Femtomolar Zn(II) Affinity in a Protein Ligand Designed to Model Thiolate-Rich Metalloprotein Active Sites

Supplementary Material

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Materials and Methods

Reagents: Trifluoroacetic acid, ethanedithiol, 1-hydroxybenzotriazole, diethyl ether, acetic anhydride, diisopropylethylamine (DIEA), piperidine were obtained from the Sigma-Aldrich Chemical Company. Natural Fmoc-protected amino acids were obtained from Bachem. HBTU, O-(1H-benzotriazole-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate, was purchased from Qbiogene. All other chemicals and solvents were reagent grade and used without further purification.

Peptide Ligand Synthesis: The peptide ligand **IGA** was synthesized on a continuous flow Applied Biosystems Pioneeer solid phase synthesizer using the Fmoc/^{t}Bu protection strategy with PAL-PEG-PS resin (0.20 mmol/g loading) at 0.2 mmole scale. Single extended coupling cycles (60 min.) with HBTU/DIEA activation chemistry were employed for all amino acids. The side chain protecting groups used are, as follows: Lys (^{t}Boc); Glu (O^{t}Bu); Cys (Trt). Each peptide was cleaved from the resin and simultaneously deprotected using 90:8:2 (v/v/v) trifluoroacetic acid : ethanedithiol : water for 2 hours. Crude peptides were triturated with cold ether, dissolved in water (0.1% v/v TFA), lyophilized, and purified to homogeneity by reversed phase C_{18} HPLC using aqueous-acetonitrile gradients containing 0.1% (v/v) TFA. After lyophilization, the identities of the resulting peptide ligands were confirmed with matrix assisted laser desorption ionization mass spectrometry (MALDI-MS).

UV-visible spectroscopy: UV-visible spectra were recorded on either a Varian Cary 100 or a Bio50 spectrophotometer using quartz cells of 1.0 cm pathlength. pH titration experiments monitored in the visible wavelength region were performed manually using an anaerobic 1.0 cm pathlength cuvette fitted with a pH electrode. Peptide

concentrations were determined spectrophotometrically using ε_{280} of 5600 M^{-1} cm^{-1} for Trp.

Fluorescence Spectroscopy: Excitation and emission fluorescence spectra were recorded on a Cary Eclipse fluorimeter using rectangular quartz cells of 1.0 cm pathlength. Excitation and emission slit widths of 5 nm were employed. pH titrations were performed using an automated titrator attached to an AVIV 215 circular dichroism spectropolarimeter with a total fluorescence attachment. The excitation wavelength was 280 nm and the total fluorescence emission was collected after a 310 nm high band pass filter. The sample was maintained at 25°C by a thermoelectric module with a ThermoNeslab refrigerated recirculating water bath as a heat sink. Peptide concentrations were between 10-30 μM as determined spectrophotometrically using ε_{280} = 5600 M^{-1} cm^{-1} for Trp.

Determination of Metal Ion Affinities by Direct Titration: Aqueous stock solutions of each metal were added in microliter aliquots to freshly prepared **IGA** peptide solutions in aqueous buffers under strictly anaerobic conditions in cuvettes of 1 cm or 10 cm pathlength. Samples were allowed to equilibrate for 3 min. before measurement of their UV-vis or fluorescence spectra. The conditional metal-ligand dissociation constants, conditional K_d values, were obtained from fitting the spectroscopic data plotted against the $[Metal_{total}]/[Peptide_{total}]$ according to the following equations for a 1:1 binding equilibrium measured by absorbance and fluorescence.

$$\textbf{IGA} + M(II)\cdot xH_2O \rightleftharpoons M(II)\text{-}\textbf{IGA} + xH_2O \qquad (S.1)$$

$$K_d^{M(II)} = [M(II)\cdot xH_2O][\textbf{IGA}]/[M(II)\text{-}\textbf{IGA}] \qquad (S.2)$$

For absorbance measurements, the data are fit to the following:

$$Abs=Abs_0+\varepsilon_B*(0.5)(x*[L_T]+K_d+[L_T]-sqrt\{(x*[L_T]+K_d+[L_T])^2-4x*[L_T]^2\}+ \varepsilon_F*(x*[L_T])-\varepsilon_F*(0.5)(x*[L_T]+K_d+[L_T])-sqrt\{(x*[L_T]+K_d+[L_T])^2-4x*[L_T]^2\} \quad (S.3)$$

Where Abs is the total absorbance, Abs_0 is the initial absorbance of the **IGA** ligand prior to addition of metal, ε_B and ε_F are the molar extinction coefficients of bound and free metal, respectively, x is the ratio of total metal to total ligand ($[M_T]/[L_T]$), or equivalents of metal added, $[L_T]$ is the total concentration of the **IGA** ligand, and K_d is the conditional dissociation constant.

