Isothermal Titration Calorimetry for Bioinorganic Chemists

Overview, Key Issues with Metals, and a Few Applications

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Outline – 2018 Penn State Workshop

- Isothermal Titration Calorimetry (ITC)
  - The Method: information, instruments, data, analysis
  - Technical Issues
  - Bioinorganic Chemistry Applications
    - Issues with Metal Ions
      - Metal-buffer interactions
      - Quantifying (de)protonation coupled to binding
    - Examples:
      - Carbonic anhydrase (Zn^{2+})
      - Glycerophosphodiesterase (GpdQ) (Co^{2+}, Mn^{2+})

ITC: Metal Ions and Proteins

Metal ions of interest to bioinorganic chemists

Group II metal ions: Mg^{2+}, Ca^{2+}

Transition metal ions: Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{1+}, Cu^{2+}, Zn^{2+}

Heavy metal ions: Cd^{2+}, Pb^{2+}, Hg^{2+}

Questions of interest

Specific binding site(s) → stoichiometry, affinity, thermodynamics

Non-specific binding and effect(s) on the protein

Metal ion contributions to protein stability or activity

Caution about the binding constant (K) and enthalpy (ΔH) from ITC

Measuring overall equilibrium (K_{ITC}) and binding enthalpy (ΔH_{ITC})

Need to consider coupled equilibria in a post-hoc analysis

Compare/confirm with results from other methods

Thermodynamics of Metal Ions Binding to Proteins

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

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

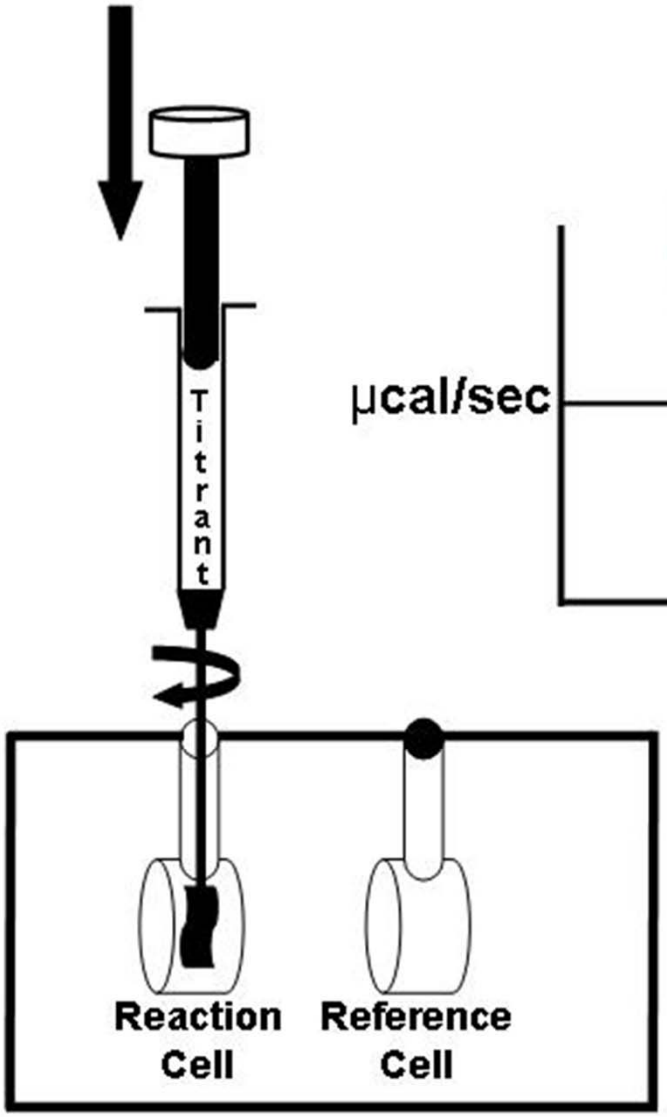
- Free Energy (ΔG) $\quad \Delta G^\circ = -R\,T\,\ln(K)$

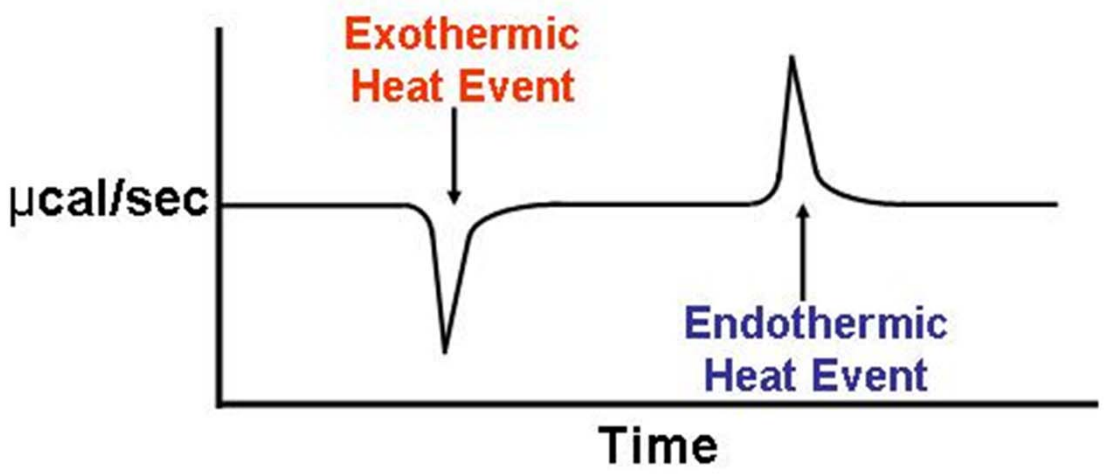
- Enthalpy (ΔH) $\quad$ calorimetry: $\Delta H^\circ = q_P$

