

Fluorescence-based thermal shift assays

Thermal shift assay

ThermoFluor®

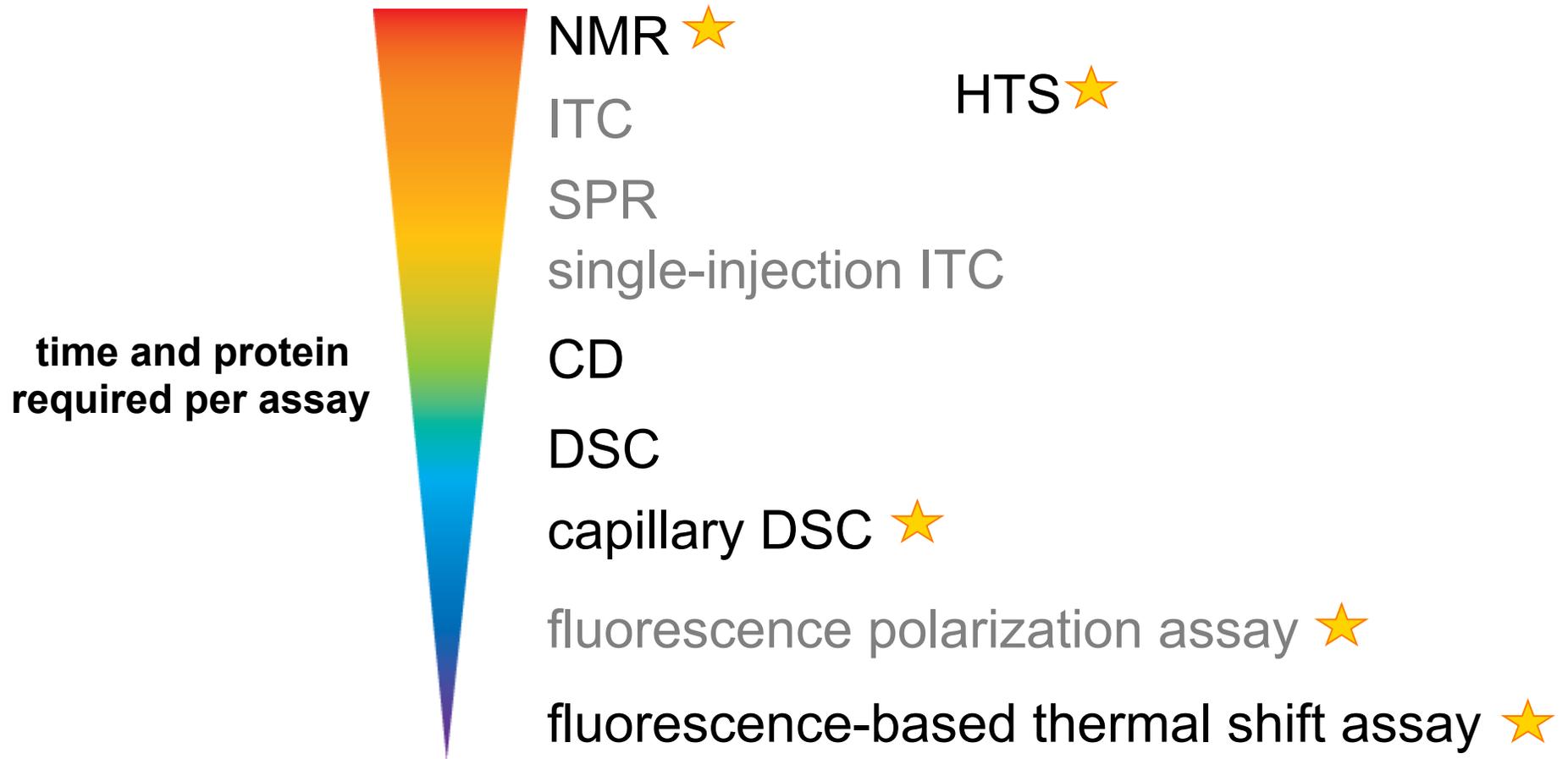
Differential scanning fluorimetry (DFS)

Carrie Partch

Dept. of Chemistry and Biochemistry

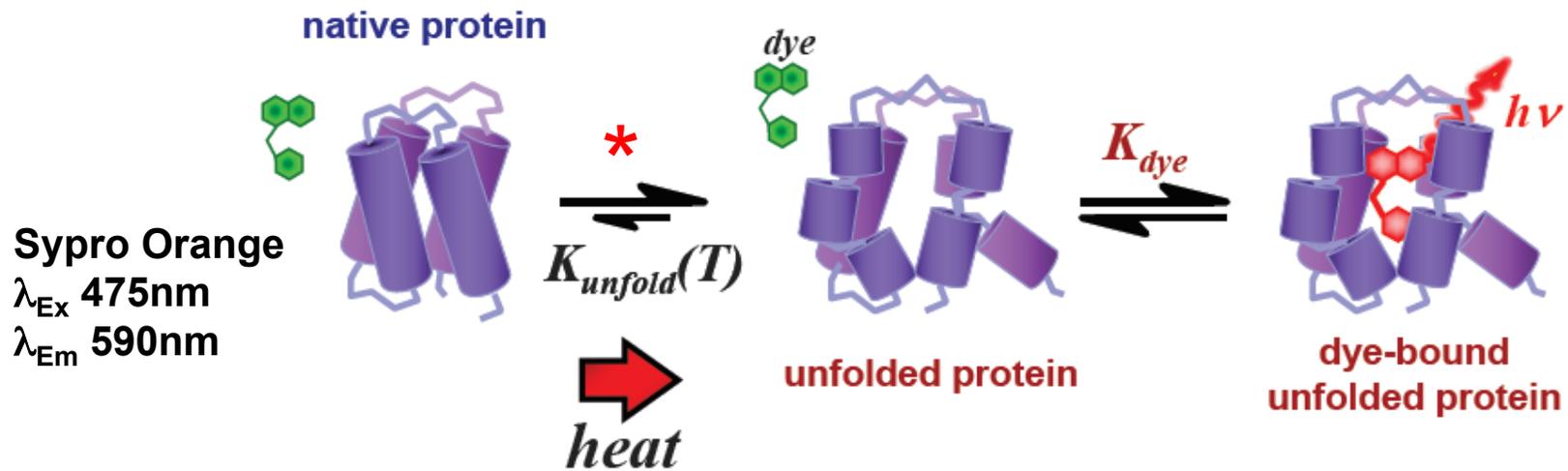
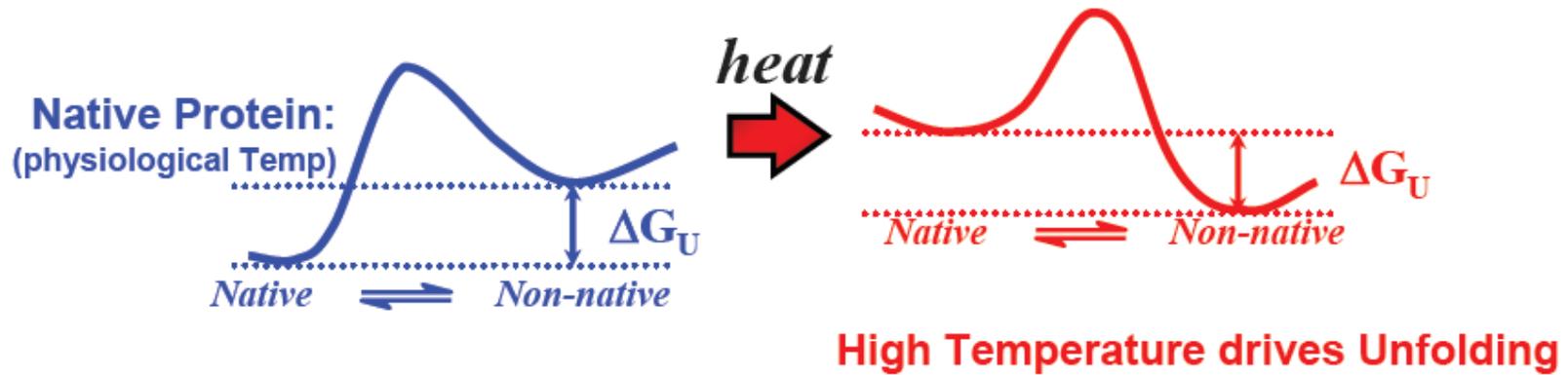
UC Santa Cruz

Common methods for characterizing/identifying ligand binding and/or protein stability



