

**Favorable Entropic Influence of Urea
Suppresses Thermal Unfolding-Mediated
Aggregation of rhIL-1R (type II)**

**Richard L. Remmele, Jr.
Scientific Director
Pharmaceuticals**

Amgen, Inc.

**One Amgen Center Drive, Thousand Oaks, CA
91320-1799**

Overview

- Hypothesis of Urea Stabilization
 - Hydrophobic Effect
 - Second Law of Thermodynamics
- Unfolding Properties of the Protein
 - Behavior in the presence of 2 M urea
 - Evidence for Endothermic Heat in the aggregation process
 - Thermal Irreversibility increases with ΔH_{agg}
 - Endothermic heat alligns with massive aggregation
 - No exothermic heat evolution
- Calculation of Solvent Entropy
 - Transfer free energies using a surrogate hydrocarbon molecule (Toluene) in 2 M urea and H₂O
 - Microcalorimetric measurment of Cp
- Proposed Mechanism of urea stabilization
- Applications to Formulation Development

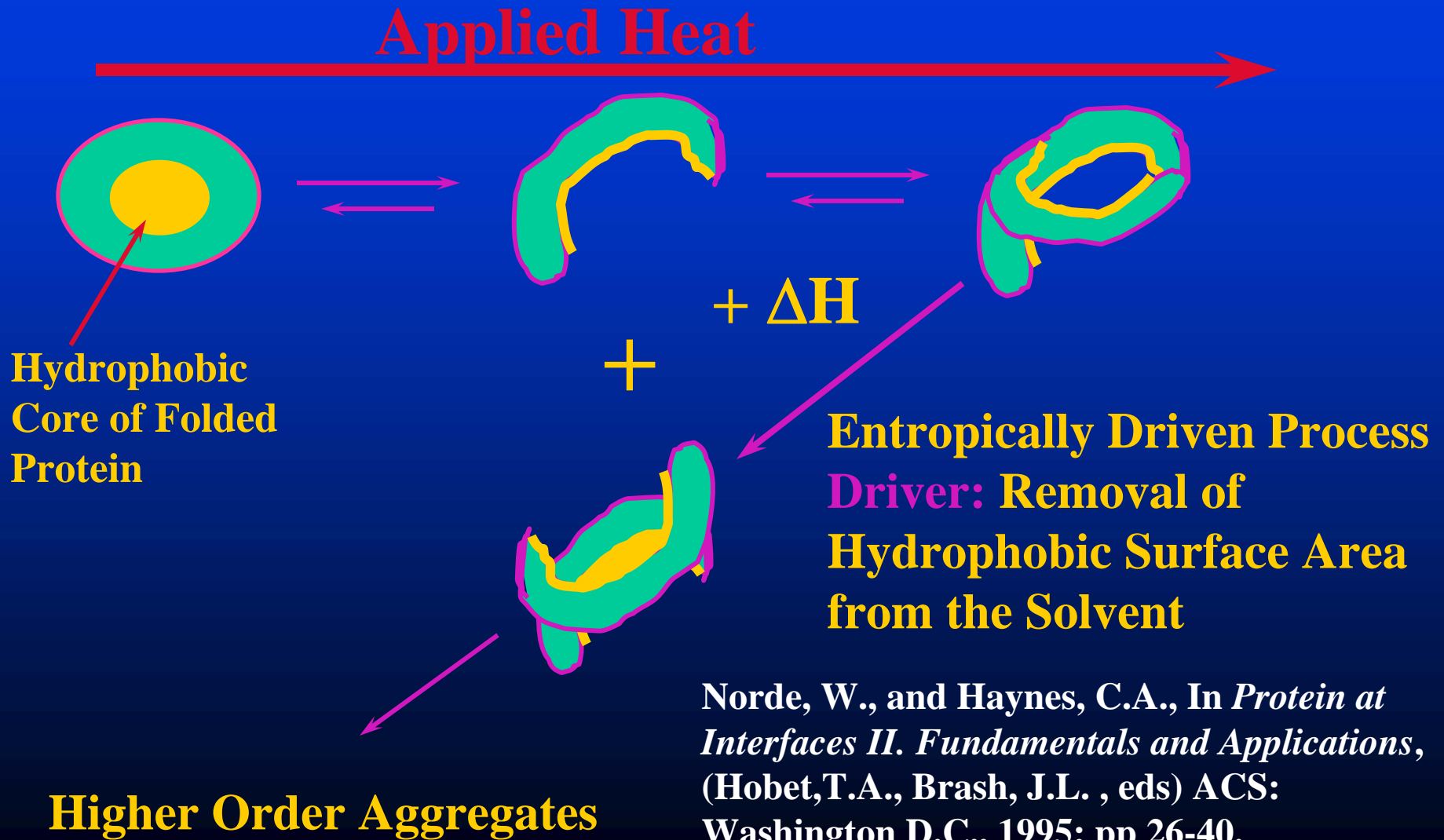
Working Hypothesis

- **Suppose Aggregation:**
 - Unfolding-Mediated
 - Entropy- Driven
 - Governed by the Hydrophobic Effect
- **Then:**
 - Protein-Protein Hydrophobic Interactions Dominate the Reaction Process
 - Entropy of the Solvent should play a Key Role

Hypothesis

“Compounds that Increase the Entropy of the Solvent in the Presence of Hydrophobic Surface (i.e., unfolded protein) Should Result in Higher Thermal Reversibilities”

Potential Role of Urea



Norde, W., and Haynes, C.A., In *Protein at Interfaces II. Fundamentals and Applications*, (Hobet, T.A., Brash, J.L., eds) ACS: Washington D.C., 1995; pp 26-40.

Observations From the Literature

- “Solvation entropy of nonpolar groups is much less negative in aqueous urea solutions than in water” – Brandts, J.F. (1964) *JACS* 86, 4302-4314
- The range from 0-6.9 M urea has been analyzed to show that sub-denaturing concentrations of urea stabilize the protein – Bhuyan (2002) *Biochemistry* 41, 13386-13394
- Urea prevents aggregation when present at low concentration in refolding buffer – Edwin et al. (2002) *Biochem. Biophys. Res. Commun.* 290, 1441-1446

Mechanism ?

- **Paradox: Currently there is no mechanism to describe the stabilization effects of urea at low concentrations (sub-denaturing) while destabilization effects of urea occur at relatively high concentrations**

Most Common Explanations of Denaturation:

- 1) Preferential Binding to the Protein Resulting in Unfolding**
- 2) Solubilization of the Hydrophobic Amino Acid Residues**

Properties of Urea at Low Concentration

- In most cases, urea binding to proteins is very weak within the 0 to 2 M concentration range.
 - ✓ Tsumoto, K., Ejima, D., Kumagai, I., and Arakawa, T. (2003) *Protein Expression and Purification* 28; 1-8.
- Urea Preferentially Interacts with Water (solvent) increasing the Solvation Entropy.
 - ✓ Brandts, J. (1964) *JACS* 86; 4302-4314.
 - ✓ Zou, Q. et al., (2002) *JACS* 124; 1192-1202.
 - ✓ Nandel, F.S. et al., (1998) *Pure & Appl. Chem.* 70; 659-664.
- Urea structure geometry reported to disrupt the cage-like structure that normally forms around nonpolar groups.
 - ✓ Barone, G., and Giancola, C. (1990) *Pure & Appl. Chem.* 62; 57-68.