- Entropy (ΔS) $\quad \Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$

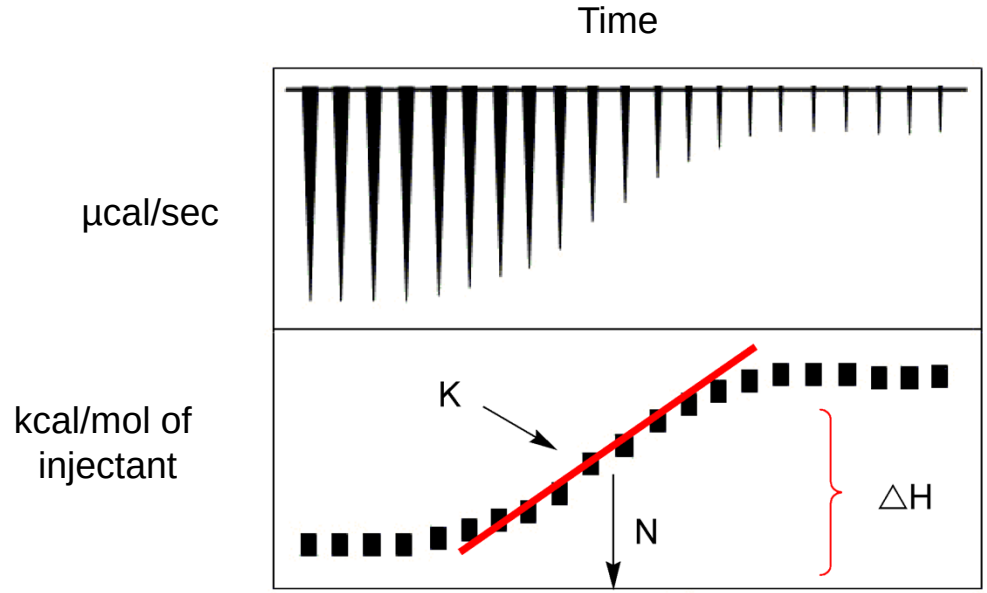
- Heat Capacity (C_P) $\quad \Delta C_P = \Delta H^\circ_{\ 0} / T_0 - \Delta H^\circ / T = \Delta\Delta H^\circ / \Delta T$

Isothermal Titration Calorimetry





Isothermal Titration Calorimetry



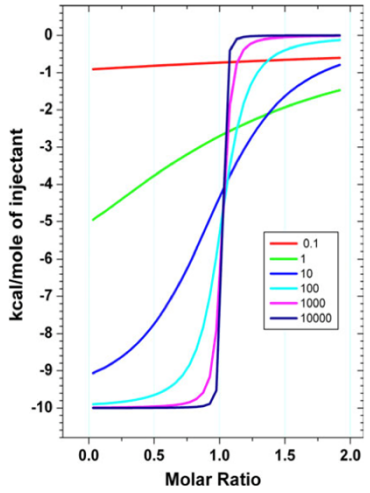
Capabilities

- determine binding stoichiometry and K value ($3 < \log K_{ITC} < 8$).

$$c = K\,[P]$$

- complete thermodynamic profile in one experiment (ΔG°, ΔH, ΔS).

- determine ΔC_p from temperature dependence of ΔH.



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 (ΔG°, ΔH°, ΔS°, ΔC_p°).

3. Can provide additional chemical insight:
   - proton competition with binding
   - 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: Uses

1. Basic

Is there binding?

What is the binding stoichiometry (n)? Caveat: generally softer number

What is the binding affinity (K_d)? Caveat: c window limitations

2. Intermediate

What is the binding enthalpy (ΔH)? Caveat: measuring net ΔH_{ITC}

Is there coupled (de)protonation that accompanies binding (n_{H+})?

What is the binding entropy (ΔS)? Caveat: from comparable ΔG, ΔH

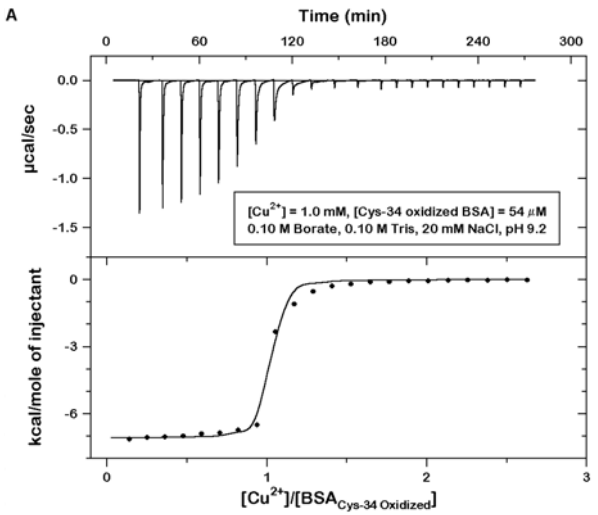
3. Advanced

What is the change in heat capacity (ΔC_p)?

What are the effects of other perturbations (ionic strength, solvent, etc.)? → $\Delta\Delta X$

Kinetics of binding?

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? enough 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
- Independent binding sites model (2)
- 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"

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!

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**

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

4. **Proton Displacement** *

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

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

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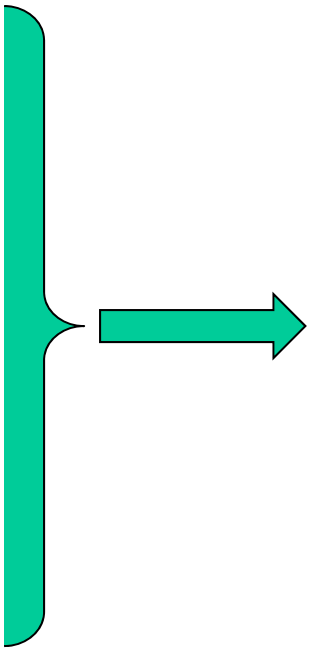

4. **Proton Displacement** *

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

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

ITC: Competing Reactions

Oxidation-reduction reactions of metal ions

When to expect them (e.g., Fe^{2+}, Cu^{+}) and when not (e.g., Zn^{2+}, Ca^{2+})?

Use anaerobic conditions if necessary

Use a reductant (e.g., DTT, TCEP)? May introduce additional heat or interact with the metal ion.

Precipitation reactions of metal ions

Observe this?

Adjust conditions (concentrations, buffer, pH, pI); avoid phosphate

Reactions with water (hydrolysis)

Often depend on concentration; see as different ΔH in forward and reverse titrations

Deliver metal as well-defined complex with a ligand or the buffer

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a) Hydrolysis

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4. Proton Displacement *

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

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

ITC of Metal-Protein Interactions: Equilibria Coupled with Metal Binding

1. **Protons.**
   - 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)protonation

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 $\Delta H^\circ_{M\text{-}buff}$ and $n_{M\text{-}buff}$

   b) interaction with H^+
      - data in different buffers quantify displaced H^+'s
      - use a buffer with larger heat of protonation to amplify signal

3. **Other species in solution?**
   - 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}
$\mathbf{M^{2+} + L \rightleftarrows ML}$		$\mathbf{\Delta H_{ML}}$

