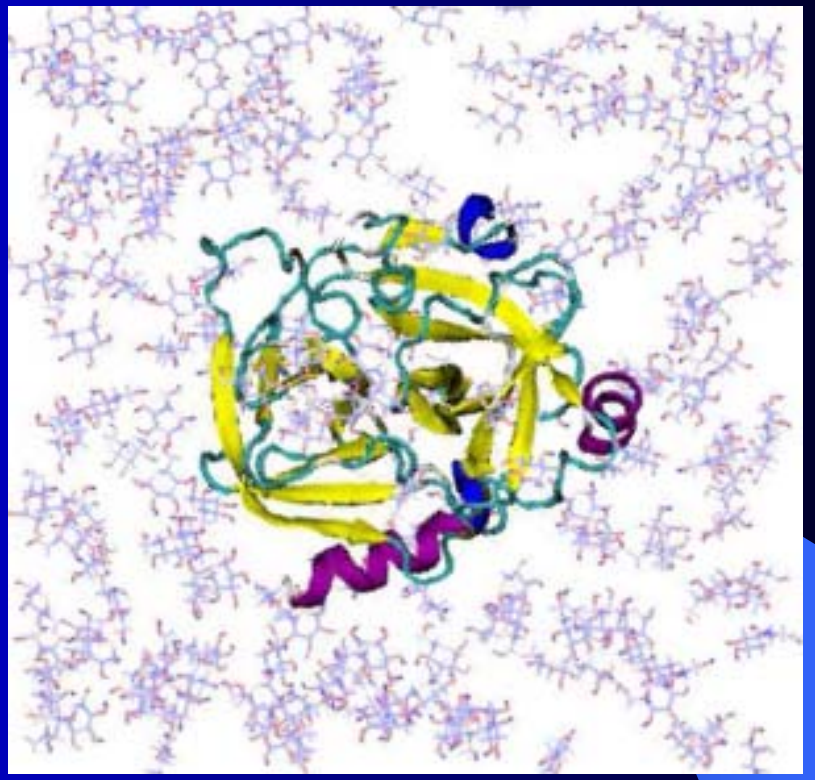
Computational Methodology



MD simulation of aqueous arginine solutions

Temperature: 278-368 K

Concentration: 0.25-2.75 molal



MD simulation of protein in aqueous arginine solution

Protein: α-Chymotripsinogen A, Lysozyme

Temperature: 298 K

46

Computing Preferential Interaction Coefficients

Preferential Interaction coefficient (Γ_{23}): excess number of additive molecules in local domain

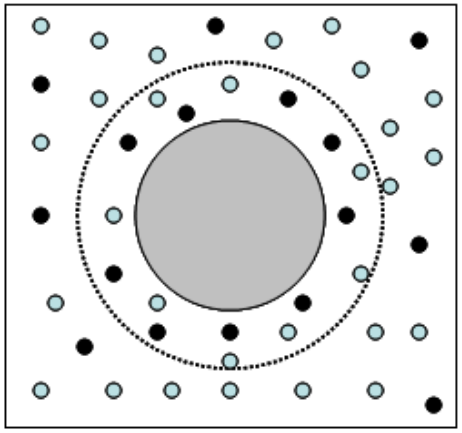
$$\Gamma_{23} = \left\langle n_3^{II} - n_1^{II}\left(\frac{n_3^I}{n_1^I}\right)\right\rangle$$
$$= \rho_3(\infty)\int (g_3(r) - g_1(r))dV$$

II -- local

I -- bulk

1-Water 2-protein 3-additive

(Scatchard notation)