For fluorescence, the equation used to fit the data is, as follows:

$$F_T=F_L+(F_{ML}-F_L/2*[L_T])*((([L_T]*x)+[L_T]+K_d) - sqrt(([L_T]*x+[L_T]+K_d)^2-(4*x*[L_T]^2))) \quad (S.4)$$

Where F_T is total fluorescence, F_L is the fluorescence of the **IGA** ligand prior to metal binding, F_{ML} is the fluorescence of the Zn(II)-**IGA** complex, x is the ratio of total metal to total ligand ($[M_T]/[L_T]$), or equivalents of metal added, $[L_T]$ is the total concentration of the **IGA** ligand, and K_d is the conditional dissociation constant.

Determination of Metal Ion Affinities by Metal Ion Competition Studies: The displacement of a metal, M_A, bound to **IGA** with another metal of higher affinity, M_B, was followed by either UV-visible or fluorescence spectroscopy. The change in signal due to displacement of M_A by M_B in **IGA** was fit to an equilibrium competition constant, $K_{comp}^{A/B}$, expressed as an equilibrium dissociation constant. The measured competition constant coupled with the dissociation constant for one of the metals was used to determine the dissociation constant for the other metal according to the relationship

$K_{comp}^{A/B} = K_d^{M_A} / K_d^{M_B}$. For the displacement of Fe(II) bound to **IGA** by Co(II), as monitored by absorption spectroscopy (Figure 5C) the following equations apply:

$$\text{Fe(II)-}\mathbf{IGA} + \text{Co(II)}\cdot x\text{H}_2\text{O} \rightleftharpoons \text{Co(II)-}\mathbf{IGA} + \text{Fe(II)}\cdot x\text{H}_2\text{O} \quad \text{(S.5)}$$

$$K_d^{Fe(II)} = [\text{Fe(II)}\cdot x\text{H}_2\text{O}][\mathbf{IGA}]/[\text{Fe(II)-}\mathbf{IGA}] \quad \text{(S.6)}$$

$$K_d^{Co(II)} = [\text{Co(II)}\cdot x\text{H}_2\text{O}][\mathbf{IGA}]/[\text{Co(II)}\mathbf{IGA}] \quad \text{(S.7)}$$

$$K_{comp}^{Fe/Co} = [\text{Fe(II)}\cdot x\text{H}_2\text{O}][\text{Co(II)-}\mathbf{IGA}]/[\text{Co(II)}\cdot x\text{H}_2\text{O}][\text{Fe(II)-}\mathbf{IGA}] \quad \text{(S.8)}$$

$$K_{comp}^{Fe/Co} = K_d^{Fe(II)} / K_d^{Co(II)} \quad \text{(S.9)}$$

$$\text{Abs} = \text{Abs}_0 + (\varepsilon_{M_AL} * L_T) + (\varepsilon_{M_BL} - \varepsilon_{M_AL}) * [-B + \text{sqrt}\{B^2 + (4 * A * (K_{comp}^{A/B}) * x * L_T^2)\}] / 2A$$
$$A = 1 - K_{comp}^{A/B}$$
$$B = M_{AT} - L_T + (K_{comp}^{A/B})(L_T) + (K_{comp}^{A/B})(x * L_T) \quad \text{(S.10)}$$

Where Abs is the total absorbance, Abs_0 is the initial absorbance of the **IGA** ligand bound to metal A prior to addition of metal B, ε_{M_AL} and ε_{M_BL} are the molar extinction coefficients of **IGA** bound to metal A and metal B, respectively, $[L_T]$ is the total concentration of the **IGA** ligand, x is the ratio of total metal B to total ligand ($[M_{BT}]/[L_T]$), or equivalents of metal B added, $[L_T]$ is the total concentration of the **IGA** ligand, $[M_{AT}]$ is the total concentration of metal A, and $K_{comp}^{A/B}$ is the conditional competition constant.

