Chemistry 5.08
Spring 2016
Recitation Week 10, April 14-15, 2016 (Nolan)
Metal-binding studies and dissociation constant determination

Understanding speciation of metal ions in biological systems is a challenge and requires (i) identification of metal-binding sites in peptides and proteins and (ii) determination of metal-ion affinities of these biomolecules. The affinity of a peptide or protein for a given metal ion is typically expressed as the dissociation constant (K_d). These values are measured *in vitro*. Today's recitation will focus on various approaches and pitfalls for determining metal-ion affinities. Detailed mathematical treatment of complex equilibria will not be covered.

Overview and reminders:

Metal ion: Lewis acid
Protein or ligand: Lewis base

Some general concepts related to metal/protein interactions:

Irving-Williams Series (1948) – the general stability of high-spin octahedral metal complexes for the replacement of water by other ligands is:

Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II)

This trend is essentially independent of the ligand.
This sequence is only for Mn(II) through Zn(II); little data for other first-row TM
Although defined for octahedral complexes, the trend is often used to describe the behavior for 4- and 5-coordinate species.

What are some implications?

For a given ligand, K_d for Mn(II) >> K_d for Zn(II) (etc.)

→ If you see values with same order of magnitude, there is likely an issue!
→ If a Zn(II) contamination, assume Zn(II) will bind to your site of interest!

Hard-Soft Acid-Base Theory -- qualitative theory to describe metal/ligand preferences

Metal-Ion: hard or soft Lewis acid
Ligand: hard or soft Lewis base

In general, hard metal ions prefer hard ligands. Soft metals prefer soft bases.

Hard:
Small atomic/ionic radius, high oxidation state, not very polarizable
Ex: Oxygen donor atoms, Mn(II), Fe(III), Ca(II)
→ siderophores (recall NRPS, enterobactin)
→ EF-hand domains for Ca(II) coordination (e.g. calmodulin)

Soft:
Large atomic/ionic radius, low oxidation state, polarizable
Ex: Sulfur donor atoms, Cu(I), Cd(II), Hg(II), Ag(I), Pt(II), Pb(II)
→ Hg(II) binding to the peptide metallothionein (20 Cys residues)

Borderline: In the middle
Ex: Histidine N donors, Fe(II), Co(II), Zn(II)
→ Zinc finger peptides (N_2S_2 coordination sphere is common)

These concepts are helpful in terms of thinking about metal/protein interactions in general terms and making predictions about the identity of the preferred metal ion. There are exceptions to these generalizations.

Some practical considerations for metal-binding studies:

Some general pitfalls that occur and cause confusion in the literature:

1. Inappropriate fitting of data
   Programs will fit data, but what does the fit mean?
   Direct titrations are often inappropriately fit (see below, stoichiometric binding)
2. Use of inappropriate buffers
   Many buffers coordinate metal ions
   Many buffers are contaminated with metal ions
   → It is also easy to contaminate the buffer with metal ions
   Note : the buffer concentration is often high relative to the concentration of protein → the buffer influences the metal speciation and equilibria!
3. Lack of pH control during experiment or experiment done at inappropriate pH
   What are the pK_a values of potential ligands?
   Are there pH requirements for the metal ion?
4. Lack of accuracy with concentrations
   Of metal-ion stock solution (what is the source of the metal?)
   Of the protein (how is this concentration determined?)
   Of the competitor if employed (where did it come from? Is it pure?)
5. Use of inappropriate concentration of protein/ligand
   → Think about K_d equation
6. Lack of temperature control (the equilibrium constant is temperature dependent!)

7. Lack of effective competition in experiments
    - Neglect to use competitor
    - Use of inappropriate competitor
    - Use of a competitor that is an appropriate one but has other problems
        - The competitor itself is not pure (organic contamination)
        - The competitor has already bound a metal (inorganic contamination)
    - Collection of data before equilibrium is reached
8. Lack of appropriate precautions to prevent peptide/protein oxidation
    - Cysteine ligands → aerobic oxidation may result in disulfide formation
    - Methionine ligands → air oxidation can occur as well
9. Lack of appropriate precautions when handling metal-ions that oxidize
    - Fe(II) will oxidize to Fe(III) in aerobic aqueous solutions
10. Use of an inappropriate technique / readout
    - If at all possible, it is always best to determine a K_d value with more than one method and then ask if the values obtained are within reasonable agreement or not.