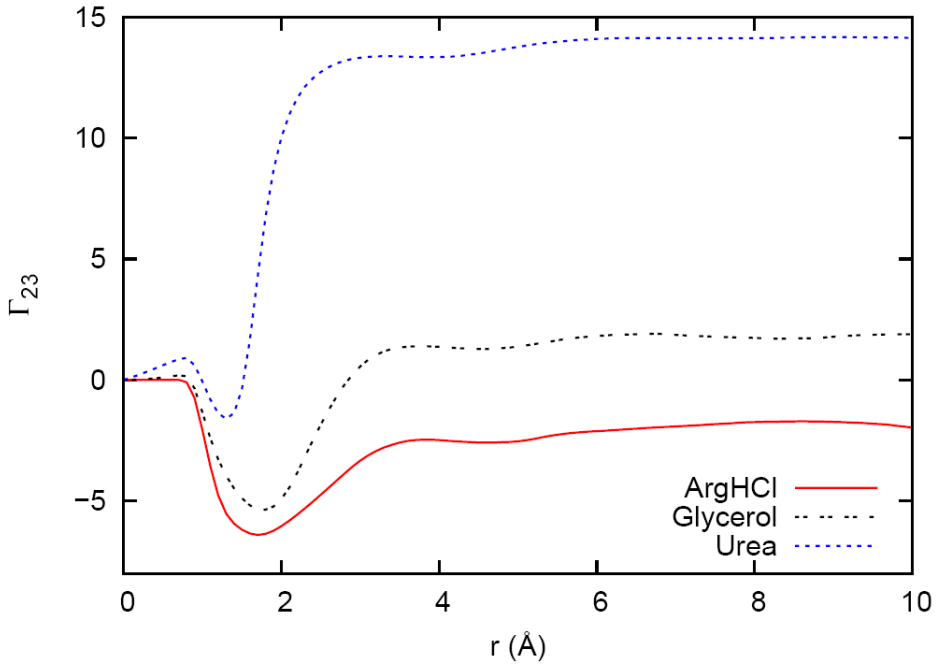


$$(g_3(r) - g_1(r)) = 0$$

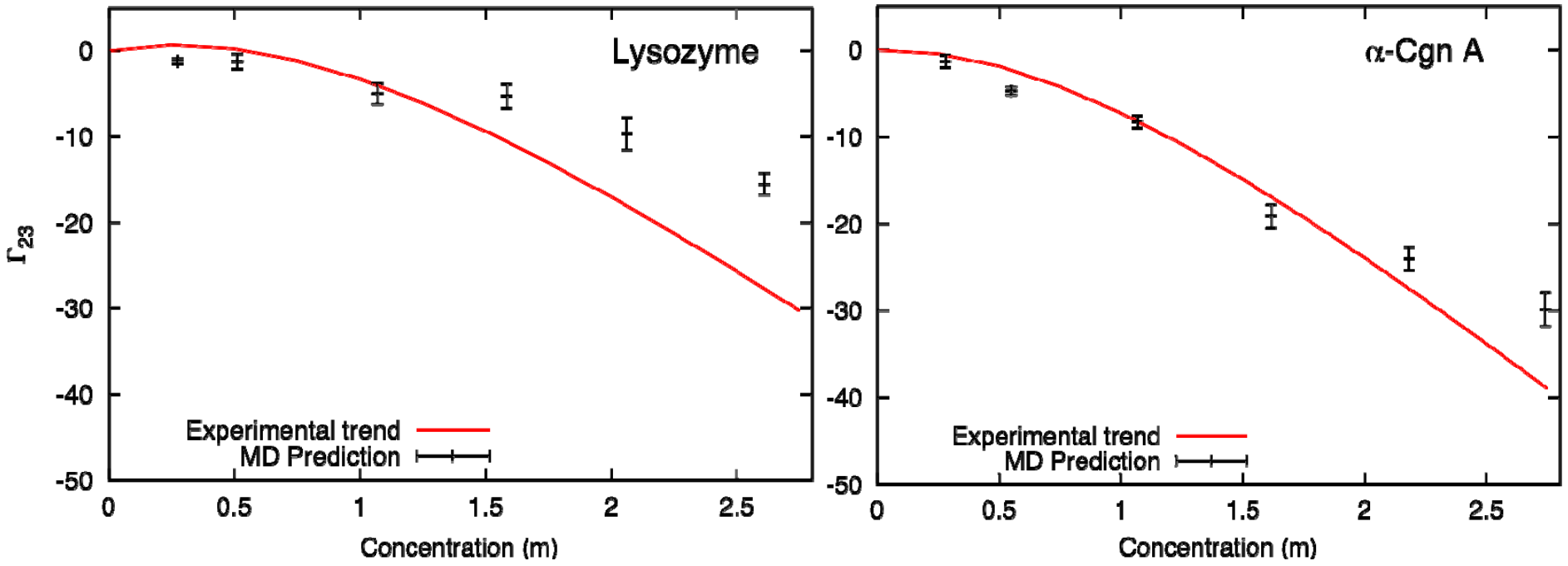
$$\Gamma_{23}(r,t) = n_3(r\ ,t) - n_1(r,t)\left(\frac{n_3 - n_3(r,t)}{n_1 - n_1(r,t)}\right)$$

n_3 total number of cosolvent molecules

n_1 total number of water molecules



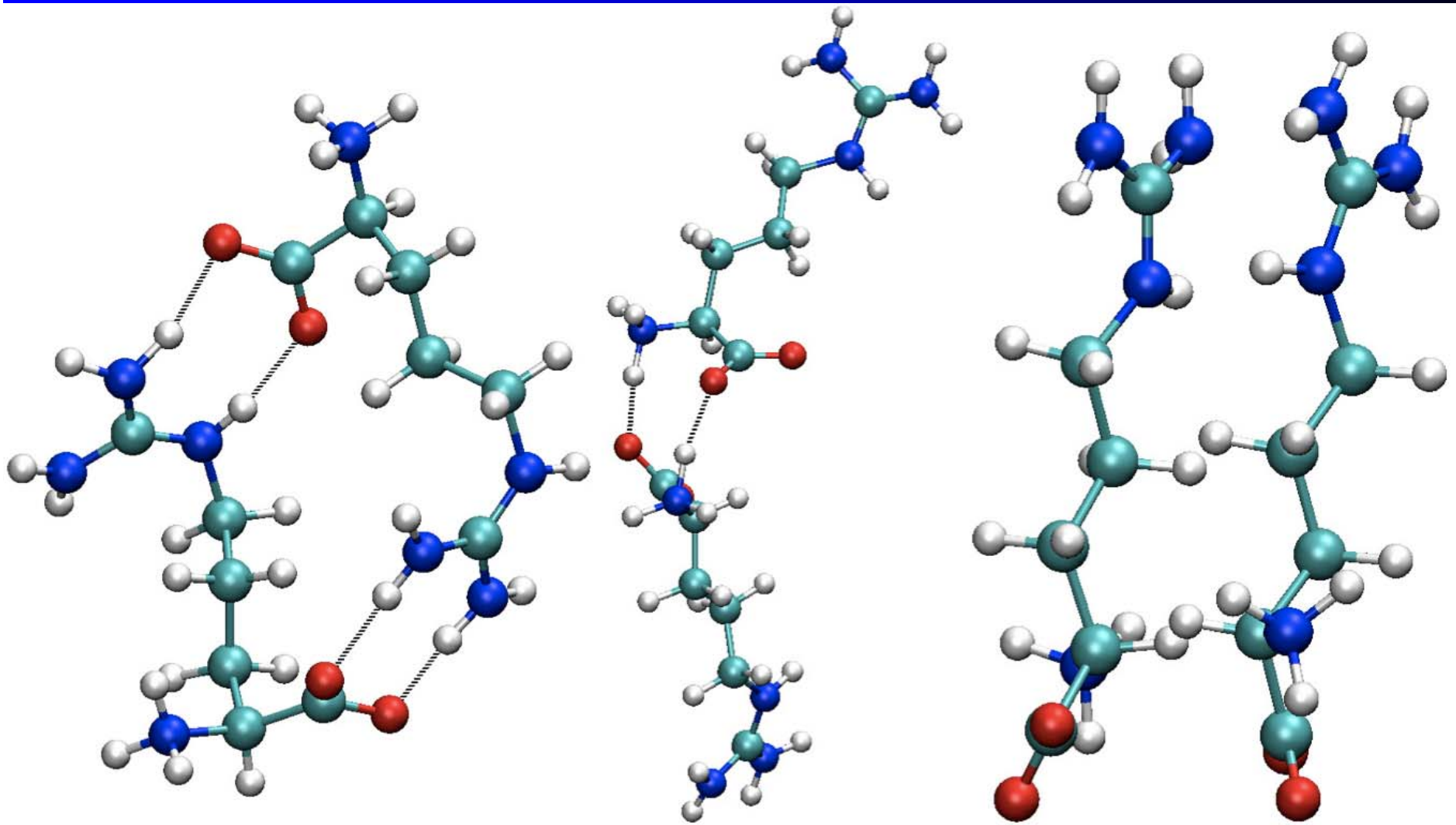
Preferential Interaction Coefficients for Arginine



experimental preferential interaction data is only available upto 0.7 molal.