variable: functional/phenotypic ligand screening assays

Fluorescence-based thermal shift assay

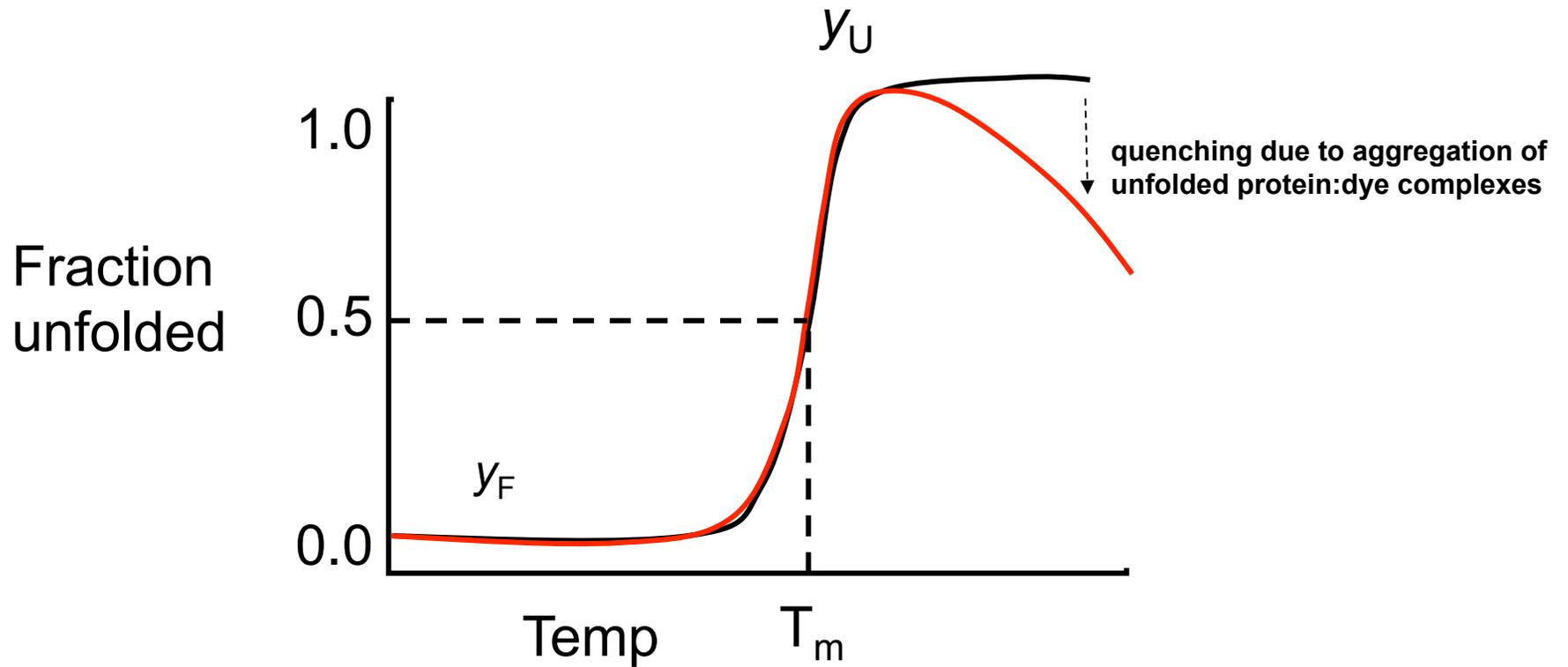


J. Kranz

Johnson & Johnson Pharmaceutical Research & Development

Fitting denaturation curves by nonlinear least-squares regression

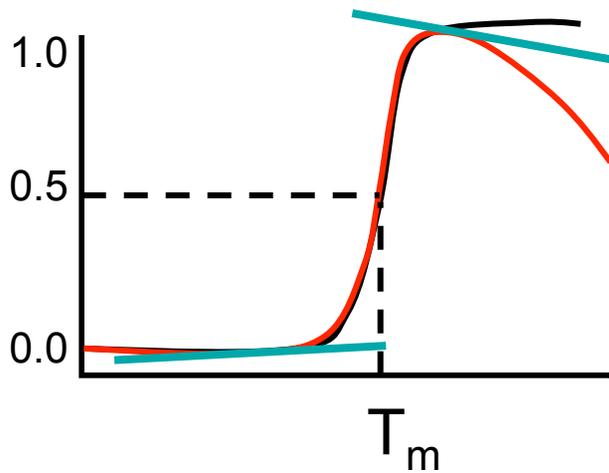
$$y(T) = y_F + \frac{y_U - y_F}{1 + e^{\Delta_U G(T)/RT}} = y_U + \frac{y_F - y_U}{1 + e^{-\Delta_U G(T)/RT}} \quad (1)$$



Obtaining thermodynamic parameters from denaturation curves

$$K_{eq} = e^{\frac{-\Delta_U G_T}{RT}} = e^{\frac{-\left(\Delta_U H_{T_r} + \Delta_U C_p(T - T_r) - T\left(\Delta_U S_{T_r} + \Delta_U C_p \ln \frac{T}{T_r}\right)\right)}{RT}}$$

$$y(T) = y_{F, T_m} + \overbrace{m_F(T - T_m)} + \frac{y_{U, T_m} - y_{F, T_m} + \overbrace{(m_U - m_F)(T - T_m)}}{1 + e^{(\Delta_U H_{T_r} + \Delta_U C_p(T - T_r) - T(\Delta_U S_{T_r} + \Delta_U C_p \ln(T/T_r)))/RT}}$$

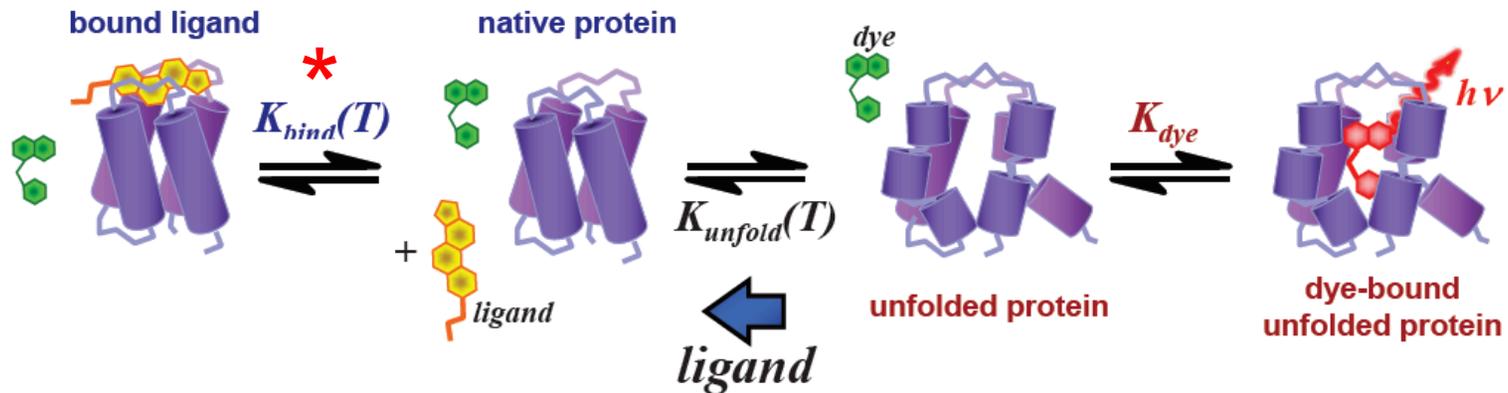
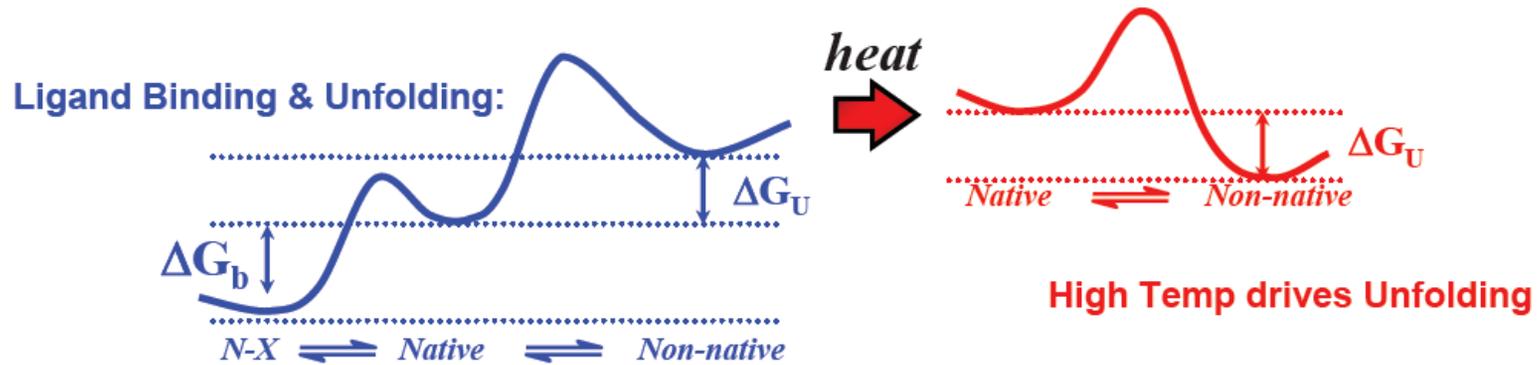


at T_m : Unfolded = Folded
 $K_{eq} = 1$ and $\Delta G^\circ = 0$

$$y(T) = y_F + \frac{y_U - y_F}{1 + e^{\Delta_U G(T)/RT}}$$

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Ligand binding shifts unfolding equilibrium towards native protein



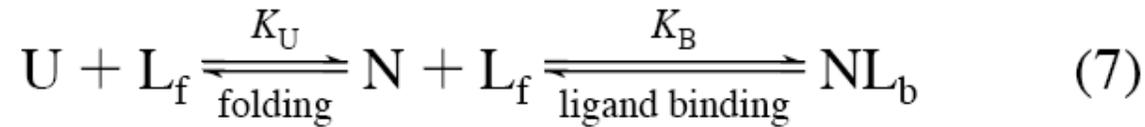
In general, ligand binding increases T_m

Brandts, J. and Lin, L. (1990) *Biochemistry* 29:6927-6940

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Equilibria: unfolding vs. ligand binding



$$K_U = \frac{[U]}{[N]} = e^{-\Delta_U G(T)/RT} = e^{-(\Delta_U H(T) - T\Delta_U S(T))/RT} =$$

$$e^{-(\Delta_U H_{T_r} + \Delta_U C_p(T - T_r) - T(\Delta_U S_{T_r} + \Delta_U C_p \ln(T/T_r)))/RT} \quad (9)$$

$$K_b = \frac{[NL_b]}{[N][L_f]} = e^{-\Delta_b G(T)/RT} = e^{-(\Delta_b H(T) - T\Delta_b S(T))/RT} =$$

$$e^{-(\Delta_b H_{T_0} + \Delta_b C_p(T - T_0) - T(\Delta_b S_{T_0} + \Delta_b C_p \ln(T/T_0)))/RT} \quad (10)$$

Making things complicated...