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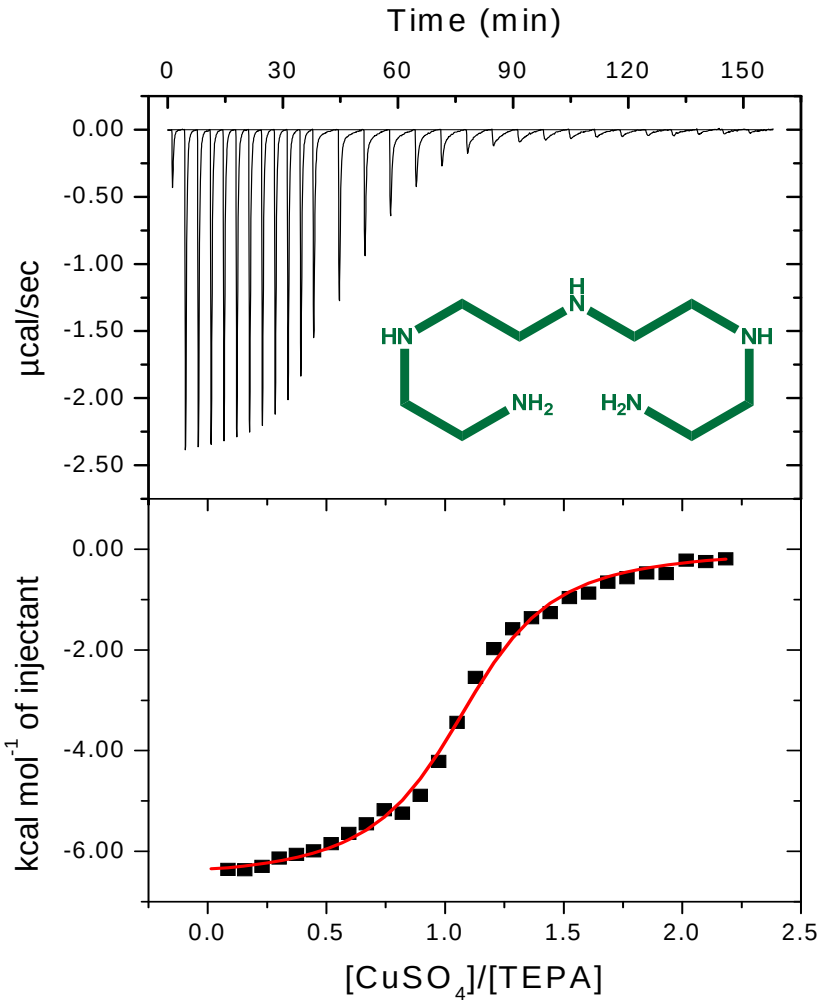
b) interaction with H^+

- data in different buffers quantify displaced H^+'s (example later)
- use a buffer with larger heat of protonation to amplify signal

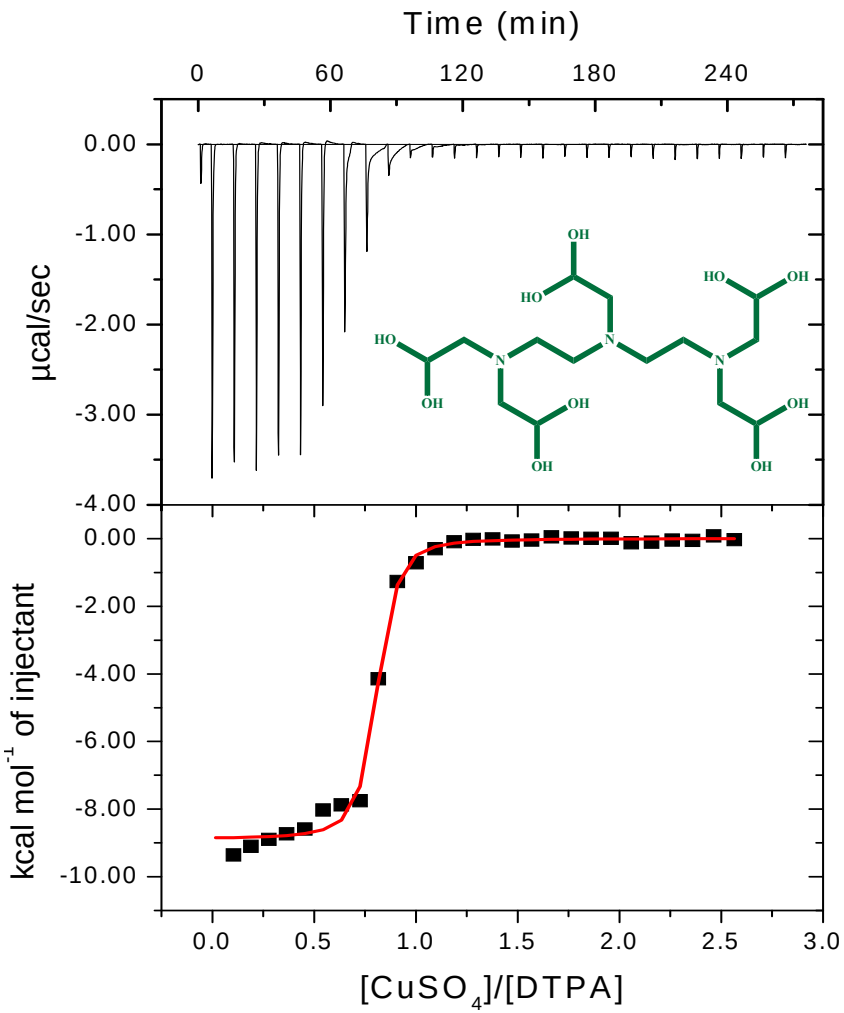
3. Other species in solution?

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

Cu^{2+}-buffer interaction: bis-Tris



1.0 mM TEPA → 0.1 mM $CuSO_4$
100 mM bis-Tris, 100 mM NaCl
(TEPA-Cu^{2+} log K = 22.8 ± 0.3)



1.2 mM DTPA→ 0.1 mM $CuSO_4$
100 mM bis-Tris, 100 mM NaCl
(DTPA-Cu^{2+} log K = 21.2 ± 0.3)

Cu^{2+}-buffer interaction (pH 7.0)

	TEPA		DTPA		Average of Both Ligands	
Buffer	**Log β_n**	**ΔH_{MB} (kcal/mol)**	**Log β_n**	**ΔH_{MB} (kcal/mol)**	**Log β_n**	**ΔH_{MB} (kcal/mol)**
bis-Tris	12.1 ± 0.4	–6.2 ± 0.7	11.8 ± 0.3	–7.9 ± 0.2	**12.0 ± 0.3**	**–7.1 ± 0.4**
TAPSO	13.0 ± 0.3	–11 ± 3	12.3 ± 0.5	–8 ± 3	**12.7 ± 0.3**	**–9 ± 2**
DIPSO	13.0 ± 0.3	–6.6 ± 0.4	10.8 ± 0.6	–5.8 ± 0.7	**11.9 ± 0.3**	**–6.2 ± 0.4**
TES	13.3 ± 0.3	–9.0 ± 0.7	13.2 ± 0.4	–9.3 ± 0.3	**13.3 ± 0.3**	**–9.2 ± 0.4**