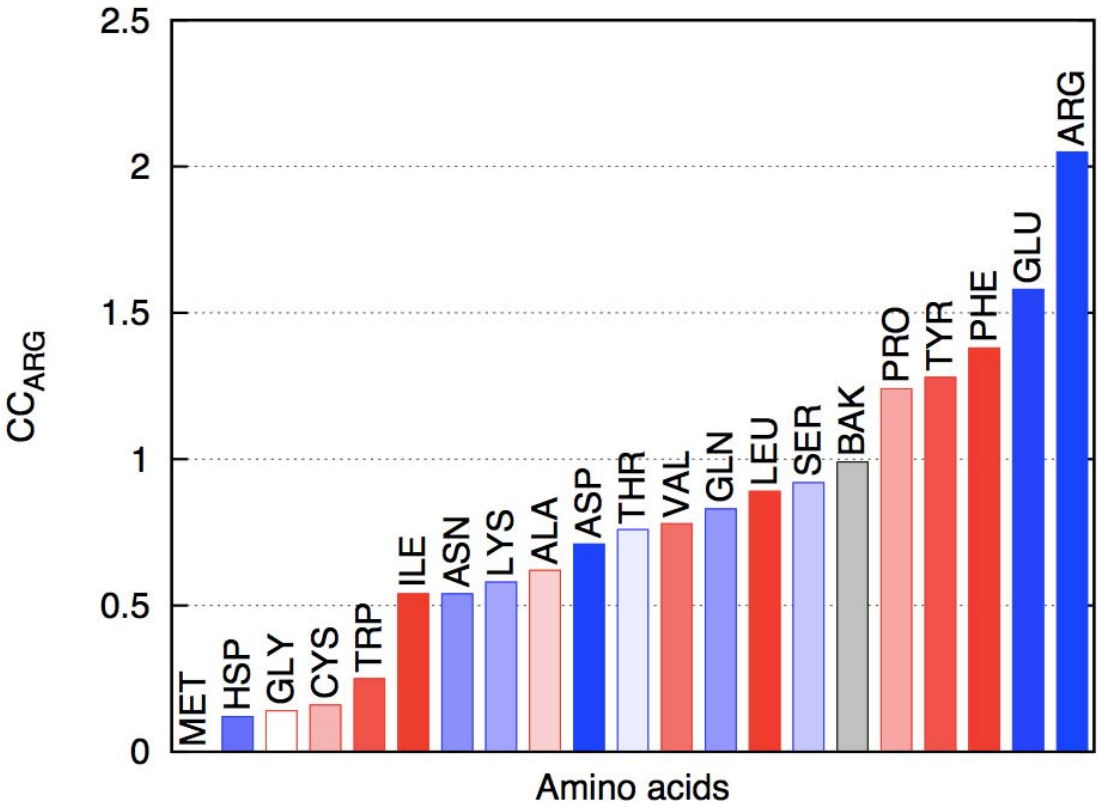
48

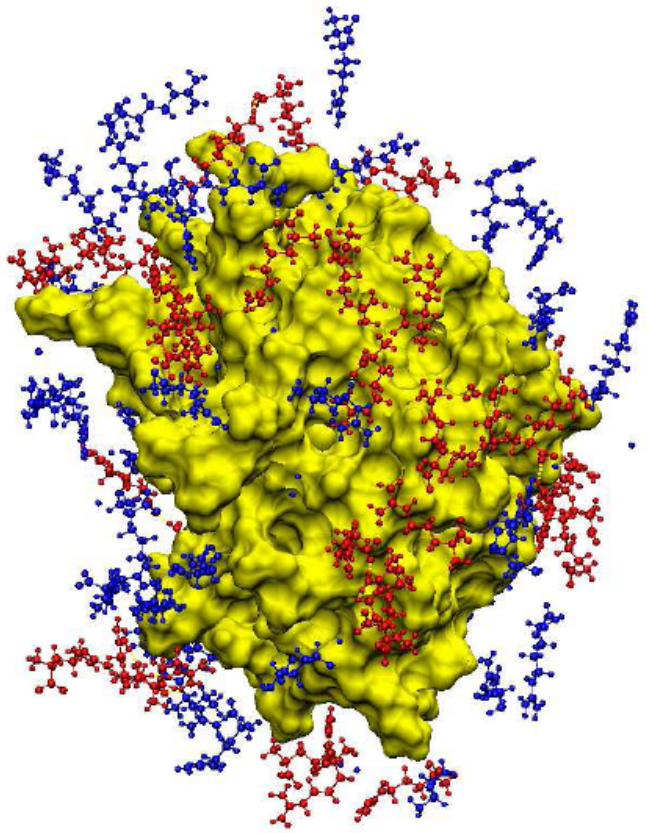
Interactions in aqueous arginine solutions



- Arginine tends to from clusters via hydrogen bonding and Gdn Stacking

Interactions between arginine and A protein

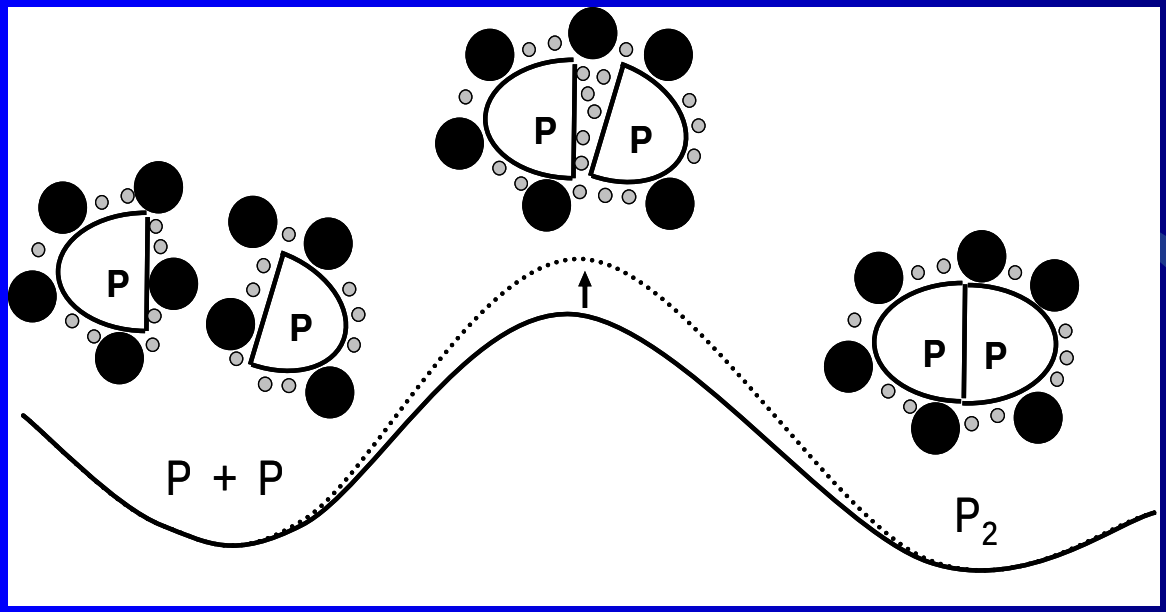




Contact coefficient = local/bulk concentration

- Arginine interacts with charged and aromatic residues.
- Interaction with aromatic residues could stabilize unfolded intermediates.
- Clustering in arginine solution leads to enhanced crowding.