Describing free/bound-folded and unfolded protein, free/bound ligand

$$P_t = [N] + [U] + [NL_b] \quad (11)$$

$$L_t = [L_f] + [NL_b] \quad (12)$$

Total concentration of ligand needed to stabilize a protein

$$L_t = (1 - K_U) \left(\frac{P_t}{2} + \frac{1}{K_U K_b} \right) \quad (13)$$

$[L_t]$ needed to raise T_m to a given value:

$$L_t = (1 - e^{-(\Delta_U H_{T_r} + \Delta_U C_p(T - T_r) - T(\Delta_U S_{T_r} + \Delta_U C_p \ln(T/T_r)))/RT}) \times \left(\frac{P_t}{2} + 1/e^{-(\Delta_U H_{T_r} + \Delta_U C_p(T - T_r) - T(\Delta_U S_{T_r} + \Delta_U C_p \ln(T/T_r)))/RT} e^{-(\Delta_b H_{T_0} + \Delta_b C_p(T - T_0) - T(\Delta_b S_{T_0} + \Delta_b C_p \ln(T/T_0)))/RT} \right) \quad (14)$$

Equation 14 cannot be solved explicitly!

Thermodynamic Stability of Carbonic Anhydrase: Measurements of Binding Affinity and Stoichiometry Using ThermoFluor

Daumantas Matulis,[‡] James K. Kranz, F. Raymond Salemme, and Matthew J. Todd*

Johnson & Johnson Pharmaceutical Research & Development, LLC, Eagleview Corporate Center, 665 Stockton Drive, Suite 104, Exton, Pennsylvania 19341

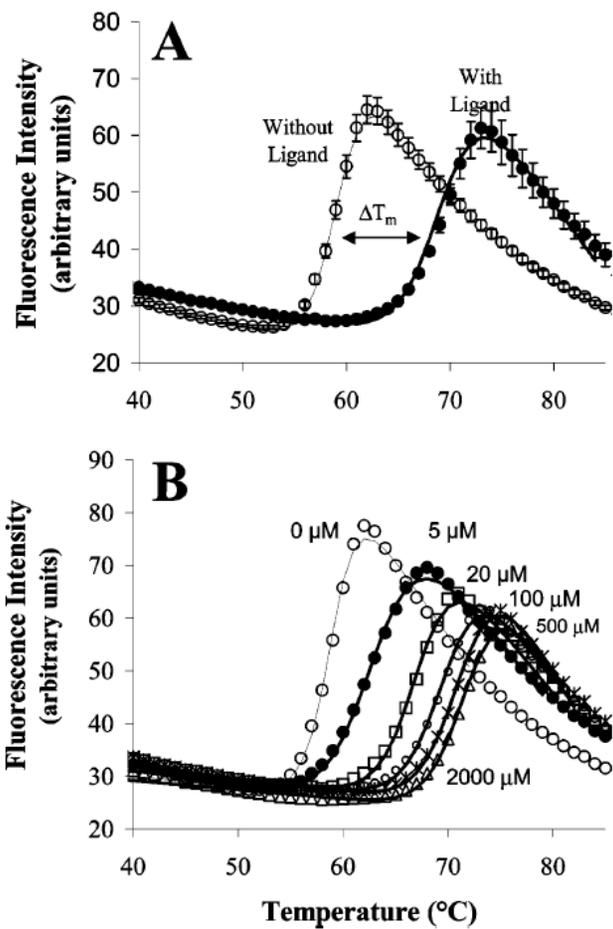
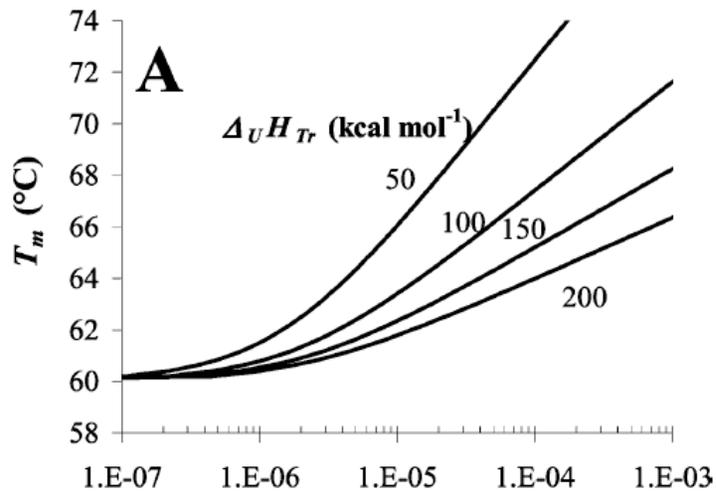


Fig. 2 Inhibitor stabilization of *h*-CA I as measured by thermal shift assay

- A. 8.3 uM *h*-CA I \pm 100 uM TFMSA
- B. TFMSA titration into 11 uM *h*-CA I

$$T_m (\text{°C}): 59.0_{\text{unliganded}} \longrightarrow 70.0_{100\text{uM}}$$

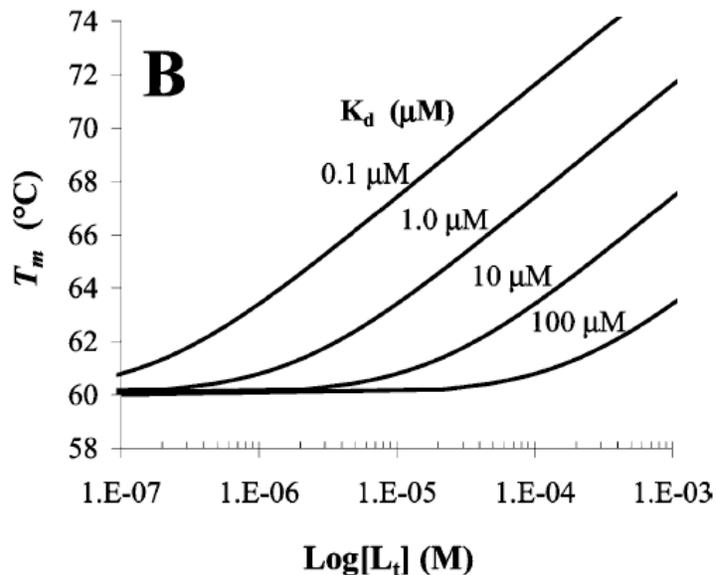
Fig. 3 Ligand effect on protein T_m is proportional to $\Delta_U H_{T_m}$



Curves simulated based on set parameters for ligand binding (K_d 1 μ M) and protein stability, except that unfolding enthalpy ($\Delta_U H_{Tr}$) at T_m is varied

- protein-specific effects of T_m shift by ligand binding

$<\Delta_U H$, then $>\Delta T_m$ with ligand



Curves simulated based on set parameters for protein stability -- K_d varied

- shows that ligand binding affinity correlates with the degree of T_m shift

Must have an accurate $\Delta_U H$ to calculate ligand binding affinities!

- can use $\Delta_U H$ calculated from melting curves (van' t Hoff enthalpy of unfolding) if you assume equilibrium two-state unfolding *

- best to obtain $\Delta_U H$ from DSC analysis (Fig.4)