Colette F. Quinn, Margaret C. Carpenter, Molly L. Croteau, Dean E. Wilcox "Isothermal Titration Calorimetry of Metal Ions Binding to Proteins", in *Methods in Enzymology*, Vol. 567, Calorimetry, A. L. Feig, ed; Academic, **2016**, pp. 3-21

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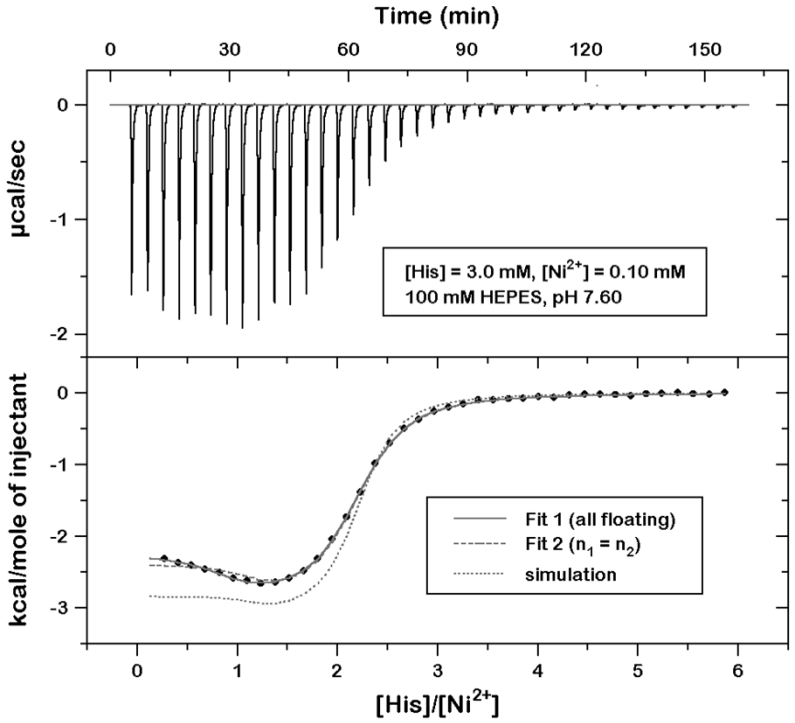
b) interaction with H^+

- data in different buffers quantify displaced H^+'s
- use a buffer with larger heat of protonation to amplify signal

3. Other species in solution?

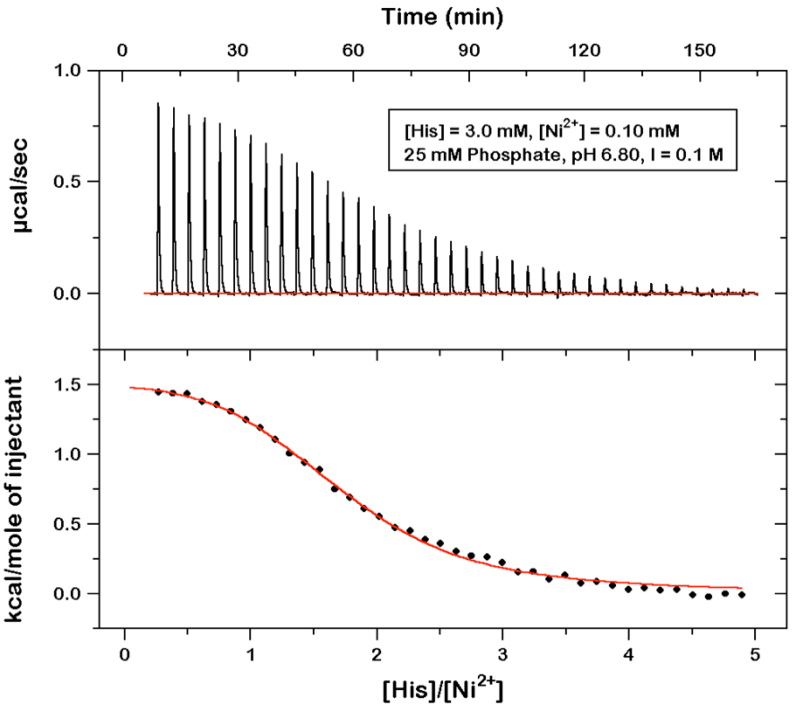
- reducing agent, complexing agent, salt, detergent, DMSO, etc.