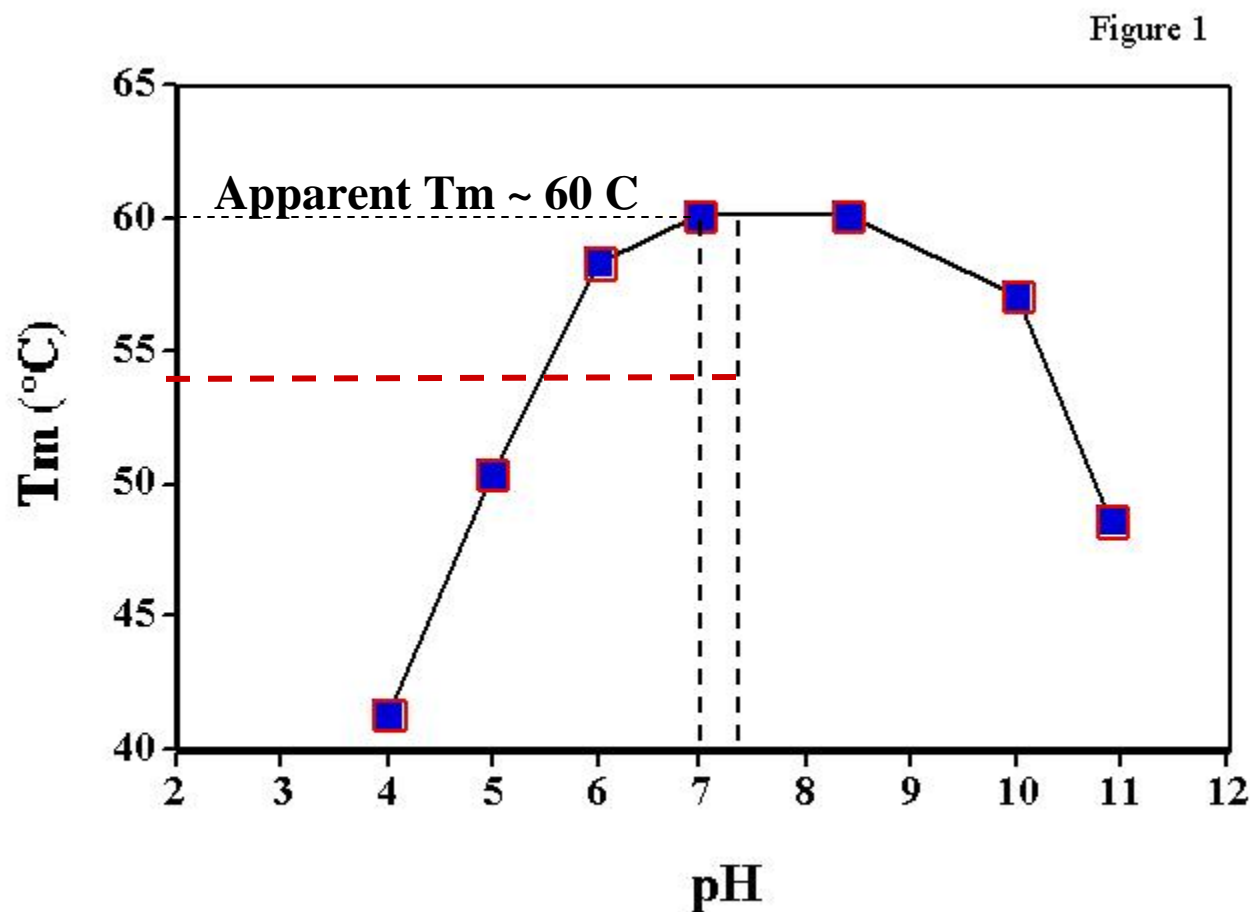
Properties of rhIL-1R (II)

- **333 amino acids**
- **Polypeptide Mw ~ 38 kDa**
- **Glycosylation (~ 20%)**
- **Predominant β -sheet Structure (~ 32%)**
- **pI ~ 3 to 7**
- **Soluble at pH 7.4**
- **C-terminus Proteolytic Sites**
- **Binds to Neutralize IL-1 β**
- **Anti-inflammatory Agent**

Microcalorimetry Experimental Conditions

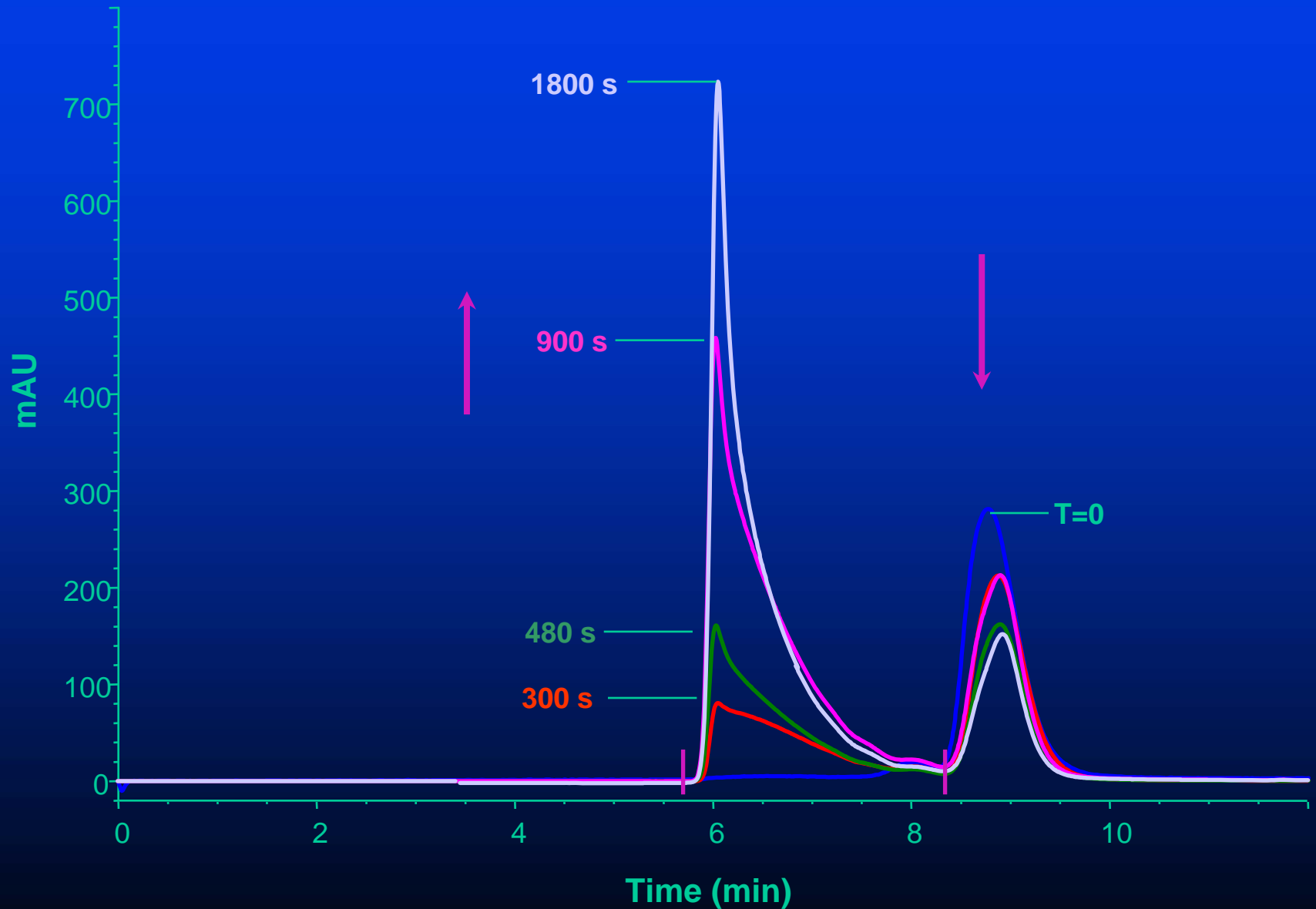
- **MicroCal vp-DSC (upscan rates of 0.25, 0.5, 1.0, and 1.5 C/min)**
- **Material: rhIL-1R (II) Mfg by Immunex (now Amgen)**
- **Solutions: PBS (Phosphate Buffered Saline); 100 mM sodium phosphate; 2M urea**
- **pH 7.4**
- **Protein Concentrations: 0.44 and 2 mg/mL**

pH Stability Profile of rhuIL-1R (II)

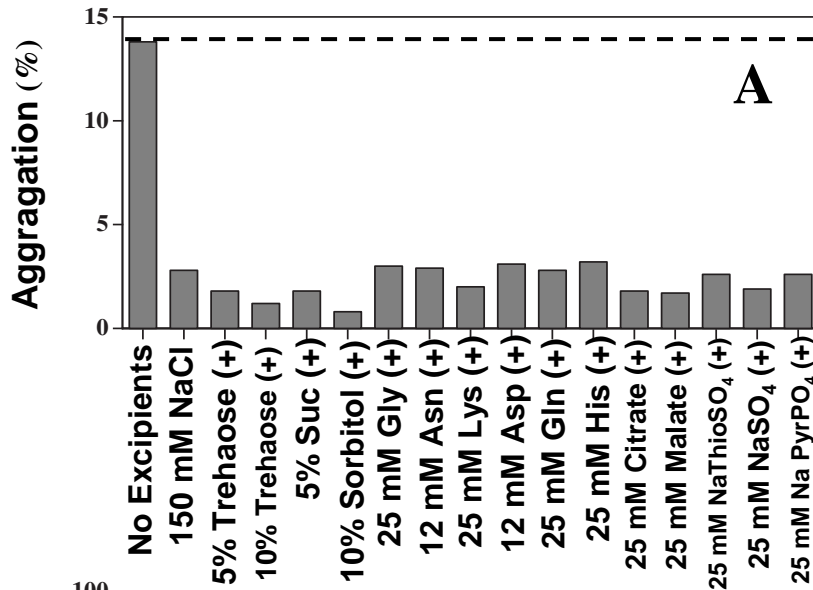


True T_m ~ **54 C** (Remmele et al., (2005) *JACS* 127, 8328-8339)