Simulated ΔT_m curves with differing ligand affinities

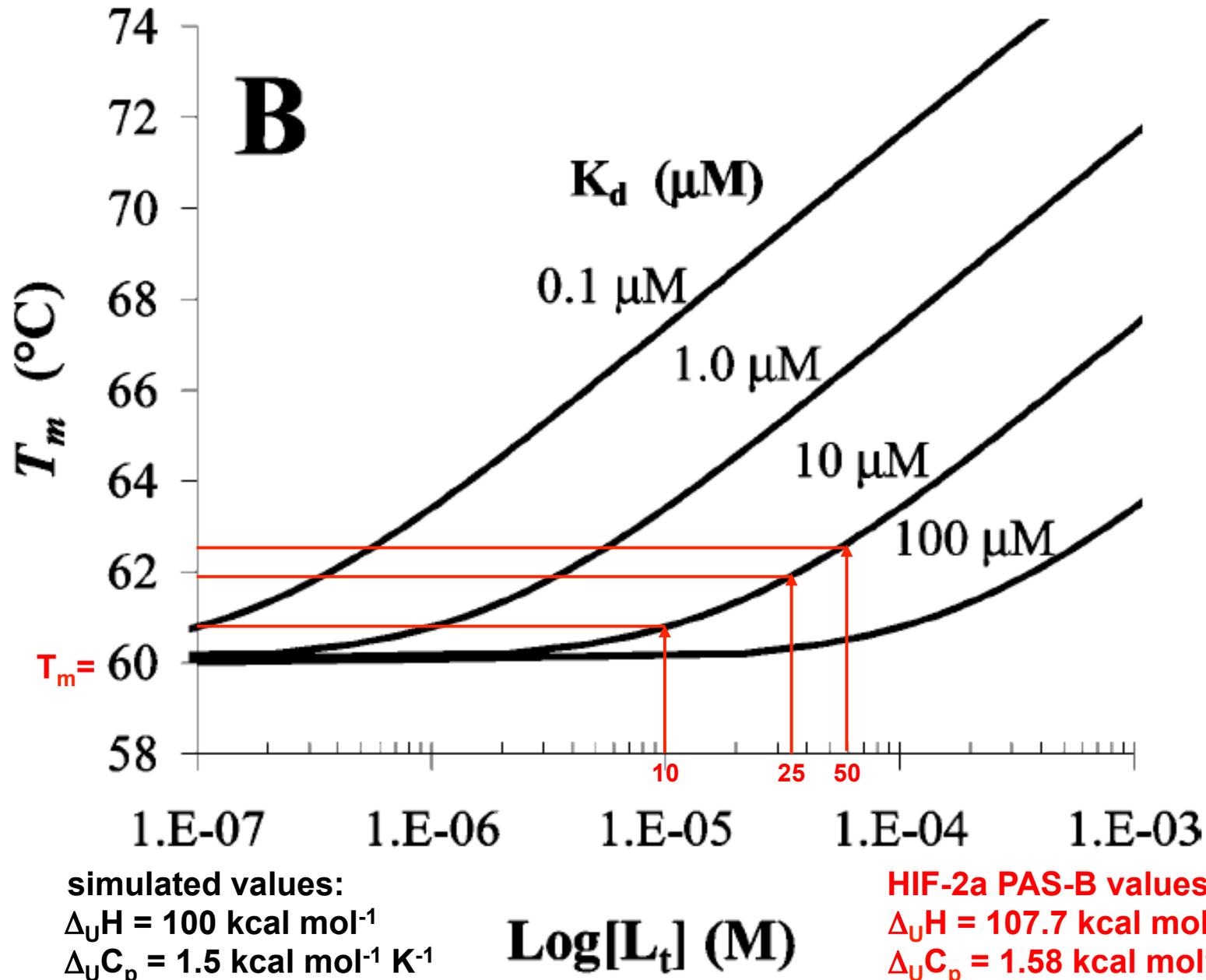


Fig. 5 Concentration/affinity effect of ligand on T_m

Compounds that bind with higher affinity shift T_m to a greater extent

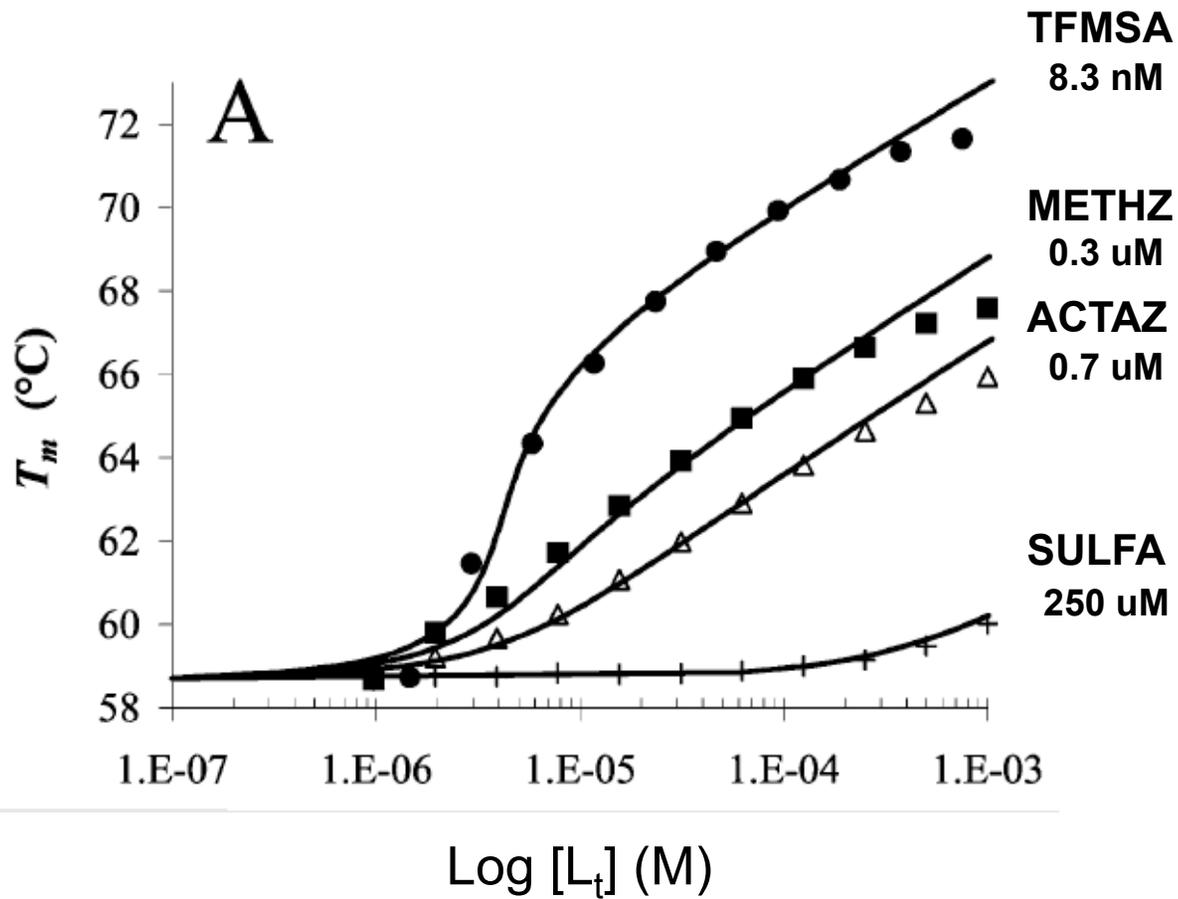
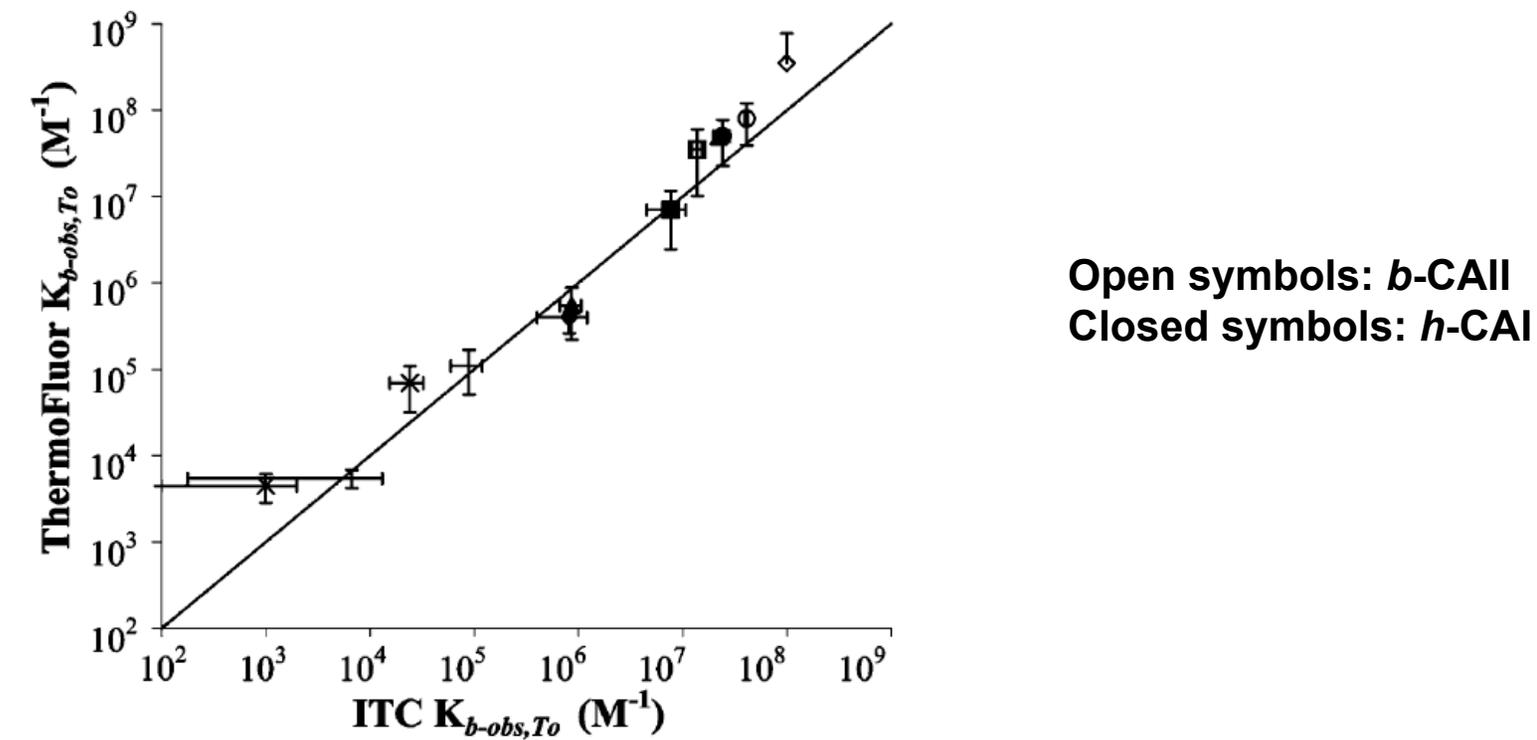
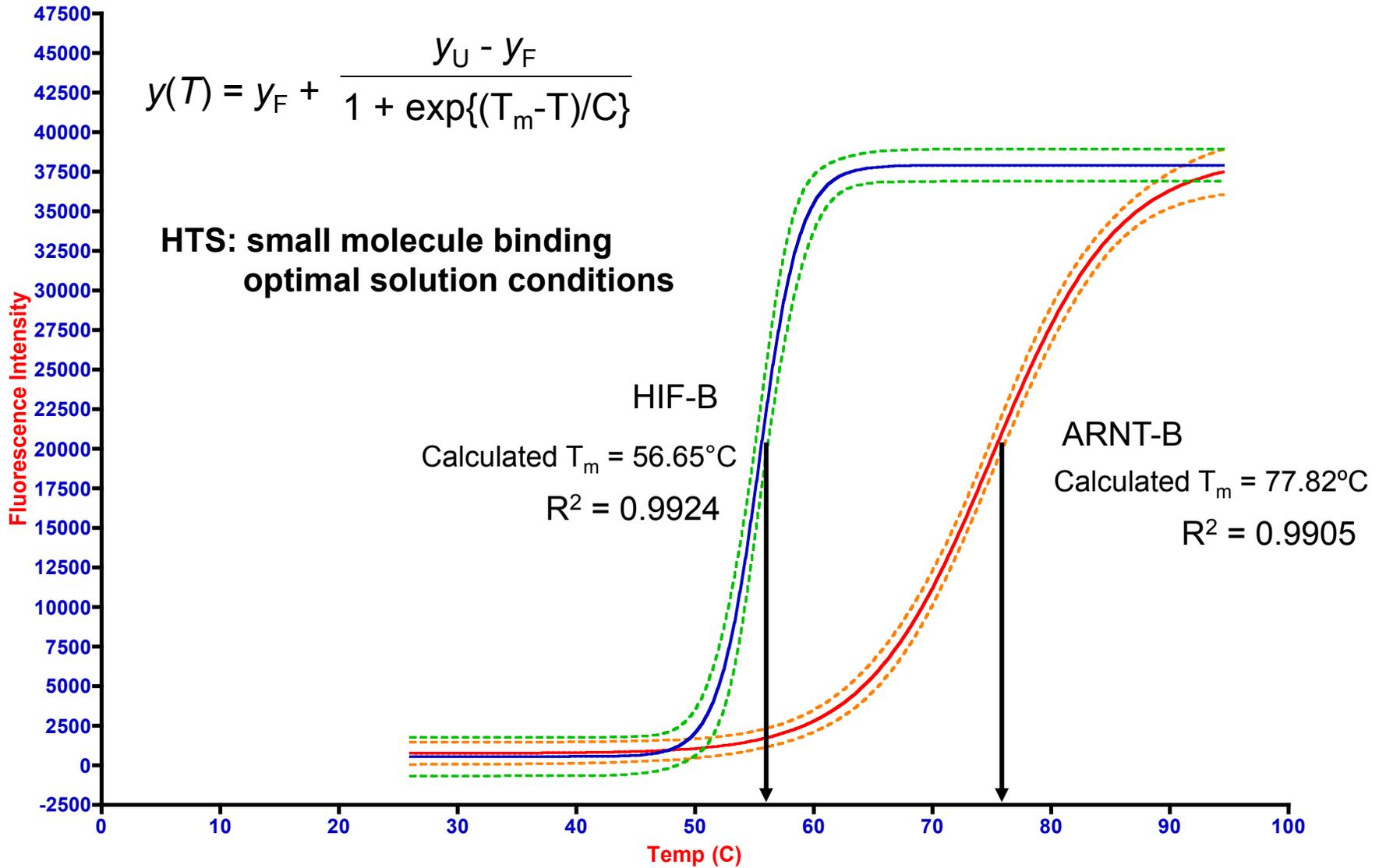


