Calorimetry for Bioinorganic Chemistry:
Isothermal Titration Calorimetry (ITC)
Differential Scanning Calorimetry (DSC)

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Outline

- Calorimetry
- Biomolecular Calorimetry
  - Isothermal Titration Calorimetry (ITC)
    - The Method: information, instruments, data, analysis
    - Bioinorganic Applications
      - Issues with metal ions
        - His (Ni^{2+}), a teaching example
      - Examples:
        - Transferrin (Fe^{3+})
        - Ferritin (Fe^{2+})
        - Albumin (Cu^{2+})
        - His-rich sequence (Zn^{2+}, Fe^{3+}, Fe^{2+}, etc.)
        - Metallothionein (Zn^{2+}, Cd^{2+}, As^{3+}, Pb^{2+})
        - UreE, a urease metallochaperone (Ni^{2+}, Cu^{2+})
        - Zinc fingers, a thermodynamic story (Zn^{2+})
  - Differential Scanning Calorimetry (DSC)
    - The Method: information, instruments, data
    - Bioinorganic Applications: Insulin (Zn^{2+}), Carbonic Anhydrase (Zn^{2+})

Calorimetry

- Measure net heat flow (q) into/out of a system (solution) for some process (e.g., mixing, heating).
  - seek the heat associated with a molecular or physical phenomenon occurring in the solution
  - bulk thermodynamic measurement (no quantum mechanics, no spectroscopy); measure all contributions to the net heat

- Isobaric conditions (constant P): $q_P = \Delta H°$

- $\Delta H°$ (measure) and $\Delta G°$ (from $K_{(d)}$) → $\Delta S°$, from $\Delta G° = \Delta H° - T\Delta S°$
  - direct measurement with calorimetry
  - indirect measurement with van't Hoff relationship:

$$\ln(K/K_0) = (\Delta H°/R)(T_0^{-1}-T^{-1}); \text{ for } \Delta C_P = 0$$

- Change in heat capacity for the process:

$$\Delta C_P = \Delta H°_0 / T_0 - \Delta H° / T = \Delta\Delta H° / \Delta T$$

Lavoisier's ice calorimeter

Calorimeters

- Various devises to measure the heats of chemical reactions:
  - Lavoisier's ice calorimeter

  - styrofoam cup calorimeter

    $Mg_{(s)} + ½\ O_{2(g)} \rightarrow MgO_{(s)}$

- Bomb calorimeter: heats of combustion

  Isochoric (constant V): $q_V = \Delta E°$

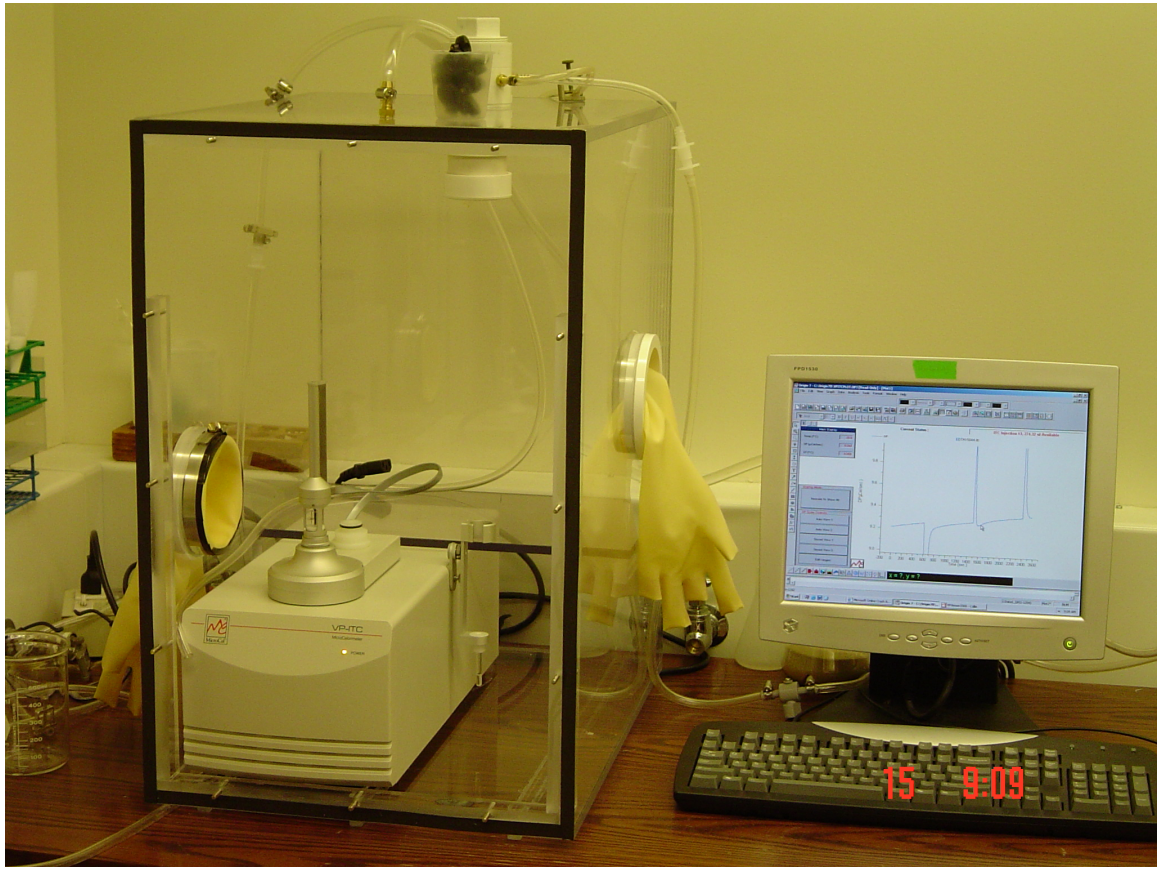
- Isothermal titration calorimeter (ITC): binding, equilibrium processes

  Isobaric (constant P): $q_P = \Delta H°$

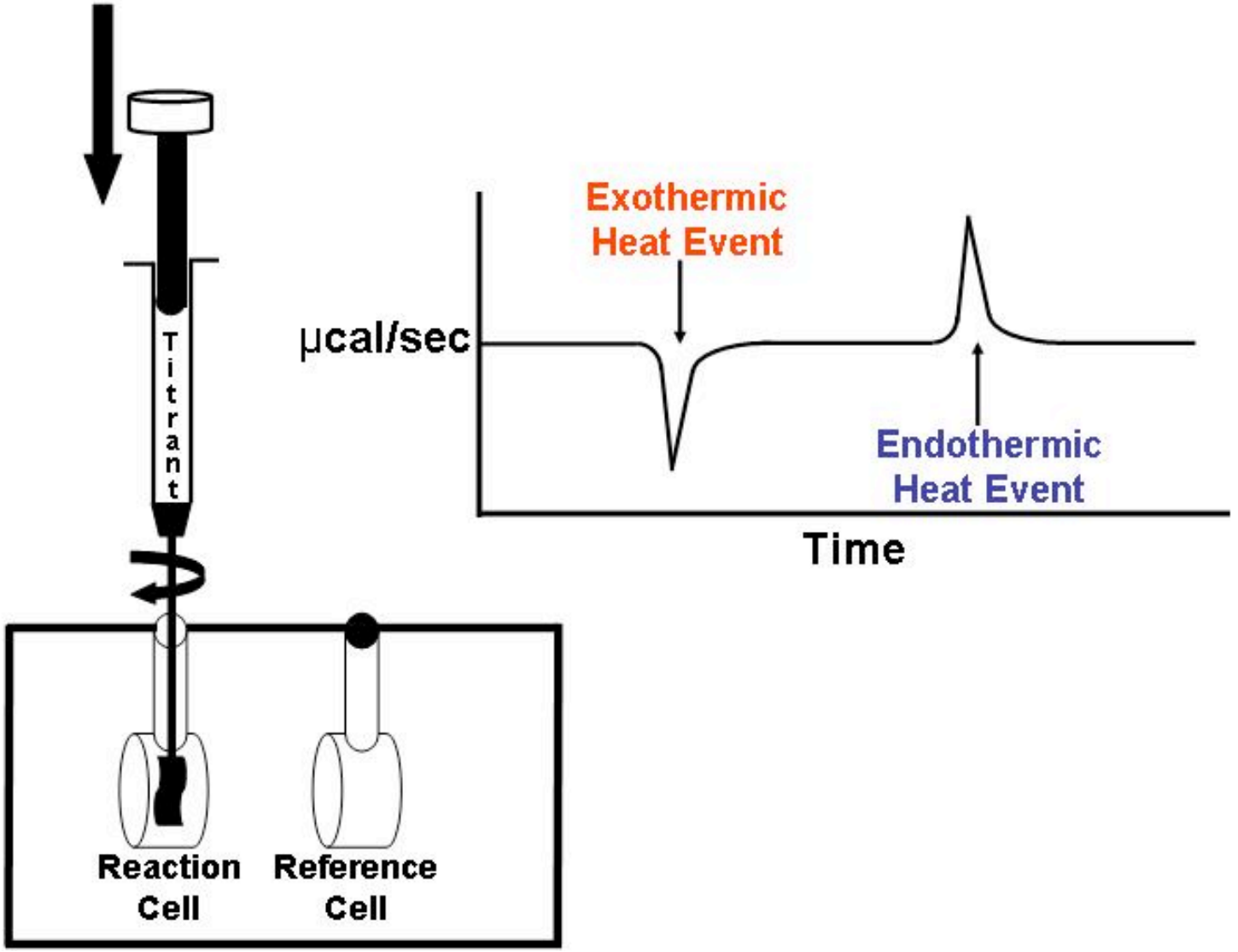
- Differential scanning calorimeter (DSC): phase changes; equilibria

  Isobaric (constant P): $q_P = \Delta H°$

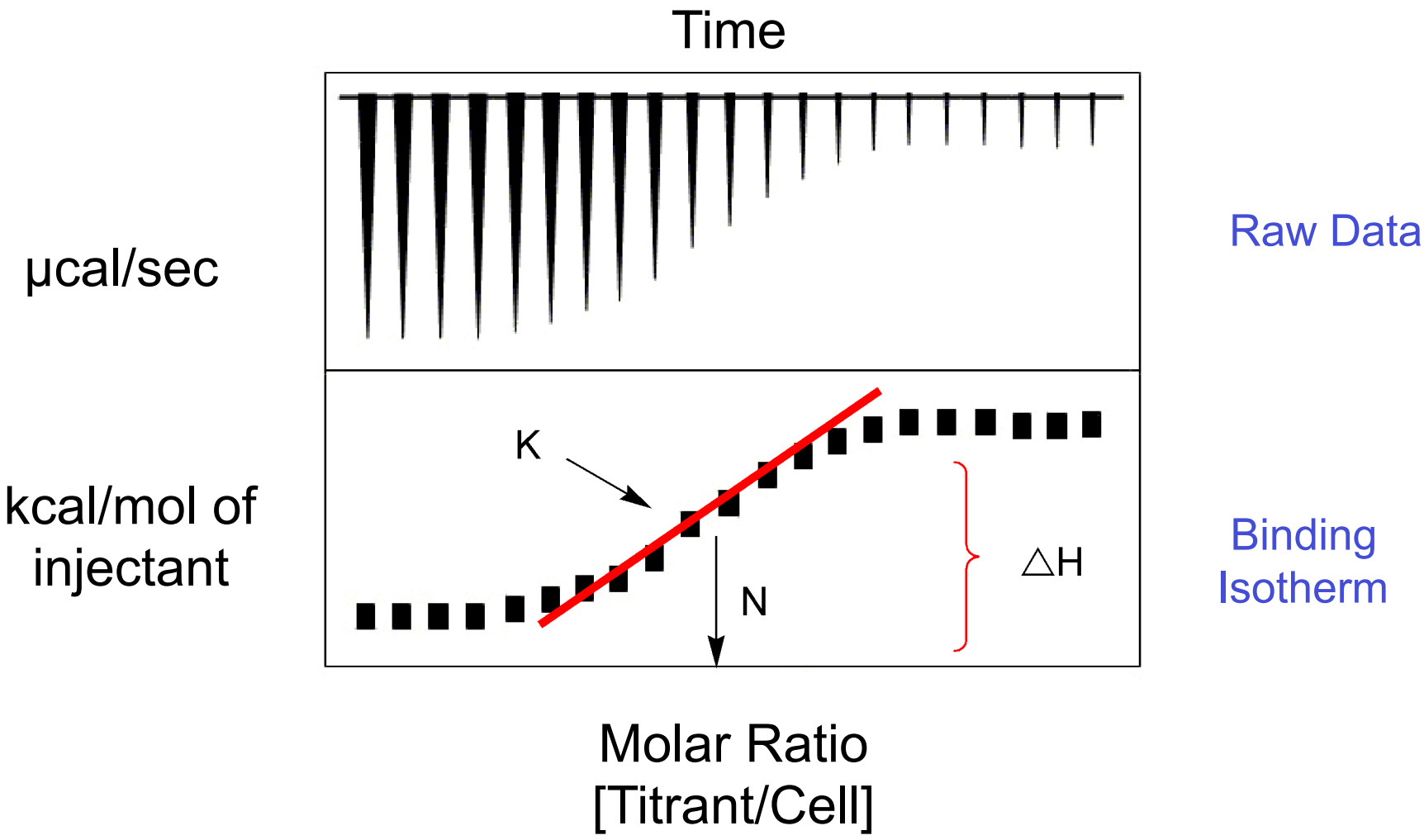
MicroCal isothermal titration calorimeter



Isothermal Titration Calorimetry



Isothermal Titration Calorimetry



The c window

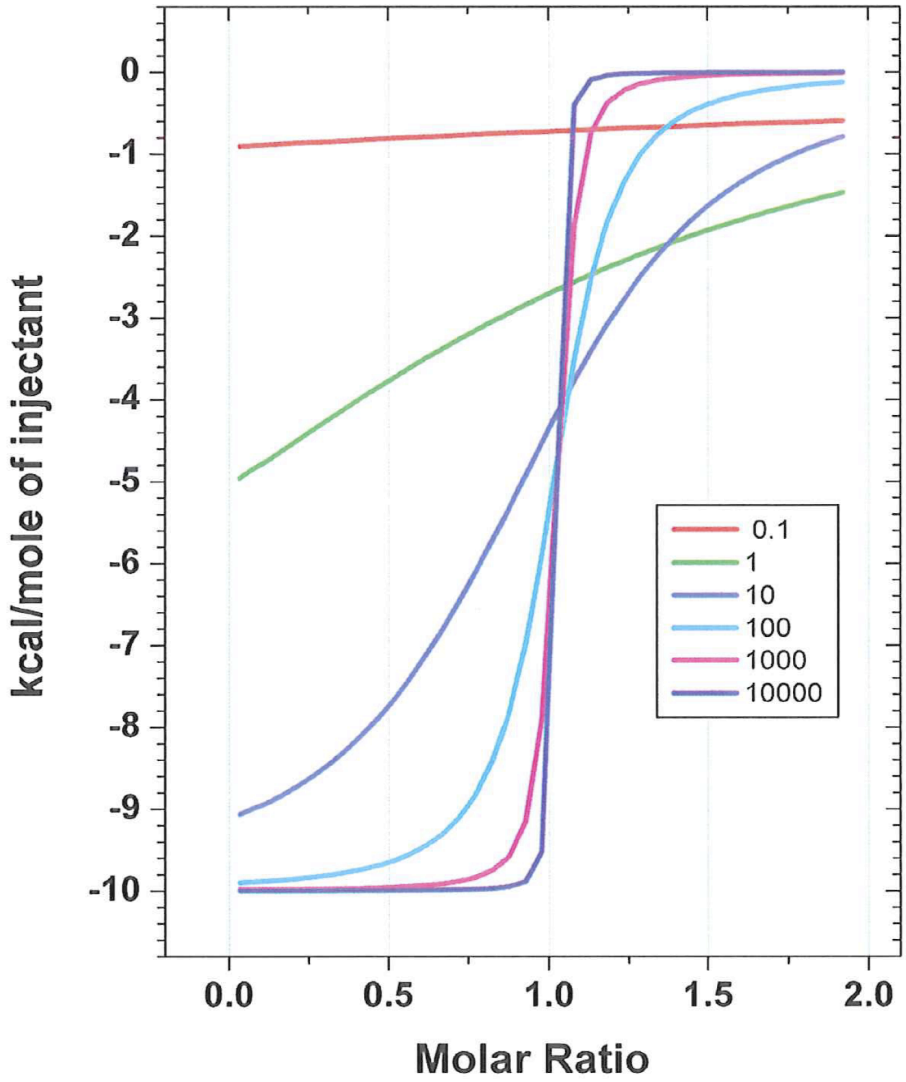
c = n K [macromolecule]

Simulated ITC isotherms with
n = 1; ΔH = -10 kcal/mol
[macromolecule] = 0.10 mM
and

$1 \times 10^3 < K < 1 \times 10^8$

corresponding to

$0.1 < c < 10{,}000$



ITC: Advantages

1. Determine binding constants ($3 < \log K_{ITC} < 8$) for a variety of molecular interactions.

2. Provides detailed thermodynamic analysis of equilibrium condition ($\Delta G°$, $\Delta H°$, $\Delta S°$, $\Delta C_p°$).

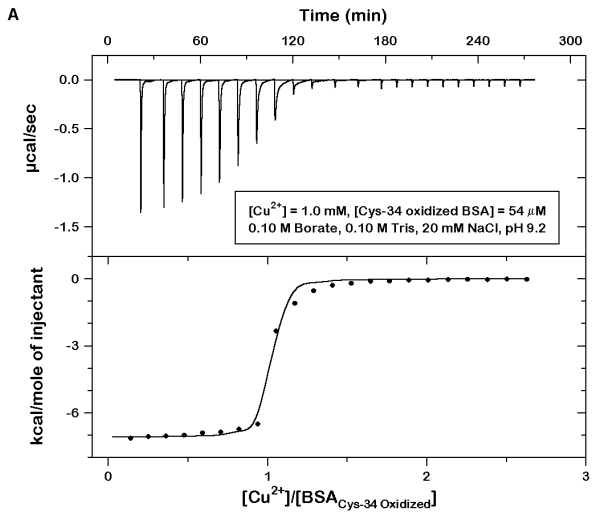
3. Can provide additional chemical insight:
   - proton competition
   - kinetics

     M. J. Todd, J. Gomez *Anal. Biochem.* **2001** *296* 179-187

     M. M. Pedroso, et al *J. Biol. Inorg. Chem.* **2014** *19* 389-398
   - redox reactions

     M. Sorlie, J. M. Chan, H. Wang, L. C. Seefeldt, V. D. Parker, *J. Biol. Inorg. Chem.* **2003** *8*, 560-566

ITC: What to do with the data?