For the displacement of Co(II) bound to **IGA** by Zn(II), as monitored by fluorescence spectroscopy (Figure 7C) the following equations apply

$$\text{Co(II)-}\mathbf{IGA} + \text{Zn(II)}\cdot x\text{H}_2\text{O} \rightleftharpoons \text{Zn(II)-}\mathbf{IGA} + \text{Co(II)}\cdot x\text{H}_2\text{O} \quad \text{(S.11)}$$

$$K_d^{Co(II)} = [\text{Co(II)}\cdot x\text{H}_2\text{O}][\mathbf{IGA}]/[\text{Co(II)-}\mathbf{IGA}] \quad \text{(S.12)}$$

$$K_d^{Zn(II)} = [\text{Zn(II)}\cdot x\text{H}_2\text{O}][\mathbf{IGA}]/[\text{Zn(II)}\mathbf{IGA}] \quad \text{(S.13)}$$

$$K_{comp}^{Co/Zn} = [\text{Co(II)}\cdot x\text{H}_2\text{O}][\text{Zn(II)-}\mathbf{IGA}]/[\text{Zn(II)}\cdot x\text{H}_2\text{O}][\text{Co(II)-}\mathbf{IGA}] \quad \text{(S.14)}$$

$$K_{comp}^{Co/Zn} = K_d^{Co(II)} / K_d^{Zn(II)} \quad \text{(S.15)}$$

$$F_T = F_o + \{\{-B + \{B^2 + (4*A*K_{comp}^{A/B}*x*L_T^2)\}^{.5}\}/ 2A*L_T\}*(F_{lim} - F_o)$$

$$A = 1 - K_{comp}^{A/B}$$
$$B = M_{AT} - L_T + (K_{comp}^{A/B})(L_T) + (K_{comp}^{A/B})(x*L_T) \quad \text{(S.16)}$$

Where F_T is the total fluorescence, F_o is the initial fluorescence of the **IGA** ligand bound to metal A prior to addition of metal B, F_{lim} is the fluorescence of the **IGA** ligand 100% bound to metal B, $[L_T]$ is the total concentration of the **IGA** ligand, x is the ratio of total metal B to total ligand ($[M_{BT}]/[L_T]$), or equivalents of metal B added, $[L_T]$ is the total concentration of the **IGA** ligand, $[M_{AT}]$ is the total concentration of metal A, and $K_{comp}^{A/B}$ is the conditional competition constant.

Determination of Zn(II) Affinities by EDTA Competition. For pH values above 7, conditional equilibrium dissociation constant determination for the Zn(II)-**IGA** complex necessitated the use of EDTA (ethylenediaminetetraacetic acid) competition. To buffered aqueous solutions (either 20mM HEPES, .1M KCl or 20mM Potassium Phosphate, .1M KCl) of 10-15 μM **IGA** and between .5-5 eq of EDTA, at pH's between 7 and 8, Zn(II) was added in microliter aliquots under strictly anaerobic conditions. The increase in fluorescence at 357nm upon the addition of Zn(II) was fit to a competition equilibrium binding model based on equation S.17.

$$\text{Zn(II)-EDTA} + \textbf{IGA} \rightleftharpoons \text{Zn(II)-}\textbf{IGA} + \text{EDTA} \quad \text{(S.17)}$$

$$K_{comp} = K_d^{Zn(II)\text{-}EDTA} / K_d^{Zn(II)\text{-}\mathbf{IGA}} \quad \text{(S.18)}$$

$$F_T = F_o + ((F_{lim} - F_o)/((2-2*K_{comp})*[L_T]))*((x*[L_T]-[EDTA_T]-K_{comp}*x*[L_T]-K_{comp}*[L_T])+sqrt((x*[L_T]-[EDTA_T]-K_{comp}*x*[L_T]-K_{comp}*[L_T])^2+4*(1-K_{comp})*[L_T]^2*x*K_{comp})) \quad \text{(S.19)}$$

Where F_T is total fluorescence, F_o is the fluorescence of the **IGA** ligand prior to Zn(II) binding, F_{lim} is the fluorescence of the **IGA** ligand 100% bound to Zn(II), x is the ratio of total metal to total ligand ($[M_T]/ [L_T]$), or equivalents of metal added, $[L_T]$ is the total concentration of the **IGA** ligand, $[EDTA_T]$ is the total concentration of EDTA, and K_{comp} is the conditional competition constant.