Fig. 7 Correlation of binding constants obtained by ThermoFluor and ITC



ThermoFluor binding constants were extrapolated to 37°C using approximate values for ligand binding enthalpy and heat capacity ($\Delta_b H_{T_0} = -5.0 \text{ kcal mol}^{-1}$, $\Delta_b C_p = -0.3 \text{ kcal mol}^{-1} \text{ K}^{-1}$)

Thermal denaturation of isolated PAS domains



Screening for small molecule binding

Set-up:

- 20 μ l 5 μ M protein
- 50 μ l 2.5X Sypro Orange Dye
- 20 μ l buffer
- 10 μ l 10X compound/solvent
- 100 μ l/well
- Screen [ligand] 10-100 μ M with duplicate wells

Run:

- Applied Biosystems 7500 Real-Time PCR machine
- Start at 25 $^{\circ}$ C, increase temp 1 $^{\circ}$ C/min for 70 cycles (26-96 $^{\circ}$ C, reading fluorescence intensity every $^{\circ}$ C)
- Save data as fluorescence intensity vs. cycle

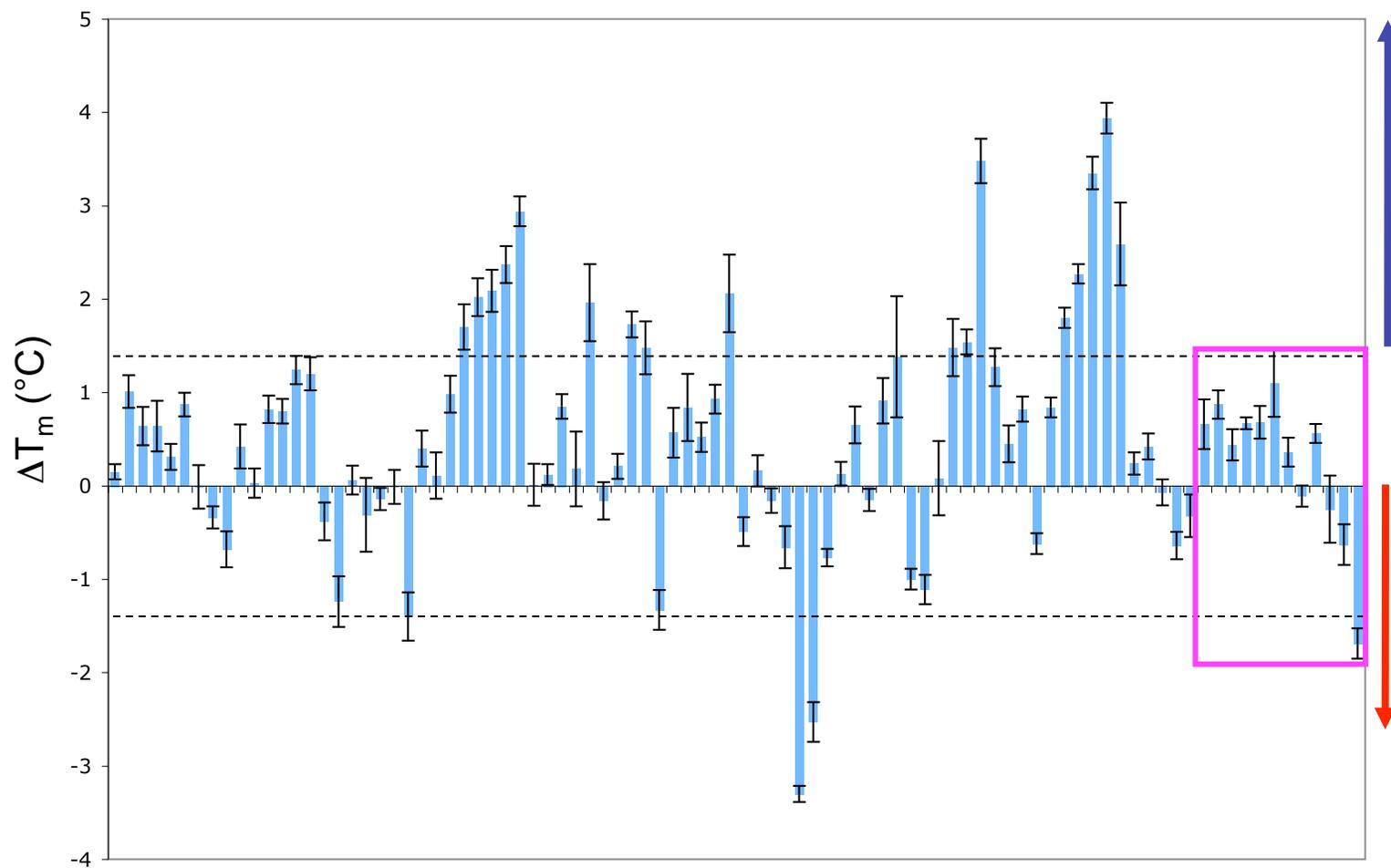
Analyze:

- Fit fluorescence intensity data to modified Boltzmann sigmoidal curve
 - obtain T_m from nonlinear regression
 - calculate $\Delta T_m = T_m^{DMSO} - T_m^{ligand}$
 - calculate propagated error: $SE_{\Delta T_m} = |SE_{T_m^{DMSO}}| + |SE_{T_m^{ligand}}|$
- Set threshold for 'significant' ΔT_m and highlight positive hits

Validation:

- Obtain thermodynamic parameters of binding by ITC or NMR

Using thermal shift assays to screen for small molecule binding



Ligand binding screen by thermal shift assay

Pros:

- Quick: ~ 1 hr set-up time, ~ 2 hr run-time, < 1 hr analysis/plate
- ΔT_m analysis is reproducible with good confidence statistics
- Requires minimal protein (<< 1mg protein/plate: 20 kDa protein: ~ 200 μ g)
- Could be scaled down to 384-well plate with proper machines (HT facility?)

Cons:

- Positive hits will be limited by K_d and compound solubility: upper limit appears to be ~ 20-50 μ M to see reproducible ΔT_m of $\geq 1^\circ\text{C}$
- Lots of pipetting required -- multichannel pipettor/HT facility to assist?

Screening:

Pantoliano, M. *et al* (2001) *J Biomolecular Screening* 6: 429-440

Weber, P. and Salemme, R. (2003) *Curr Opin Struct Biol* 13: 115-121

Lo, M. *et al* (2004) *Analytical Biochem* 332: 153-159

Assay validation:

Matulis, D. *et al* (2005) *Biochemistry* 44: 5258-5266

Klinger, A. *et al* (2006) *J Med Chem* 49: 3496-3500

Structural/functional studies:

Carver, T. *et al* (2005) *J Biol Chem* 280: 11704-11712 - identifying ligands/possible fxn for unknown protein

Bullock, A. *et al* (2005) *J Med Chem* 48: 7604-7614 - structural basis of PIM-1 kinase inhibitor specificity

Yanchunas, Jr. J. *et al* (2005) *Antimicrob Agents* 49: 3825-3832 - HIV variants and susceptibility to PI

Koblish, K. *et al* (2006) *Mol Cancer Therapeutics* 5: 160-169 - identification of MDM2/p53-SM antagonists

Using thermal shift analysis to probe protein stability

Factors influencing protein stability

Temperature:

- Parabolic dependence on ΔG (cold and heat denaturation).
- High Temperature can result in **irreversible unfolding**.

Preservatives (formulation):

- Added to ensure sample sterility.
- Can induce aggregation in the absence of additional stabilizers.

Surfactants:

- Added to prevent aggregation and adsorption to surfaces.
- Can destabilize native protein, while kinetically inhibiting aggregation.