SEC Results of IL-1R (II) @ 58 °C



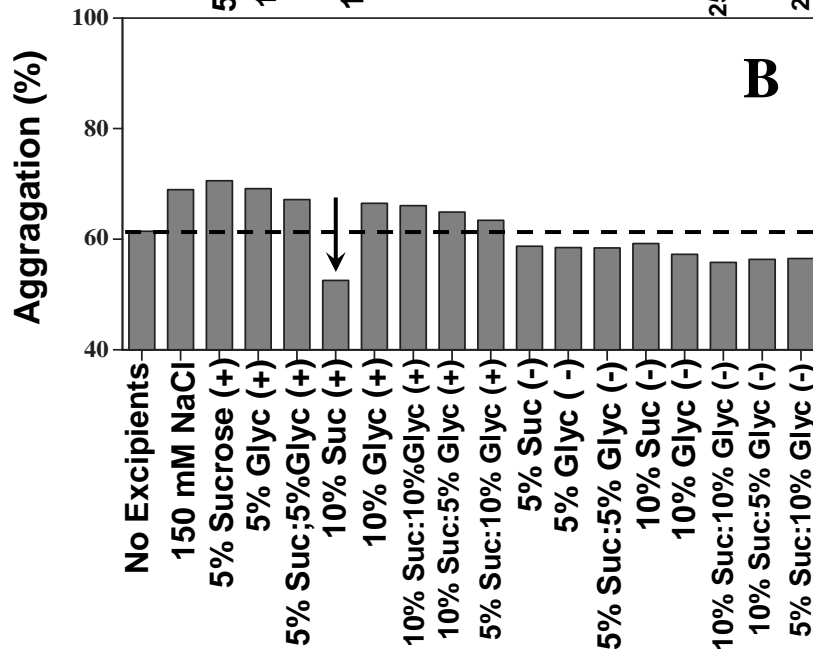
Excipient Screening at 2 mg/mL



6 hr @ 44 C

Below the T_m, NaCl suppresses formation of soluble aggregates

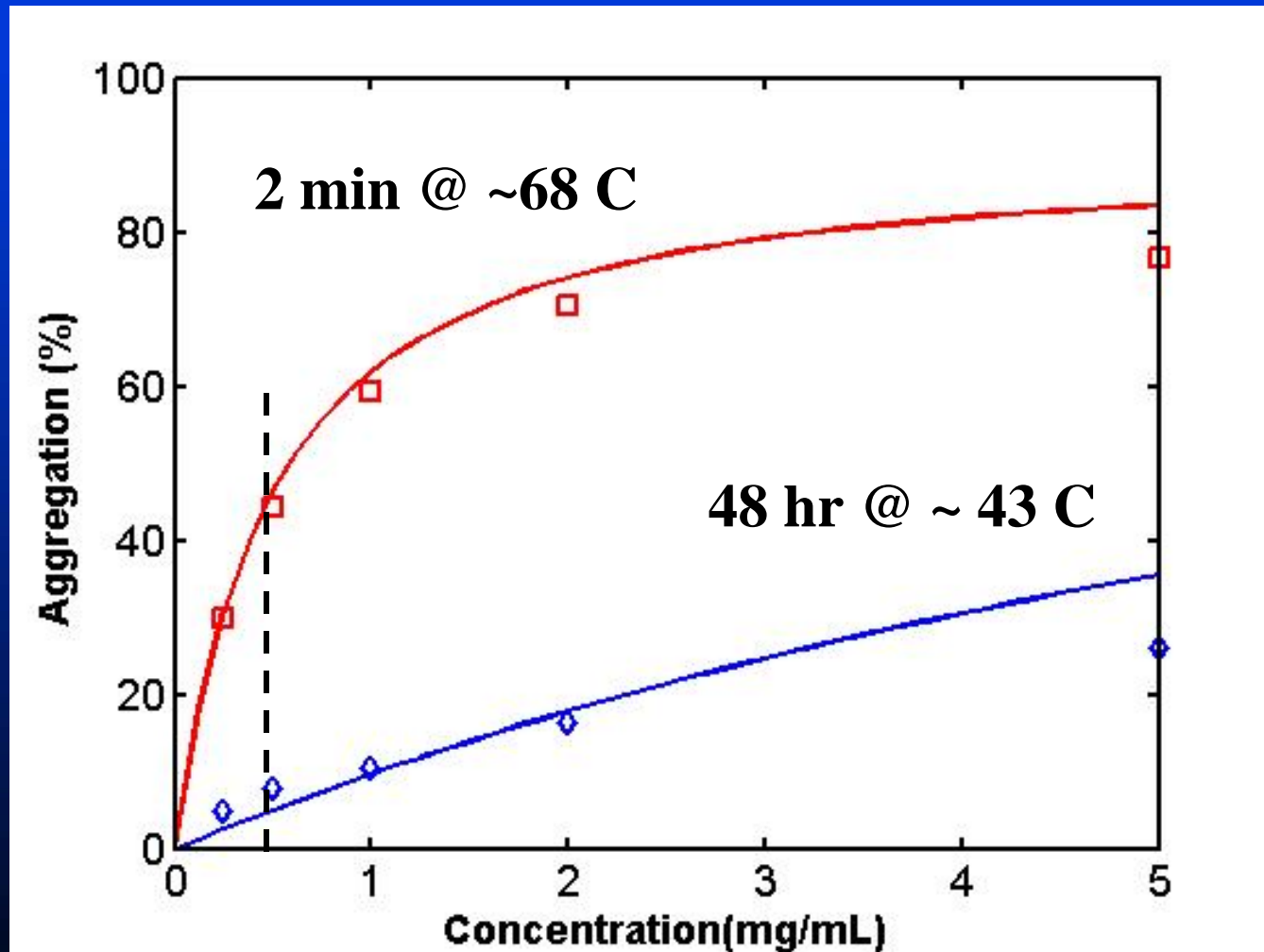
{T_m ~ 54 C}



2 min @ 70 C

Above the T_m, NaCl augments formation of soluble aggregates

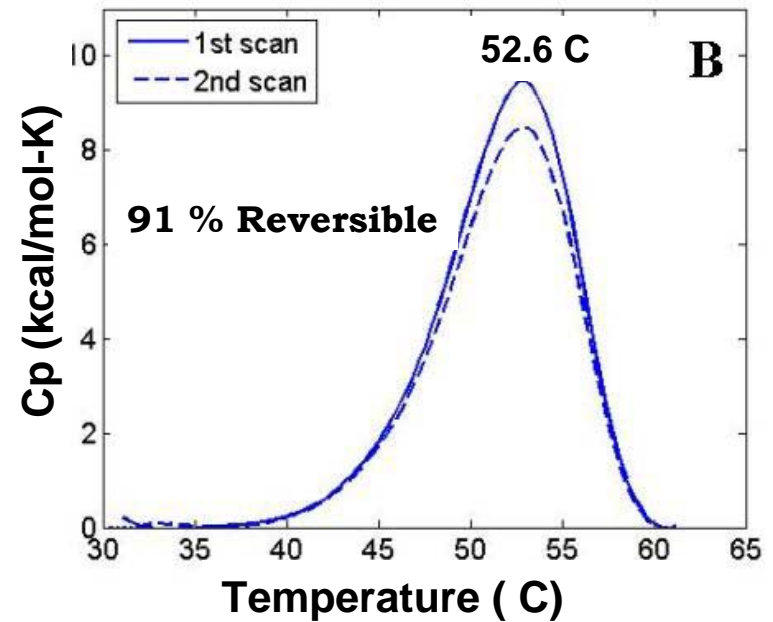
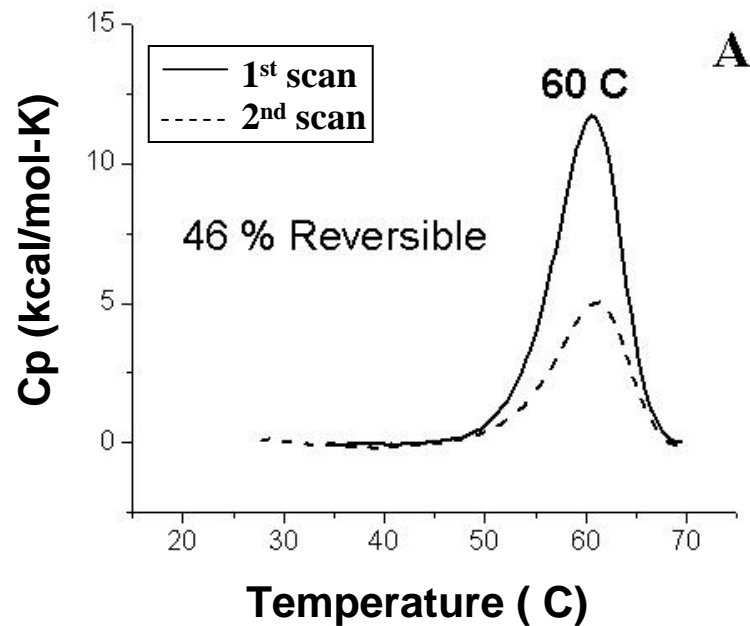
Concentration Dependence of Aggregation