1. Manipulation of the data.
    - baseline correction
    - subtract heat of dilution
        - control titration
        - extended region of experimental data
    - integration
2. Qualitative analysis of the data (critical evaluation).
    - injection peaks? stoichiometry? c-window? heat? (concentration, conditions)
    - (initial data → better data → optimal data → reproducible data)
3. Quantitative analysis of the data.
    a) Fitting to a model
        - Single binding site model → n, K, ΔH, (ΔS)
        - Independent binding sites model
        - Sequential binding sites model
        - Competition binding model → known K_A, ΔH_A; unknown K_B, ΔH_B
    b) Post-hoc analysis of K_{ITC} and ΔH_{ITC} values
        - (condition-dependent values → "condition-independent" values)

ITC: "Dirty Secrets Slide"

Many laboratories have ITC instruments from MicroCal (GE Healthcare) or TA Instruments (~$50K - $100K).

Some have been frustrated in their attempts to obtain useful ITC data (you are not alone!)

Some have published ITC data with errors in analysis (and interpretation) (they are not alone)

Issues: instruments are very sensitive
contaminating species → care and cleanliness
competing reactions/processes → eliminate or account for them
more complications when metal ions are involved!

Thermodynamics of Metal Ions Binding to Proteins

$$\mathbf{M^{n+} + Protein \rightleftarrows M^{n+}Protein}$$

- Equilibrium (K) $K = [M^{n+}Protein] / [M^{n+}][Protein]$

- Free Energy (ΔG) $\Delta G° = -R\ T\ \ln(K)$

- Enthalpy (ΔH) calorimetry: $\Delta H° = q_P$

- Entropy (ΔS) $\Delta G° = \Delta H° - T\Delta S°$

- Heat Capacity (C_P) $\Delta C_P = \Delta H°_0 / T_0 - \Delta H° / T = \Delta\Delta H° / \Delta T$

ITC of Metal-Protein Interactions: Complications

1. Metal-Buffer Interactions *

$$M(buffer)_x^{+n} \rightleftarrows M^{+n} + X\ buffer$$

2. Metal Redox Reactions

$$M^{+n} + [O] \rightleftarrows M^{+(n+1)} + [R]$$

3. Metal Solution Chemistry

a) Hydrolysis

$$L_nM^{+n}\text{-}H_2O \rightleftarrows L_nM^{+n}\text{-}OH^- + H^+$$

b) Precipitation / Dissolution

$$L_nM_{(aq)} \rightleftarrows L_nM_{(s)}$$

4. Proton Displacement *

$$M^{+n} + Protein \rightleftarrows M^{+n}Protein + n\ H^+$$

$$n\ H^+ + n\ buffer \rightleftarrows n\ bufferH^+$$

ITC of Metal-Protein Interactions: Experimental Design

1. pH.

- metal ions are Lewis acids and compete with H^+
- coupled equilibria involving H^+ are common with metal ions
- need to account for heat associated with coupled (de)protonations

2. Buffer.

a) interaction with the metal ion

- can suppress side reactions of the metal ion
- can provide a competing ligand for the metal ion
- need to know $K_{M\text{-}buff}$ and $\Delta H°_{M\text{-}buff}$ and $n_{M\text{-}buff}$

b) interaction with H^+

- $\Delta H°_{buff\text{-}H+}$ can be used to quantify H^+'s in coupled equilibria
- use a buffer with larger heat of protonation to amplify signal

3. Other species?

- reducing agent, complexing agent, salt, detergent, DMSO, etc.

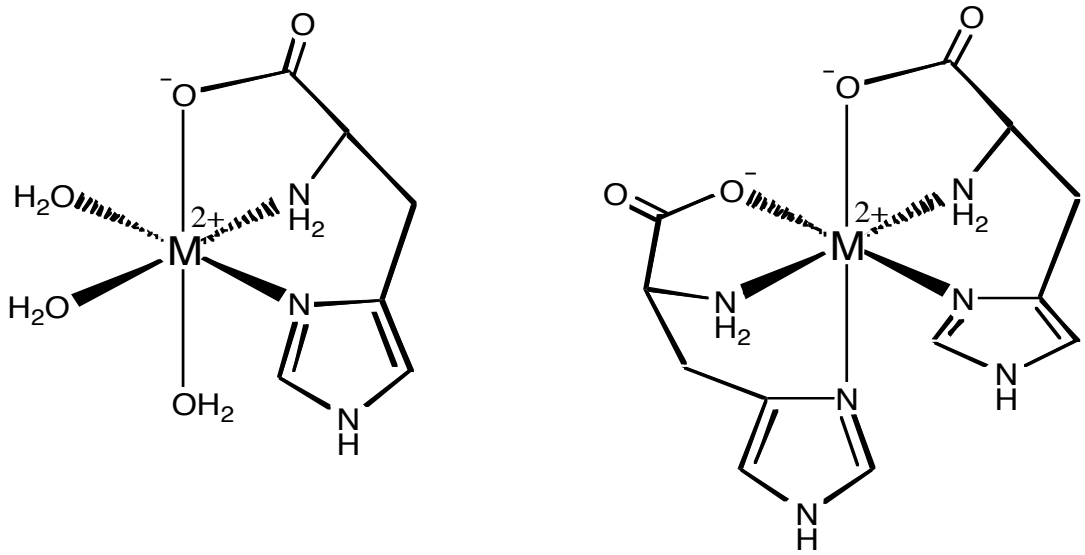
Overall: $(1\text{-}z)\mathbf{M^{2+}} + z\mathrm{MB^{2+}} + (1\text{-}x)\mathbf{L} + x\mathrm{HL^{+}} + (x\text{-}z)\mathrm{B} \rightleftarrows \mathbf{ML^{2+}} + x\mathrm{HB^{+}}$

Buffer- and pH-Independent Equilibrium: $\mathbf{M^{2+} + L \rightleftarrows ML^{2+}}$

Thermodynamic cycles are required to obtain condition-independent values from condition-dependent experimental values

$(1\text{-}z)\mathbf{M^{2+}} + z\mathrm{MB^{2+}} + (1\text{-}x)\mathbf{L} + x\mathrm{HL^{+}} + (x\text{-}z)\mathrm{B} \rightleftarrows \mathbf{ML^{2+}} + x\mathrm{HB^{+}}$		ΔH_{ITC}
$\mathrm{MB^{2+}} \rightleftarrows \mathrm{M^{2+}} + \mathrm{B}$	z	$-\Delta H_{MB}$
$\mathrm{HL^{+}} \rightleftarrows \mathrm{H^{+}} + \mathrm{L}$	x	$-\Delta H_{HL}$
$\mathrm{H^{+}} + \mathrm{B} \rightleftarrows \mathrm{HB^{+}}$	x	ΔH_{HB}
$\mathbf{M^{2+} + L \rightleftarrows ML}$		$\mathbf{\Delta H_{ML}}$

Ni^{2+} + His



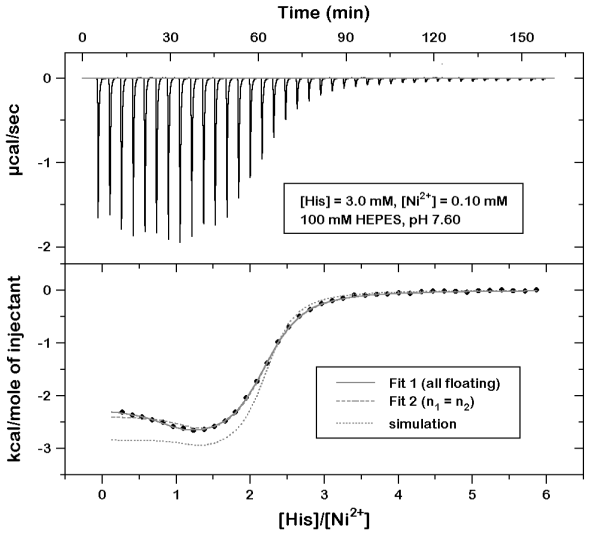
M-His and M-His_2 structures (M : Cu^{2+}, Ni^{2+})

Ligand Binding Equilibria

$$M^{2+} + His^{-} \rightleftarrows M(His)^{+}$$

$$M(His)^{+} + His^{-} \rightleftarrows M(His)_2$$

Ni^{2+} + His

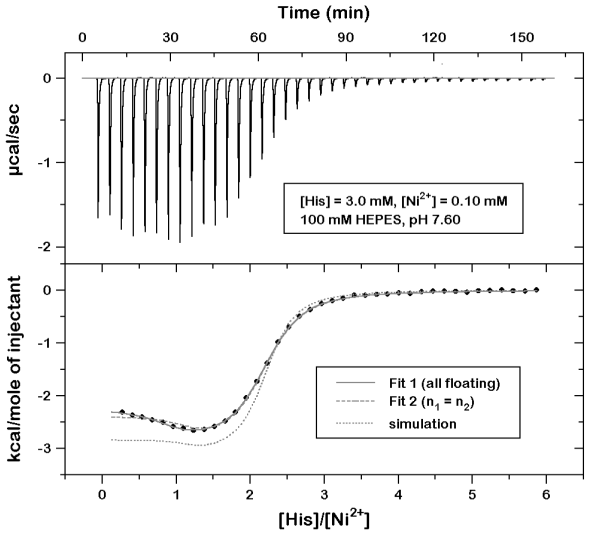


His → Ni^{2+} Titration

$His^- + Ni^{2+} \rightleftarrows Ni(His)^+$ K_1

$His^- + Ni(His)^+ \rightleftarrows Ni(His)_2$ K_2

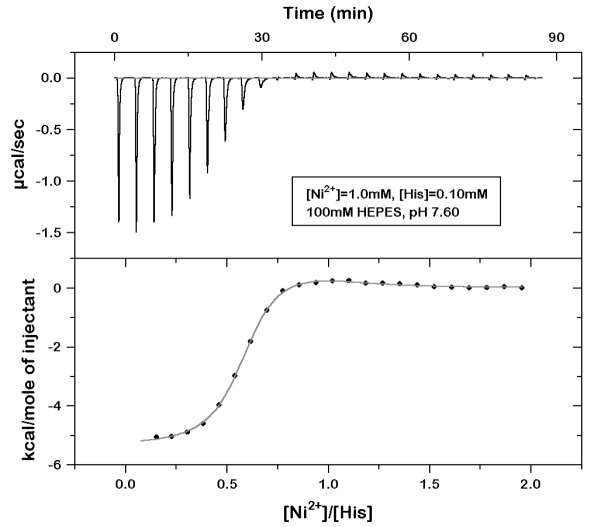
Ni^{2+} + His



His → Ni^{2+} Titration

$$His^- + Ni^{2+} \rightleftarrows Ni(His)^+ \qquad K_1$$

$$His^- + Ni(His)^+ \rightleftarrows Ni(His)_2 \qquad K_2$$



Ni^{2+} → His Titration

$$Ni^{2+} + 2\,His^- \rightleftarrows Ni(His)_2 \qquad K = K_1 \cdot K_2$$

$$Ni^{2+} + Ni(His)_2 \rightleftarrows 2\,Ni(His)^+ \qquad K = K_1 / K_2$$

Ni^{2+} + His

Two Equilibria of Interest

$$Ni^{2+} + His^{-} \rightleftarrows Ni(His)^{+}$$

$$Ni(His)^{+} + His^{-} \rightleftarrows Ni(His)_2$$

First Equilibrium in a Buffered ITC Measurement

$$Ni(buffer)_x^{2+} \rightleftarrows Ni^{+2} + X\ buffer$$

$$HisH \rightleftarrows His^{-} + H^{+}$$

$$Ni^{2+} + His^{-} \rightleftarrows Ni(His)^{+}$$

$$H^{+} + buffer \rightleftarrows bufferH^{+}$$

$$Ni(buffer)_x^{2+} + HisH \rightleftarrows Ni(His)^{+} + bufferH^{+} + (X\text{-}1)\ buffer$$

Ni^{2+} + His

Two Equilibria of Interest

$$Ni^{2+} + His^{-} \rightleftarrows Ni(His)^{+}$$

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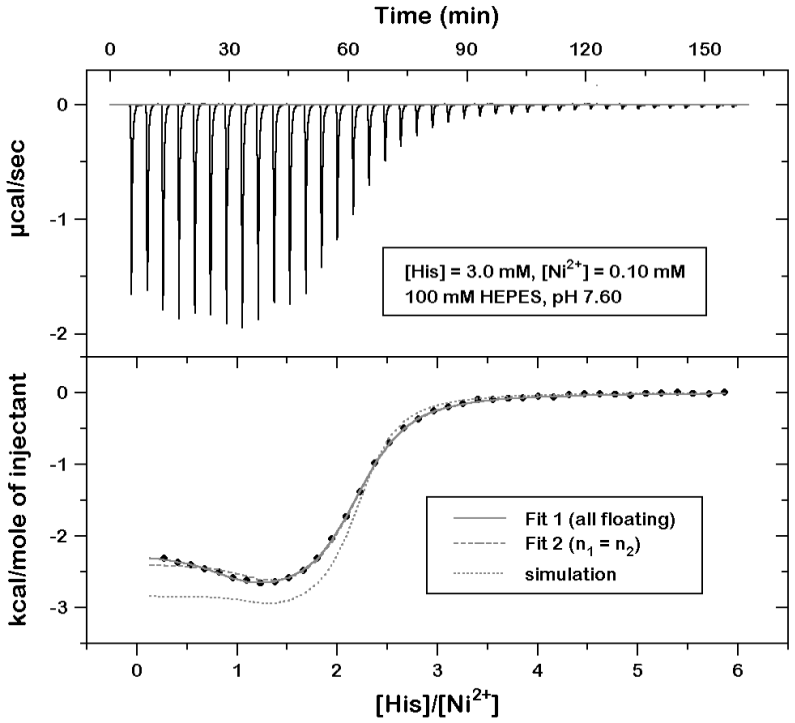
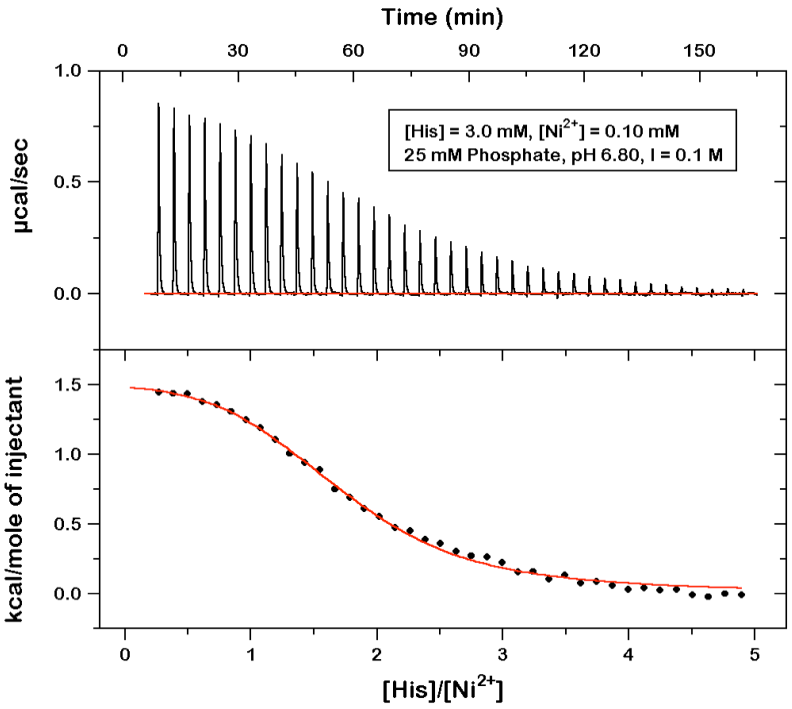
Ni^{2+} + His



His → Ni^{2+}
100 mM HEPES pH 7.6



His → Ni^{2+}
25 mM phosphate pH 6.8

- Buffer protonation enthalpy can dominate ΔH_{ITC}

Ni^{2+} + His

Two Equilibria of Interest

$$Ni^{2+} + His^{-} \rightleftarrows Ni(His)^{+}$$

$$Ni(His)^{+} + His^{-} \rightleftarrows Ni(His)_2$$

First Equilibrium in a Buffered ITC Measurement

$$Ni(buffer)_x^{2+} \rightleftarrows Ni^{+2} + X\ buffer$$

$$HisH \rightleftarrows His^{-} + H^{+}$$

$$Ni^{2+} + His^{-} \rightleftarrows Ni(His)^{+}$$

$$H^{+} + buffer \rightleftarrows bufferH^{+}$$

$$Ni(buffer)_x^{2+} + HisH \rightleftarrows Ni(His)^{+} + bufferH^{+} + (X\text{-}1)\ buffer$$

Ni^{2+} + His

Best fit parameters of the His → Ni(II) ITC data to a two-site model

K_1 and K_2 are the pH-independent binding constants calculated from $K_{1,ITC}$ and $K_{2,ITC}$, which also account for Ni(II) interaction with the buffer in the case of Tris and phosphate

Buffer (pH)	$n_{1,ITC}$	log $K_{1,ITC}$	$\Delta H°_{1,ITC}$[a] ($\Delta H°_{1,Calc}$)[a, b]	log K_1	$n_{2,ITC}$	log $K_{2,ITC}$	$\Delta H°_{2,ITC}$[a] ($\Delta H°_{2,Calc}$)[a, b]	log K_2	log (K_1K_2)
100 mM HEPES (7.60)	$n_{2,ITC}$	6.69	-2.39 (-2.84)	8.21	1.09	5.22	-3.0 (-3.14)	6.74	14.95
100 mM Tris (8.10)	$n_{2,ITC}$	6.67	-6.16 (-8.91)	8.47	1.08	4.82	-5.80 (-9.21)	7.19	15.66
25 mM Phosphate (6.80, I = 0.1 M)	$n_{2,ITC}$	5.89	1.50 (2.09)	8.66	1.13	4.59	1.02 (1.79)	6.95	15.61
20 mM HEPES (7.50, I = 0.1 M)	$n_{2,ITC}$	6.19	-2.16 (-2.79)	7.81	1.04	5.05	-3.00 (-3.09)	6.67	14.48
25 mM Tris (8.23, I = 0.1 M)	$n_{2,ITC}$	6.38	-6.99 (-8.86)	7.74	0.92	5.27	-6.66 (-9.16)	7.02	14.77
Reported Values [c]				**8.66**				**6.86**	**15.52**

a. kcal/mol

b. ΔH calculated from ΔH for His deprotonation, metal-His binding and buffer protonation.

c. A. E. Martell, R. M. Smith, V. I. Simeon, *Critical Stability Constants*, Plenum, New York, 1989.

- Metal-buffer interaction needs to be included in data analysis

Ni^{2+} + His

Two Equilibria of Interest

$$Ni^{2+} + His^{-} \rightleftarrows Ni(His)^{+}$$

$$Ni(His)^{+} + His^{-} \rightleftarrows Ni(His)_2$$

First Equilibrium in a Buffered ITC Measurement

$$Ni(buffer)_x^{2+} \rightleftarrows Ni^{+2} + X\ buffer$$

$$HisH \rightleftarrows His^{-} + H^{+}$$

$$Ni^{2+} + His^{-} \rightleftarrows Ni(His)^{+}$$

$$H^{+} + buffer \rightleftarrows bufferH^{+}$$

$$Ni(buffer)_x^{2+} + HisH \rightleftarrows Ni(His)^{+} + bufferH^{+} + (X\text{-}1)\ buffer$$

Ni^{2+} + His

Thermodynamic parameters for His binding to Ni^{+2} at 25°C.