Neutral Crowder Excipients



$$\Gamma_{XP} \cong 0$$

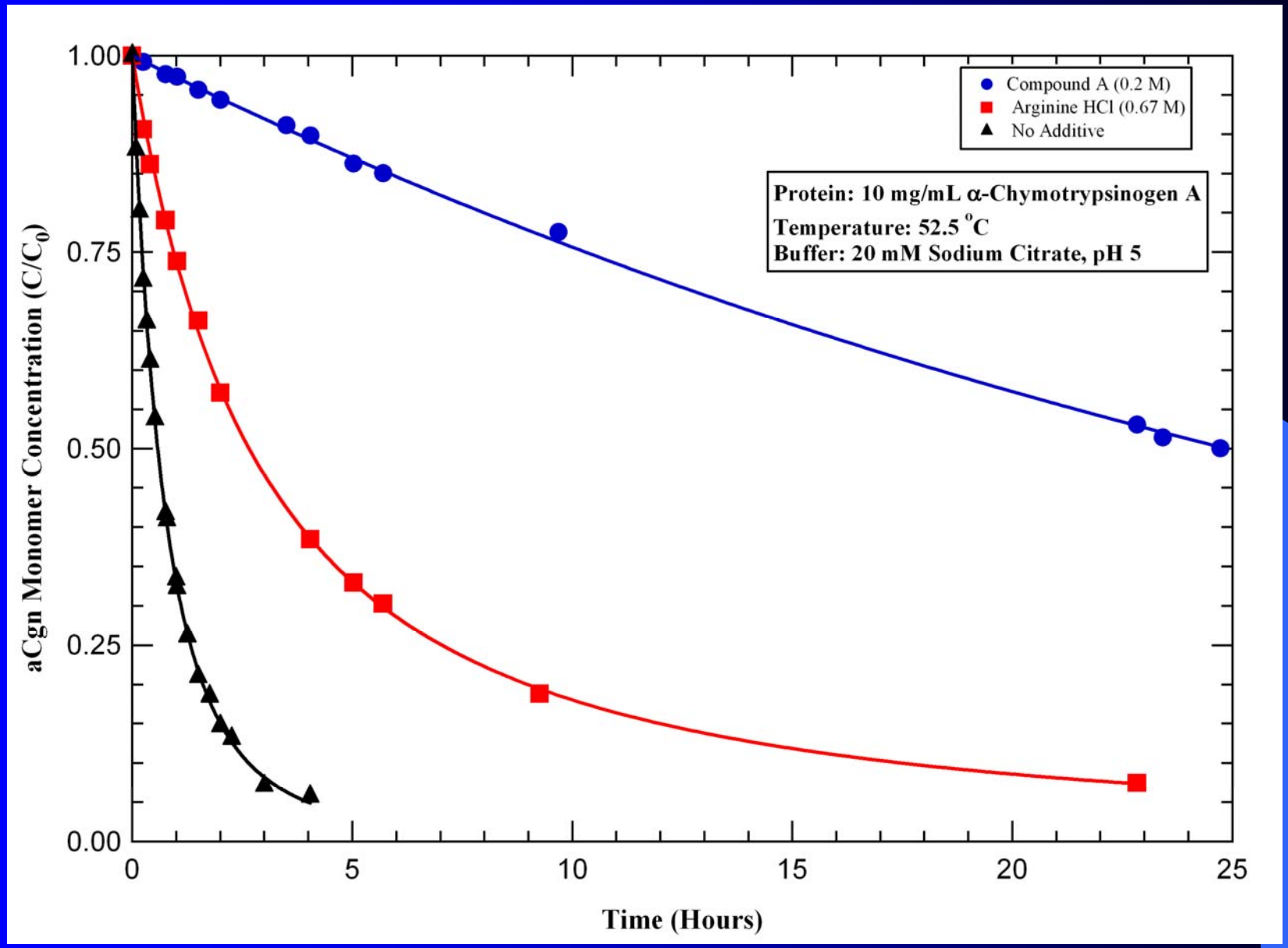
$$\delta\Delta G_u^{\circ} \cong 0$$

- We have created novel compounds that:
  - Solvate proteins much like water
  - Have little influence on the folding equilibrium
  - Specifically inhibit protein association
- We call such excipients “neutral crowders”.

Baynes, B.M. and B.L. Trout, Biophysical Journal, 2004. **87**(3): p. 1631-1639.
Baynes, B.M., D.I.C. Wang, and B.L. Trout, Biochemistry, 2005. **44**(12): p. 4919-4925.