Ni^{2+} + His



His → Ni^{2+}

100 mM HEPES pH 7.6

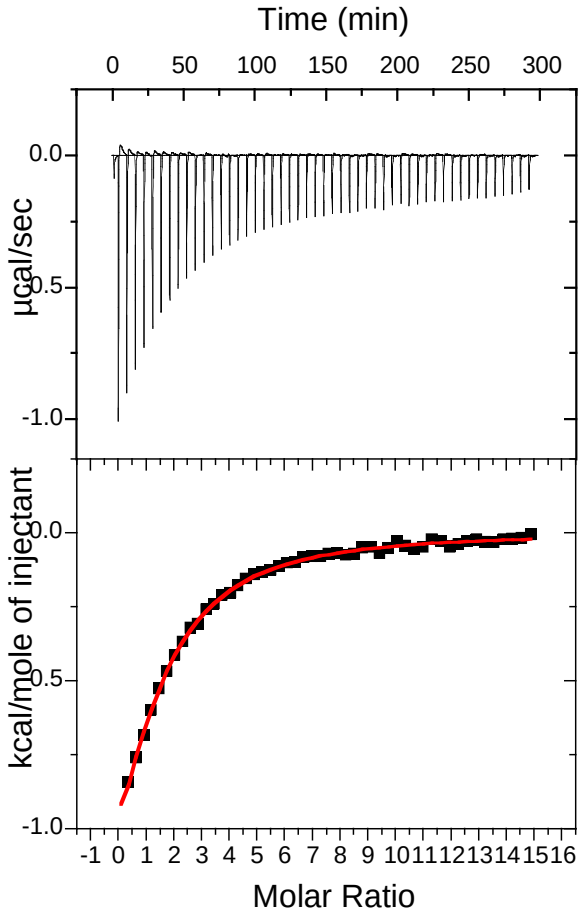


His → Ni^{2+}

25 mM phosphate pH 6.8

- Buffer protonation enthalpy can dominate ΔH_{ITC}

IRT1pep: $P(HG)_4P$



Zn^{2+} → $P(HG)_4P$

25 mM ACES, pH 7.25

n = 1.07 ± 0.02

K_{ITC} = 8.6 ± 0.4 x 10^3

ΔH_{ITC} = -2.3 ± 0.2 kcal/mol

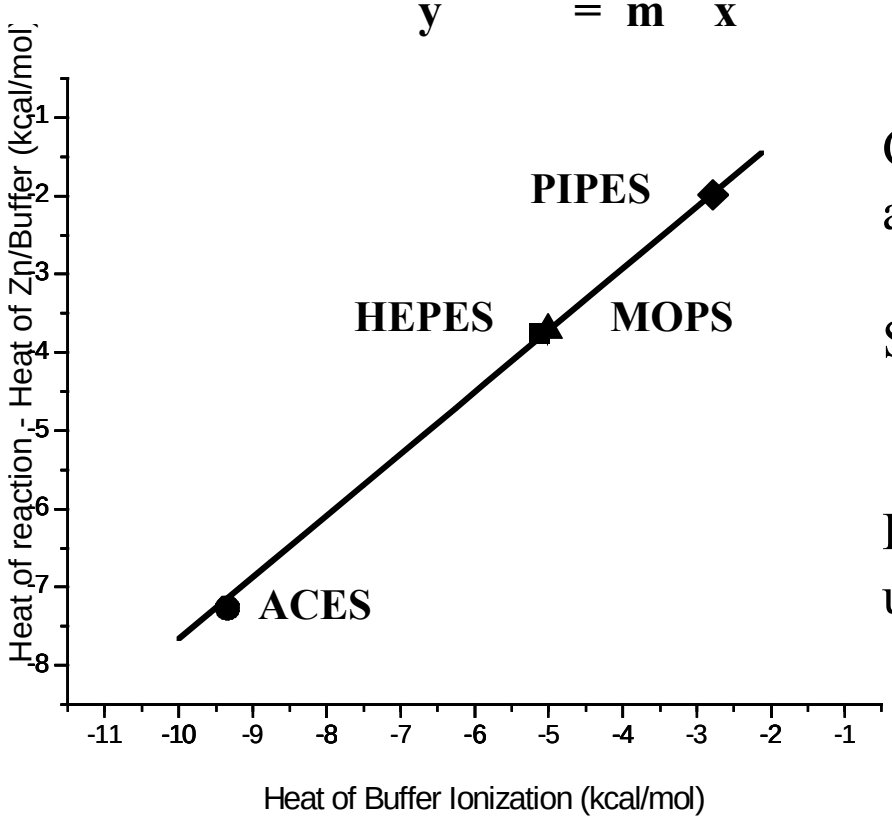
$$\Delta H^\circ_{ITC} = -\Delta H^\circ_{M\text{-}buff} - \Delta H^\circ_{Pep\text{-}H+} + n_{H+}(\Delta H^\circ_{buff\text{-}H+}) + \Delta H^\circ_{M\text{-}Pep}$$

Quantifing proton release upon metal binding

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

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

$$\mathbf{y = m\ x + b}$$



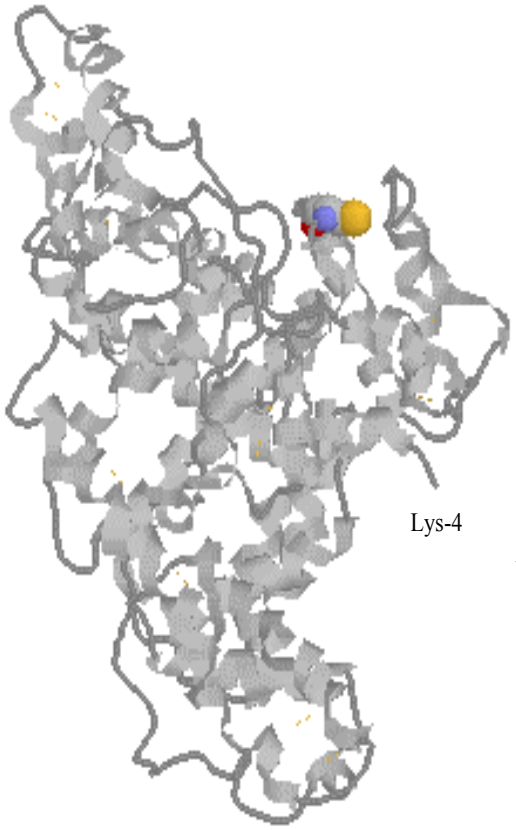
Collect ITC data in ≥2 buffers at the experimental pH.

Slope = n_{H+}
= 0.79 ± 0.02

Protons displaced from $P(HG)_4P$ upon Zn^{2+} binding at pH 7.25

Buffer dependence of the binding enthalpy can be used to quantify the number of protons that are displaced from a protein upon metal binding.

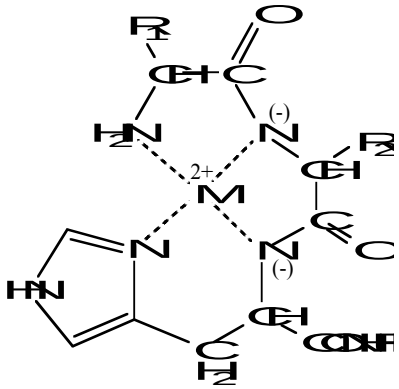
Penn State Workshop Tutorial:
Cu^{2+} → albumin



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

Most abundant circulatory protein (~ 0.6 mM).