Calorimetric data without and with 2M urea

Figure 3A

Figure 3B



Endotherm that appears to correlate with Aggregation

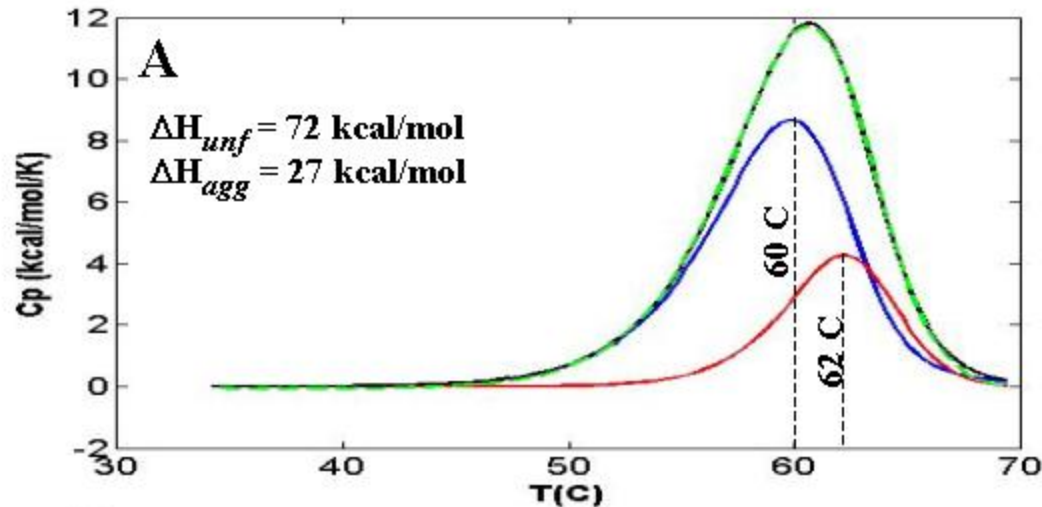
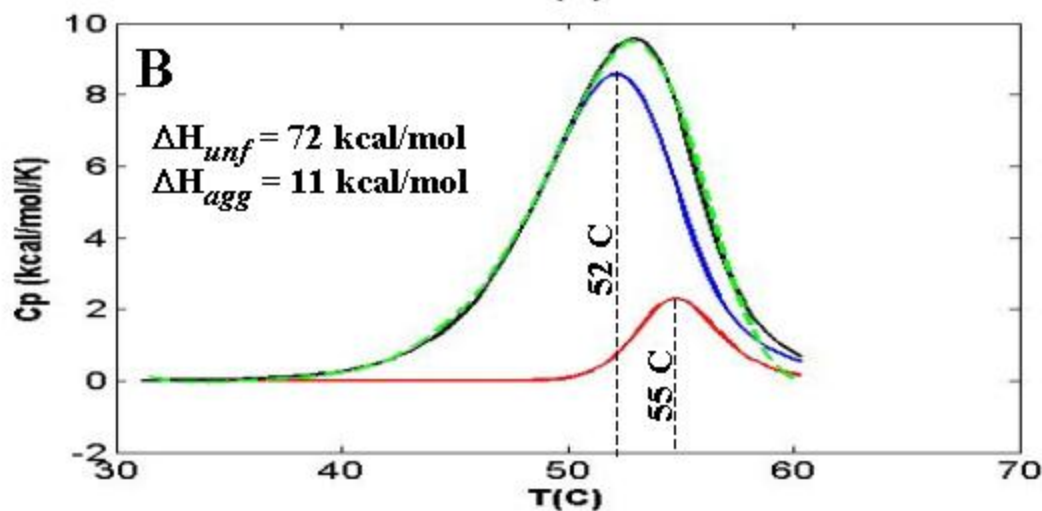


Figure 4

Without Urea

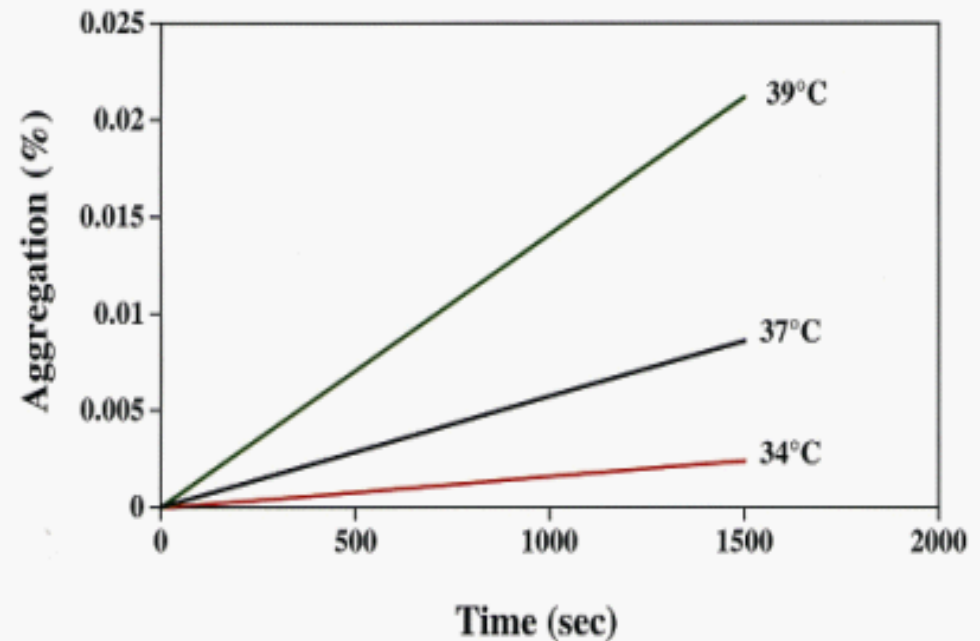
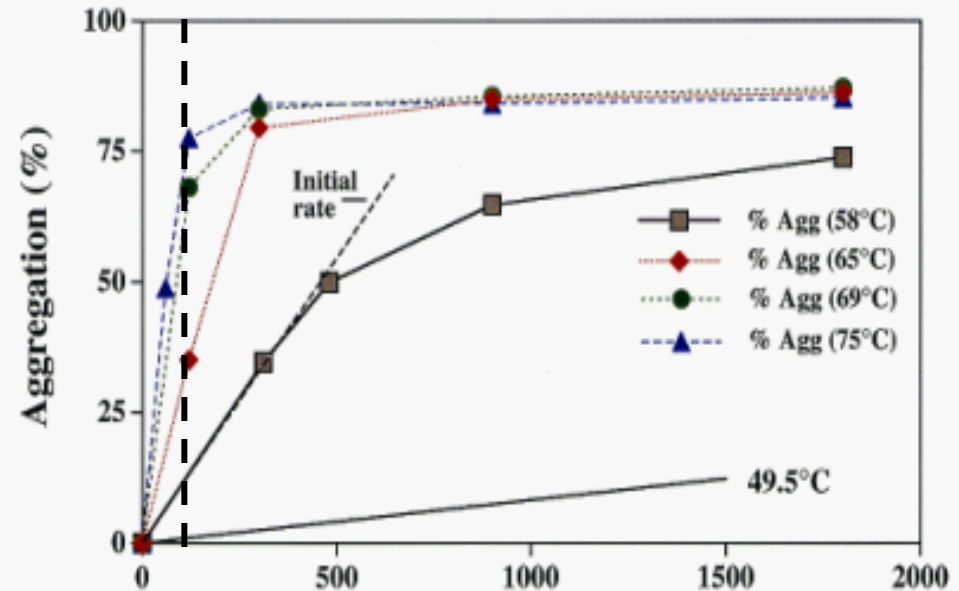