Parameters in bold are derived from a thermodynamic cycle consisting of measured values.

Reactions	log K	ΔG^{o} [a]	ΔH^{o} [a]	ΔS^{o} [b]
x = 0.82, y = 0.90 and z = 0.0075 for 100 mM Tris at pH 8.10				
Overall ITC Experiment Equilibrium:				
$xNi(Tris)_2^{2+} + (1-x)Ni(Tris)^{2+} + 2yHis + 2zHis^{+} + 2(1-y-z)His^{-} \rightleftarrows Ni(His)_2 + 2(y+2z)TrisH^{+} + (1+x-2y-4z)Tris$	9.63 [c]	-13.15	-11.96	3.99
Literature Equilibria: [d]				
2y ($His \rightleftarrows His^{-} + H^{+}$	-9.09	12.41	10.50	-6.41)
2z ($His^{+} \rightleftarrows His^{-} + 2H^{+}$	-15.11	20.63	17.50	-10.51)
x ($Ni(Tris)_2^{2+} \rightleftarrows Ni^{2+} + 2Tris$	-4.60	6.28	**6.77**	**1.64** [e])
(1-x) ($Ni(Tris)^{2+} \rightleftarrows Ni^{2+} + Tris$	-2.63	3.59	**3.42**	**-0.57** [e])
1 ($Ni^{2+} + 2His^{-} \rightleftarrows Ni(His)_2$	15.54	-21.22	-16.50	15.83)
2(y + 2z) ($H^{+} + Tris \rightleftarrows TrisH^{+}$	8.10	-11.06	-11.36	-1.01)
	9.53	**-13.01**		

- kcal/mol
- cal/(mol•K)
- $K = K_{1,ITC}K_{2,ITC}f(x, y, z)$, where $f(x, y, z)$ is a function that accounts for the relative contributions
- of different His protonation species and metal-Tris complexes to the overall equilibrium.
- A. E. Martell, R. M. Smith, V. I. Simeon, *Critical Stability Constants*, Plenum, New York, 1989.
- derived from $\Delta G^{o} = \Delta H^{o} - T\Delta S^{o}$
- negligible contribution

- Account for all of the complexes and their protonation states.

ITC Studies of Metal Ions Binding to Proteins

1. Fe^{3+} binding to Transferrin: Brandts, Woodworth, Mason

2. Fe^{2+} binding to Ferritin: Chasteen, Bou-Abdallah

3. Cu^{2+} binding to Albumin

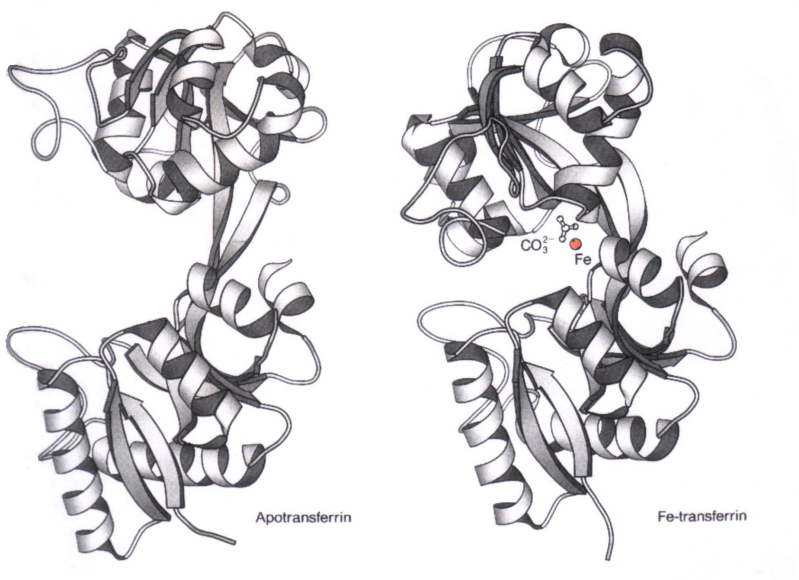
4. Metal ions binding to the His-rich sequence of IRT1

5. Metal ions binding to Metallothionein

6. Cu^{2+} binding to the urease metallochaperone UreE

Transport: Transferrin

Transferrin



N-terminal Domain of
Apo and Fe^{3+}-bound Lactoferrin
(from Priniciples of Bioinorganic Chemistry
by Lippard and Berg)

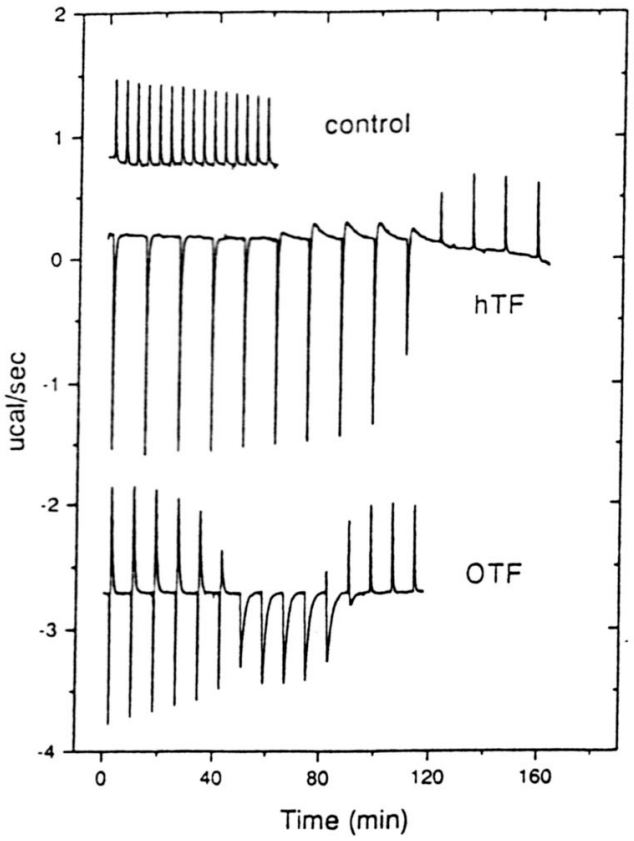
Iron transporting ~80 kDa circulatory glycoprotein.

Consists of similar N- and C-terminal domains that each bind one Fe^{3+} and a synergistic CO_3^{2-} ion.

Octahedral Fe^{3+} coordination consists of Asp-60, Tyr-92, Tyr-192, His-253, and the CO_3^{2-} ion.

Binds Fe^{3+} tightly with $K_{apparent}$ ~10^{20}.

Transport: Transferrin



L.-N. Lin, A. B. Mason, R. C. Woodworth, J. F. Brandts, *Biochemistry*, 30, 11660-11669, 1991, and *Biochemistry*, 32, 9398-9406, 1993.

ITC thermograms of Fe(NTA) binding to both ovotransferrin (OTF) and human transferrin (hTF) show different thermodynamics for the N-terminal and C-terminal domains.

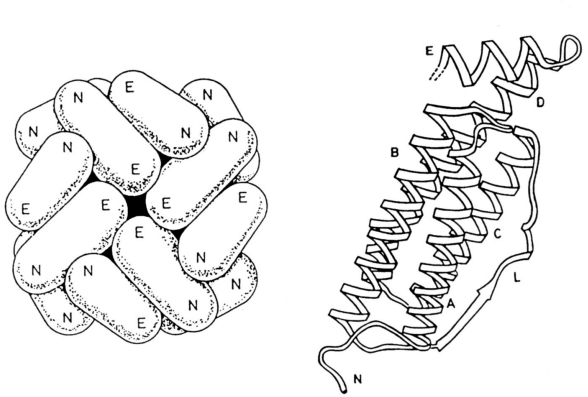
Injection peaks show fast initial contact binding followed by slow CO_3^{2-} substitution for NTA^{3-}.

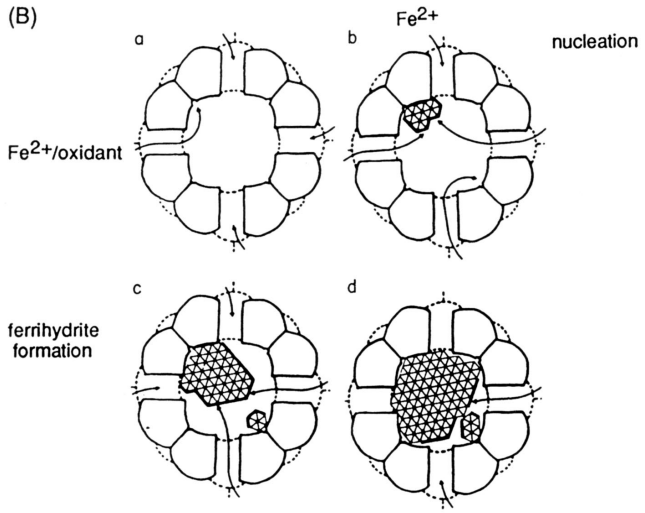
Data analysis indicates $K_N > K_C$, for OTF, while $K_C > K_N$ for hTF, which correlates with the enthalpy of Fe^{3+} and CO_3^{2-} binding.

$\Delta H°$ of binding is very temperature dependent, resulting in a large negative value of ΔC_P for both domains.

The thermodynamics of interaction between the two sites, which are separated by ~40 Å, were quantified.

Storage: Ferritin





(from Inorganic Biochemistry by Cowan)

Ferritin

Protein shell consisting of 24 H (heavy, 21 kDa) and/or L (light, 18.5 kDa) subunits.

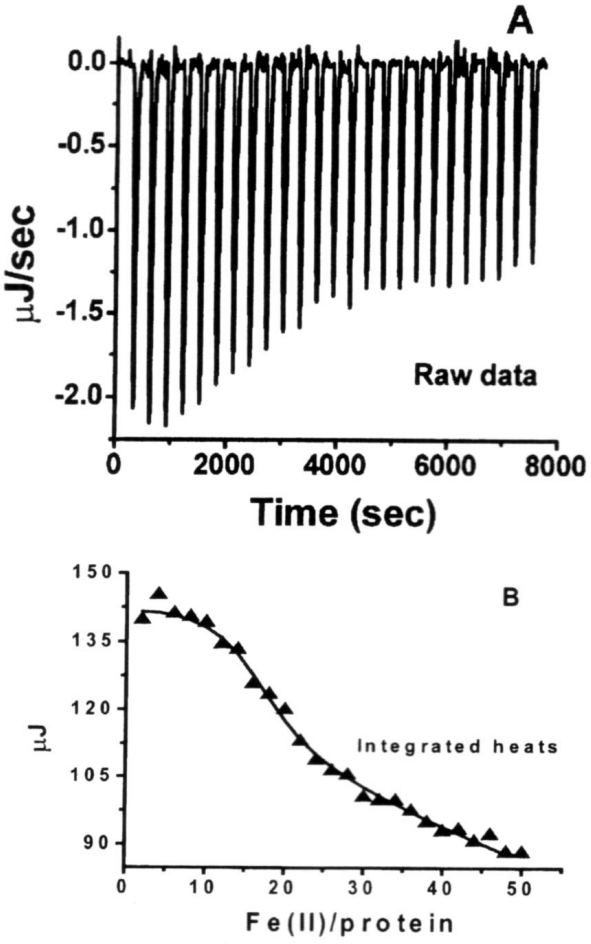
The ~75 Å diameter core binds up to ~4500 Fe^{3+} ions in a ferrihydrate mineral.

Access to the core is through 3-fold hydrophilic and 4-fold hydrophobic channels.

Fe^{2+} ions bind initially to dinuclear ferroxidase sites on the H subunits, where they are oxidized and subsequently nucleate formation of the mineral core.

Release of iron involves reduction to labile Fe^{2+} ions, possibly by organic reductants that gain access to the core through the hydrophobic channels

Storage: Ferritin



F. Bou-Abdallah, P. Arosio, P. Santambrogio, X. Yang, C. Janus-Chandler, N. D. Chasteen *Biochemistry*, 41, 11184-11191, 2002.

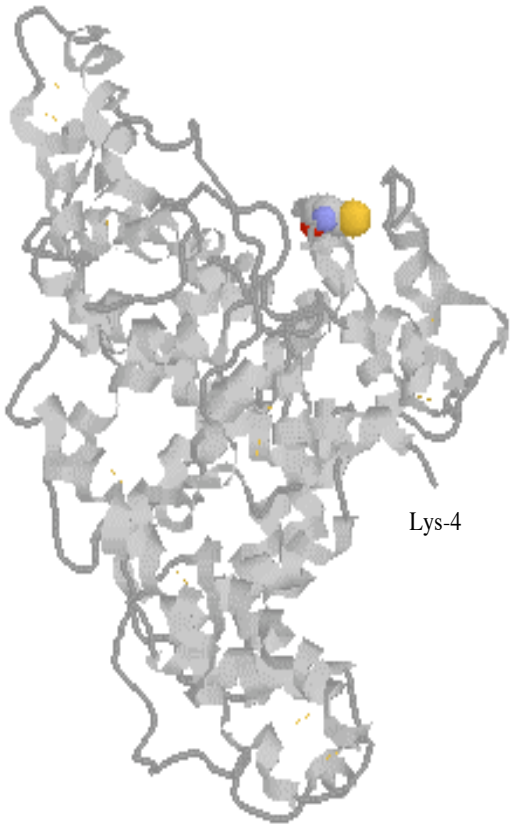
ITC studies by Chasteen and co-workers have shown that one Fe^{2+} ion binds to each of the ferroxidase sites in the absence of an oxidant, with additional Fe^{2+} ions binding more weakly to the protein.

Fe^{2+} binding is endothermic and entropically favored (fit parameters for data at left in 50 mM Mops, pH 7.43 are n = 23, K = 2 x 10^5, $\Delta G° = -7.2$ kcal/mol, $\Delta H° = 2.1$ kcal/mol, and $\Delta S° = 31.2$ cal/mol K.

Reduced binding stiochiometry with 3-fold channel mutants suggests Fe^{2+} gains access through these channels.

CSC instrument was used.

Albumin



Human Serum Albumin
(PDB file 1UOR(HSA))

Most abundant circulatory protein (~ 0.6 mM).

Binds and transports amino acids, fatty acids, metal ions, drugs.

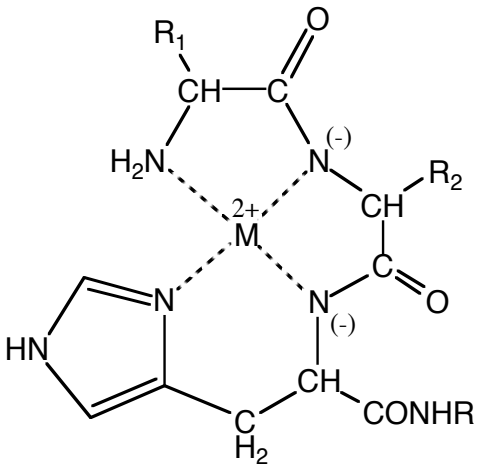
35 Cys residues: 17 disulfide bonds; 1 free thiol, Cys-34 (highlighted).

Binds and transports Cu^{+2} and Ni^{+2} at its N-terminal X-X-His- binding site (not resolved in X-ray structure).

Cu^{2+} Binding to Albumin

Human	Asp Ala	**His** Lys Ser Glu Val Ala His Arg Phe Lys Asp
Macaque	Asp Thr	**His** Lys Ser Glu Val Ala His Arg Phe Lys Asp
Horse	Asp Thr	**His** Lys Ser Glu Ile Ala His Arg Phe Asn Asp
Bovine	Asp Thr	**His** Lys Ser Glu Ile Ala His Arg Phe Lys Asp
Sheep	Asp Thr	**His** Lys Ser Glu Ile Ala His Arg Phe Lys Asp
Rat	Glu Ala	**His** Lys Ser Glu Ile Ala His Arg Phe Lys Asp
Dog	Glu Ala	*Tyr* Lys Ser Glu Ile Ala His Arg Tyr Asn Asp
Pig	Asp Thr	*Tyr* Lys Ser Glu Ile Ala His Arg Phe Lys Asp
Chicken	Asp Ala *Glu*	His Lys Ser Glu Ile Ala His Arg Tyr Asn Asp

N-terminal sequence of serum albumin from nine species.



- N-terminal sequence with His in the third position provides a strong square planar coordination for Cu^{+2} and Ni^{+2} with deprotonation of the first two amides.

Cu^{2+} Binding to Albumin

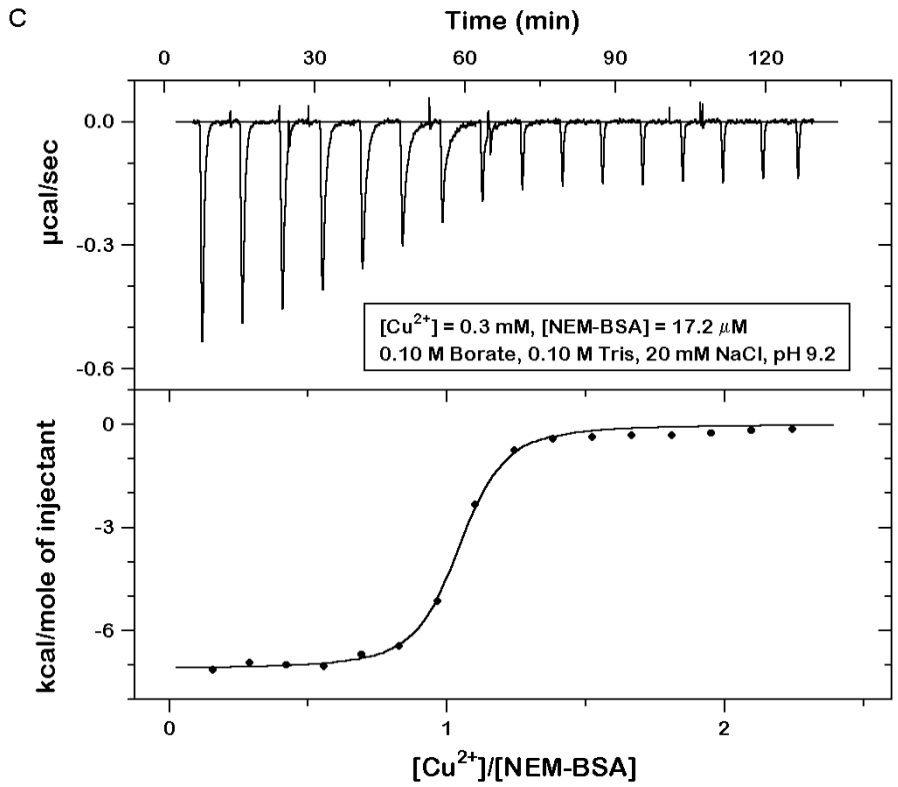
Cu^{+2} binding to bovine serum albumin (BSA)
(reported, pH-independent, and pH 7.4 equilibrium constants

Method	pH	Buffer	Competing Ligand	log $K_{reported}$ [a]	log $K_{calc..}$ [b]	log $K_{pH\ 7.4}$ [c]	Reference
Ultrafiltration	7.5 [c]	MOPS (50 mM)	Gly	13.2	-1.26	13.0	Giroux and Schoun, 1981
Dialysis	7.4			12.04	-2.12	12.2	Ryall 1974
	7.0 [d]	HEPES (30 mM)	His	11.12	-1.79	12.5	Saltman, et al, 1993
	8.5 [d]	HEPPS (30 mM)	His	11.12	-5.80	8.5	Saltman, et al, 1993
Ion-selective electrode	7.3	HEPES (20 mM)		13.2	-0.67	13.6	Ljones, et al, 1986
	7.3	BisTris (46 mM)		12.6	-1.27	13.0	Ljones, et al, 1986
	5.9	Acetate (25 mM)		11.2	2.36	16.7	Ljones, et al, 1986

a) $K_{reported} = [Cu(BSA)]/[Cu^{2+}][BSA]$; reported apparent binding constant at the given pH and experimental conditions.
b) $K_{calc..} = [Cu(BSA)][H^+]^2/[Cu^{2+}][BSA]$; intrinsic equilibrium constant for the reaction: $Cu^{2+} + BSA \rightleftarrows Cu(BSA) + 2H^+$
c) $K_{pH\ 7.4} = [Cu(BSA)]/[Cu^{2+}][BSA]$; apparent binding constant at pH 7.4.