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



Cu^{2+} Binding to Albumin

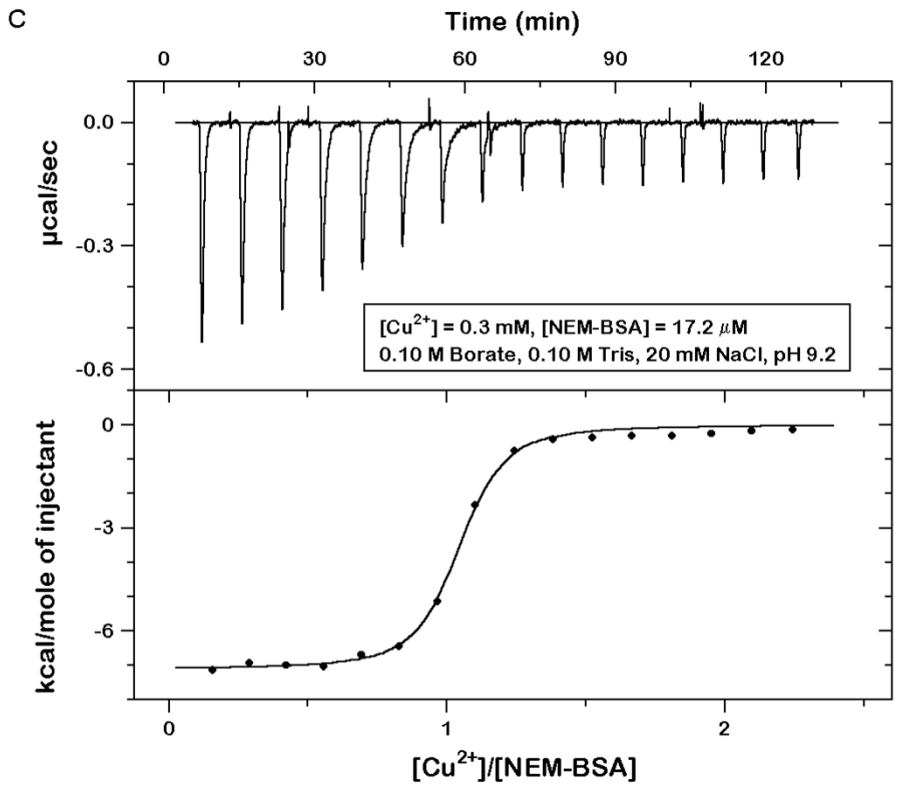
Cu^{+2} binding to bovine serum albumin (BSA)
(reported, pH-independent, and pH 7.4 equilibrium constants

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 buffer (e.g. Tris) to measure high affinity Cu^{2+} binding.

Cu^{2+} Binding to Albumin



Cu^{2+} → BSA

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

ITC: Useful Titrations with Metal Ions

1. Forward vs Reverse Titration (if possible)

- M^{n+} → Protein (dilution of the metal ion)
- Protein → M^{n+} (dilution of the protein)

2. Displacement Titration

- One metal ion (M_2) competes with another (M_1) for the protein
- M_2 → M_1-Protein
- $M_2 + M_1\text{-Protein} \rightleftarrows M_2\text{-Protein} + M_1$ $\qquad K_{P\text{-}M2} > K_{P\text{-}M1}$

3. Chelation Titration

- Ligand (L) competes with the protein for the metal ion
- L → M-Protein
- $L + M\text{-Protein} \rightleftarrows L\text{-}M + \text{Protein}$ $\qquad K_{L\text{-}M} > K_{P\text{-}M}$

ITC: Examples of Metal-Binding Measurements

1. Zn^{2+} binding to carbonic anhydrase

   Toone, Fierke and co-workers; Biochemistry **2001**, *40*, 5338

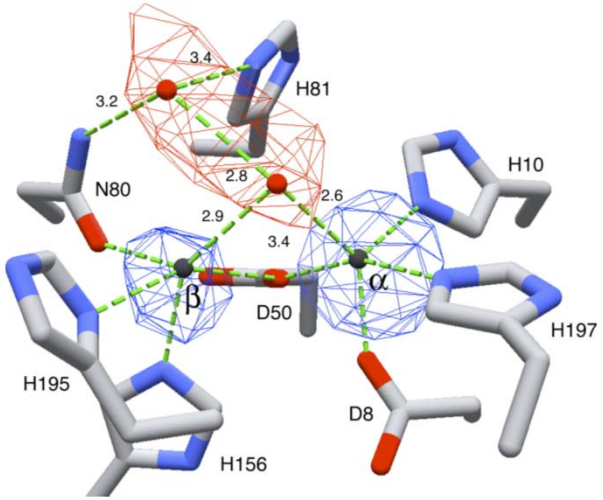
   "Thermodynamics of Metal Ion Binding. 1. Metal Ion Binding by Wild-Type Carbonic Anhydrase"

   Emerson, Lewis and co-workers, Inorg. Chem. **2012**, *51*, 11098

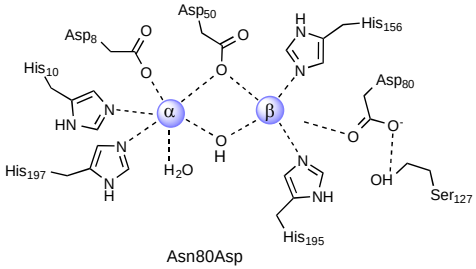
   "Revisiting Zinc Coordination in Human Carbonic Anhydrase II"

2. Co^{2+} and Mn^{2+} binding to glycerophosphodiesterase

Glycerophosphodiesterase (GpdQ) from *Enterobacter aerogenes*

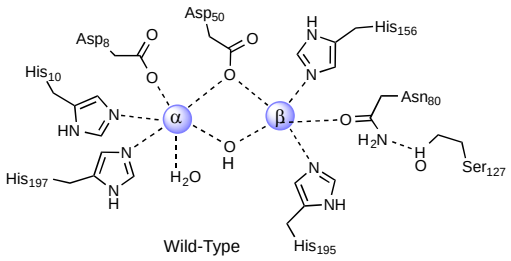


C. J. Jackson, P. D. Carr, J-W. Liu, S. J. Watt, J. L. Beck, D. L. Ollis,
J. Mol. Biol. **2007** *367*, 1047-1062

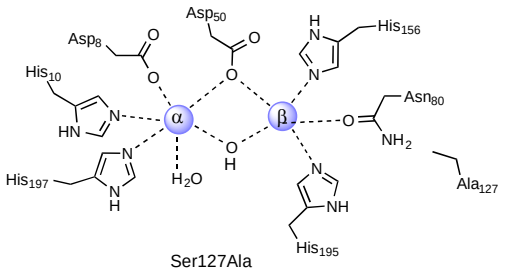


Asn80Asp

1st coordination sphere variant



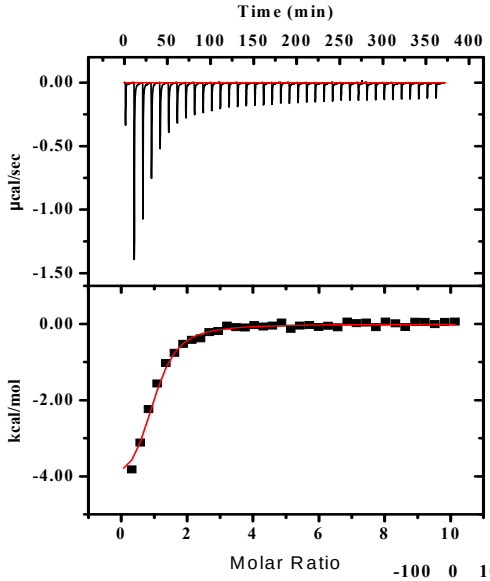
Wild-Type



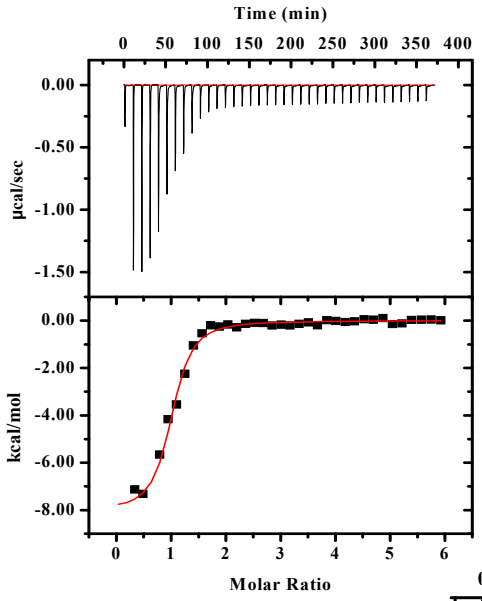
Ser127Ala

2nd coordination sphere variant (high activity)