The K_{comp} value, coupled with the conditional equilibrium dissociation constant value of Zn(II)-EDTA, $K'_{Zn(II)\text{-}EDTA}$, given by equation (S.20), gives the conditional equilibrium dissociation constant value for Zn(II)-**IGA**.

$$K'_{Zn(II)\text{-}EDTA} = K_{Zn(II)\text{-}EDTA} * \alpha_L \quad \text{(S.20)}$$

$$\alpha_L = K_1K_2K_3K_4K_5K_6/(K_1K_2K_3K_4K_5K_6+K_1K_2K_3K_4K_5[H^+]+K_1K_2K_3K_4[H^+]^2 +K_1K_2K_3[H^+]^3+ K_1K_2[H^+]^4+K_1[H^+]^5+[H^+]^6) \quad \text{(S.21)}$$

Where α_L is the mole fraction of fully deprotonated EDTA, $K_{Zn(II)\text{-}EDTA}$ ($=10^{16.5}$) is the formation constant of fully deprotonated EDTA for Zn(II),[1] and $K_{(1\text{-}6)}$ are the proton dissociation constants of EDTA[1]; $K_1 = 1$, $K_2 = .031$, $K_3 = .01$, $K_4 = .0022$, $K_5 = 6.92*10^{-7}$, $K_6 = 5.75*10^{-11}$.

Proton Competition Studies: Under anaerobic conditions, a dilute solution of HCl in water was added in microliter aliquots to solutions of each metal complex of **IGA** prepared in a cuvette fitted with a pH electrode. The absorbance or fluorescence data as a function of pH results from the following step-wise protonation equilibria.

$$pK_a^{MLH_4} \qquad M(II)\text{-}\mathbf{IGA}\text{-}4H^+ \rightleftharpoons M(II)\text{-}\mathbf{IGA}\text{-}3H^+ + H^+ \qquad \text{(S.22)}$$

$$pK_a^{MLH_3} \qquad M(II)\text{-}\mathbf{IGA}\text{-}3H^+ \rightleftharpoons M(II)\text{-}\mathbf{IGA}\text{-}2H^+ + H^+ \qquad \text{(S.23)}$$

$$pK_a^{MLH_2} \qquad M(II)\text{-}\mathbf{IGA}\text{-}2H^+ \rightleftharpoons M(II)\text{-}\mathbf{IGA}\text{-}H^+ + H^+ \qquad \text{(S.24)}$$

$$pK_a^{MLH_1} \qquad M(II)\text{-}\mathbf{IGA}\text{-}H^+ \rightleftharpoons M(II)\text{-}\mathbf{IGA} + H^+ \qquad \text{(S.25)}$$

Thus, there are five possible protonation states, MLH_x where x = 0-4, of the metal-ligand complex. The observed spectroscopic signal (S_T) is the sum of the intrinsic signals of each MLH_x species, S_{MLH_x} x = 0 - 4, weighted by its mole fraction in solution, $\_MLH_x$ (the fraction of $[MLH_x]$ present over all forms of metal ligand species, ΣMLH_x).

$$S_T = (S_{ML}*\_ML) + (S_{MLH}*\_MLH) + (S_{MLH_2}*\_MLH_2) + (S_{MLH_3}*\_MLH_3) + (S_{MLH_4}*\_MLH_4) \qquad \text{(S.26)}$$

The mole fractions of each MLH_x species, $\_{MLH_x}$, are given as function of solution pH, their respective $pK_a^{MLH_x}$ (x = 0 - 4) values and the values of the $pK_a^{LH_x}$ (x = 0 - 4) value of the **IGA** ligand, LH_4.

$$\_{MLH_4} = 10^{(-4*pH)} / \Sigma MLH_x \quad \text{(S.27)}$$

$$\_{MLH_3} = 10^{(-3*pH - pK_a^{MLH4})} / \Sigma MLH_x \quad \text{(S.28)}$$

$$\_{MLH_2} = 10^{(-2*pH - pK_a^{MLH4} - pK_a^{MLH3})} / \Sigma MLH_x \quad \text{(S.29)}$$

$$\_{MLH} = 10^{(-pH - pK_a^{MLH4} - pK_a^{MLH3} - pK_a^{MLH2})} / \Sigma MLH_x \quad \text{(S.30)}$$

$$\_{ML} = 10^{(-pK_a^{MLH4} - pK_a^{MLH3} - pK_a^{MLH2} - pK_a^{MLH})} / \Sigma MLH_x \quad \text{(S.31)}$$

$$\Sigma MLH_x = [ML] + [MLH_1] + [MLH_2] + [MLH_3] + [MLH_4] \quad \text{(S.32)}$$

$$\Sigma MLH_x = 10^{(-4*pH)} + 10^{(-3*pH - pK_a^{LH4})} + 10^{(-2*pH - pK_a^{MLH4} - pK_a^{MLH3})} + 10^{(-pH - pK_a^{MLH4} - pK_a^{MLH3} - pK_a^{MLH2})} + 10^{(-pK_a^{MLH4} - pK_a^{MLH3} - pK_a^{MLH2} - pK_a^{MLH})} \quad \text{(S.33)}$$