- Use competition with coordinating buffer (e.g. Tris) to measure high affinity Cu^{2+} binding to BSA by ITC.

Cu^{2+} Binding to Albumin



Cu^{2+} → BSA

100 mM Tris pH 9.2
(100 mM borate, 20 mM NaCl)

Cu^{2+} Binding to Albumin

Thermodynamic parameters for Cu^{+2} binding to

Cys-34 oxidized, Cys-34 reduced, and Cys-34 blocked BSA

(100 mM borate and 100 mM Tris buffer, pH 9.2, 20 mM NaCl, 25°C)

Overall ITC Equilibrium: $Cu(Tris)_4^{2+} + BSA \rightleftarrows Cu(BSA) + 2TrisH^+ + 2Tris$

Individual Equilibria: $Cu(Tris)_4^{2+} \rightleftarrows Cu^{2+} + 4Tris$

$2Tris + 2H^+ \rightleftarrows 2TrisH^+$

$Cu^{2+} + BSA \rightleftarrows Cu(BSA) + 2H^+$

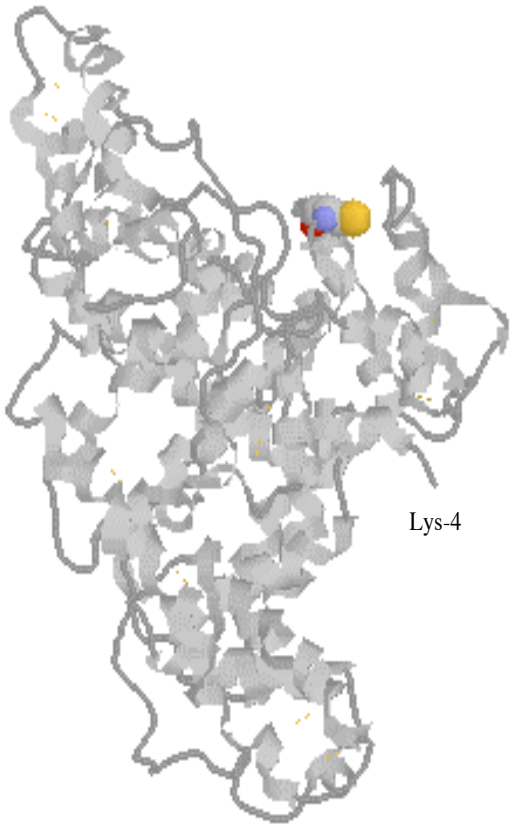
	log K	ΔG^{o} [a]	ΔH^{o} [a]	ΔS^{o} [b]	log K^{*} (pH 7.4)
Cys-34 Oxidized BSA	**-1.34**	**1.83**	**0.88**	**-3.18**	**12.9**
Cys-34 Reduced BSA	**-0.49**	**0.67**	**0.70**	**0.1**	**13.8**
Cys-34 Blocked BSA	**-1.40**	**1.91**	**0.85**	**-3.55**	**12.9**
GlyGlyHis [c]	-1.62	2.21	-1.85	-13.62	

a. kcal/mol
b. cal/(mol•K)
c. Y. Zhang, S. Akilesh, D. E. Wilcox (2000) *Inorganic Chemistry* **39** 3057-3064.

- Higher Cu^{2+} affinity of albumin with reduced Cys-34 is predominantly (~90%) due to entropic ($\Delta S°$) factors.

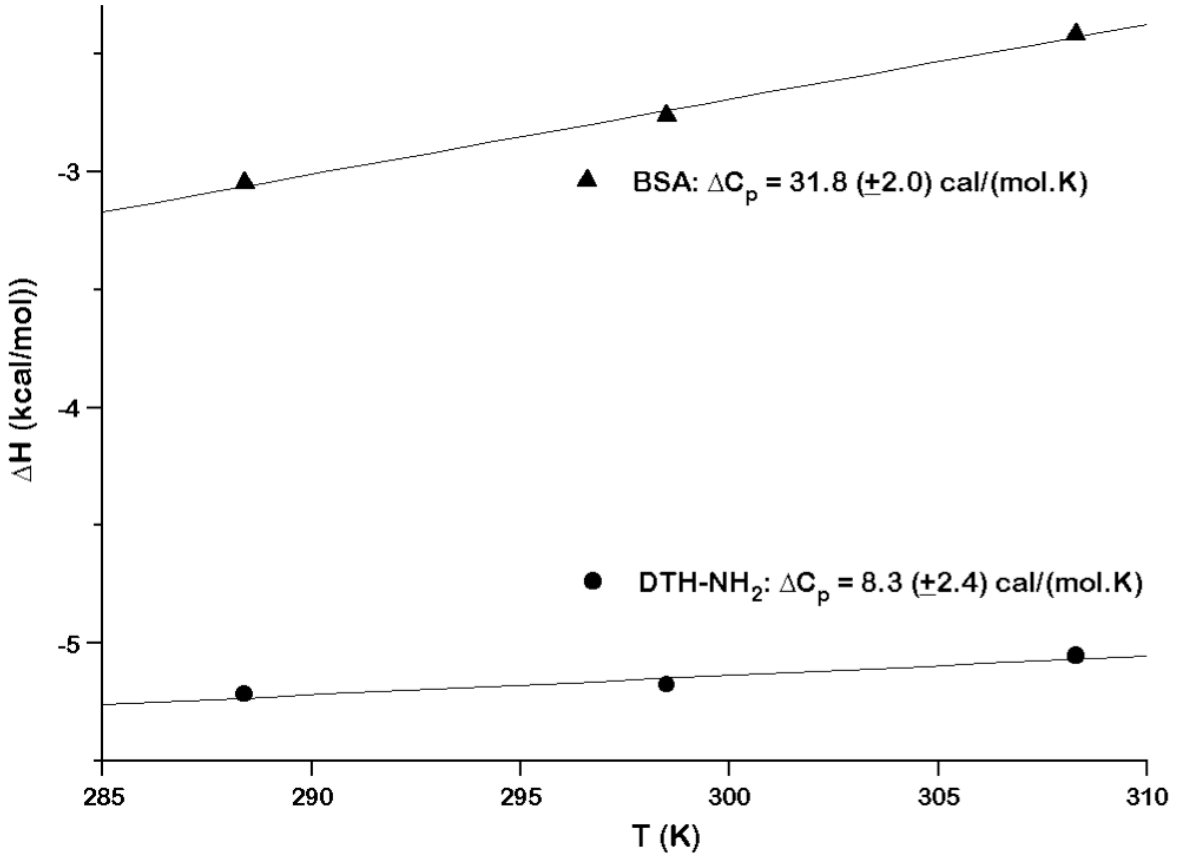
(Y. Zhang, D. E. Wilcox *J. Biol. Inorg. Chem.* **2002** *7*, 327-337)

Cu^{2+} Binding to Albumin



Redox status of Cys-34 affects Cu^{2+} (and Ni^{2+}) affinity
at N-terminal binding site ~20 Å away

Cu^{2+} Binding to Albumin: ΔC_p measurements



Cu^{2+} Binding to Albumin

ΔCp° for Metal Ions Binding to Proteins

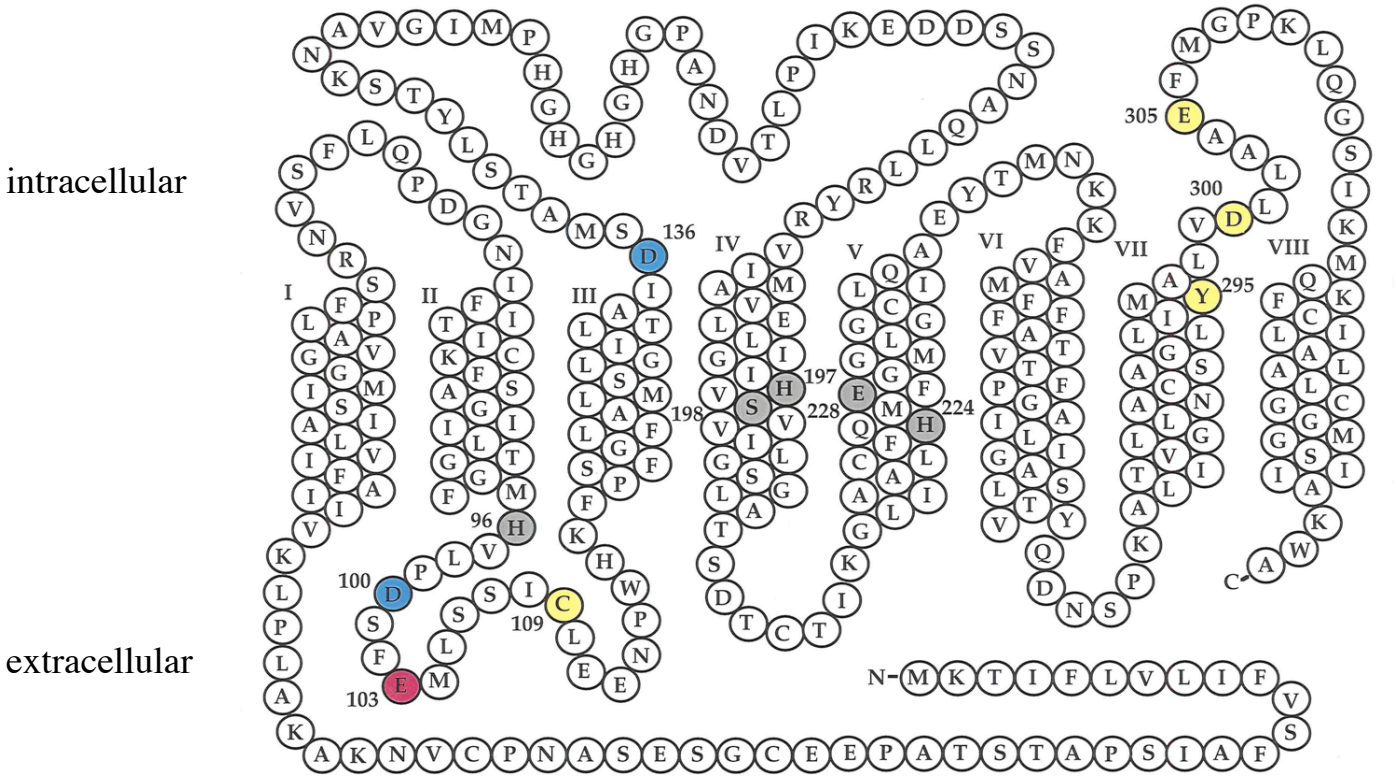
Protein	Metal ion	Buffer	**ΔCp°** (cal/mol·K)	Reference
Albumin (BSA)	Cu^{+2}	Tris	32 ± 2	Wilcox, et al, 2003
AspThrHis-NH_2	Cu^{+2}	Tris	8 ± 2	Wilcox, et al, 2003
Carbonic Anhydrase	Co^{+2}	ACES	1 ± 33	Toone, et al, 2001
Carbonic Anhydrase	Cu^{+2}	ACES	-57 ± 8	Toone, et al, 2001
Carbonic Anhydrase	Zn^{+2}	ACES	-117 ± 10	Toone, et al, 2001
Transferrin (C site)	Fe^{+3}	HEPES	-70	Brandts, et al, 1993
Transferrin (C site)	Fe^{+3}	Tris	-220	Brandts, et al, 1993
Transferrin (N site)	Fe^{+3}	HEPES	-200	Brandts, et al, 1993
Transferrin (N site)	Fe^{+3}	Tris	-520	Brandts, et al, 1993
Ovoransferrin (N site)	Fe^{+3}	HEPES	-335	Brandts, et al, 1991
Ovotransferrin (C site)	Fe^{+3}	HEPES	-440	Brandts, et al, 1991
Concanavalin A (S3 site)	Cd^{+2}	DMG	-342 ± 98	Schwarz, et al, 1998

- Larger negative value of $\Delta C_p°$ appears to be associated with major protein structural changes upon metal binding.

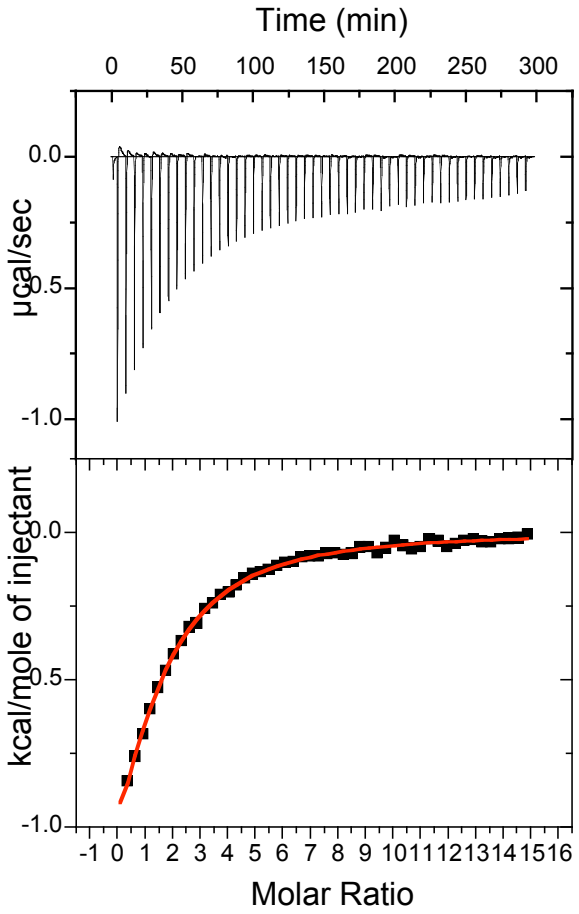
Iron-Regulated Transporter (IRT1)
from *Arabidopsis thaliana*

Membrane spanning metal transporter in roots of plants.

Unique His-rich sequence, PHGHGHGHGP, in long intracellular loop.



IRT1pep: PHGHGHGHGP



Zn^{2+} → IRT1pep

25 mM ACES, pH 7.25

$n = 1.07 \pm 0.02$

$K_{ITC} = 8.6 \pm 0.4 \times 10^3$

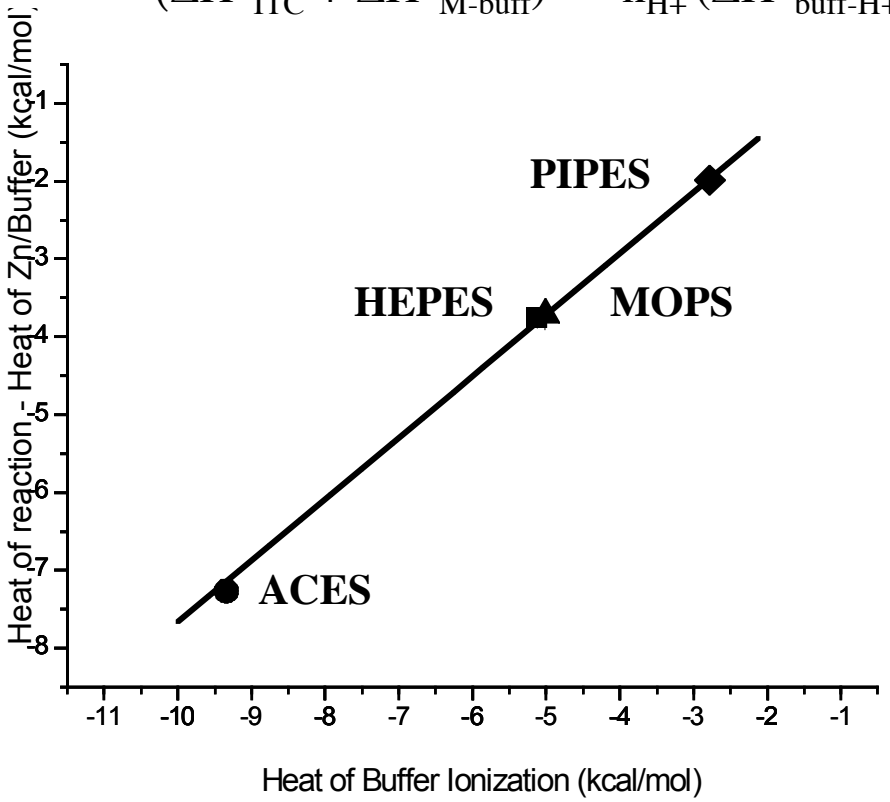
$\Delta H_{ITC} = -2.3 \pm 0.2$ kcal/mol

Lower affinity binding requires careful subtraction of background enthalpy of dilution.