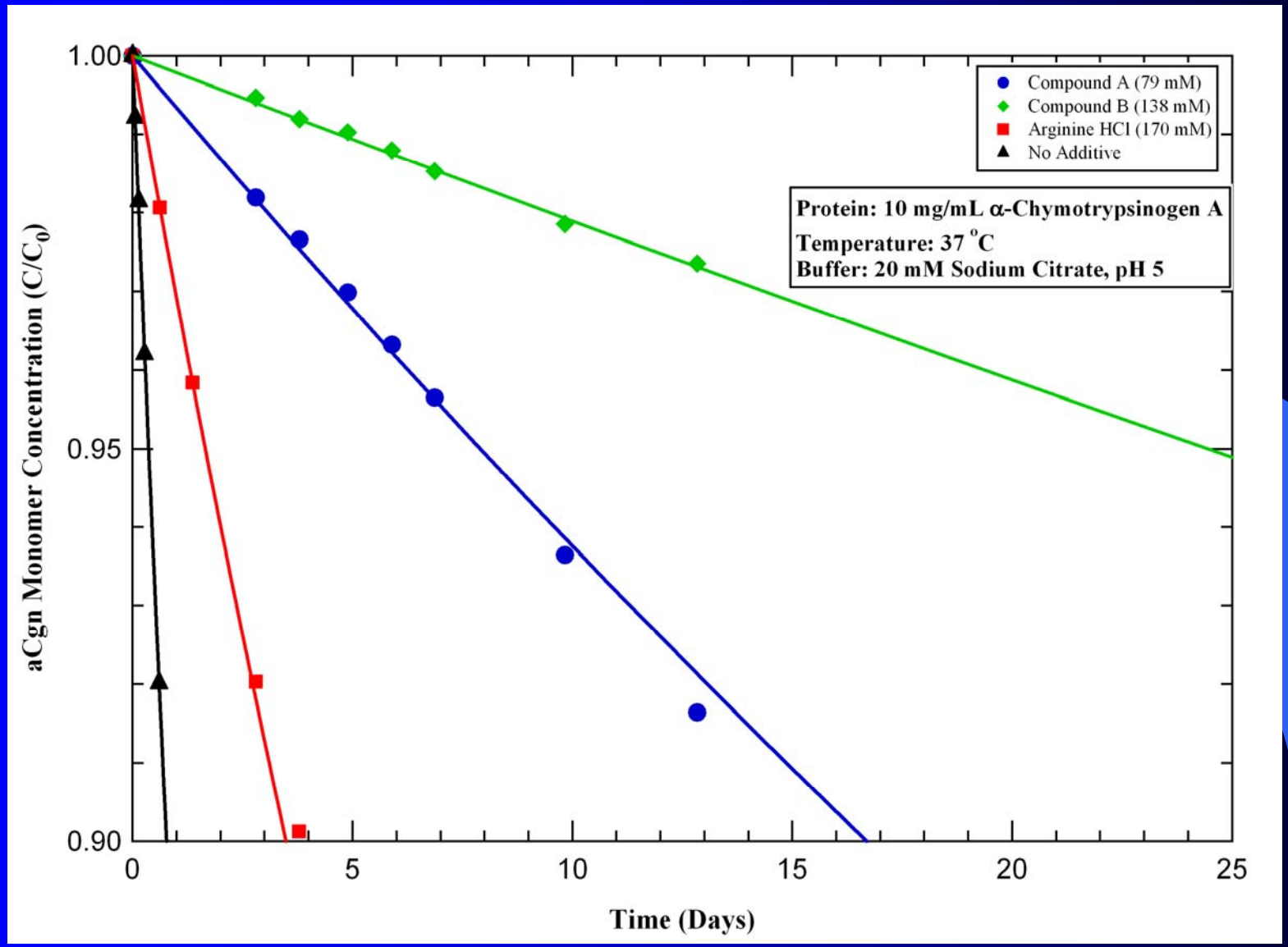
51

Aggregation: High Temperature



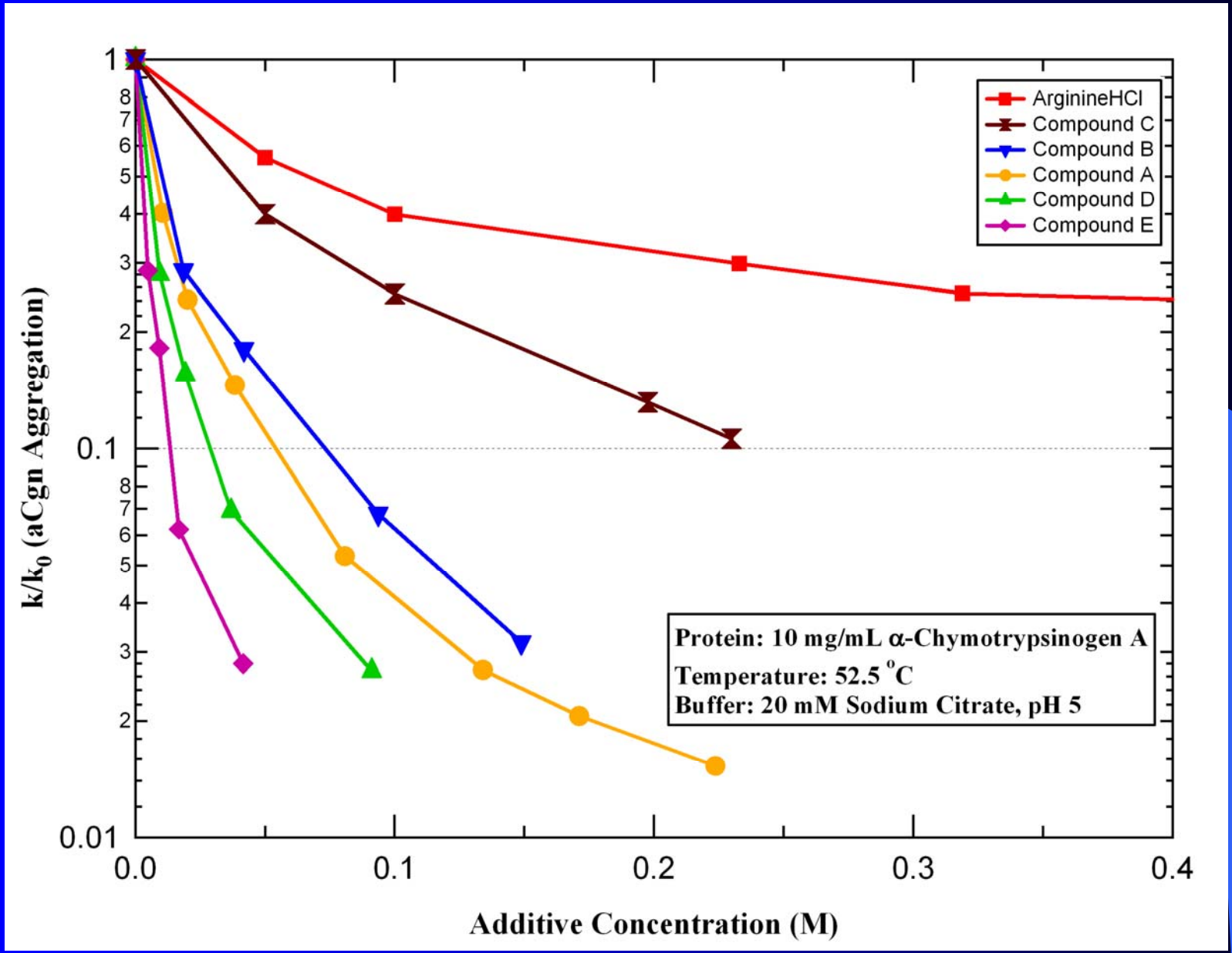
52

Aggregation: Body Temperature



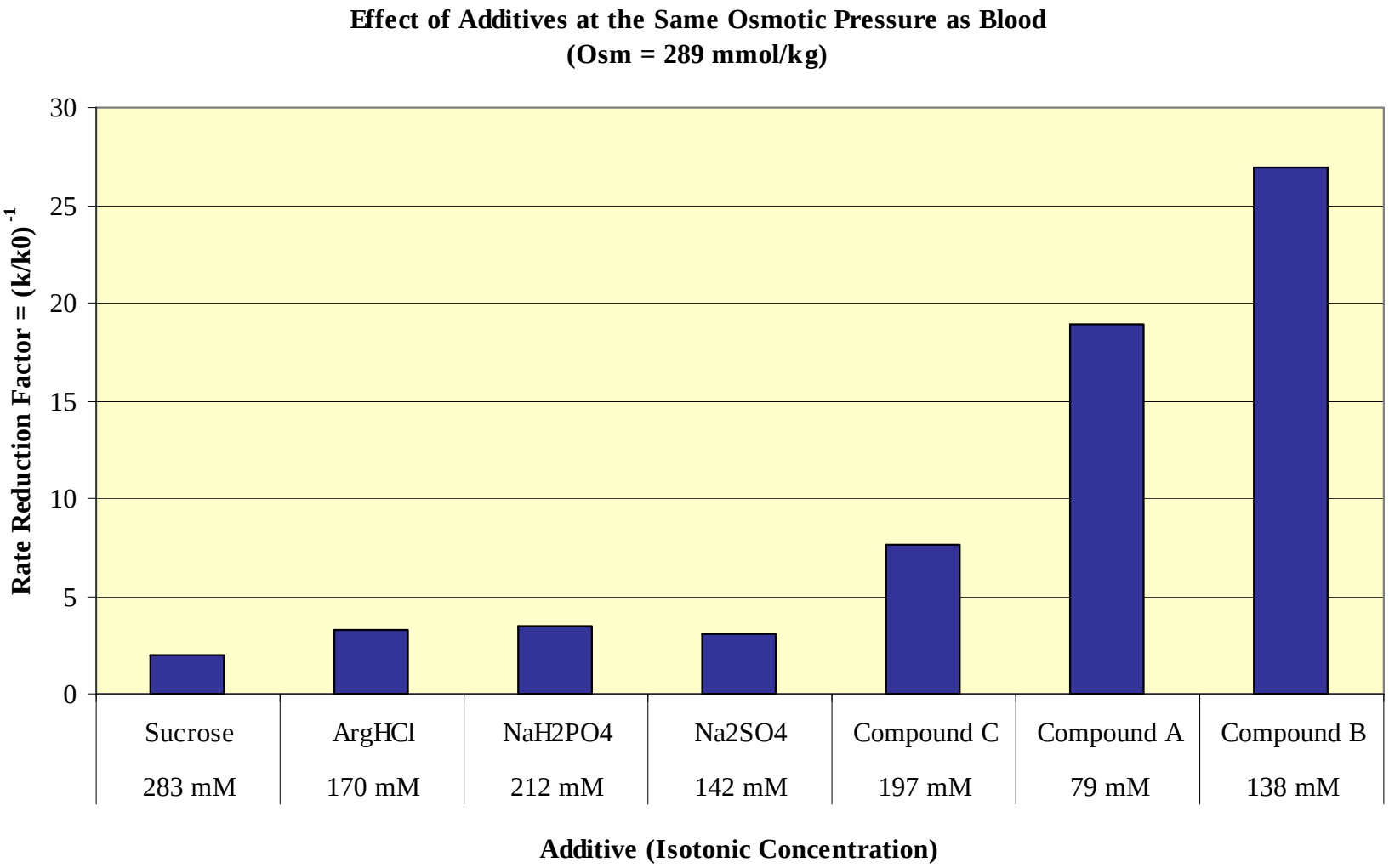
53

Aggregation Rates vs. Concentration



54

Other Excipients



T = 52.5 °C, 20 mM Sodium Citrate, pH 5

55

Shelf Life Predictions (5% Loss)