★ Salt Type and Concentration:

- Complex effects on protein stability, solubility, and aggregation rates.
- Net effect on protein stability is a balance of multiple mechanisms.

★ Solution pH:

- Determines total charge on a protein.
- **Strong influence** of pH on protein **aggregation rates**.

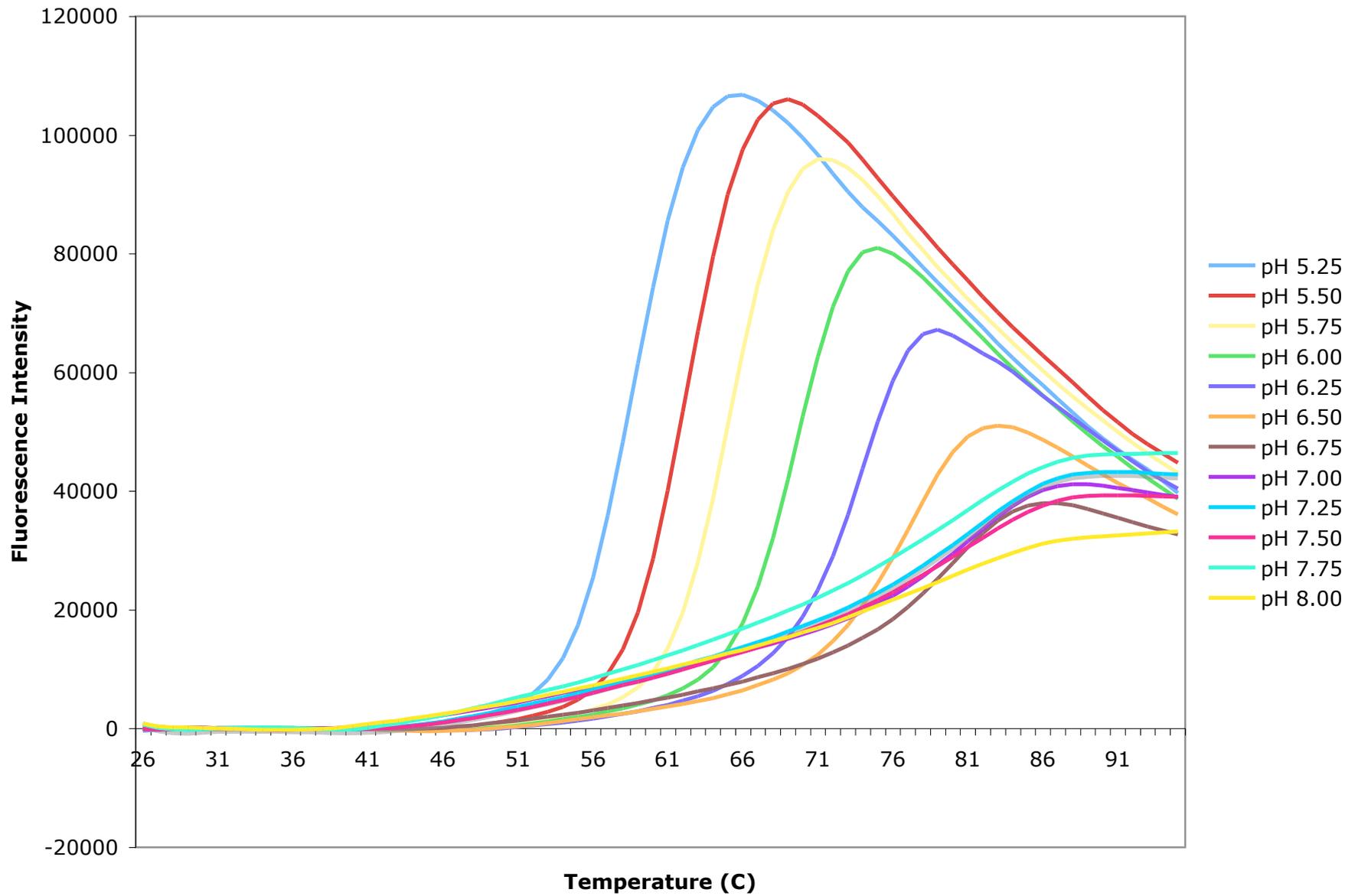
★ Ligands & Cosolutes:

- Compound binding *generally* will stabilize native protein.
- Preferential hydration by cosolutes can prevent unfolding.