With
2 M Urea

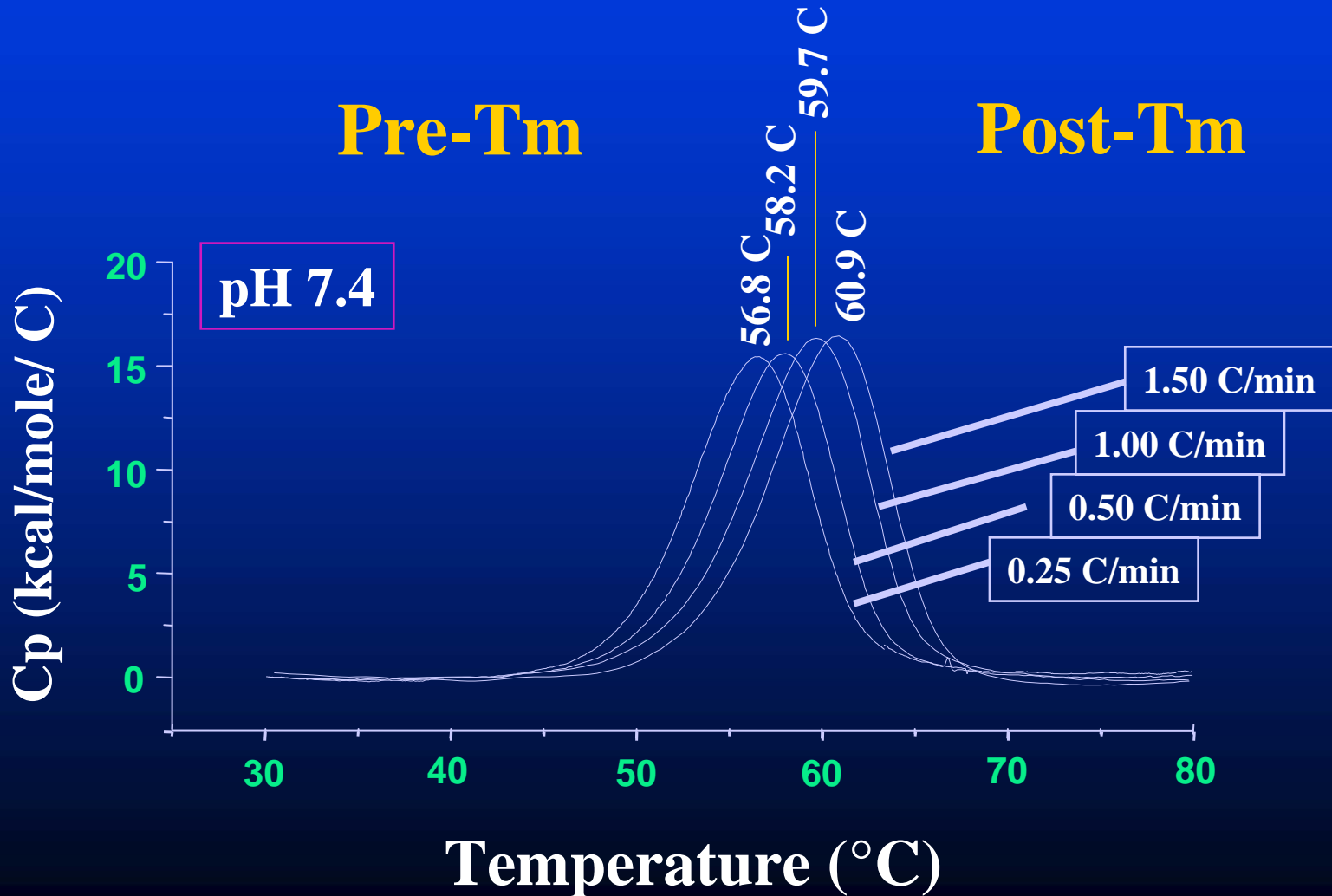
All aggregate forms remain soluble

Kinetic Curves of IL-1R (II)

pH 7.4



DSC Scan-Rate Dependence



Scan-Rate Influences in the Absence of Urea

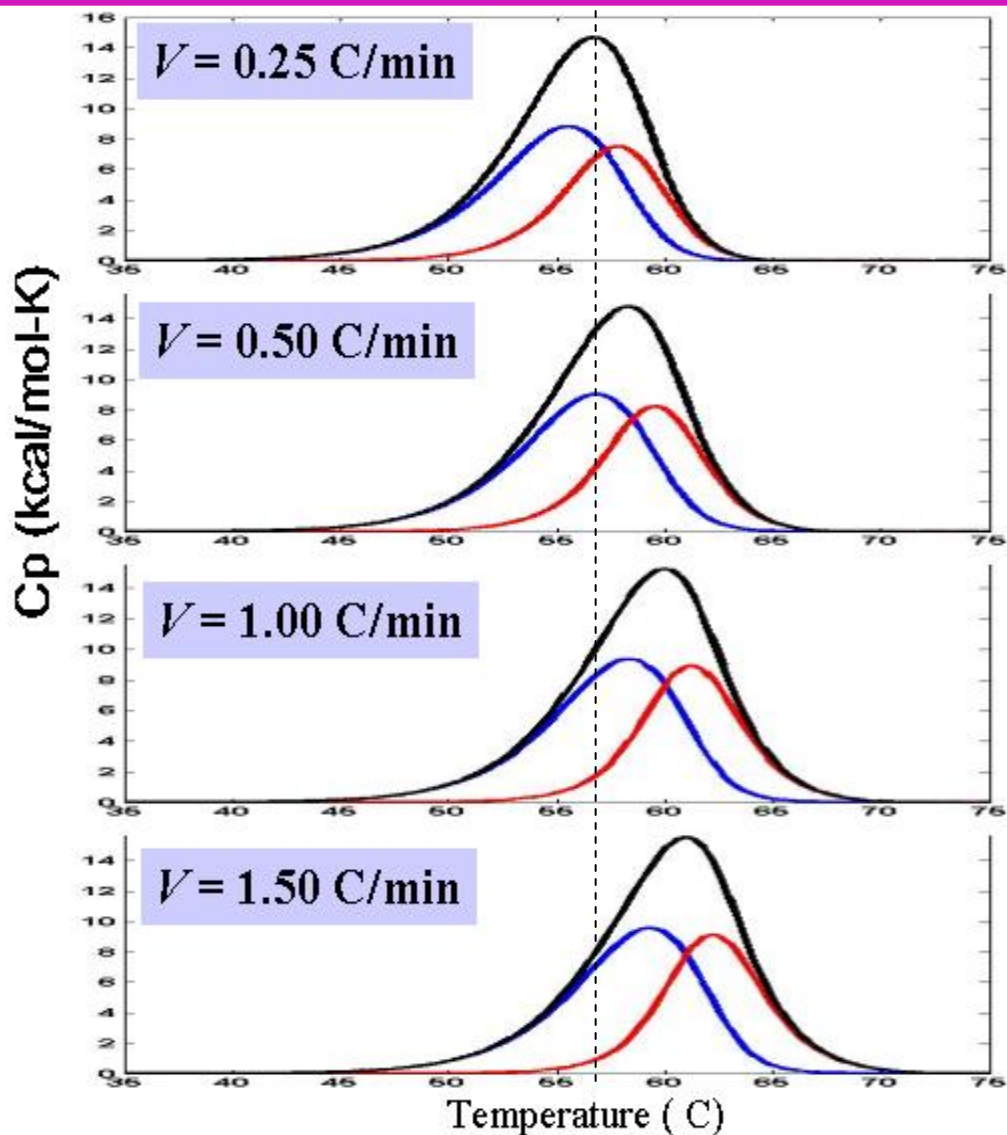


Figure 5

Table I. Parameters of Thermal Irreversibility and Enthalpy of Aggregation

Sample	Irreversibility (%)	ΔH_{agg} (kcal/mole)
w Urea @ 0.44 mg/mL	9 ± 2	11 ± 2
w/o Urea @ 0.44 mg/mL	54 ± 3	27 ± 3
w/o Urea @ 2 mg/mL	80 ± 6	50 ± 5

The Enthalpy of Aggregation Correlates with Thermal Irreversibility!