k/k_0 (aCgn Aggregation)					
	10 mg/mL aCgn			40 mg/mL aCgn	
	37 °C	45 °C	52.5 °C	37 °C	45 °C
Compound A	4.6%	6.1%	5.3%	5.9%	6.6%
Compound B	1.5%	2.5%	3.7%	2.7%	1.8%
ArgHCl	23.7%	27.0%	30.3%	21.7%	36.0%

Aggregation suppression is fairly constant at various temperatures and concentrations.

Shelf Life extended from a few days to several months.

t_{95}:alpha-Chymotrypsinogen A Monomer Loss (5%)						
T (°C)	**No Additive**		**Arginine k/k_0 = 0.25**		**Compound B k/k_0 = 0.025**	
52.5	2	Minutes	8	Minutes	1.3	Hours
45	2.1	Hours	8.4	Hours	3.5	Days
37	8.6	Hours	1.4	Days	14	Days
25*	3.4	Days	12	Days	5	Months

10 mg/mL aCgn, 20 mM Sodium Citrate, pH 5
*Predicted Value (Arrhenius Plot of Low Temperature Data)

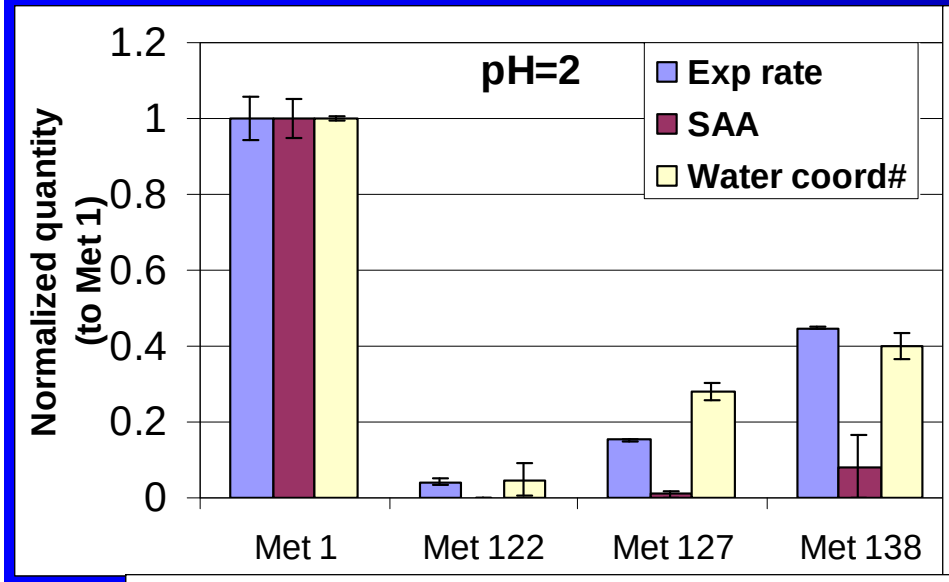
Summary of Molecular Simulation Approaches for Cosolutes

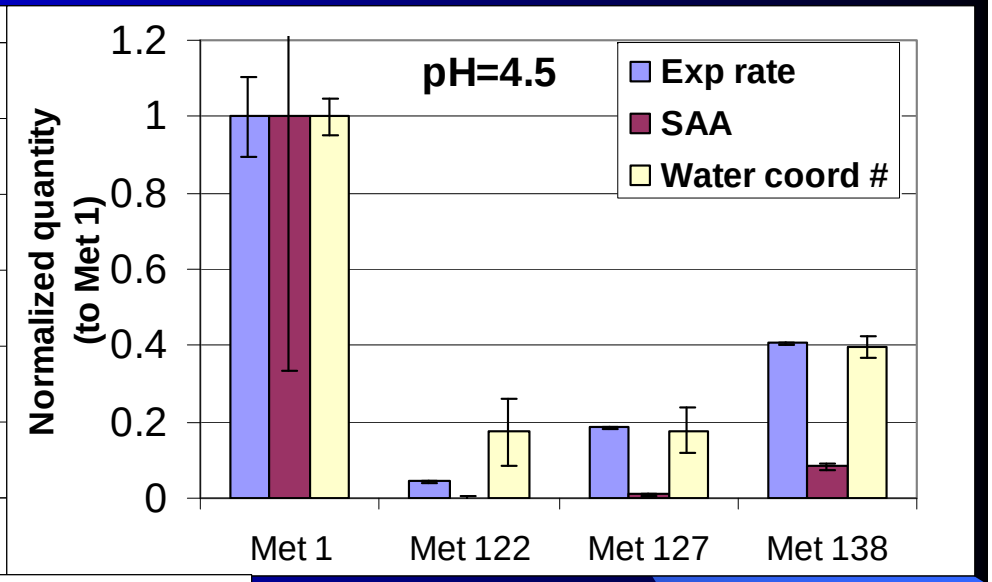
- Gain Mechanistic Understanding
- Allow Rational Design
  - E.g. additives
  - Buffers