The simplest model that adequately fit the data sets for Fe(II), Co(II) and Zn(II) complexes of **IGA** is one in which there are two acid dissociation constants, one represents a one proton event at pK_{a1}^{eff} and the other a cooperative three proton event at pK_{a2}^{eff}.

$$pK_{a1}^{eff} \qquad M(II)\text{-}\mathbf{IGA}\text{-}H^+ \rightleftharpoons M(II)\text{-}\mathbf{IGA} + H^+ \quad \text{(S.34)}$$

$$pK_{a2}^{eff} \qquad M(II)\text{-}\mathbf{IGA}\text{-}4H^+ \rightleftharpoons M(II)\text{-}\mathbf{IGA}\text{-}H^+ + 3\ H^+ \quad \text{(S.35)}$$

Thus, the general equation above reduces to the following,

$$S_T = (S_{ML} * \_{ML}) + (S_{MLH} * \_{MLH}) + (S_{MLH4} * \_{MLH4}) \quad \text{(S.36)}$$

$$\_{MLH_4} = 10^{(-4*pH)} / \Sigma MLH_x^{coop} \quad \text{(S.37)}$$

$$\_{MLH} = 10^{(-pH - 3*pK_{a2}^{eff})} / \Sigma MLH_x^{coop} \quad \text{(S.38)}$$

$$\_{ML} = 10^{(-pK_{a2}^{eff}*3 - pK_{a1}^{eff})} / \Sigma MLH_x^{coop} \quad \text{(S.39)}$$

$$\Sigma MLH_x^{coop} = [ML] + [MLH_1] + [MLH_4] \quad (S.40)$$

$$\Sigma MLH_x^{coop} = 10^{(-4*pH)} + 10^{(-pH - pK_{a2}^{eff}*3)} + 10^{(-pK_{a2}^{eff}*3 - pK_{a1}^{eff})} \quad (S.41)$$

Determination of Metal Ion Affinities as a Function of pH: Due to the expected $[H^+]^4$ dependence of the **IGA** ligand's conditional K_d value, K_d's were measured at a series of pH values. The K_d value at each pH was determined as above using either UV-visible or fluorescence spectroscopies. The resulting plots of -log K_d versus measured pH are fit to the following equation:

$$\log K_f' = K_f'^{MLH_4} + K_f'^{MLH} + K_f'^{ML} \quad (S.42)$$

Where K_f', the observed conditional formation constant at any given solution pH, is a sum of the conditional formation constants of the various protonated metal-ligand species, $K_f'^{MLH_4}$, $K_f'^{MLH}$, and $K_f'^{ML}$.

$$\log K_f' = (\_LH_4 * K_f^{MLH_4} / \_MLH_4) + (\_LH * K_f^{MLH} / \_MLH) + (\_L * K_f^{ML} / \_ML) \quad (S.43)$$

$$\log K_f' = \log\{((\_LH_4 * (10^{(-pK_a^{LH} + pK_a^{MLH})}) * ((10^{(-pK_a^{LH}4*3 + pK_a^{MLH}4*3)}) * K_f^{ML}) / \_MLH_4) + ((\_LH * (10^{(-pK_a^{LH} + pK_a^{MLH})}) * K_f^{ML}) / \_MLH) + (\_L * K_f^{ML} / \_ML)\} \quad (S.44)$$

where

$$\_LH_4 = 10^{(-4*pH)} / \_Lcoop \quad (S.45)$$

$$\_LH = 10^{(-pH - 3*pK_a^{LH})} / \_Lcoop \quad (S.46)$$

$$\_L = 10^{(-pK_a^{LH}4*3 - pK_a^{LH})} / \_Lcoop \quad (S.47)$$

$$\_Lcoop = 10^{(-4*pH)} + 10^{(-pH - pK_a^{LH}4*3)} + 10^{(-pK_a^{LH}4*3 - pK_a^{LH})} \quad (S.48)$$

$$\_MLH_4 = 10^{(-4*pH)} / \_MLcoop \quad (S.49)$$

$$\_MLH = 10^{(-pH - 3*pK_a^{MLH}4)} / \_MLcoop \quad (S.50)$$

$$\_ML = 10^{(-pK_a^{MLH}4*3 - pK_a^{MLH})} / \_MLcoop \quad (S.51)$$

$$\_MLcoop = 10^{(-4*pH)} + 10^{(-pH - pK_a^{MLH}4*3)} + 10^{(-pK_a^{MLH}4*3 - pK_a^{MLH})} \quad (S.52)$$