IRT1pep: PHGHGHGHGP

$$\Delta H^\circ_{ITC} = -\Delta H^\circ_{M\text{-}buff} - \Delta H^\circ_{Pep\text{-}H+} + n_{H+}(\Delta H^\circ_{buff\text{-}H+}) + \Delta H^\circ_{M\text{-}Pep}$$

$$(\Delta H^\circ_{ITC} + \Delta H^\circ_{M\text{-}buff}) = n_{H+}(\Delta H^\circ_{buff\text{-}H+}) + (\Delta H^\circ_{M\text{-}Pep} - \Delta H^\circ_{Pep\text{-}H+})$$

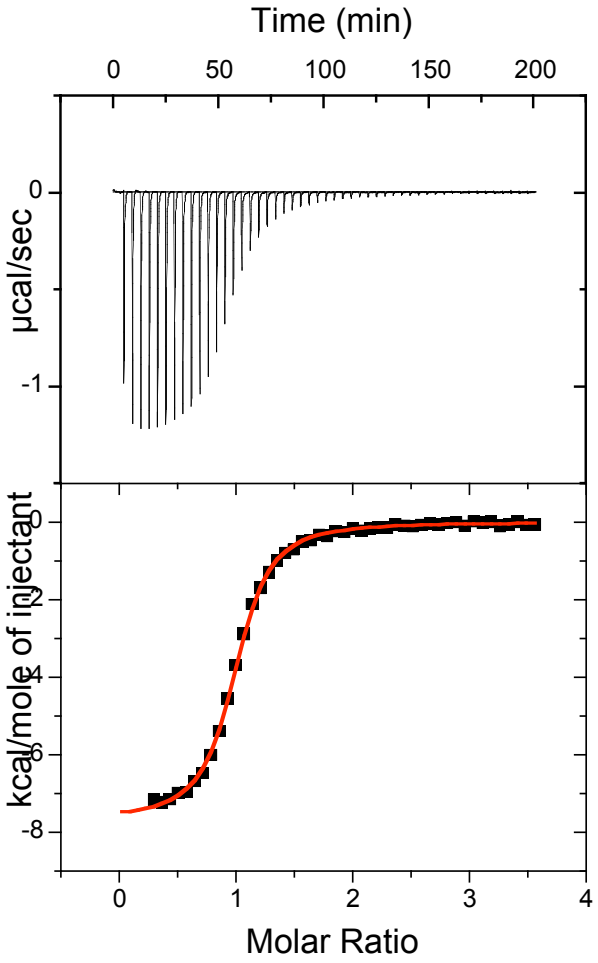


Slope = n_{H+}
= 0.79 ± 0.02

protons displaced from IRT1pep upon Zn^{2+} binding at pH 7.25

Buffer dependence of the binding enthalpy can be used to quantify the number of protons displaced upon metal binding.

IRT1pep: PHGHGHGHGP



EDTA → 6.5 µM Fe^{3+}
+ 5 mM IRT1pep

pH 6.45

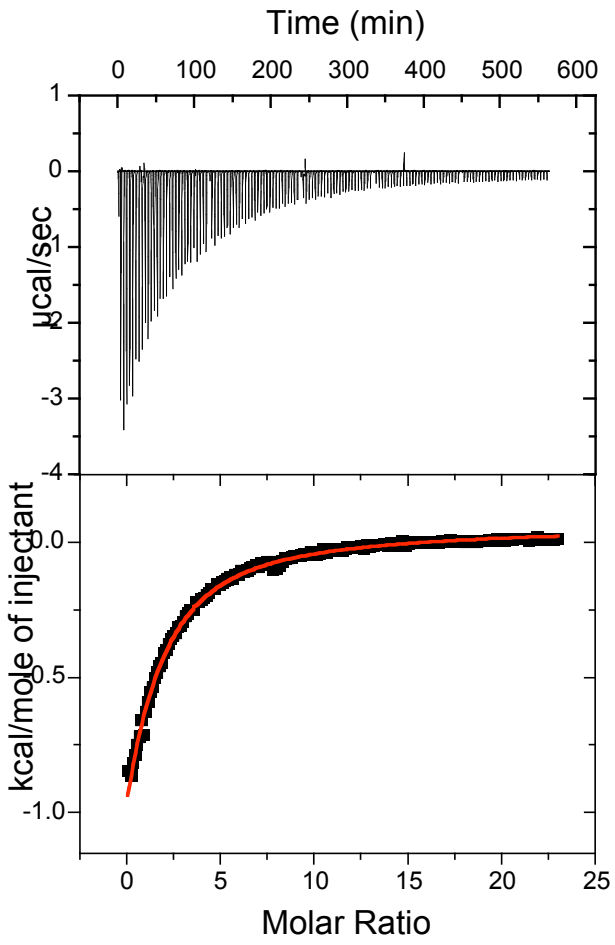
n = 0.98 ± 0.01

K_{ITC} = 3.1 ± 0.3 x 10^6

ΔH_{ITC} = -7.4 ± 0.1 kcal/mol

Fe^{3+} can be studied with ITC by chelation measurements.

IRT1pep: PHGHGHGHGP



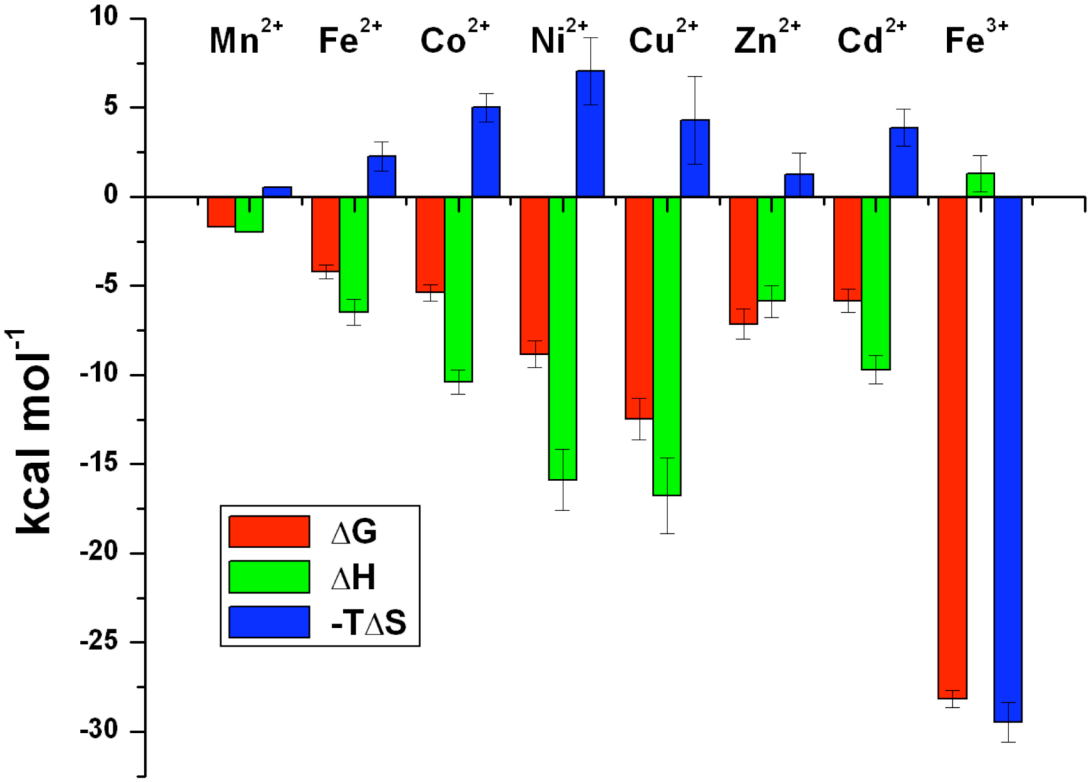
Fe^{2+} → IRT1pep

25 mM MOPS, pH 7.25
(strictly anaerobic conditions)

n = 1 (known and fixed)
K_{ITC} = 311 ± 7
ΔH_{ITC} = -4.47 ± 0.06 kcal/mol

Low affinity binding requires extended ITC data (concatination).

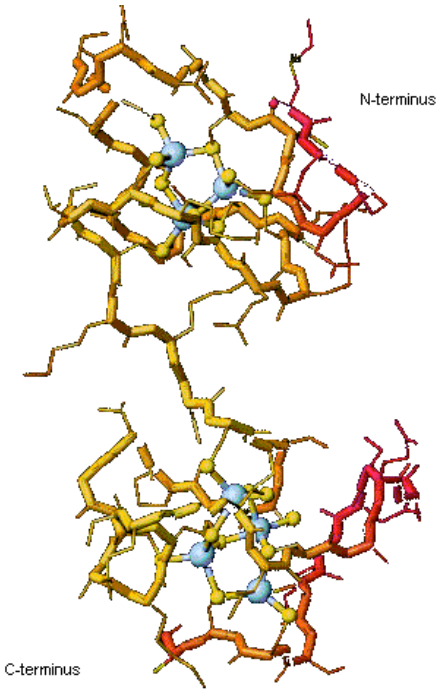
Thermodynamics of metal ions binding to IRT1pep

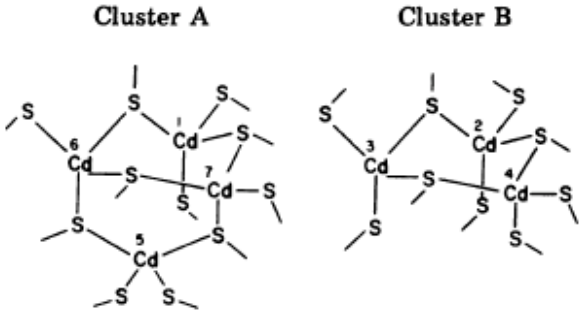


N. E. Grossoehme, S. Akilesh, M. L. Guerinot, D. E. Wilcox, *Inorganic Chemistry* **2006** *45*, 8500-8508

Metallothionein

MDPNCSCAADGACTCATSCKCKECKCTSCKKSCCSCCPSGCAKCAQGCICKGASDKCSCCA





Relative metal ion affinity:
$Ni^{2+} \sim Co^{2+} < Zn^{2+} < Cd^{2+} \sim Pb^{2+} < Ag^{+} \sim Cu^{+} < Hg^{2+}$

Average Zn^{2+} affinity: 3×10^{11} to 1×10^{12}

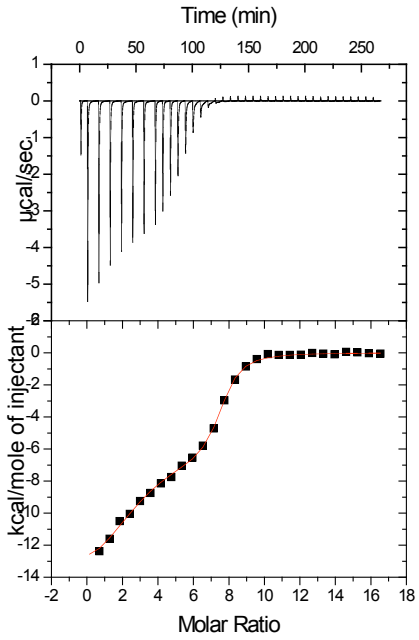
Recently Krezel and Maret have reported that 4 Zn^{2+} bind with $K = 6 \times 10^{11}$, 2 Zn^{2+} bind with $K \sim 10^{10}$ and 1 Zn^{2+} binds with $K = 5 \times 10^{7}$.

Metallothionein

Zn^{2+} → apo MT

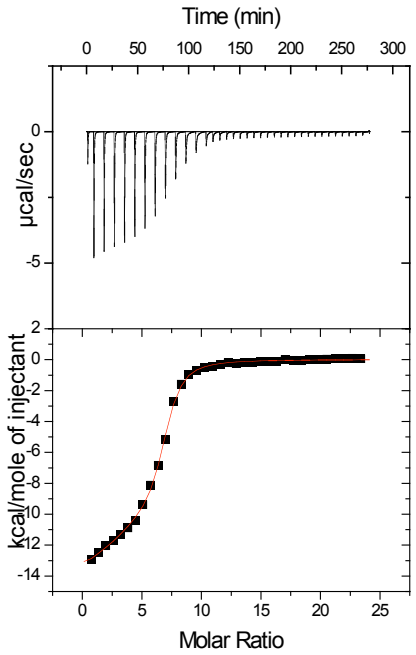
pH 7.4 , 0.56 mM $S_2O_4^{2-}$

100 mM **ACES**



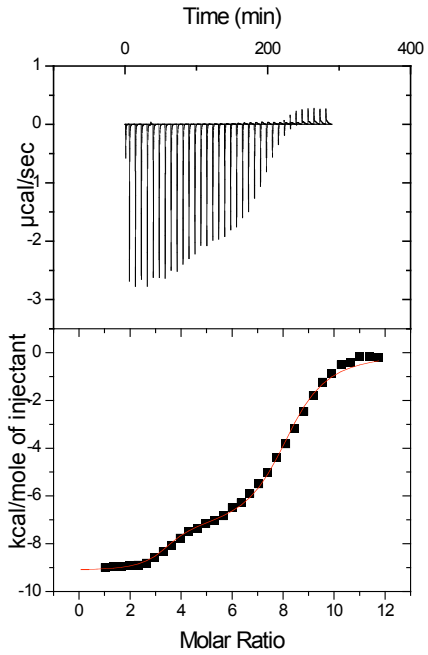
$n_1 = 2.3 \pm 0.2$
$K_1 = 5 \pm 1 \times 10^6$
$\Delta H_1 = -15 \pm 1$ kcal/mol
$n_2 = 5.0 \pm 0.2$
$K_2 = 8 \pm 1 \times 10^5$
$\Delta H_2 = -6.3 \pm 0.6$ kcal/mol

100 mM **Bis-Tris**



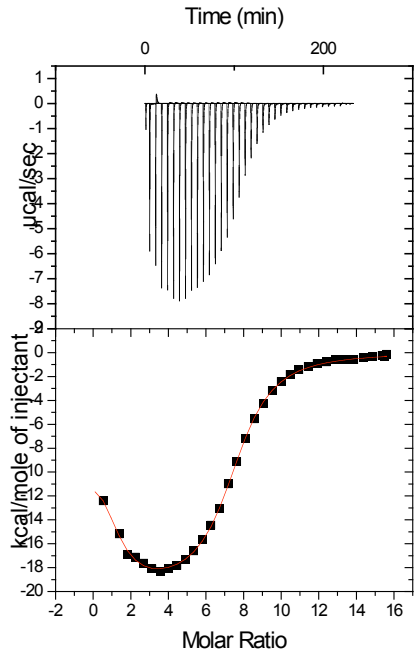
$n_1 = 4 \pm 1$
$K_1 = 2 \pm 1 \times 10^6$
$\Delta H_1 = -15 \pm 2$ kcal/mol
$n_2 = 3 \pm 1$
$K_2 = 5.9 \pm 0.8 \times 10^5$
$\Delta H_2 = -7 \pm 5$ kcal/mol

100 mM **HEPES**



$n_1 = 3.4 \pm 0.1$
$K_1 = 1.1 \pm 0.4 \times 10^7$
$\Delta H_1 = -9.1 \pm 0.1$ kcal/mol
$n_2 = 4.0 \pm 0.1$
$K_2 = 2.8 \pm 0.3 \times 10^5$
$\Delta H_2 = -7.1 \pm 0.2$ kcal/mol

100 mM **Tris**



$n_1 = 1.2 \pm 0.1$
$K_1 = 5 \pm 1 \times 10^6$
$\Delta H_1 = -9 \pm 1$
$n_2 = 6.3 \pm 0.1$
$K_2 = 2.8 \pm 0.1 \times 10^5$
$\Delta H_2 = -20.1 \pm 0.2$ kcal/mol

Metallothionein

First set of Zn^{2+} ions binding:

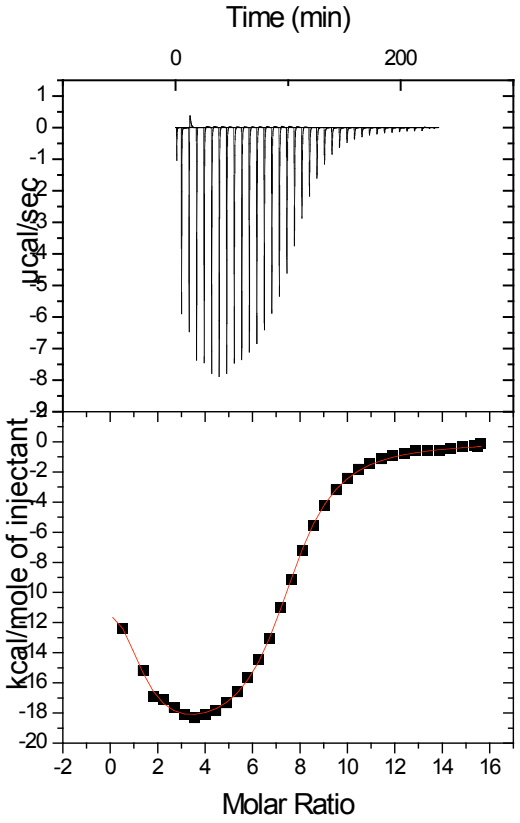
$$4\ Zn^{2+} + MT(H^+)_{16} \rightleftarrows Zn_4MT(H^+)_4 + 12\ H^+$$

Second set of Zn^{2+} ions binding:

$$3\ Zn^{2+} + Zn_4MT(H^+)_4 \rightleftarrows Zn_4MT + 4\ H^+$$

	Buffer	Log K	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol K)
1st Event (n =4)	HEPES	9.7	-13.2	5.7	63
	Bis-Tris	11.2	-15.3	6.1	72
	ACES	11.4	-15.6	5.8	72
	Average	10.8 ± 0.6	-14.7 ± 0.8	5.9 ± 0.1	69 ± 3
2nd Event (n=3)	HEPES	8.8	-12.0	0.67	42
	Bis-Tris	9.2	-12.9	0.97	47
	ACES	9.5	-12.6	0.85	45
	Average	9.2 ± 0.2	-12.5 ± 0.3	0.8 ± 0.1	45 ± 1

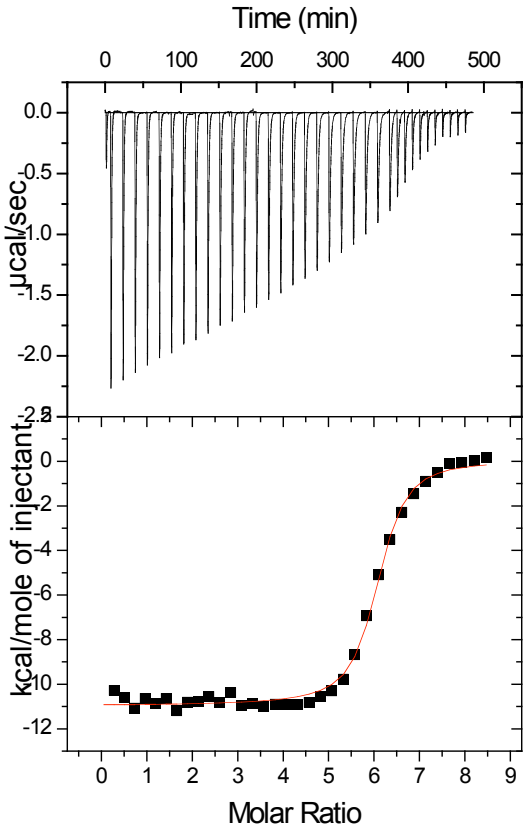
Metallothionein



Zn^{2+} → apo MT

100 mM Tris, pH 7.4

$n_1 = 1.2 \pm 0.1$
$K_1 = 5 \pm 1 \times 10^6$
$\Delta H_1 = -9 \pm 1$
$n_2 = 6.3 \pm 0.1$
$K_2 = 2.8 \pm 0.1 \times 10^5$
$\Delta H_2 = -20.1 \pm 0.2$ kcal/mol