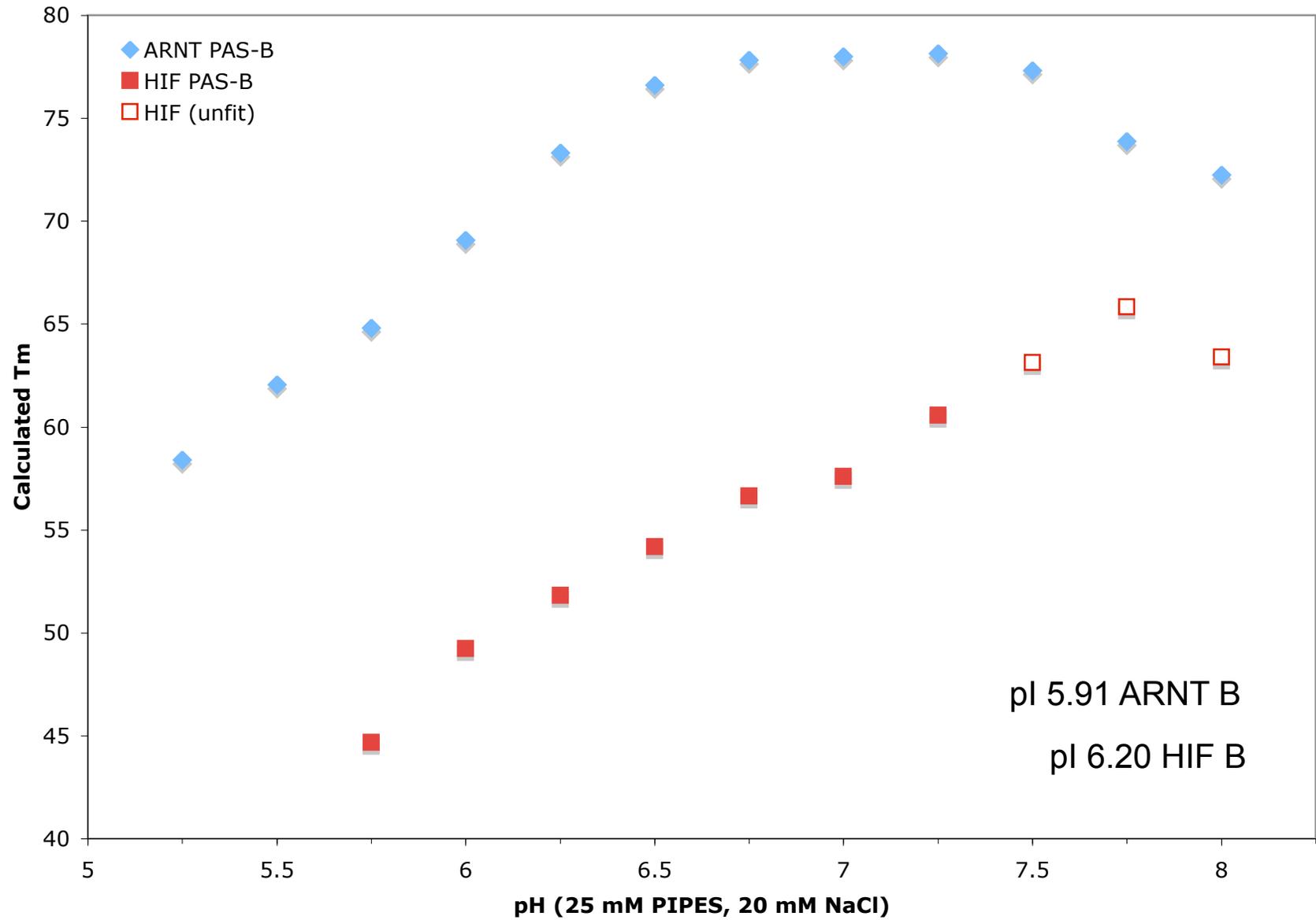
J. Kranz

Johnson & Johnson Pharmaceutical Research & Development

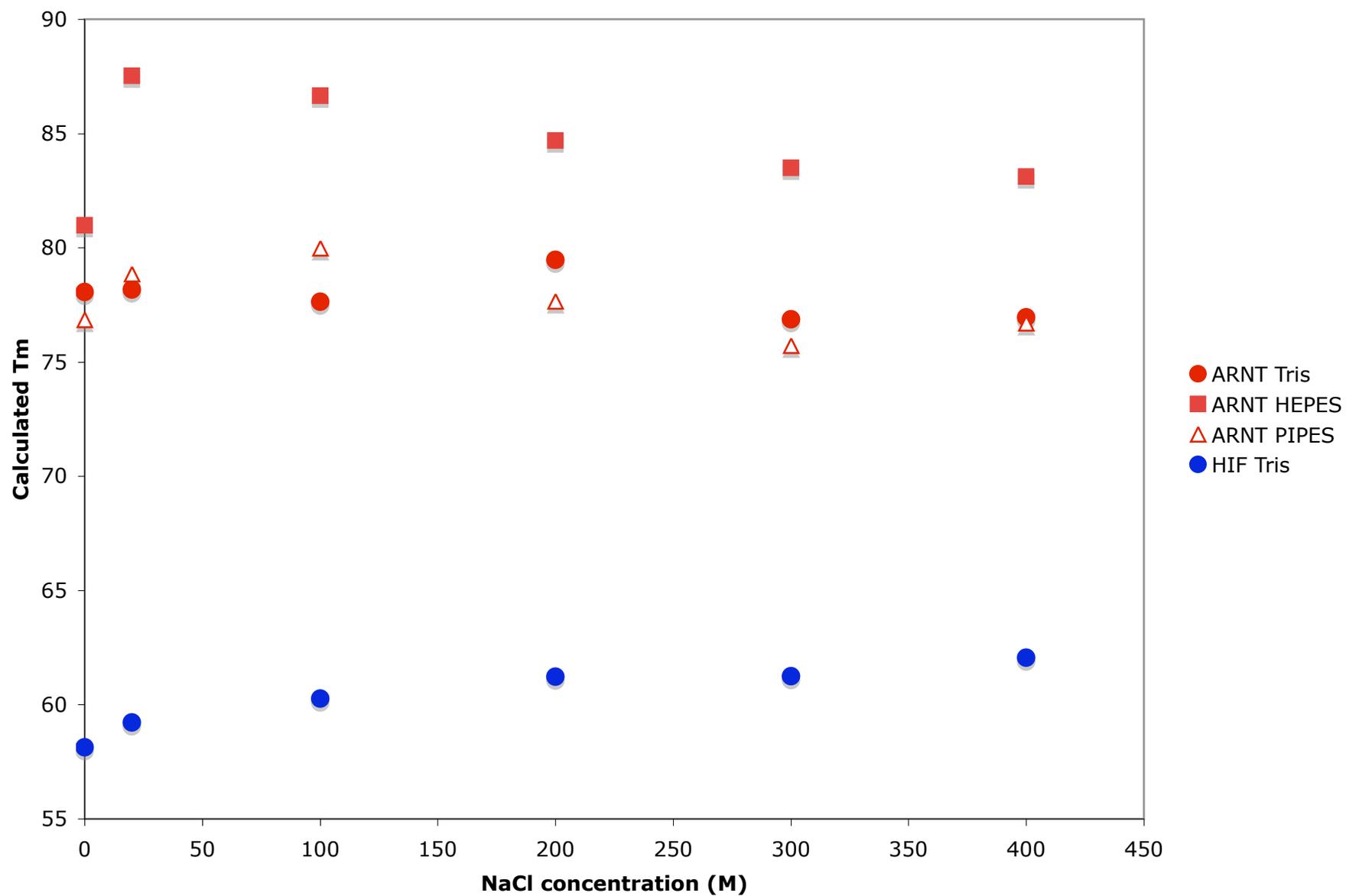
Effect of pH on thermal stability of ARNT-B



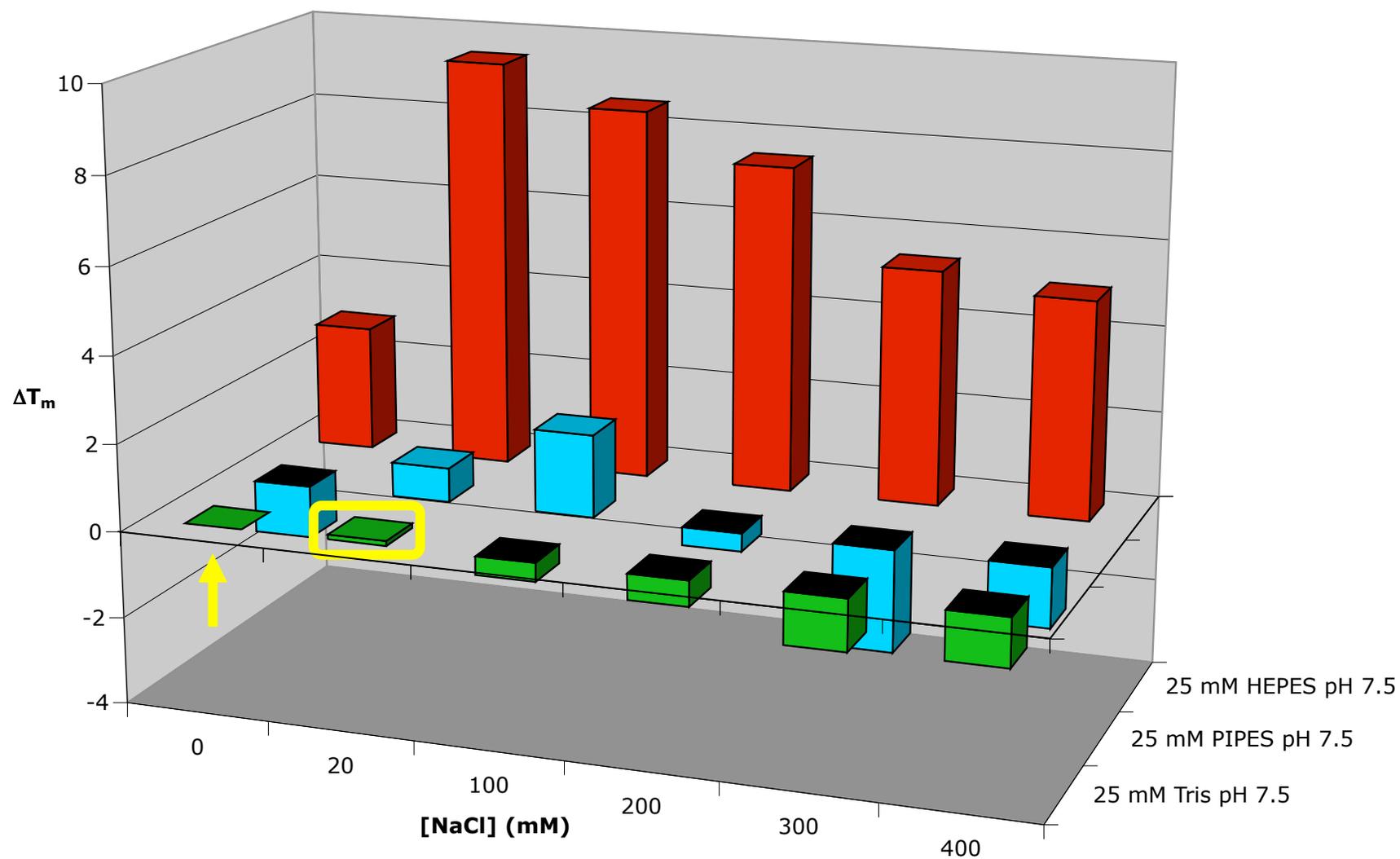
pH-dependent changes in PAS domain stability



Effect of buffer and ionic strength on PAS domain stability



Effect of buffer and salt on ARNT-B stability



Increases in thermal stability result in

- increased yield of purified protein
 - increased solubility
 - decreased degradation
- increased crystallizability

Yeh, A. *et al* (2006) *Acta Cryst D* D62: 451-457
Ericsson, U. *et al* (2006) *Analytical Biochem* In press
Vedadi, M. *et al* (2006) *PNAS* In press

Develop 96-well assay conditions for initial screen of optimal buffer

Pick 5-6 buffers (Na-P_i, Citrate, MES, HEPES, Tris, etc.) and assay:

- pH range (I.e. 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) at 20-100 mM NaCl
- Salt (NaCl at 0, 20, 50, 100, 250, 500mM) at pKa of buffer
- Use last two rows of plate to screen for addition of other small molecules (NADP, NADH, ATP, AMP-PNP, MgAc, MnCl₂, Chaps, Glycine, etc)

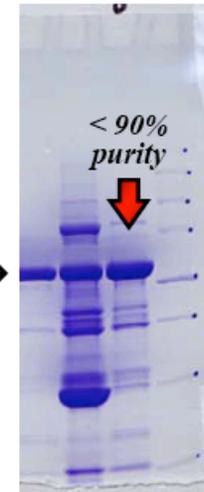
Kinase#1: Protein Purification challenges

□ Expression/Purification

- Expressed as GST-fusion protein
- Purified off GSH-resin, thrombin cleavage
- Described procedure suggests handling at pH 8.0
 - < 90% pure
 - Significant quantities of aggregates present

50mM Tris pH 8.0
150mM NaCl
1mM DTT
10% Glycerol

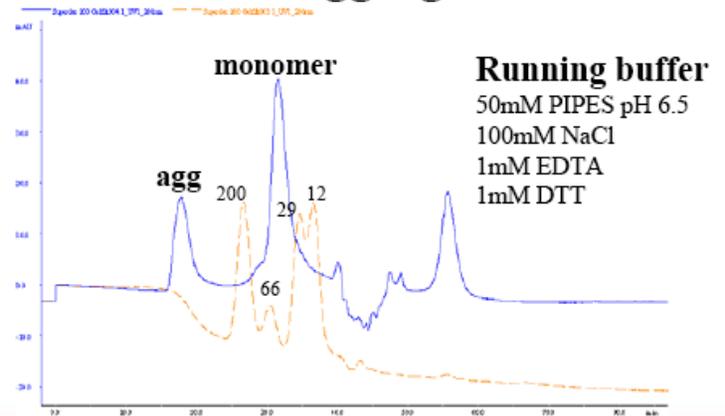
pure protein →



□ PSP suggests

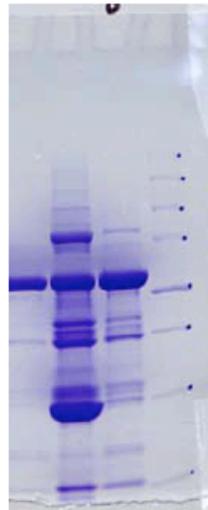
- use lower pH
- use HEPES
- low ionic strength

30% soluble aggregate(1.2mg/ml)