Where $pK_a^{LH_x}$ is the pK_a value of the **IGA** ligand (assumed to be 8.3 or that of free cysteine), $pK_a^{MLH_x}$ values are the effective pK_a values of the individual metal-ligand species as derived from proton competition studies (pK_{a1}^{eff} and pK_{a2}^{eff}), K_f^{ML} ($=\beta_{110}$) is the formation constant of the ML complex from metal and deprotonated ligand (L^{4-}), and K_f' is the conditional formation constant of the metal-ligand species at a particular pH value. The value of β_{110} ($=K_f^{ML}$) is related to the overall formation constant, β_{114}, which describes the formation of MLH_4 from one M(II), one **IGA**, and 4 H^+, by a factor of $(1/ K_{a1}^{eff})*(1/ K_{a2}^{eff})^3$, the acid association constants of the metal bound cysteine ligands.

$\beta_{110} = K_f^{ML}$ M(II) + **IGA** $\rightleftharpoons$ M(II)-**IGA** (S.53)

β_{114} M(II) + **IGA** + 4 H^+ $\rightleftharpoons$ M(II)-**IGA**-4H^+ (S.54)

$\beta_{114} = \beta_{110} * (1/K_{a1}^{eff}) * (1/K_{a2}^{eff})^3$ (S.55)

Derivations of Equilibrium Binding Models

pH Dependent Chemical Speciation of the Ferredoxin Maquettes

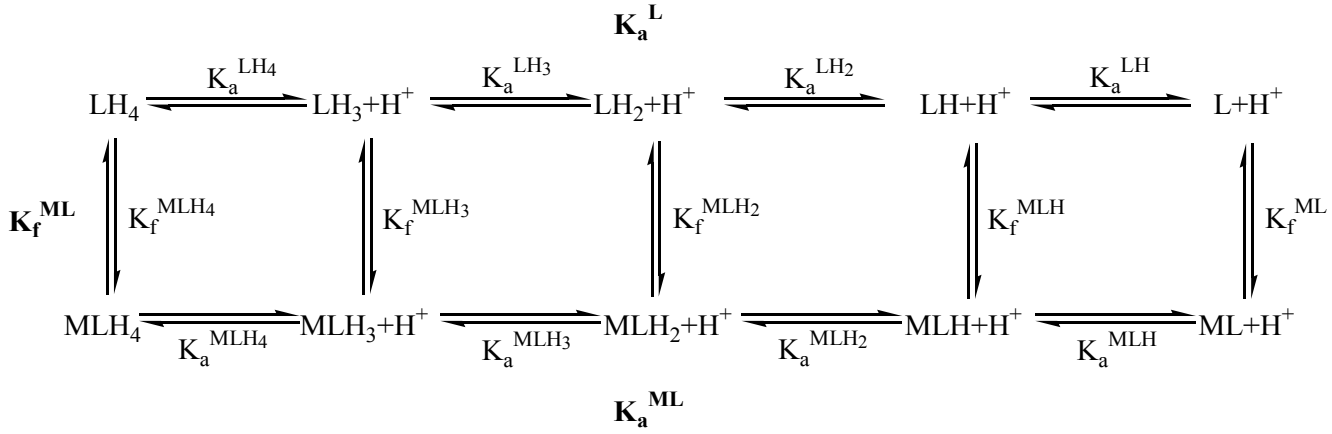

Figure 1: Stepwise formation, $\mathbf{K_f^{ML}}$, and proton dissociation, $\mathbf{K_a^{L}}$ and $\mathbf{K_a^{ML}}$, constants relevant to the complex equilibria of metal substituted Ferredoxin Maquettes.