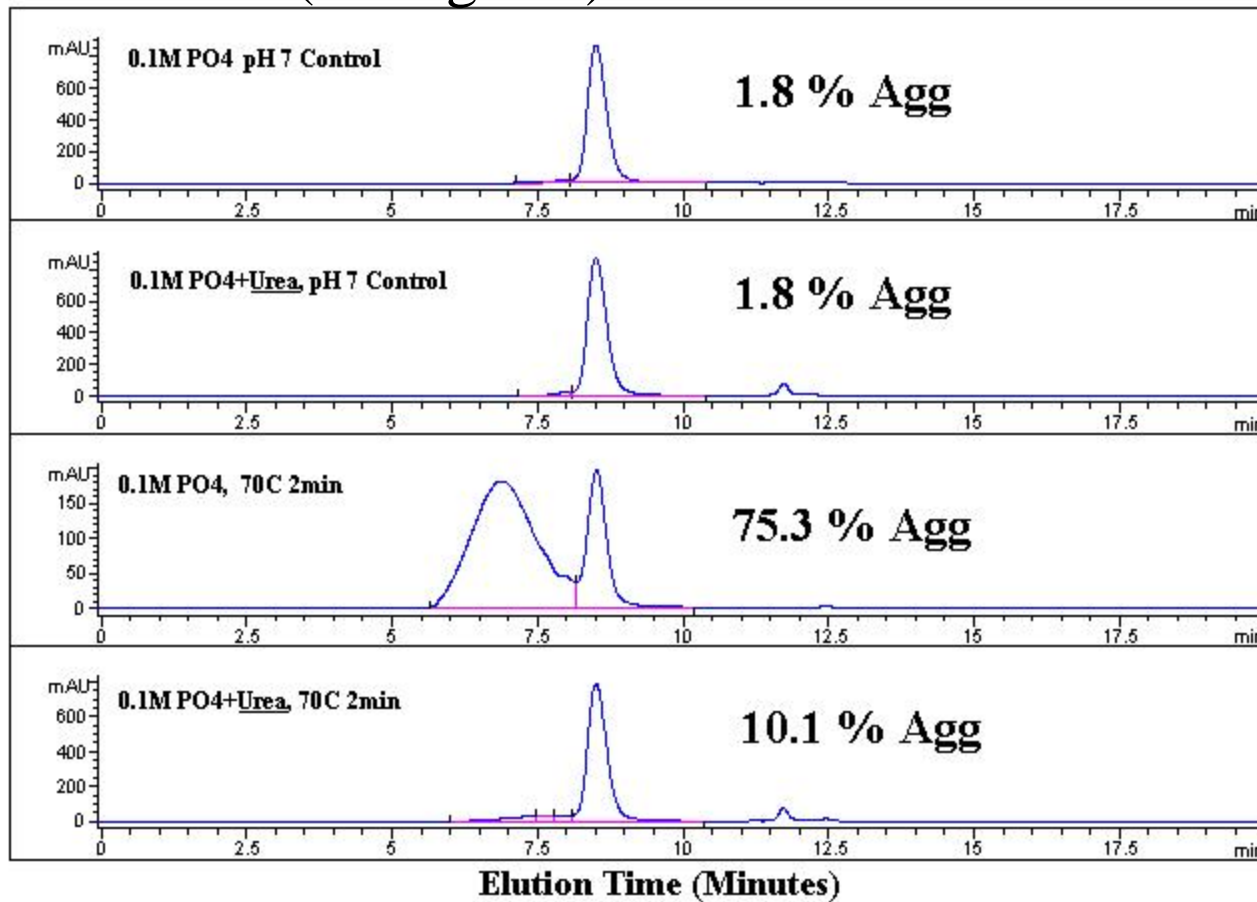
IL-1R (II) Aggregation Governed by Hydrophobic Effect

- Aggregation Reaction is Unfolding-Mediated (massive aggregation occurs above the unfolding transition)
- Endothermic Heat is consistent with a reaction that is “Entropy-Driven”
- Entropy-Driven Reactions are associated with Protein-Protein Hydrophobic Interactions
- The Hydrophobic Effect must be in Play

Size Exclusion Evidence of Aggregate Suppression Above the T_m with 2 M Urea

Figure 6

Protein (~ 2mg/mL) Heated 70 C for 2 min.



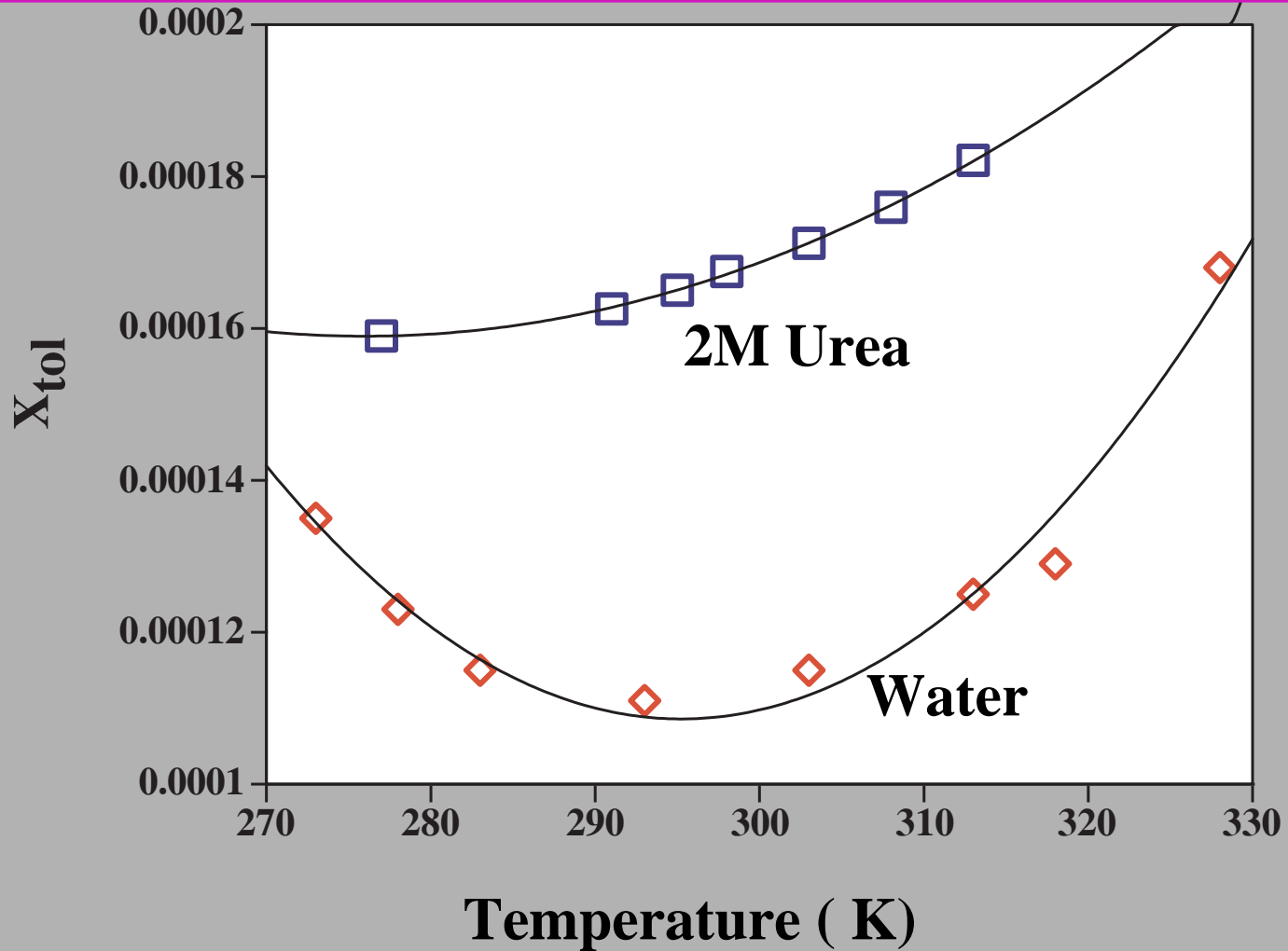
Evaluate Solvent Entropy and Estimate Relative Entropic Force

- Using a surrogate hydrocarbon (toluene) determine in 2 M urea:
 - $\Delta G_{tr} = -RT \ln X_{tol}$
 - $\Delta C_p (T) = C_{p_{tol/urea}}(T) - C_{p_{tol}}(T)$
 - $\Delta H = \Delta C_p (T - T_h)$
 - $\Delta S = (\Delta G_{tr} - \Delta H) / -T$
- Determine relative entropic force:
 - $E_f = \Delta S_{tol/urea} / \Delta S_{tol/w}$

Equilibrium Solubilities

- **Measured Spectrophotometrically in a constant temperature cell at 260 nm**
 - Calibration using standard compositions of toluene volumetrically prepared in n-ButOH
 - Sealed Cuvette Method (Smith, R.R. et al., (1989) *J. Phys. Chem.* 93; 5938-5943)
- **Solubilities of Toluene in Water obtained from: IUPAC, Solubility Data Series Vol 37 (1989); Hydrocarbons with Water and Seawater Part I: Hydrocarbons C5 to C7; Pergamon Press, New York.**
- **Densimeter measurements at temperature (Anton Paar DMA 5000)**