The bottom line:

1. Understanding the chemistry of the system and consideration of all possible complications before setting up a titration is very important. Optimization takes time.
2. Be thoughtful about the method of choice!
3. Do many titrations to sort out the affinity.
4. Be patient and persistent!

Two general ways to determine the metal-ion affinity of a protein:

1. **Direct approach** – look at a change in a some biophysical property
    - Optical absorption (e.g. cobalt binding)
    - Intrinsic fluorescence (e.g. changes in Trp emission)
    - EPR spectroscopy (e.g. binding of EPR active metal like Cu(II) or Mn(II))
    - NMR spectroscopy (e.g. Zn(II) binding to Zn(II) finger peptide)

    This approach works well for relatively low affinity sites. Why?
    - What is a relatively low affinity site?

    Note: many proteins bind metal ions adventitiously
    → What amino acid side chains contribute?

    This approach is not appropriate for a high-affinity site. Why?
    - Direct titration will provide information on stoichiometry.
    - Direct titration will only provide a limit on the K_d value.
    - (See example below)

2. **Indirect approach** – set up a competition between the protein and another chelator with a known affinity for the metal-ion of interest; monitor an observable and fit data to a series of equations to determine affinity for protein of interest.
    - Compete protein and colorimetric indicator for the metal of interest
    - Compete protein with a ligand like EDTA or EGTA and monitor a change in some biophysical property of the protein
    - → buffers that control the concentration of free metal

Direct titrations – be aware of the issue of stoichiometric binding when high-affinity complexes form (Figure taken from *Anal. Chem.* **2003**, *320*, 39-54).

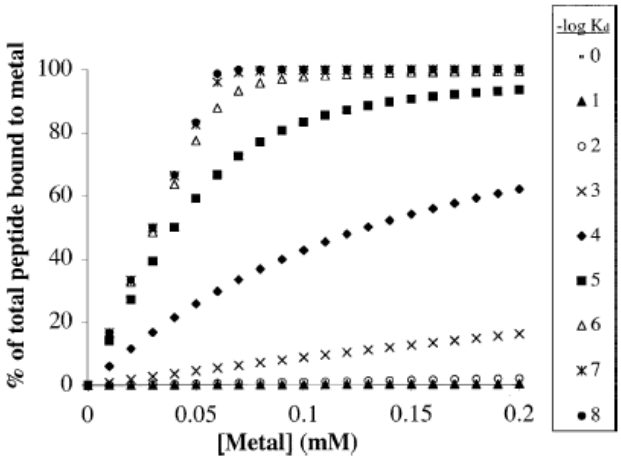

Fig. 3. Calculated binding curves for a direct forward titration of 60 μM protein. For all $K_d \leqslant 10^{-7}$, the curves are essentially identical.

Another example highlighting this same problem is shown in Figure 5 of the assigned Giedroc reading (simulated ITC data).

Competition titrations – a way to determine a K_d for a high-affinity site

1. Compete two metals for the same protein ligand
   Ex. Co(II) and Zn(II)
2. Compete two ligands for the same metal
   Ex. Protein of interest and a colorimetric indicator with known affinity for the metal
   Ex. Prepare a buffer that contains a chelator that allows for buffering of the free metal concentration

Other Methods for determining metal ion affinities – many possibilities

Equilibrium dialysis
Immunoprecipitation
Size exclusion chromatography

In these cases, the amount of metal-bound form must be determined. The protein can be quantified (how?) and metal concentration determined.

Methods to determine metal concentration include:

Atomic absorption spectroscopy
ICP-MS
A colorimetric assay (e.g. ferrozine assay for iron)
Radioactivity

Simple binding problem – occupancy calculation

The calcium(II) sensor Fura-2 forms a 1:1 complex with Ca(II) and a K_d value of 1 μM.

If the [Ca(II)] in solution is 1 μM, what fraction of Fura-2 will be occupied with Ca(II) if

[Fura-2] = 10 nM

[Fura-2] = 1 μM

[Fura-2] = 100 μM

How to set up and solve this problem? Think about K_d equation(s) and what is measurable or known.

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