$\mathbf{K_a^{L}}$

$$K_a^{LH_4} = [LH_3]\,[H^+] / [LH_4]$$
$$K_a^{LH_3} = [LH_2]\,[H^+] / [LH_3]$$
$$K_a^{LH_2} = [LH]\,[H^+] / [LH_2]$$
$$K_a^{LH} = [L]\,[H^+] / [LH]$$

$\mathbf{K_a^{ML}}$

$$K_a^{MLH_4} = [MLH_3]\,[H^+] / [MLH_4]$$
$$K_a^{MLH_3} = [MLH_2]\,[H^+] / [MLH_3]$$
$$K_a^{MLH_2} = [MLH]\,[H^+] / [MLH_2]$$
$$K_a^{MLH} = [ML]\,[H^+] / [MLH]$$

$\mathbf{K_f^{ML}}$

$$K_f^{MLH_4} = [MLH_4] / [M][LH_4]$$
$$K_f^{MLH_3} = [MLH_3] / [M][LH_3]$$
$$K_f^{MLH_2} = [MLH_2] / [M][LH_2]$$
$$K_f^{MLH} = [MLH] / [M][LH]$$
$$K_f^{ML} = [ML] / [M][L]$$

I. Modeling the pH Dependency of the Conditional Formation Constant, K_f^{ML}.

The solution pH dictates the extent to which various protonated metal-ligand complexes, $ML(H)_x$ or M(II)-**IGA**-$(H)_x$, are able to form, i.e. ML, MLH, MLH_2, MLH_3, MLH_4. If one wishes to determine an equilibrium binding constant for ML, two conditions must be satisfied; the experiment must be performed 1) at a pH where ML is the predominant species present, and 2) the ligand concentration must be on the order of the equilibrium dissociation constant, K_d (= 1 / K_f). In many cases, it is nearly impossible to meet "condition 2". For example, many biological macromolecules have nanomolar or tighter affinities for metal at a given pH, as does **IGA** for Zn(II). However, it is often times very difficult to measure changes in experimental signal at such low concentrations. One method workers in the field employ is to perform equilibrium formation constant measurements at lower pH's, where protons compete with the metal for ligand binding. This effectively lowers the ligand's affinity for metal, thereby allowing the researcher to perform experiments at higher, more reasonable ligand concentrations. However, this methodology violates "condition 1"; the formation constant measured at a lower pH is no longer the formation constant for ML, but rather any number of other protonated metal-ligand complexes in equilibrium, i.e. MLH, MLH_2, MLH_3, MLH_4. In order to obtain a true equilibrium formation constant for ML (when it's affinity for metal is too tight to be measured at a given pH), one should generate a plot of the conditional formation constant as a function of pH. Proper fitting of this plot will allow one to derive an equilibrium formation constant for ML. The following is a derivation of the function used to fit the pH dependence of the conditional formation constant of Fe(II), Co(II), and Zn(II) substituted **IGA**.

Over the relevant pH range (4-9), the $ML(H)_x$ species in solution are ML, MLH, MLH_2, MLH_3, and MLH_4. Their formation constants are given by:

$$K_f^{MLH_4} = [MLH_4] / [M][LH_4] \quad \text{(I.1)}$$

$$K_f^{MLH_3} = [MLH_3] / [M][LH_3] \quad \text{(I.2)}$$

$$K_f^{MLH_2} = [MLH_2] / [M][LH_2] \quad \text{(I.3)}$$

$$K_f^{MLH} = [MLH] / [M][LH] \quad \text{(I.4)}$$

$$K_f^{ML} = [ML] / [M][L] \quad \text{(I.5)}$$

Because the formation of each of these species is pH dependent, one must define a set of conditional formation constants, $\mathbf{K_f'}$, that are functions of pH. We do this by first defining $\mathbf{K_f'}$ in terms of a quantity _, which is defined to be the mole fraction of a particular species present in solution.

$$K_f'^{MLH4} = ([MLH_4] / \__{MLH4}) / ([M][LH_4] / \__{LH4}) \quad \text{(I.6)}$$

$$K_f'^{MLH_3} = ([MLH_3] / \alpha_{MLH_3}) / ([M][LH_3] / \alpha_{LH_3}) \quad (I.7)$$

$$K_f'^{MLH_2} = ([MLH_2] / \alpha_{MLH_2}) / ([M][LH_2] / \alpha_{LH_2}) \quad (I.8)$$

$$K_f'^{MLH} = ([MLH] / \alpha_{MLH}) / ([M][LH] / \alpha_{LH}) \quad (I.9)$$

$$K_f'^{ML} = ([ML] / \alpha_{ML}) / ([M][L] / \alpha_{L}) \quad (I.10)$$

$$L_T = [LH_4] + [LH_3] + [LH_2] + [LH] + [L] \quad (I.11)$$

where L_T is the mass balance for all forms of free ligand, L.