Solubility in 2M Urea



Heat Capacity of Toluene in 2M Urea Solution

- **Determined calorimetrically:**
 - $C_{p_{\text{tol/urea}}} = C_{p_{\text{urea}}}(v_2/v_1) - \Delta C_{p,\phi}/m$
 - $\Delta C_{p,\phi}$ → microcalorimetry measured
 - $C_{p_{\text{urea}}}$ → specific heat capacity of 2M urea
 - v_1 → molar specific volume of 2M urea
 - v_2 → partial specific volume of toluene
- **Use existing data for $C_{p_{\text{urea}}}$**
 - Hakin, A.W. et al.,(1996) *J. Chem Soc., Faraday Trans.* 92; 207-214

Determination of Partial Specific Volume of Toluene in 2M urea

- **Equation:**

- $v_2 = [1 - (\rho / \rho_1) + (m / \rho_1 V)] (V/m)$
- $V =$ matched cell volume (0.51961 mL)
- $\rho =$ density of the tol/urea solution
- $\rho_1 =$ density of 2M urea
- $m =$ mass of solute in cell (grams)

Note: this equation can also be used to determine v_1 (partial specific volume of 2M urea)

Calculated ΔC_p

- Equation:

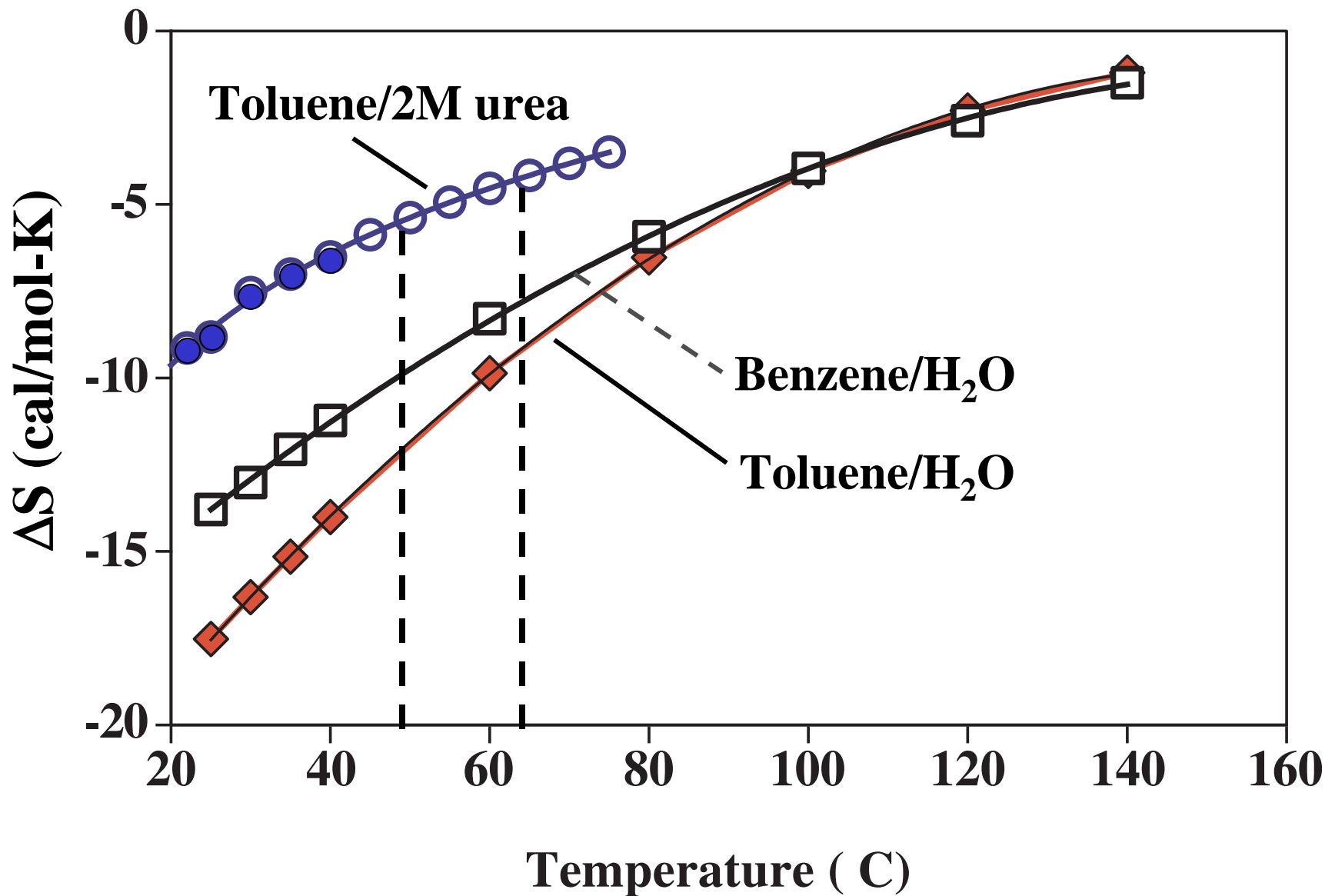
$$\Delta C_p = C_{p_{\text{tol/urea}}} - C_{p_{\text{tol}}}$$

- Use existing data for pure toluene heat capacity ($C_{p_{\text{tol}}}$)
 - Makhatadze, G.I., & Privalov, P.L. (1988) *J. Chem. Thermodynamics* 20; 405-412

Thermodynamic Quantities

T (K)	Sol $X_{\text{tol}} \times 10^{-4}$	ΔG_{tr} cal/mol	ΔH cal/mol	ΔC_p cal/mol-K	ΔS cal/mol-K	ΔS_w cal/mol-K	E_f
291	1.626	5045	(2106)	(150)	(-10.1)	(-19.4)	0.51
295	1.650	5106	2417	134.3	-9.1	(-18.3)	0.50
298	1.675	5149	2526	120.3	-8.8	-17.5	0.50
303	1.712	5222	2941	113.1	-7.5	-16.3	0.46
308	1.760	5291	3135	119.4	-7.0	-15.1	0.46
313	1.821	5356	3321	92.2	-6.5	-14.0	0.46
318	(1.892)	(5417)	(3541)	(86.4)	(-5.9)	-12.9	0.46
323	(1.974)	(5475)	(3731)	(81.1)	(-5.4)	-11.8	0.46
333	(2.180)	(5579)	(4081)	(72.9)	(-4.5)	-9.9	0.45
343	(2.452)	(5666)	(4363)	(66.1)	(-3.8)	-8.1	0.47
348	(2.619)	(5703)	(4485)	(63.2)	(-3.5)	-7.3	0.48