ITC of Mn^{2+} and Co^{2+} Binding to GpdQ ± phosphate

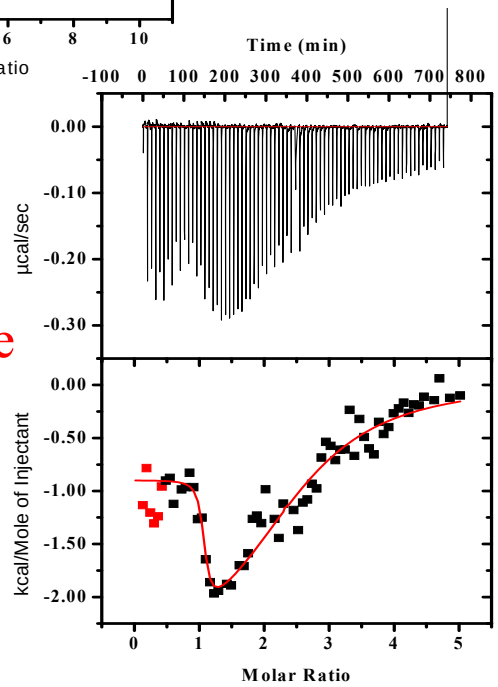


Mn^{2+}

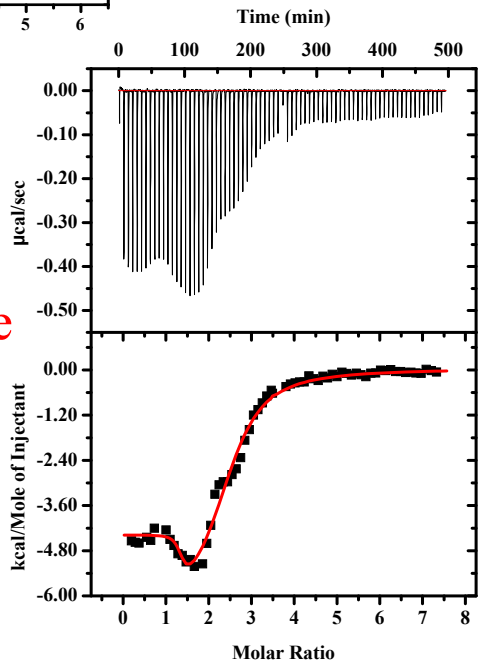


Co^{2+}

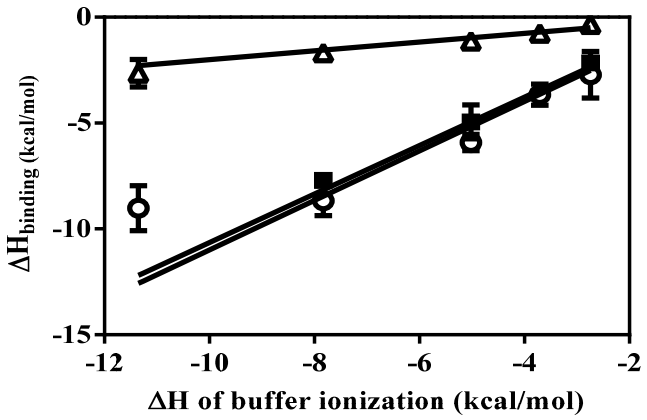
+ phosphate



+ phosphate



Thermodynamics of Co^{2+} binding to wild-type GpdQ



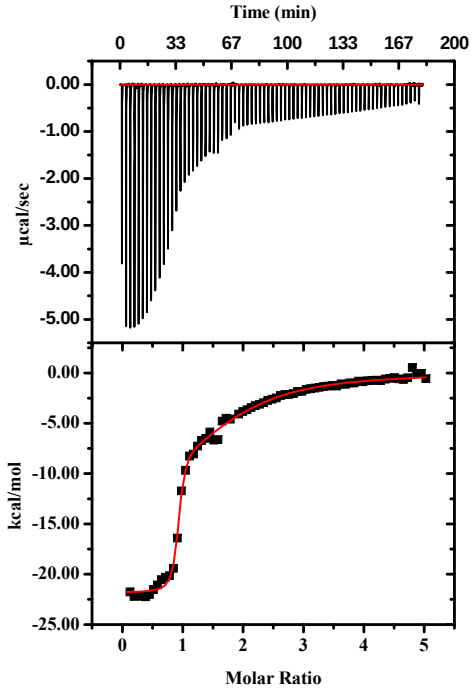
Calorimetric determination of the number of protons displaced from the α and β sites of GpdQ upon addition of Co(II) at pH 7.25 in the absence and presence of 1.5 mM K_2HPO_4. The buffers, and their protonation enthalpies (kcal/mol), used for these data are Tris (-11.35), TES (-7.83), HEPES (-5.02), MES (-3.71) and Pipes (-2.74). The slopes (number of displaced protons) from the linear regression are: α site (-phosphate), open circles: 1.15 ± 0.09; α site (+phosphate), solid squares: 1.01 ± 0.01; β site (+phosphate), open triangles: 0.19 ± 0.01.