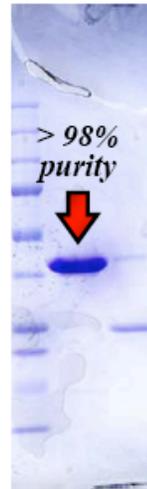
Kinase#1: Protein Purification solutions

pH 8.0
higher salt

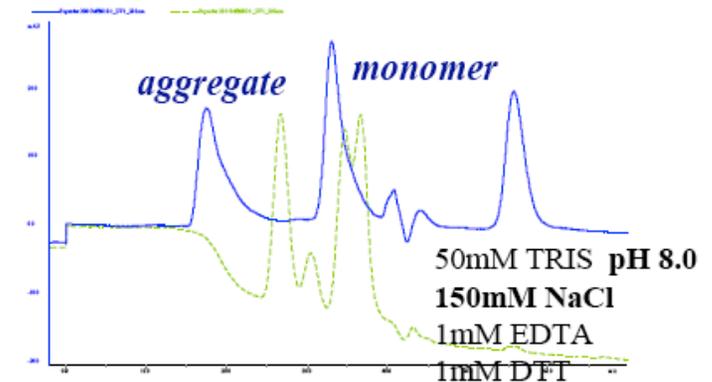


protein band →

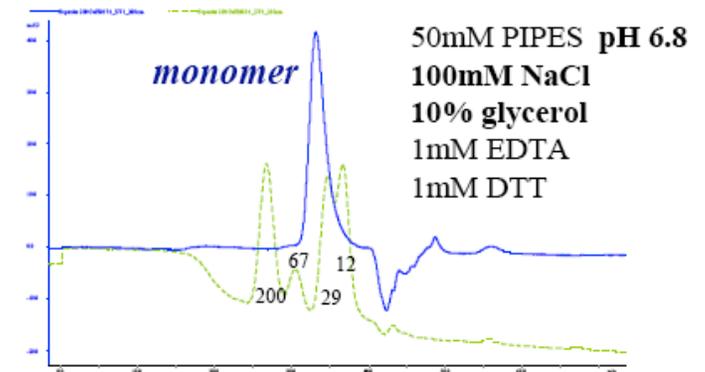
pH 6.8
lower salt
10% glycerol



Original (Published) Protocol



Revised Procedure

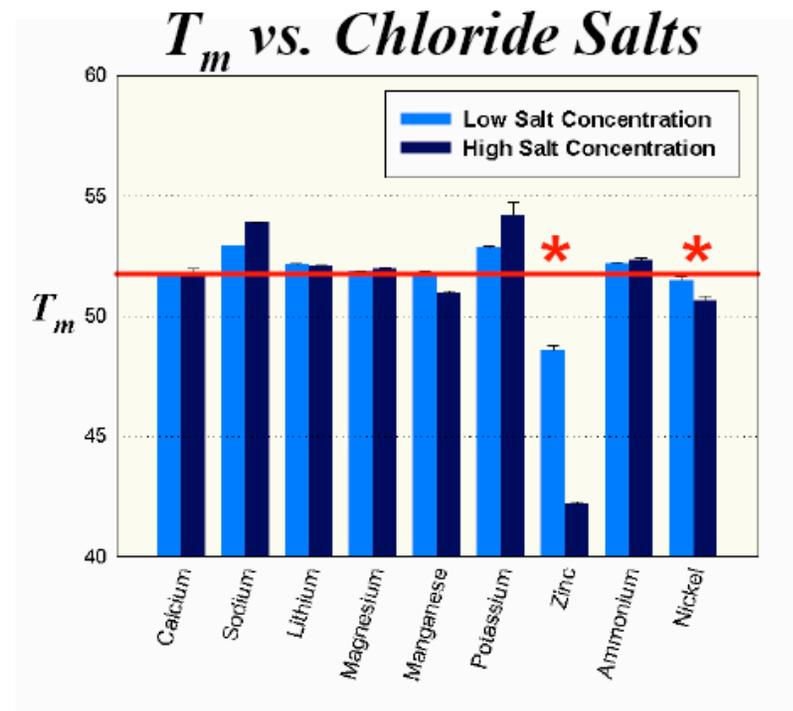
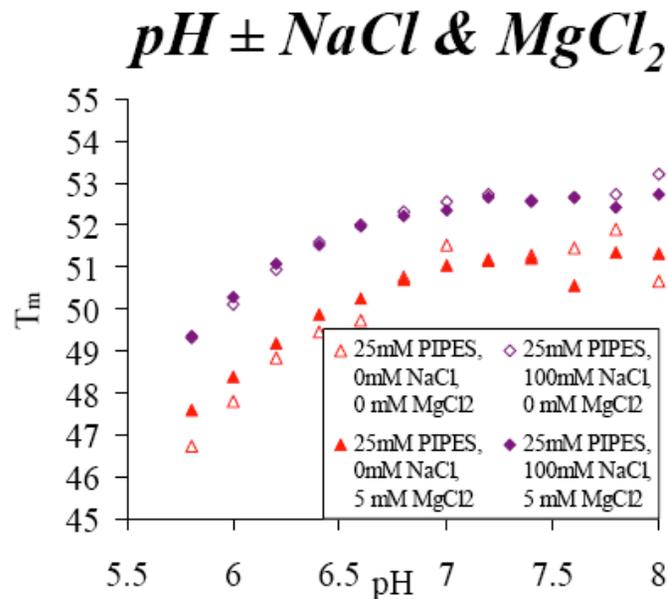


□ Using conditions from PSP

- altered thrombin cleavage kinetics
- significantly improved protein purity
- prevented aggregate formation

Protein Stability Profiling:

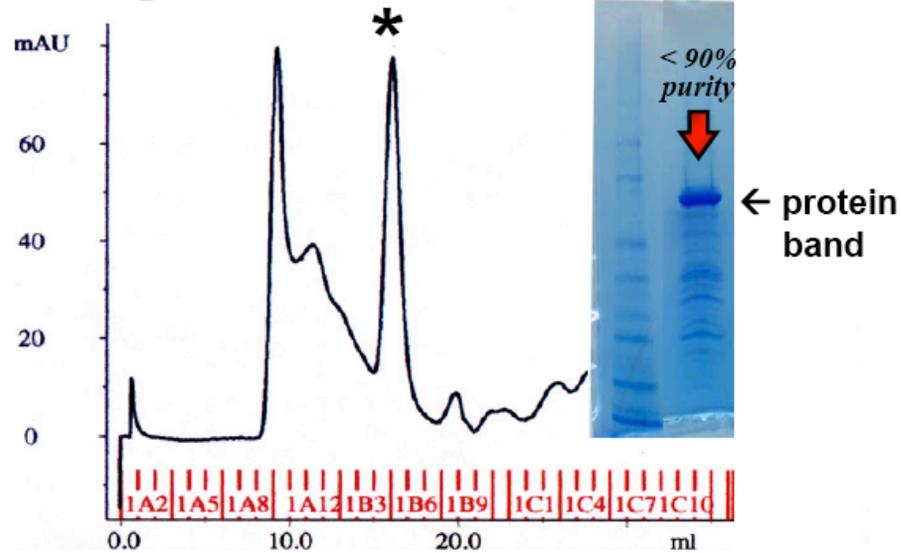
Kinase #2 – pH, Salt, & Buffer effects on stability



- ❑ High Salt stabilizes the kinase domain (also Phosphate Buffer).
- ❑ Protein is destabilized by Zinc and by Nickel (also imidazole).
- ❑ **Combination of NiCl₂ & HEPES Buffer used initially in prep.**

Kinase #2: Protein Purification Challenges

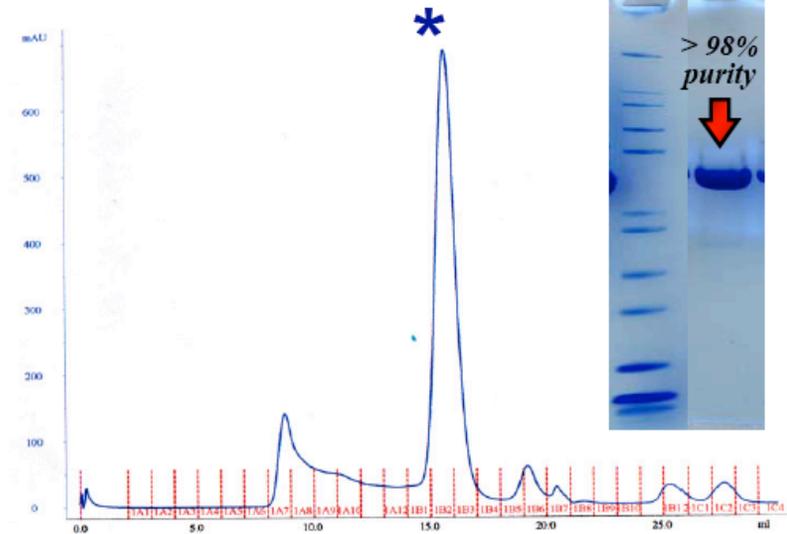
Original Protocol



Gel filtration analysis of protein eluted from Nickel-NTA column in HEPES buffer.

* peak corresponding to gel fraction.

Revised Procedure



Gel filtration analysis of protein eluted from Talon column in phosphate buffer.

* peak corresponding to gel fraction.

Change of column type minimized exposure of protein to Nickel.

Kinase Protein Stability Profiling

Kinase #1

- ❑ **Original conditions:**
 - Tris Buffer, typical salt & reductant, GST-column purification
 - **Aggregation was biggest challenge**

- ❑ **Protein Stability Profile:**
 - pH profile - maximum at pH ~ 6.5
 - Salt profile – prefers low salt, polyols
 - Buffer profile – HEPES preferable to Phosphate, PIPES, MOPS
 - Metals - divalents are destabilizing

- ❑ **PSP-Altered Purification:**
 - **Changed to HEPES Buffer**
 - **Added 10% Glycerol to thrombin cleavage & column elution buffer**
 - **Minimized Aggregation**

Kinase #2

- ❑ **Original conditions:**
 - HEPES Buffer, typical salt, Nickel-column purification
 - **Aggregation was biggest challenge**

- ❑ **Protein Stability Profile:**
 - pH profile - maximum at pH > 7
 - Salt profile – stabilized by high salt
 - Buffer profile - Phosphate buffers uniquely stabilizing
 - Metals - Nickel is destabilizing

- ❑ **PSP-Altered Purification:**
 - **Changed to Phosphate Buffer**
 - **Substituted Talon Column for Ni-NTA column**
 - **Minimized Aggregation**