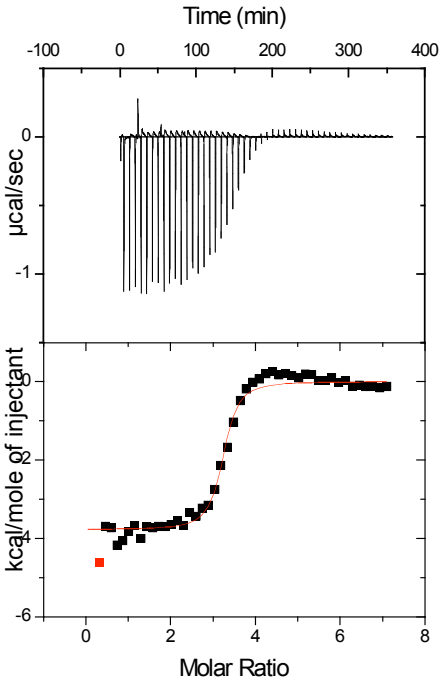


As^{3+} → apo MT

100 mM Tris, pH 7.4

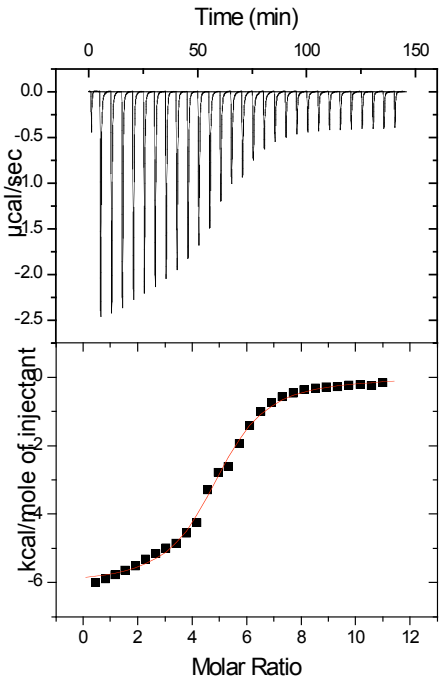
$n = 6.0 \pm 0.1$
$K_{ITC} = 1.6 \pm 0.2 \times 10^6$
$\Delta H_{ITC} = -10.9 \pm 0.1$ kcal/mol

Metallothionein



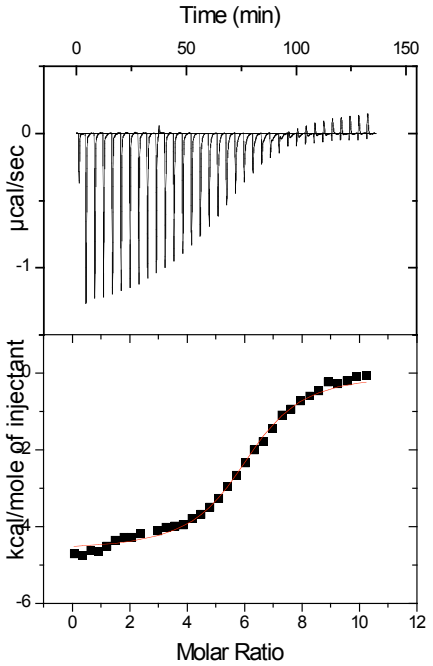
Cd^{2+} → Zn_7MT
100 mM **HEPES**, pH 7.4

n = 3.2 ± 0.1
K_{ITC} = 3.3 ± 0.8 x 10^6
ΔH_{ITC} = -3.8 ± 0.1 kcal/mol



Cd^{2+} → Zn_7MT
100 mM **Tris,** pH 7.4

n = 4.9 ± 0.1
K_{ITC} = 2.2 ± 0.2 x 10^5
ΔH_{ITC} = -6.1 ± 0.1 kcal/mol



Pb^{2+} → Zn_7MT
100 mM MES, **pH 6.1**

n = 6.9 ± 0.1
K_{ITC} = 3.1 ± 0.3 x 10^5
ΔH_{ITC} = -4.6 ± 0.1 kcal/mol

UreE from *Klebsiella aerogenes*

- Encoded by the *ure* operon, UreE is the metallochaperone protein that delivers Ni^{2+} to the apo urease enzyme.

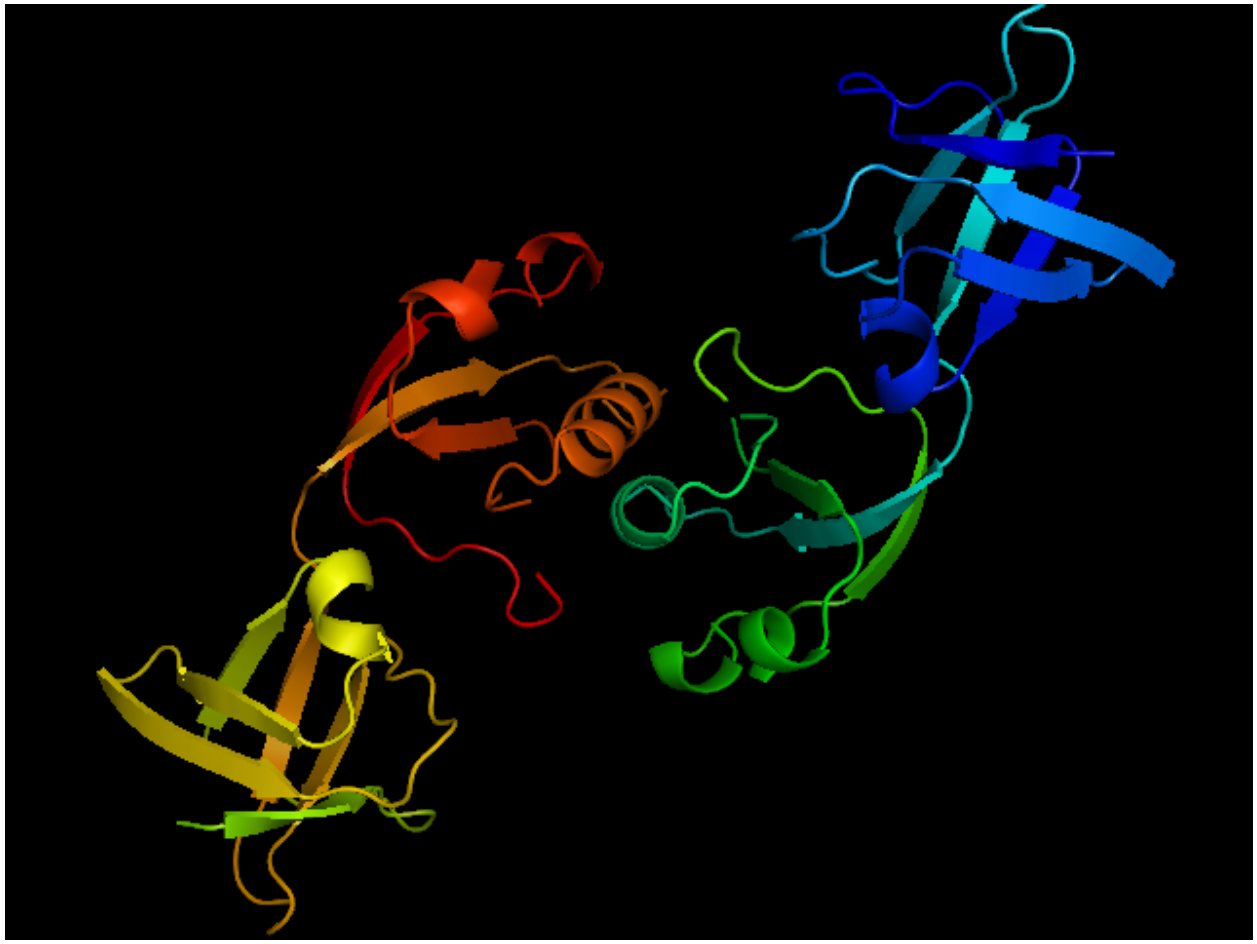
- *Ka*UreE lacking its 15-residue His-rich C-terminal tail is functional both *in vivo* and *in vitro*; this truncated form is known as H144*UreE.

- H144*UreE binds 2 Ni^{2+} ions (K_d = 1.5 and 50 μM) or 2 Cu^{2+} ions with ~2 His ligands each, as indicated by EXAFS and paramagnetic NMR.

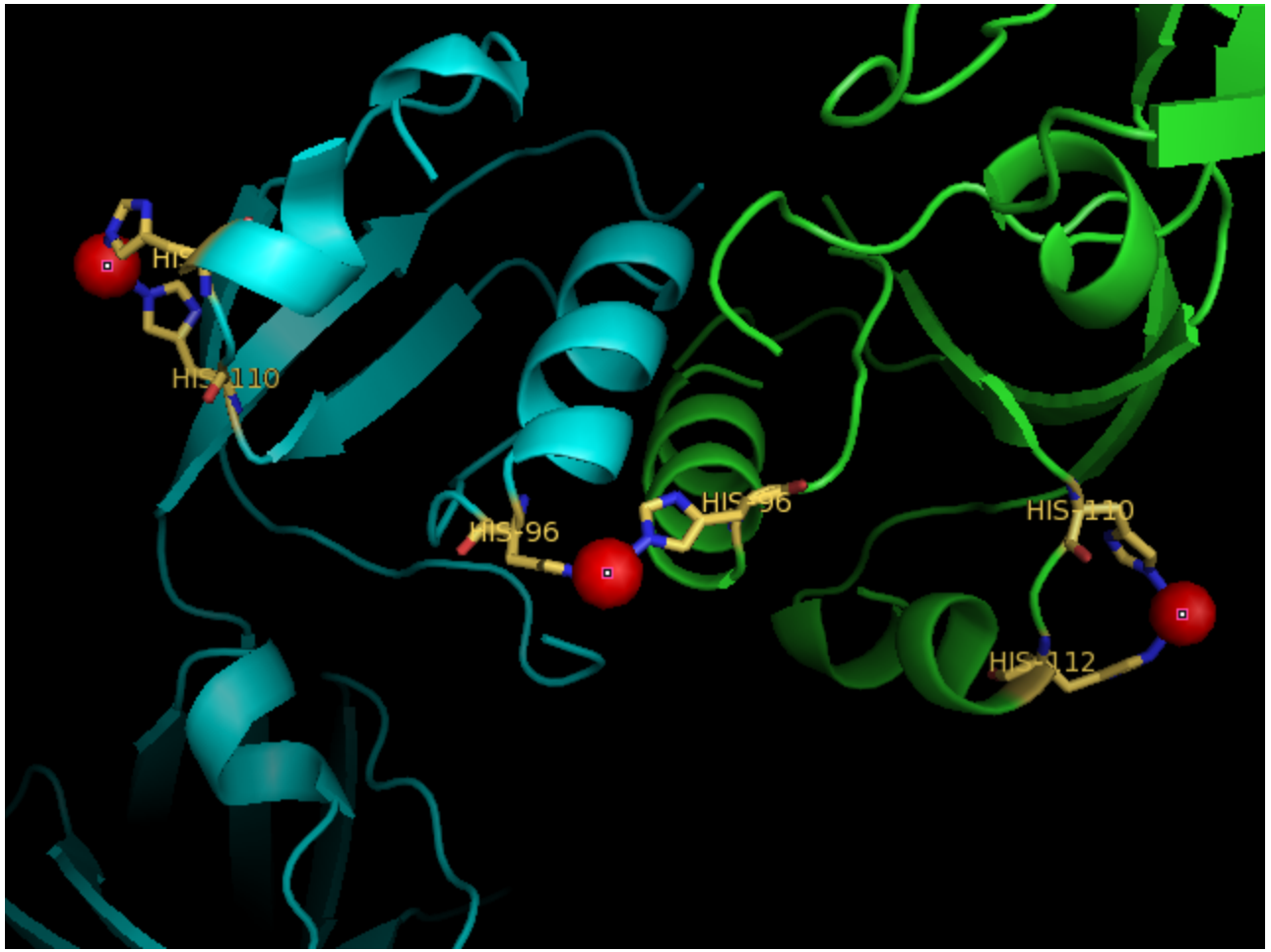
- X-ray crystallography of H144*UreE shows that each subunit consists of two domains, a metal-binding Atx1-like domain and an Hsp40-like domain, and it forms a head-to-head dimer structure. (H. K. Song, S. B. Mulrooney, R. Huber, R. P. Hausinger *J. Biol. Chem.* **2001** *276*, 49359-49364).

- Studied with ITC: N. E. Grossoehme, S. B. Mulrooney, R. P. Hausinger, D. E. Wilcox *Biochemistry* **2007** *46*, 10506-10516.

H144*UreE X-ray Structure



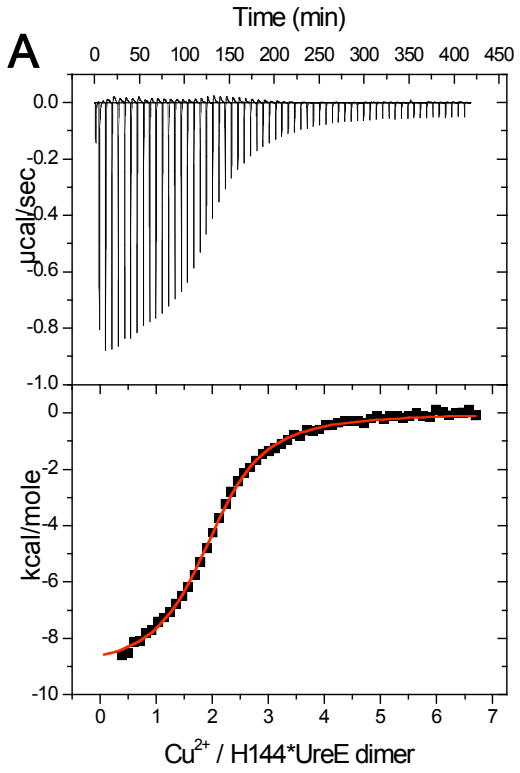
H144*UreE Crystallographic Cu^{2+} Sites



Addition of Cu^{2+} to protein crystals indicates **3** binding sites!

Cu^{2+} binding to H144*UreE

100 mM Tris, pH 7.45, 25°C

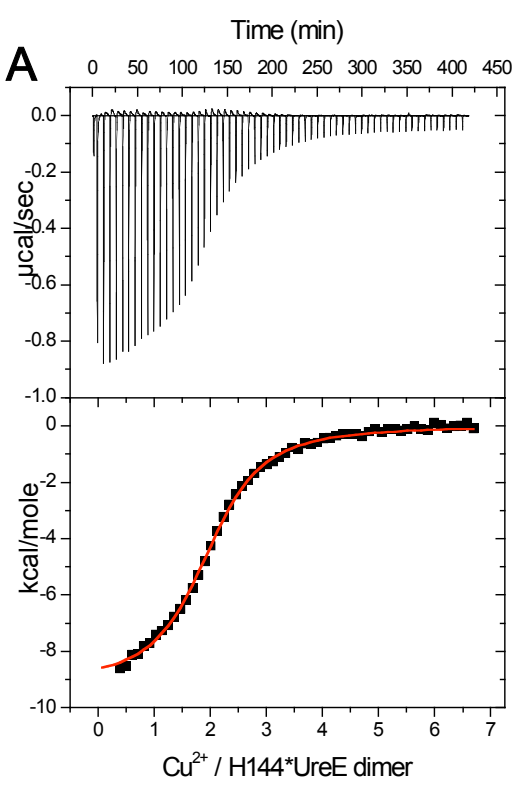


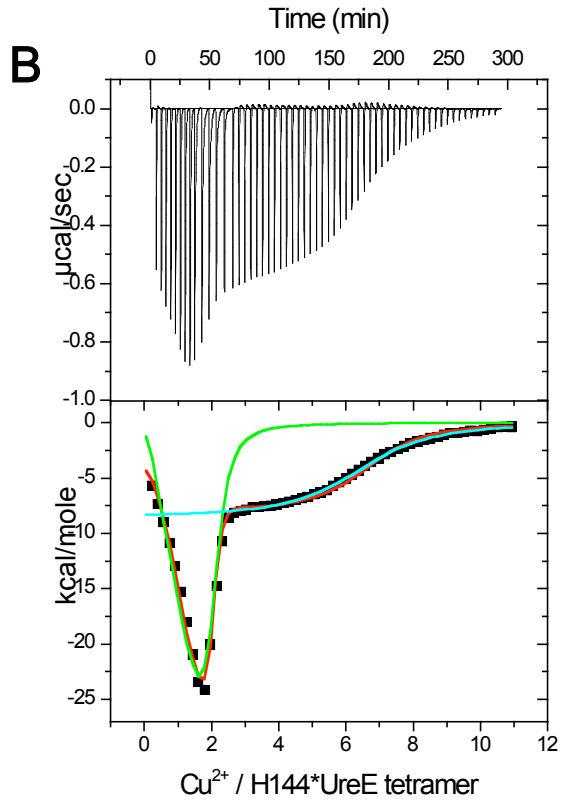
Cu^{2+} → **6 µM** H144*UreE

n = 2.0
K = 4.3 ± 0.2 x 10^5
ΔH = -9.2 ± 0.1 kcal/mol

Cu^{2+} binding to H144*UreE

100 mM Tris, pH 7.45, 25°C





Cu^{2+} → **6 µM** H144*UreE

n = 2.0
K = 4.3 ± 0.2 x 10^5
ΔH = -9.2 ± 0.1 kcal/mol

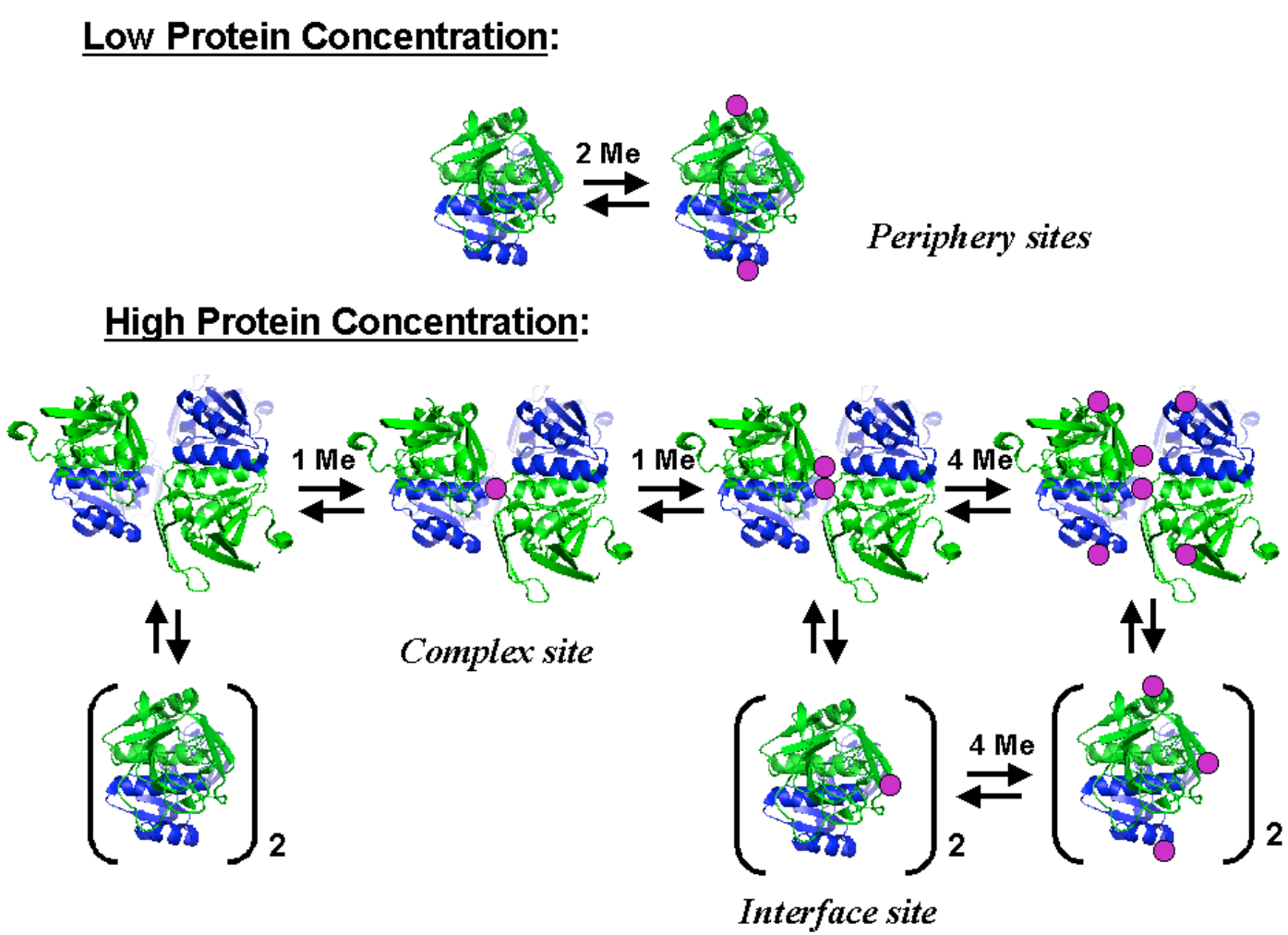
Cu^{2+} → **25 µM** H144*UreE

n_1 = 0.5 (/dimer)
K_1 = 1.1 ± 0.6 x 10^7
ΔH_1 = -0.1 ± 3.9 (kcal/mol)