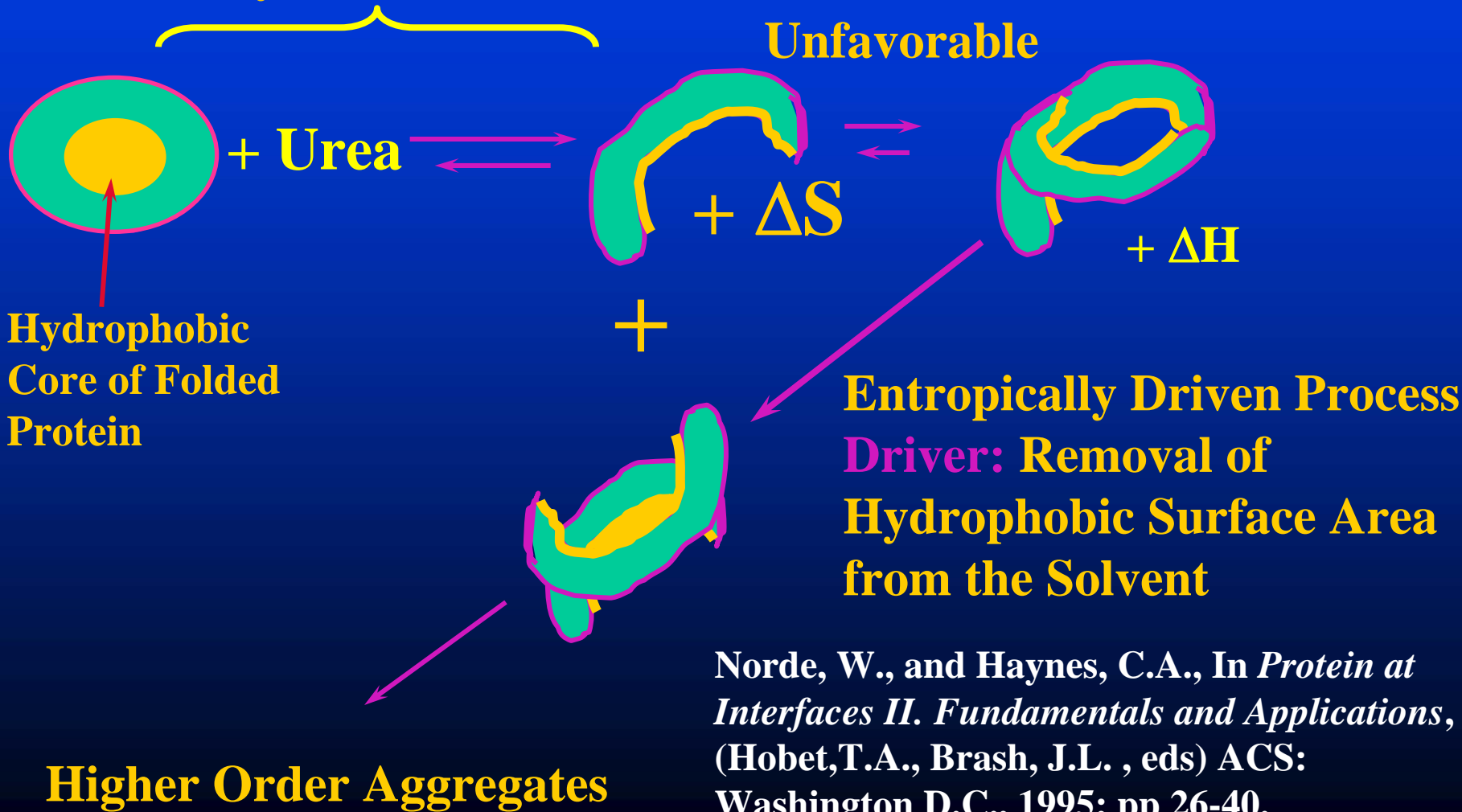
Solvent Entropies



Proposed Mechanism

Applied Heat

Thermodynamic Renaturation



Norde, W., and Haynes, C.A., In *Protein at Interfaces II. Fundamentals and Applications*, (Hobet, T.A., Brash, J.L., eds) ACS: Washington D.C., 1995; pp 26-40.

Conclusions

- Aggregation pathway has been shown to depend upon protein unfolding
- Additional endothermic heat correlates with massive aggregation and ΔH_{agg} on the high temp side of unfolding endotherm
- An Entropy driven process can explain the endothermic heat observed from the aggregation reaction
- Toluene probe studies confirm an increase in solvent entropy in the temperature regime where the unfolding of the protein occurs in 2M urea
- A significant reduction in propensity to form aggregate can be explained by the increase in solvent entropy where unfolded protein predominate (proposed mechanism)

Application

- **Would excipient compounds that afford greater thermal reversibility in systems that follow an unfolding-mediated aggregation process be better stabilizers?**
- **Would such stabilizers increase solvation entropy of the solution?**

Acknowledgements

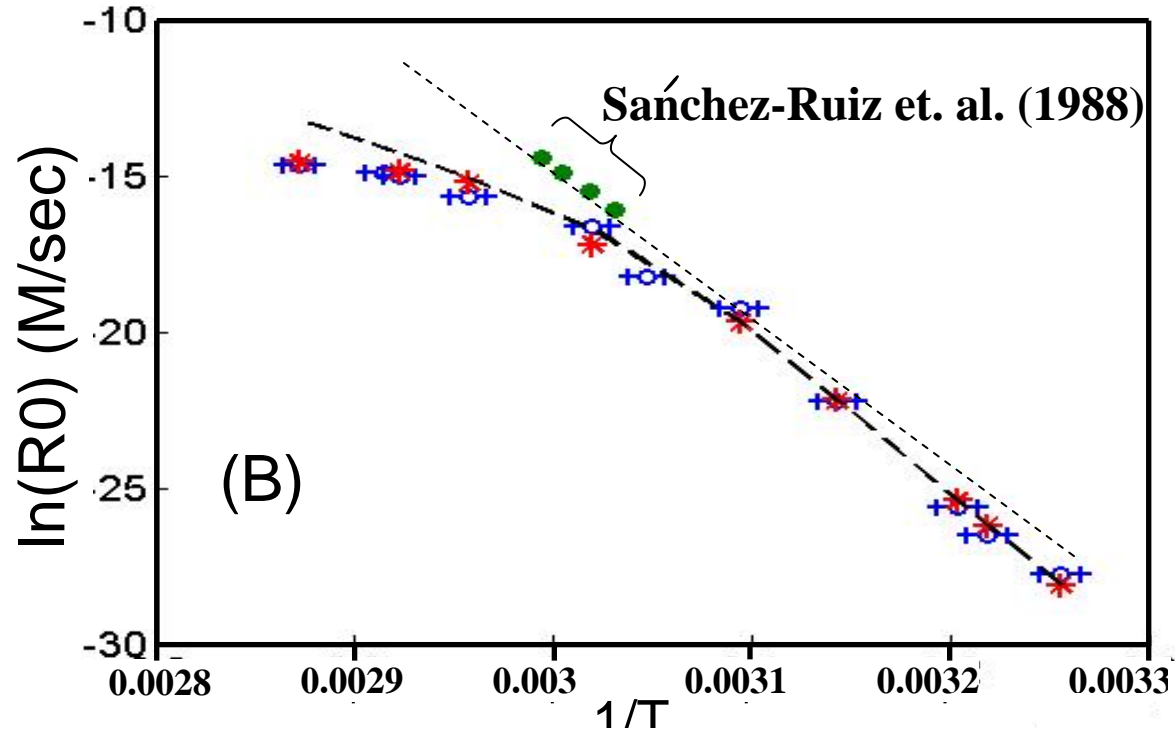
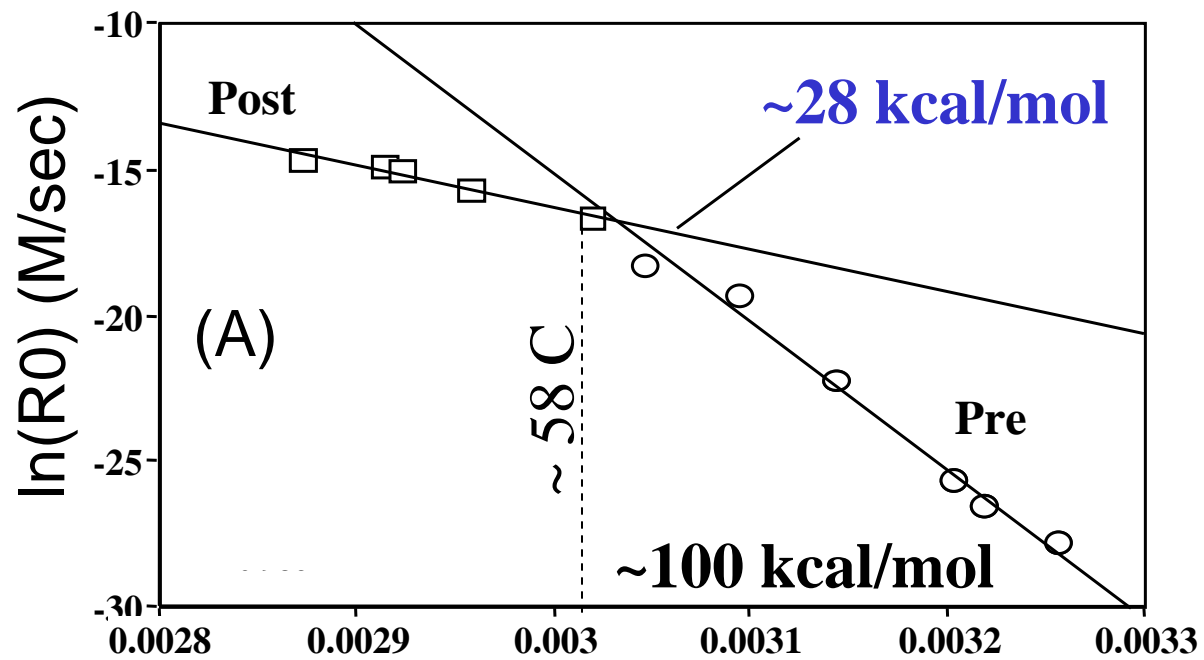
Amgen

- Dr. Jian Zhang-van Enk (Systems Informatics, T.O.)
- Duke Phan (Analytical Sciences, AWA)
- Bryan (Lei) Yu (Pharmaceutics, T.O.)

Penn State College of Medicine

- Professor George Makhatadze

Back-up Slides



Reference: Remmele
et al., (2005) *JACS*
127, 8328-8339

Dielectric Effect?: Toluene in H2O and Butanol