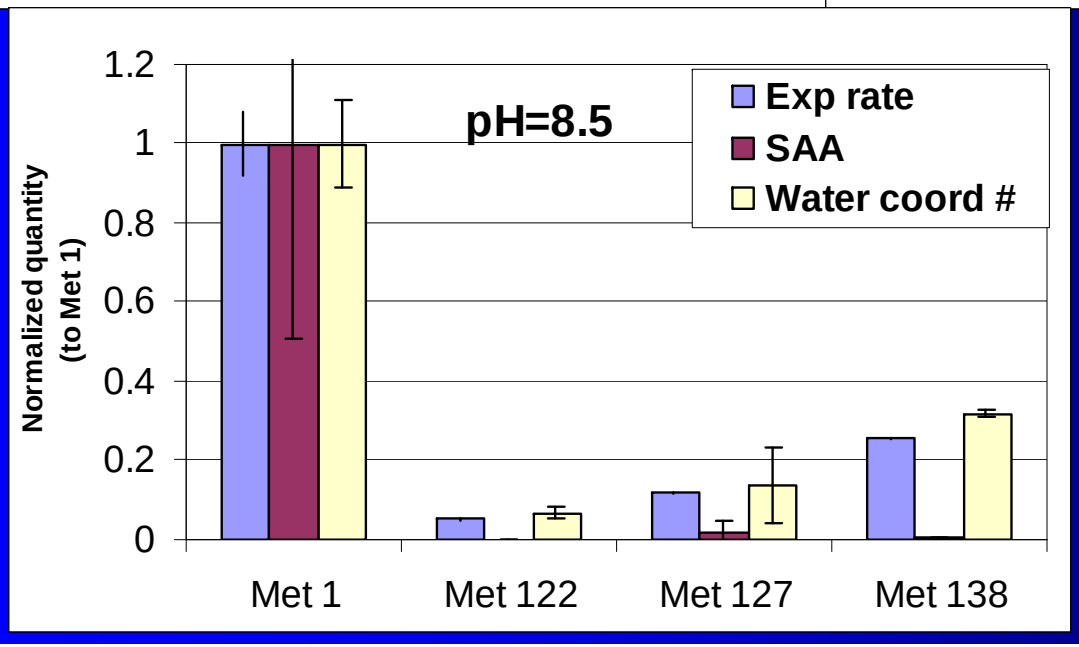
A very brief summary of the oxidation of therapeutic antibodies

58

Correlation between WCN and the Relative Rates of Oxidation







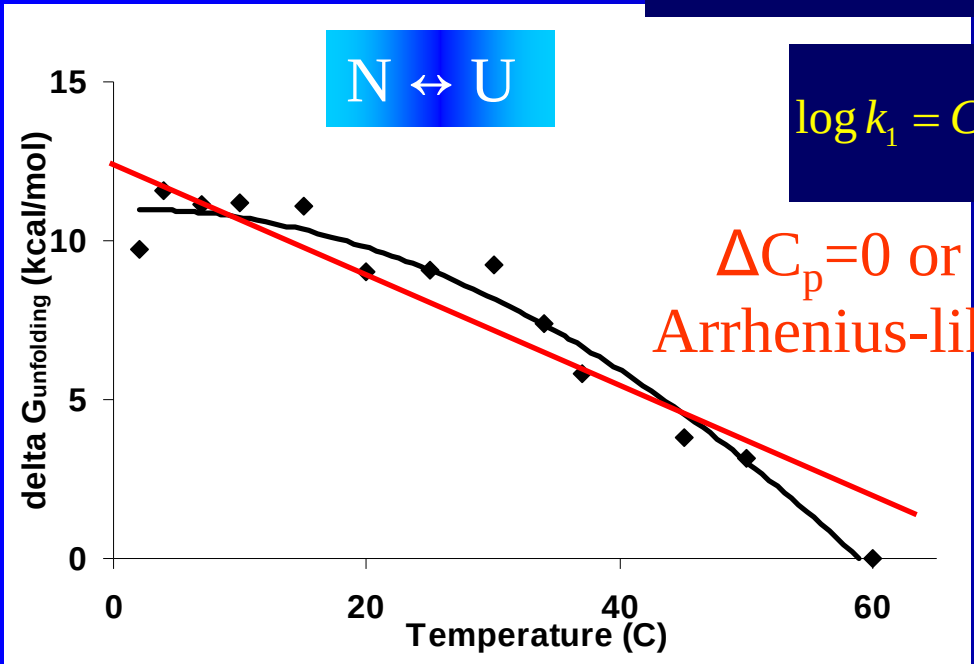
Solves puzzle of 0 SAA from X-ray structure.

Expected Structural Effect

Fit to a two-state protein unfolding model N ↔ U

Gibbs-Helmholtz equation

$$\frac{\Delta G_{(unfolding)}}{RT} = \frac{\Delta H^{0}_{(unf)}}{RT_m} - \frac{\Delta H^{0}_{(unf)} - \Delta C_p (T - T_m)}{RT} + \frac{\Delta C_p}{R} \log \frac{T}{T_m}$$



Equilibrium denaturation monitored by fluorescence

$$\log k_1 = C' - \frac{\Delta^{\neq}_r H(T_m) - \Delta^{\neq}_r C_p T_m}{RT} + \frac{\Delta^{\neq}_r C_p}{R} \log \frac{T}{T_m}$$