n_2 = 0.5 (/dimer)
K_2 = 3 ± 1 x 10^6
ΔH_2 = -37 ± 3 (kcal/mol)

n_3 = 2.1 (/dimer)
K_3 = 3.5 ± 0.1 x 10^5
ΔH_3 = -9.2 ± 0.1 (kcal/mol)

Model for Ni^{2+}/Cu^{2+} Binding to H144*UreE

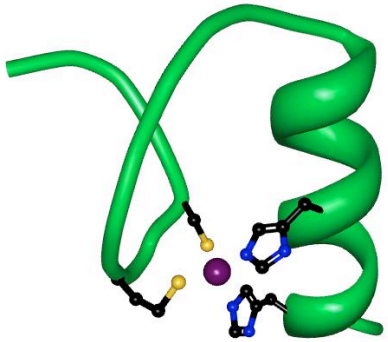


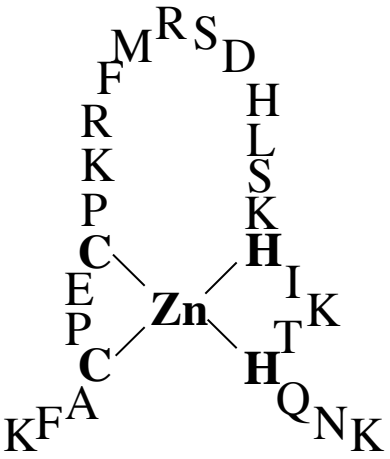
ITC of Metal-Protein Interactions

1. **General**
   - know the metal chemistry (e.g., disproportionation of Cu^{+}; solubility)
2. **Albumin**
   - delivery of metal in well-defined complex with buffer or chelate avoids unwanted (unknown) reactions, and allows high affinity binding sites to be studied; compare to other measurements of K
3. **IRT1pep**
   - number of protons displaced upon metal binding at a given pH can be quantified with ITC data in different buffers
   - Fe^{3+} could be studied by EDTA chelation from the peptide; also used to study Zn^{2+} binding and stabilization of the insulin hexamer
   - low affinity binding needs extended ITC data and known stoichiometry
4. **Metallothionein**
   - multiple metal binding requires analysis with different binding models
5. **UreE**
   - deconvolution of complex isotherms requires accurate stoichiometries

(N. E. Grossoehme, A. M. Spuches, D. E. Wilcox *J. Biol. Inorg. Chem.* **2010** *15*, 1183-1191)

Zinc Fingers: Sequences and Structures

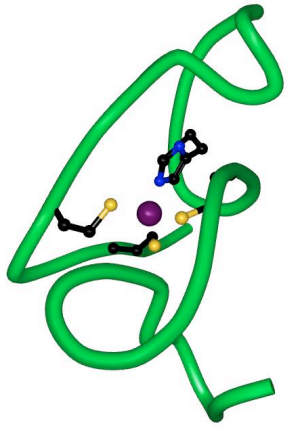




Sp1-3

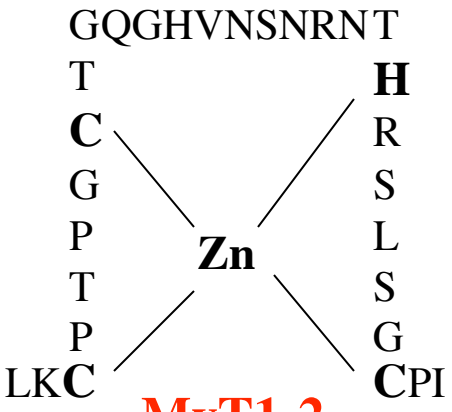
3rd Zinc Finger of Sp1

(PDB file 1SP2)



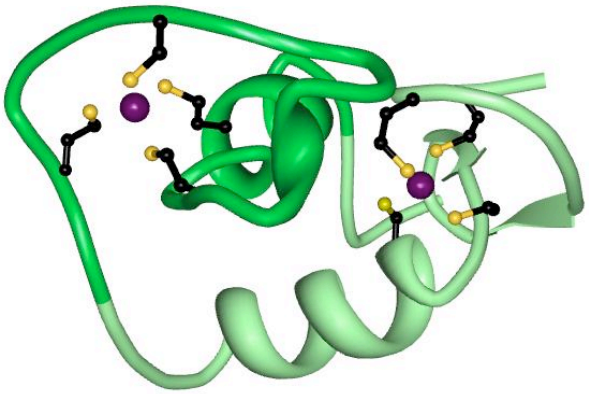
NZF-1

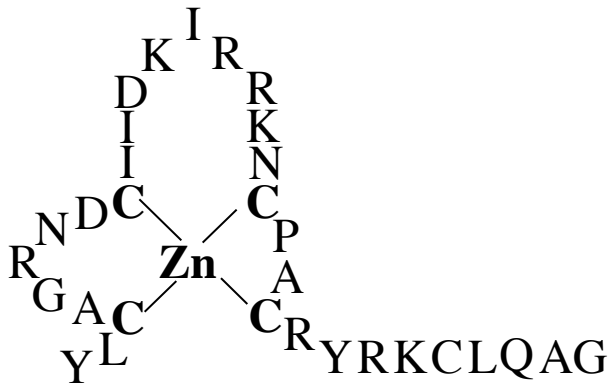
(PDB file 1PXE)



MyT1-2

2nd Zinc Finger of MyT1-2 (homologous to NZF-1)



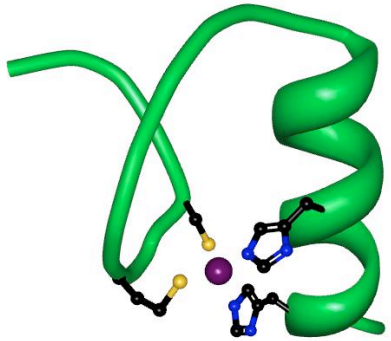


GR-2

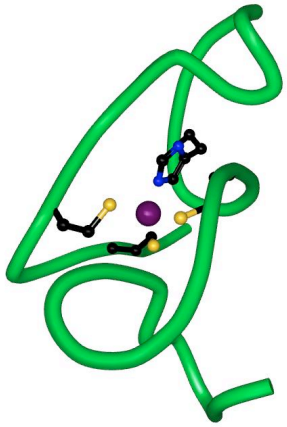
2nd Zinc Binding Site of Glucocorticoid Receptor DBD

(PDB file 2GDA)

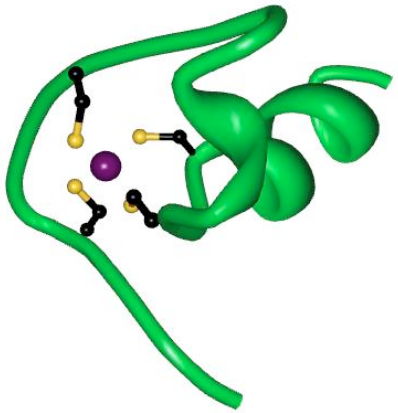
Zinc Fingers: Secondary Structure



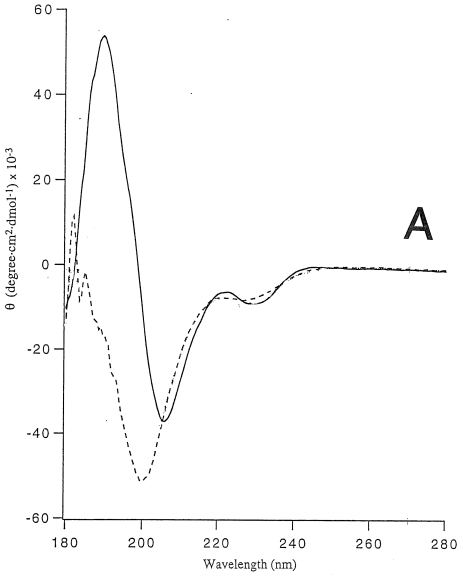
A: Sp1-3 (C_2H_2)

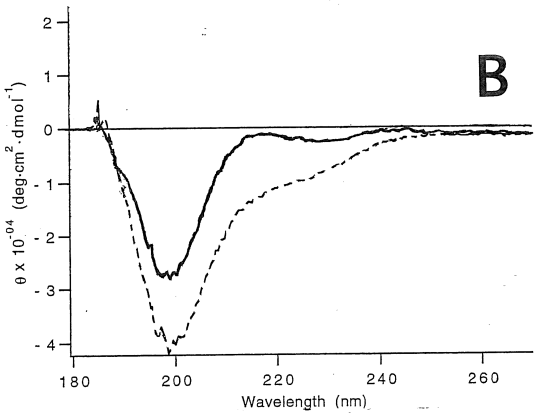


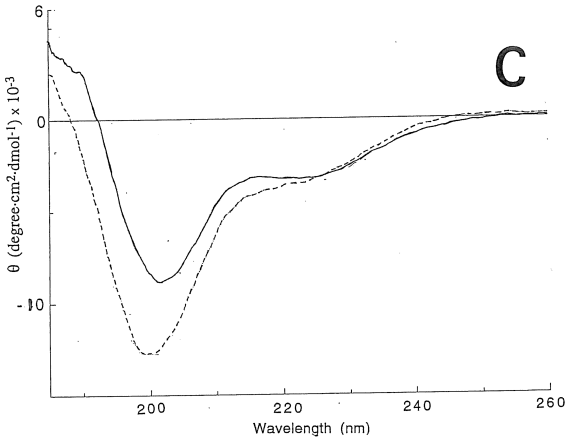
B: MyT1-2 (C_2HC)



C: GR-2 (C_4)

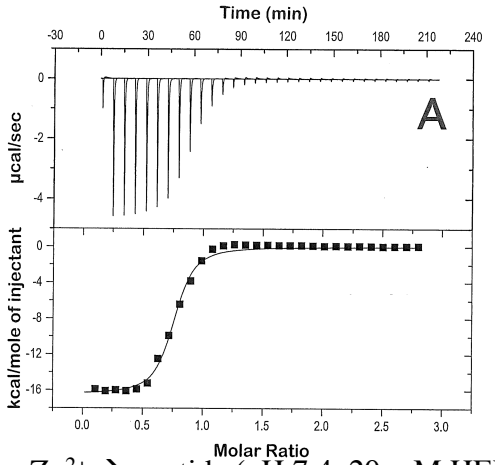


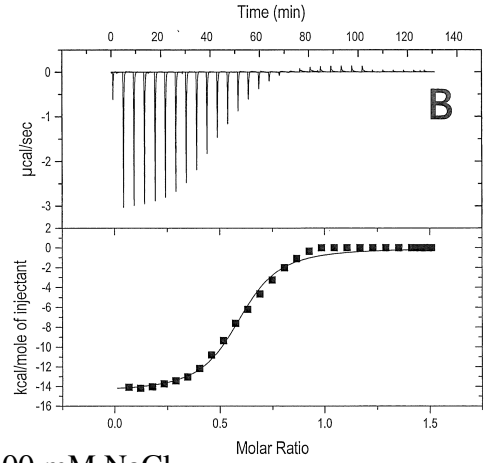


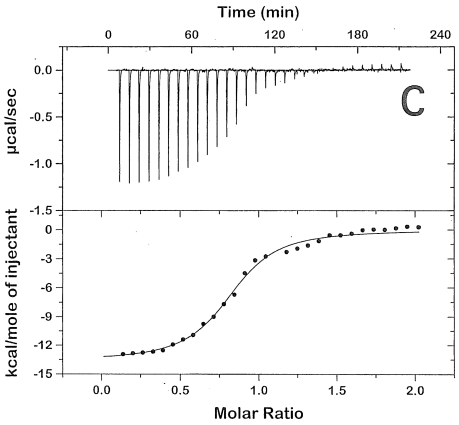


- - - apo
----- Zn-bound

Zinc Fingers: ITC Measurements







Zn^{2+} → peptide (pH 7.4, 20 mM HEPES, 100 mM NaCl

Average best fit parameters for ITC data from Zn^{2+} and Co^{2+} binding to Sp1-3, MyT1-2 and GR-2 in HEPES, PIPES and Tris buffers at pH 7.4 and 25 °C.

		Sp1-3 (C_2H_2)	**MyT1-2** (C_2HC)	**GR-2** (C_4)
Zn^{2+} 20 mM HEPES	K_{ITC}	$1.2 \pm 0.3 \times 10^6$	$7 \pm 1 \times 10^5$	$4 \pm 3 \times 10^6$
	ΔH_{ITC} [a]	-16.5 ± 0.2	-14.5 ± 0.3	-10 ± 2
Zn^{2+} 20 mM PIPES	K_{ITC}	$6 \pm 4 \times 10^6$	$8 \pm 2 \times 10^5$	
	ΔH_{ITC} [a]	-12 ± 1	-11.5 ± 0.5	
Zn^{2+} 500 mM Tris	K_{ITC}	$1.1 \pm 0.3 \times 10^6$	$4.6 \pm 0.5 \times 10^5$ [b]	3×10^5
	ΔH_{ITC} [a]	-29 ± 1	-33 ± 1 [b]	-34

a. kcal/mol; *b*. 100 mM Tris; *c*. 500 mM Tris; *d*. 20 mM HEPES

Buffer-independent thermodynamics [a] of Zn^{2+} binding to Sp1-3, MyT1-2 and GR-2 in the indicated buffers at pH 7.4 and 298 K.

		Sp1-3 (C_2H_2)	**MyT1-2** (C_2HC)	**GR-2** (C_4)
Zn^{2+} (20 mM HEPES)	$\Delta G°$	-10.2	-9.8	-10.8
	$\Delta H°$	-5.0 ± 0.5	+0.1 ± 0.5	+10 ± 2
	$-T\Delta S°$	-5.2	-9.9	-20.8
Zn^{2+} (20 mM PIPES)	$\Delta G°$	-11.0	-9.8	
	$\Delta H°$	-5.4 ± 0.5	-3.2 ± 0.2	
	$-T\Delta S°$	-5.6	-6.6	
Zn^{2+} (500 mM Tris)	$\Delta G°$	-10.4	-8.7 [b]	-9.5
	$\Delta H°$	-5.3 ± 0.5	-2.5 ± 0.1 [b]	+10
	$-T\Delta S°$	-5.1	-6.2 [b]	-19.5
Average	$\Delta G°$	-10.5 ± 0.3	-9.4 ± 0.4	-10 ± 1
	$\Delta H°$	-5.2 ± 0.2	-2 ± 1	+10 ± 2
	$-T\Delta S°$	-5.3 ± 0.2	-7.5 ± 1.5	-20 ± 2

a. kcal/mol; *b*. 100 mM Tris; *c*. 500 mM Tris; *d*. 20 mM HEPES

Thermodynamics of Zn^{2+} binding to zinc finger peptides

Peptides	K (K_d)	ΔG° kcal/mol	ΔH° kcal/mol	-TΔS° kcal/mol	ΔS° cal/mol K	n_{H+} (H^+ displaced by Zn^{2+})	$\Delta H°_{Zn\text{-}Pep}$ kcal/mol (Cys thiolate
Cys_2His_2							
Sp1-3	$6 \pm 3 \times 10^7$ (17 nM)	-10.5 ± 0.3	-5.2 ± 0.2	-5.3 ± 0.2	+17	2.3 ± 0.3	-25
$Cys_2HisCys$							
MyT1-2	$1.1 \pm 0.8 \times 10^7$ (91 nM)	-9.4 ± 0.4	-2 ± 1	-7.5 ± 1.5	+25	2.9 ± 0.2	-27
Cys_4							
GR-2	$5 \pm 4 \times 10^7$ (20 nM)	-10	+10	-20	+70	4.1 ± 0.4	-25

Similar affinities (ΔG) but different enthalpic (ΔH) and entropic (ΔS) components

- Zn^{+2} binding to Sp1-3 ("classical" zinc finger) is both enthalpically and entropically driven.
- Zn^{+2} binding to GR-2 (Cys_4 site) is entropically driven with enthalpic penalty
- Example of **Enthalpy-Entropy Compensation (EEC)**