	- phosphate		+ phosphate	
	α site	**β site**	**α site**	**β site**
K_d	0.7 μM	-	9 nM	5 μM
n_{H+}	1.1	-	1.0	0.2
ΔG° (kcal/mol)	-8.5	-	-11	-7
ΔH (kcal/mol)	1.2	-	6	-7
$-T\Delta S$ (kcal/mol)	-9.7	-	-17	0

- loss of a proton *only* upon metal binding to the α site
- phosphate (substrate analog/inhibitor) increases affinity at both sites; entropic benefit at α site and enthalpic benefit at β site

ITC of Co^{2+} Binding to GpdQ Variants (no phosphate)

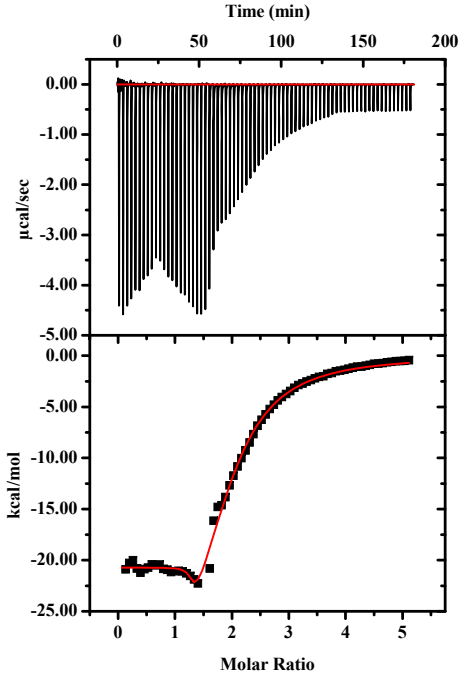
Asn80Asp (1st sphere)



	α site	β site
K_d	0.1 µM	120 µM
n_{H+}	1.1	0.1
ΔG° *	-10	-5
ΔH^*	-12	-13
$-T\Delta S^*$	3	8

(* kcal/mol)

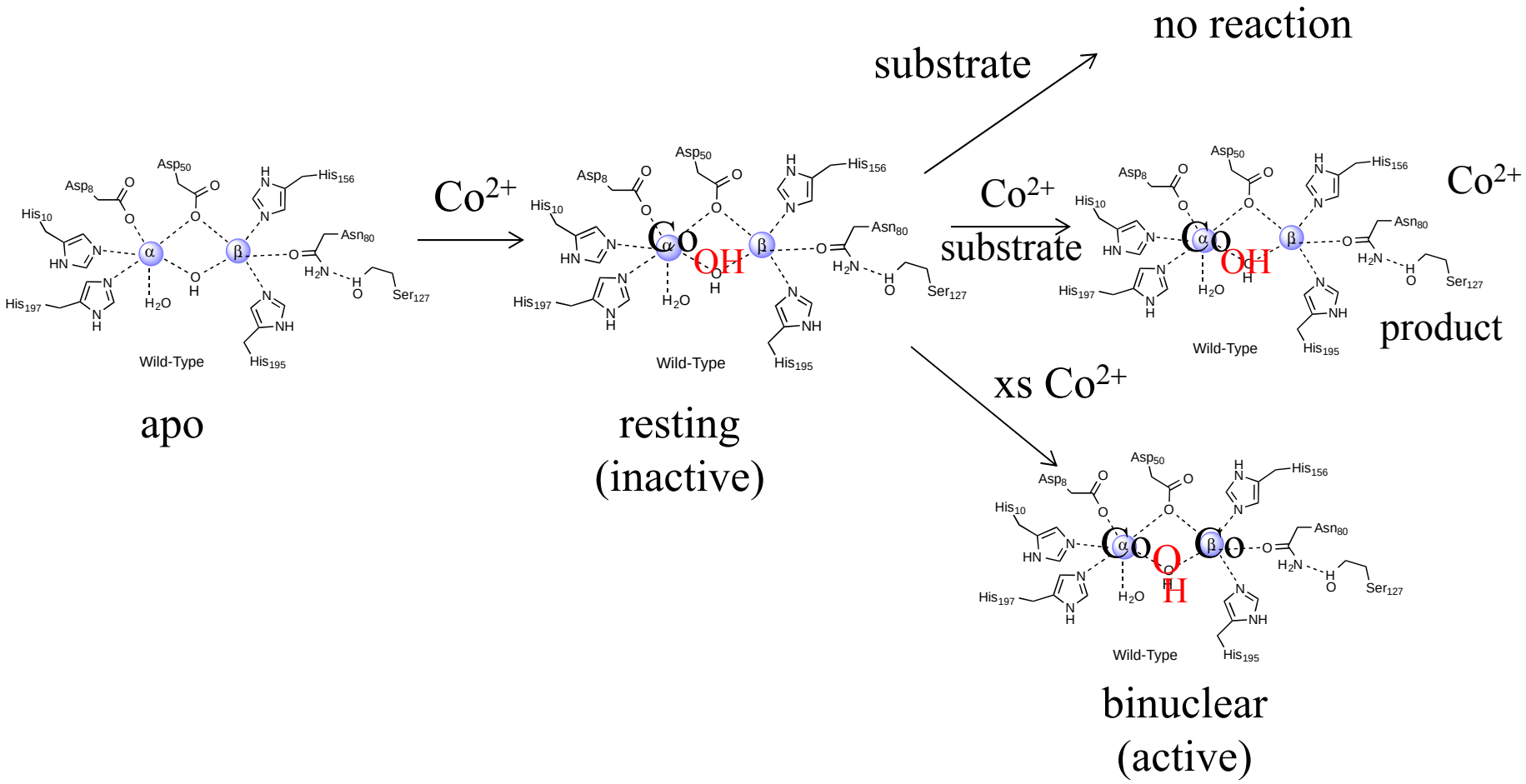
Ser127Ala (2nd sphere)



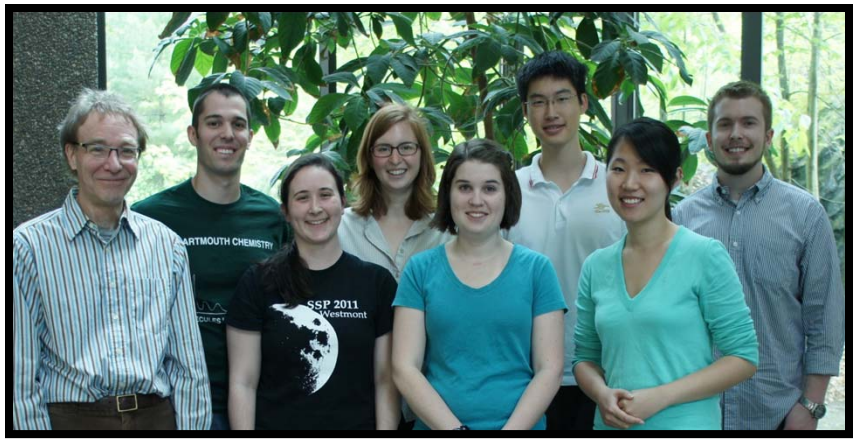
	α site	β site
K_d	70 nM	67 µM
n_{H+}	1.1	0.2
ΔG° *	-10	-6
ΔH^*	-11	-37
$-T\Delta S^*$	1	31

(* kcal/mol)

Assembly and Reactivity of the GpdQ Active Site



Acknowledgements



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