$\Delta C_p = 0$ or Arrhenius-like

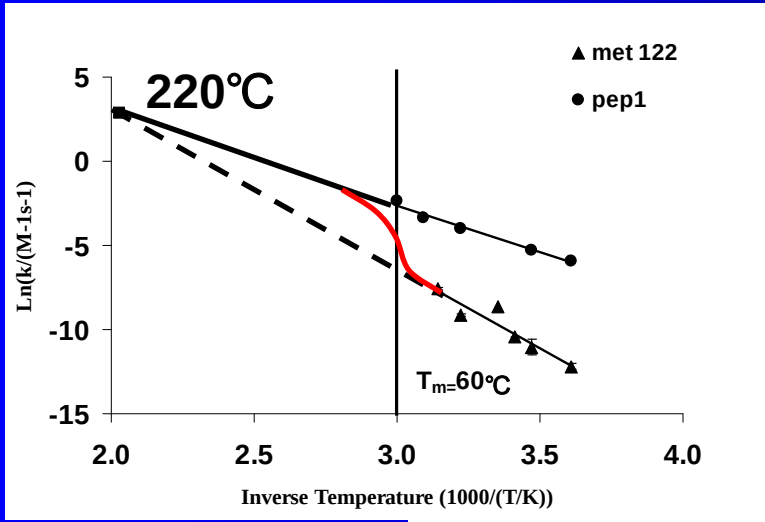
$\Delta C_p \neq 0$

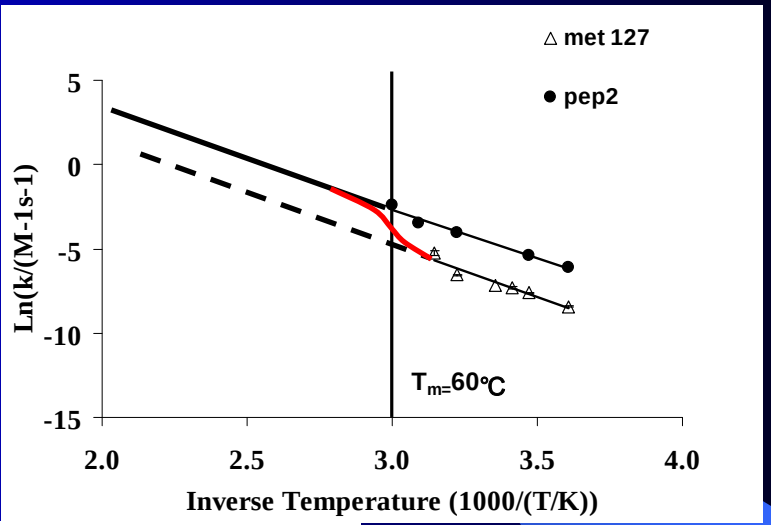
Non-Arrhenius-like structural change

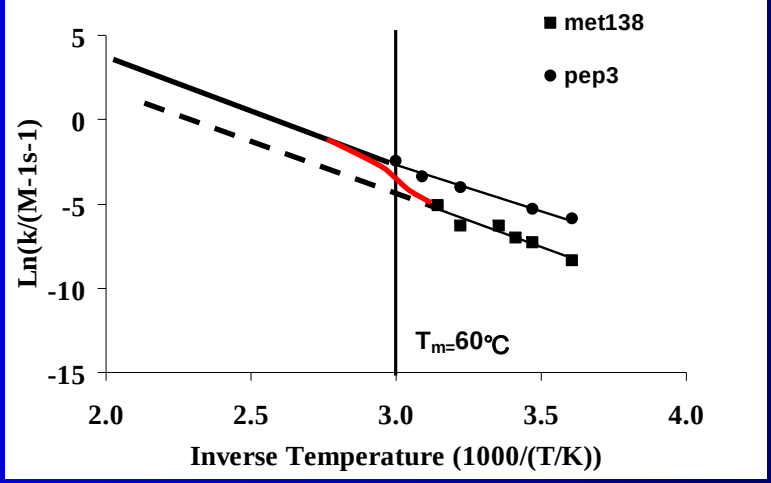
Possibly non-Arrhenius-like oxidation kinetics!

Extrapolation Analysis

On the physical basis when there is no structural effect



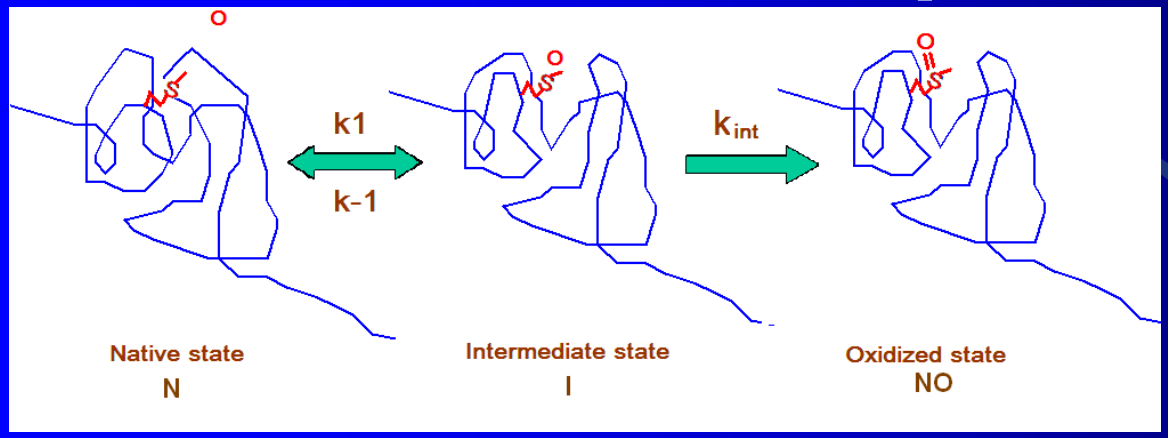




Expect a non-Arrhenius behavior connecting high T and low T regions

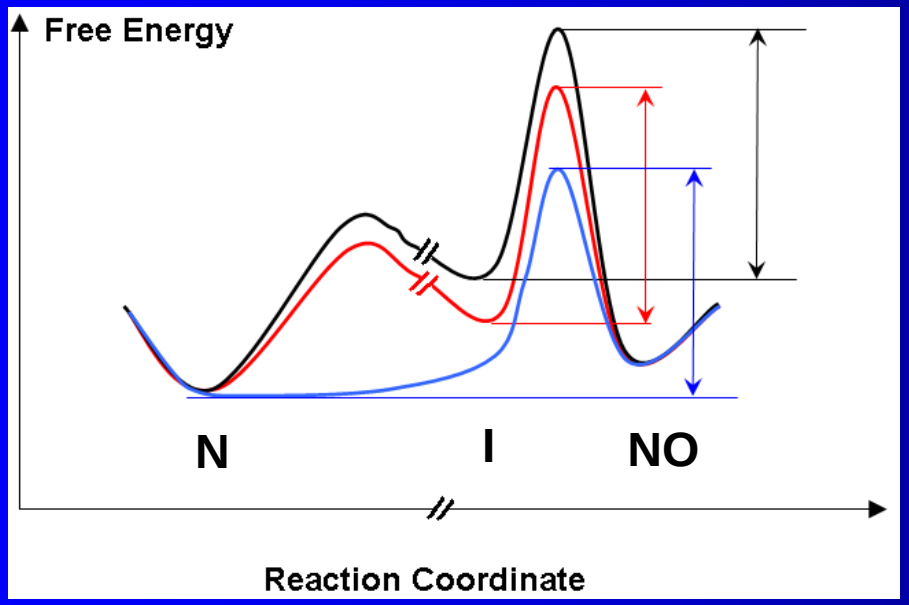
A Phenomenological Model

One of the several models we developed



$$O + N \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} I$$

$$I \xrightarrow{k_{int}} NO$$



More buried met

More exposed met

Met in peptides

Structural effect is an activated process

Expression for Rate Constant

Use equilibrium condition, mass balance and kinetic expressions

$$k_{apparent} == \frac{1}{[O]_0} \frac{Ae^{-\frac{\Delta E^{\dagger}}{RT}}}{1+\frac{c^{\varnothing}}{[O]_0} e^{\frac{\Delta G^{(l)}}{RT}}}$$

A pre-factor

$[O]_0$ initial oxidant concentration

c^{Φ} standard concentration

$\Delta E^{\dagger}$ intrinsic reaction free energy barrier

$\Delta G^{(l)}$ Gibbs free energy change of local structural change

Classification of structural effect

No structural dependence when $\Delta G^{(l)} << RT$

$$k_{apparent} = A' e^{-\frac{\Delta E^{\dagger}}{RT}}$$

Local structural dependence when $\Delta G^{(l)} \sim RT$

Global structural dependence when $\Delta G^{(l)} >> RT$

$$k_{apparent} = A'' e^{-\frac{\Delta E^{\dagger}+\Delta G^{(l)}}{RT}} = A'' e^{-\frac{\Delta E_{apparent}}{RT}}$$

Can be simplified into Arrhenius equation

Only when temperature is near the local T_m, structural effect results in non-Arrhenius

Conclusions

- New Strategic Approach: Molecular QbD for Integration of Discovery, Development, and Manufacturing. Objective: reduce over all time from Discovery to Market Delivery
- Areas of Impact:
  - Discovery
    - Developability/Manufacturability
      - Aggregation
      - Oxidation
      - Deamidation
      - Fragmentation
    - Payload Conjugation
  - Development

Conclusions

- Areas of Impact:
  - Discovery
    - Developability/Manufacturability
      - Aggregation
      - Oxidation
      - Deamidation
      - Fragmentation
    - Payload Conjugation
  - Development
    - Formulation
    - Stability modeling

MIT Summer Professional Course

July 12-14 MIT Short Course on Formulation and Stabilization of Biotherapeutics

http://web.mit.edu/professional/short-programs/courses/formulation_stabilization_biotherapeutics.html

66