Zn^{2+} displacement of Cys protons is origin of enthalpic differences

$$Zn^{2+} + n\,RSH \rightarrow Zn^{2+}(^{-}SR)_n + n\,H^+$$

Thermodynamic comparison with other zinc finger peptides

Peptides	K_d	ΔG° kcal/mol	ΔH° kcal/mol	-TΔS° kcal/mol	ΔS° cal/mol K
Cys_2His_2					
Sp1-3	**17 nM**	**-10.5 ± 0.3**	**-5.2 ± 0.2**	**-5.3 ± 0.2**	**+17**
CP-1 (Berg)	6 pM	-15.3 ± 0.5	-21.1 ± 1.0	+5.8 ± 1.0	-19
WT1-3 (Weiss)	0.15 nM	-13.4 ± 0.1	-7.8 ± 0.4	-5.6 ± 0.4	+19
ZFY-6 (Weiss)	24 nM	-10.4	+4.0 ± 0.1	-14.4 ± 0.1	+48
$Cys_2HisCys$					
MyT1-2	**91 nM**	**-9.4 ± 0.4**	**-2 ± 1**	**-7.5 ± 1.5**	**+25**
NC-1 (McLendon)	2 fM	-20	-6.5	-13.5	+45
NCp7-N (Mely)	32 fM	-18.4 ± 0.1	-8.1 ± 0.7	-10.3 ± 0.7	+35
NCp7-C (Mely)	100 fM	-17.7 ± 0.1	-8.4 ± 0.2	-9.3 ± 0.2	+32
Cys_4					
GR-2	**20 nM**	**-10**	**+10**	**-20**	**+70**

Zn^{2+} binding is entropically favored with variable enthalpic contributions

Thermodynamic contributions of the metal and the peptide

Peptides	ΔG° kcal/mol	ΔH° kcal/mol	-TΔS° kcal/mol	ΔS° cal/mol K
Cys_2His_2				
Sp1-3	-10.5 ± 0.3	-5.2 ± 0.2	-5.3 ± 0.2	+17
GGG-H_2C_2 (Gibney)	-16.3	-0.1	-16.2	+54
$Cys_2HisCys$				
MyT1-2	-9.4 ± 0.4	-2 ± 1	-7.5 ± 1.5	+25
GGG-HC_3 (Gibney)	-17.3	+1.0	-18.3	+61
Cys_4				
GR-2	-10	+10	-20	+70
GGG-C_4 (Gibney)	-17.4	+5.6	-23.0	+77

Peptides	ΔΔG° kcal/mol	ΔΔH° kcal/mol	-TΔΔS° kcal/mol	ΔΔS° cal/mol K
Cys_2His_2				
Sp1-3	+5.8	-5.1	+10.9	-37
$Cys_2HisCys$				
MyT1-2	+7.9	-3	+10.8	-36
Cys_4				
GR-2	+7	+4	+3	-10

Gibney's GGG peptides allow the metal contributions to be subtracted

Zn^{2+} binding is enthalpically neutral (Cys deprotonation cancels Zn-Cys/His bonds)

Zn^{2+} binding is entropically favored (displaced H^+ from Cys; desolvation)

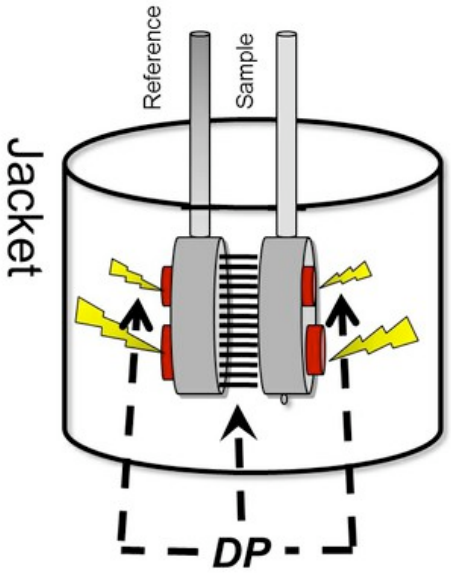
Entropic penalty associated with longer peptides

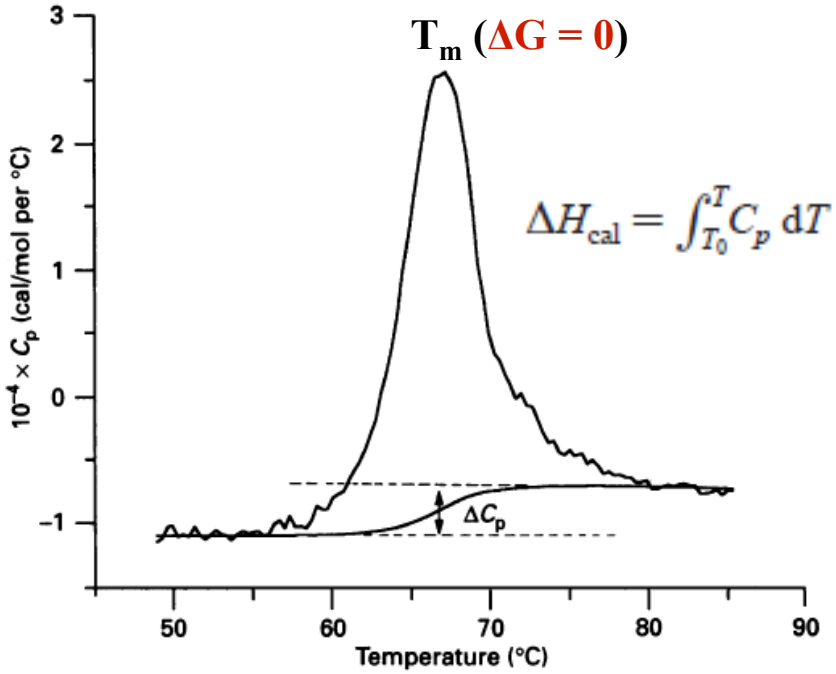
Protein contributions do not correlate with the amount of secondary structure

(A. M. Rich, et al. *J. Am. Chem. Soc.* **2012** *134*, 10405-10418)

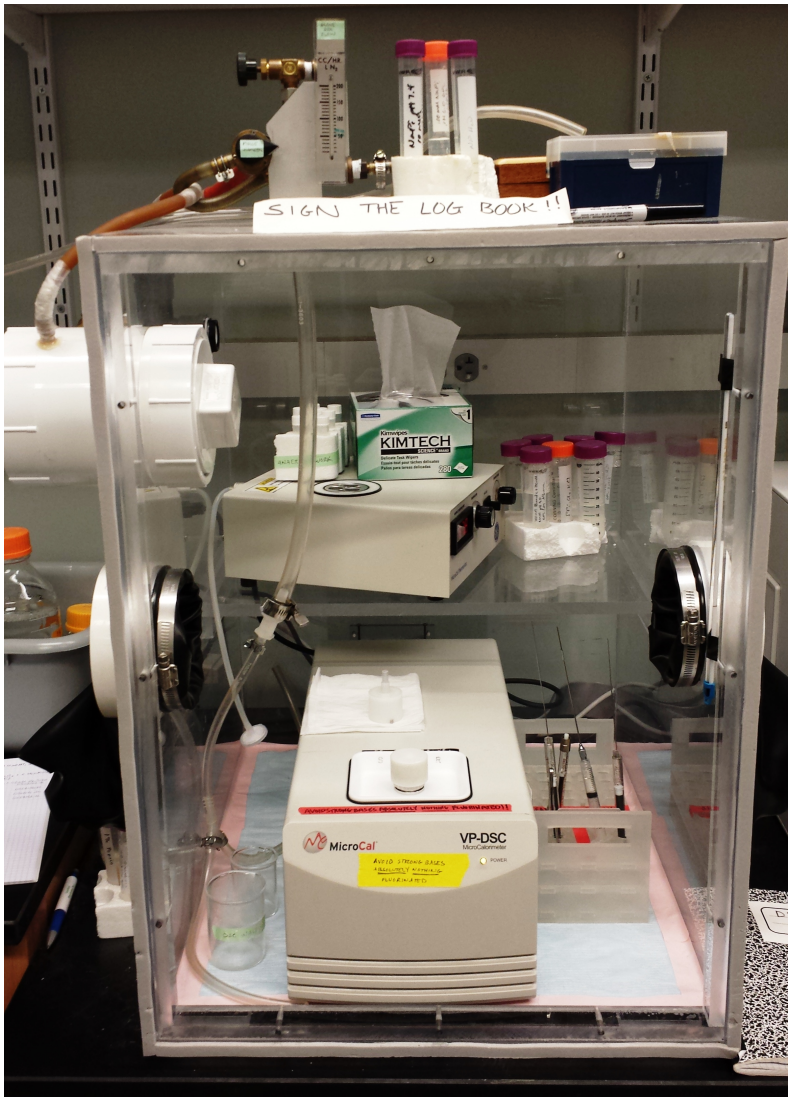
Differential Scanning Calorimetry (DSC)

1. **Measure the differential heat flow from/to a sample undergoing a phase transition.**
2. **Biological examples include protein unfolding, DNA melting, phase transitions of membranes.**
3. **Quantified by the temperature, T_m, where the two phases are in equal amounts (K = 1) and in equilibrium.**
4. **Also measure the total excess heat (ΔH_{cal}) and the change in heat capacity (ΔC_p) associated with the transition.**

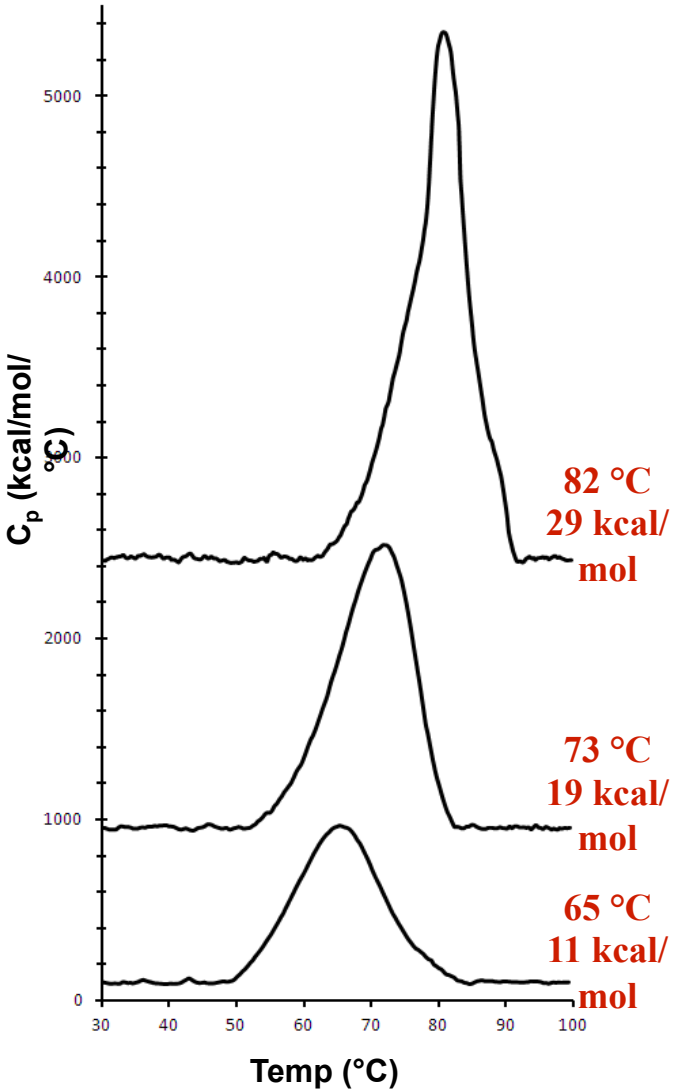




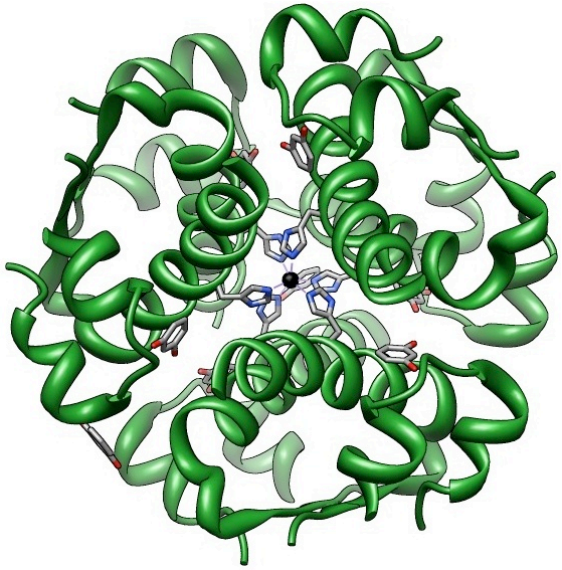
MicroCal differential scanning calorimeter



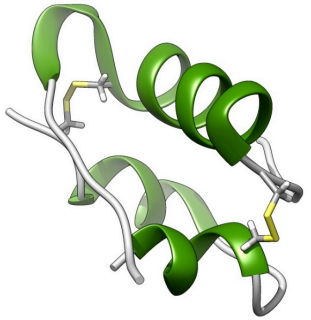
Zn^{2+} Stabilization of the Insulin Hexamer



Zn_2Ins_6 + xs Zn^{2+}

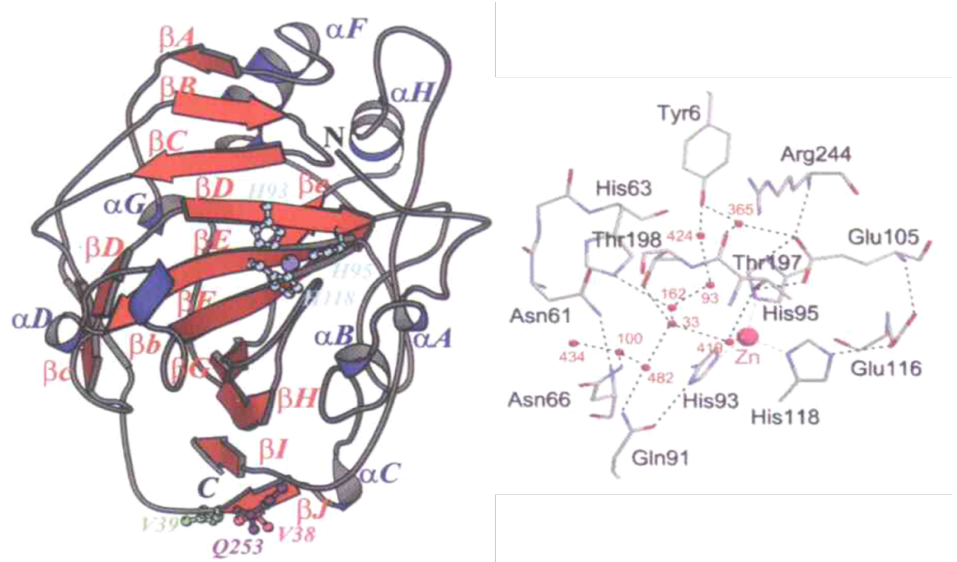


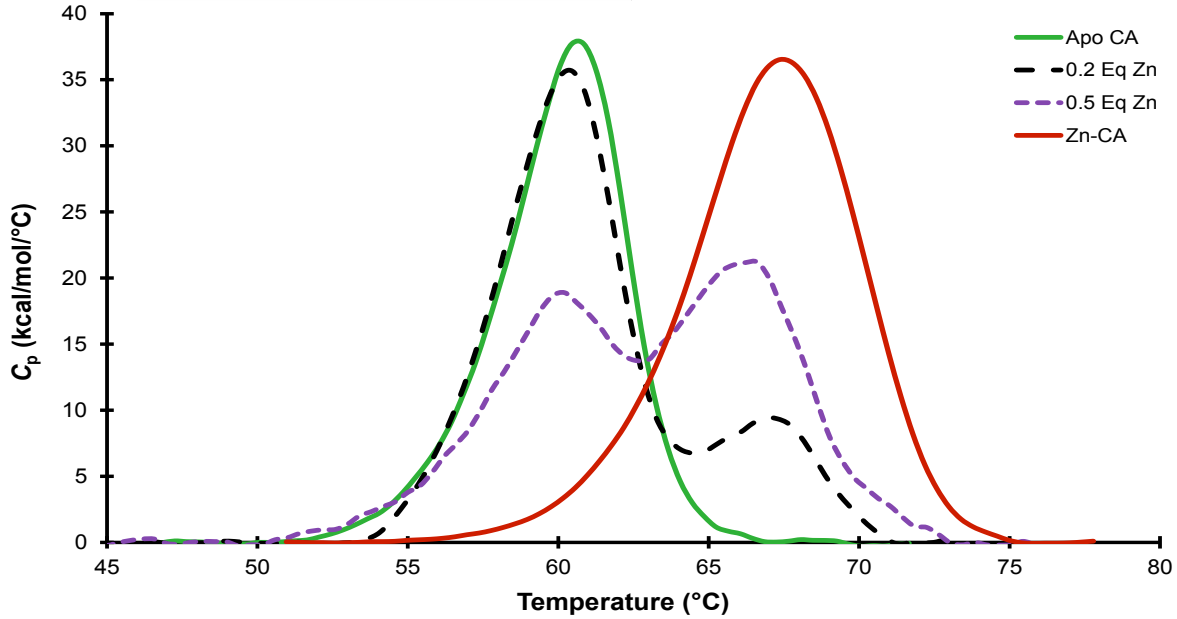
Zn_2Ins_6



Insulin
(predominantly dimer)

Zn^{2+} Stabilization of Carbonic Anhydrase





DSC of Metal-Protein Interactions

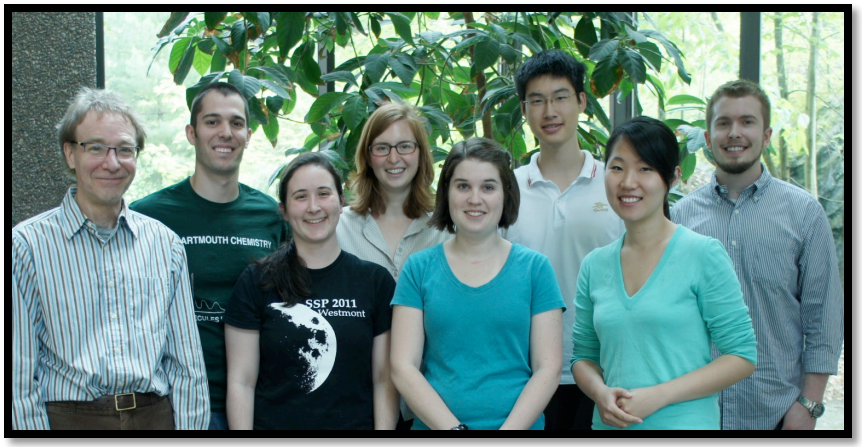
1. **Measure the perturbation of protein thermal unfolding by metal ion(s).**

2. **Quantify the thermodynamic contributions of metal ions to protein stability by comparison to the apo form of the protein (i.e., ΔT_m, $\Delta\Delta H_{cal}$).**

3. **Correlate with other measurements of metal perturbation of the thermal stability of proteins (e.g, CD, fluorescence).**

4. **Correlate the thermodynamics of metal ions binding to a protein (ITC) with the thermodynamics of the metal ions stabilizing the protein (DSC).**

Acknowledgements



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