$$ML_T = [MLH_4] + [MLH_3] + [MLH_2] + [MLH] + [ML] \quad (I.12)$$

where ML_T is the mass balance for all forms of bound metal (metal-ligand complex), ML.

$$\alpha_{MLH_4} = [MLH_4] / ML_T \quad (I.13)$$

$$\alpha_{MLH_3} = [MLH_3] / ML_T \quad (I.14)$$

$$\alpha_{MLH_2} = [MLH_2] / ML_T \quad (I.15)$$

$$\alpha_{MLH} = [MLH] / ML_T \quad (I.16)$$

$$\alpha_{ML} = [ML] / ML_T \quad (I.17)$$

$$\alpha_{LH_4} = [LH_4] / L_T \quad (I.18)$$

$$\alpha_{LH_3} = [LH_3] / L_T \quad (I.19)$$

$$\alpha_{LH_2} = [LH_2] / L_T \quad (I.20)$$

$$\alpha_{LH} = [LH] / L_T \quad (I.21)$$

$$\alpha_{L} = [L] / L_T \quad (I.22)$$

As $\alpha \rightarrow 1$, $\mathbf{K_f' \rightarrow K_f}$;(the conditional formation constant, $\mathbf{K_f'}$, approaches the true formation constant, $\mathbf{K_f}$.) Consider Eq. I.10. When the fractions of L and ML in solution, α_L and α_{ML}, are 1, the true equilibrium formation constant (I.5) is obtained. As soon as α_L and α_{ML} drop below 1, the conditional formation constant, $K_f'^{ML}$, becomes a function of L_T and ML_T. The only way the fraction of a given species, α, can change, is if the pH changes. Thus, α of a given species can be put in terms of pH. However, before this is done, let us first relate the conditional formation constant, $\mathbf{K_f'}$, to the true, unconditional formation constant $\mathbf{K_f}$. After all, the eventual goal is to obtain $\mathbf{K_f^{ML}}$ from a plot of $\mathbf{K_f'}$ vs. pH.

To relate $\mathbf{K_f'}$ to $\mathbf{K_f}$, plug equations (I.1-I.5) into (I.6-I.10). This gives

$$K_f'^{MLH_4} = (\alpha_{LH_4} * K_f^{MLH_4}) / \alpha_{MLH_4} \quad (I.23)$$

$$K_f'^{MLH_3} = (\alpha_{LH_3} * K_f^{MLH_3}) / \alpha_{MLH_3} \quad (I.24)$$

$$K_f'^{MLH_2} = (\alpha_{LH_2} * K_f^{MLH_2}) / \alpha_{MLH_2} \quad (I.25)$$

$$K_f{}'^{MLH} = (\_{}_{LH} * K_f{}^{MLH}) / \_{}_{MLH} \quad \text{(I.26)}$$

$$K_f{}'^{ML} = (\_{}_{L} * K_f{}^{ML}) / \_{}_{ML} \quad \text{(I.27)}$$

In order to put _ in terms of pH, let us first generate a series of expressions that define L_T and ML_T in terms of all forms of free ligand, L, and metal-ligand complex, ML, respectively. We do this by relating the equilibria highlighted in the thermodynamic scheme in Figure 1 to the free ligand and metal-ligand complex mass balances, I.11 and I.12.

$$L_T = [L] * \{([H^+]^4 / K_a{}^{LH4}*K_a{}^{LH3}*K_a{}^{LH2}*K_a{}^{LH}) + ([H^+]^3 / K_a{}^{LH3}*K_a{}^{LH2}*K_a{}^{LH}) + ([H^+]^2 / K_a{}^{LH2}*K_a{}^{LH}) + ([H^+] / K_a{}^{LH}) +1\} \quad \text{(I.28)}$$

$$L_T = [LH] * \{([H^+]^3 / K_a{}^{LH3}*K_a{}^{LH2}*K_a{}^{LH}) + ([H^+]^2 / K_a{}^{LH3}*K_a{}^{LH2}) + ([H^+] / K_a{}^{LH3}) + (K_a{}^{LH4} / [H^+]) + 1\} \quad \text{(I.29)}$$

$$L_T = [LH_2] * \{([H^+]^2 / K_a{}^{LH3}*K_a{}^{LH4}) + ([H^+] / K_a{}^{LH3}) + (K_a{}^{LH2} / [H^+]) + (K_a{}^{LH}*K_a{}^{LH2} / [H^+]^2) + 1\} \quad \text{(I.30)}$$

$$L_T = [LH_3] * \{(K_a{}^{LH3}*K_a{}^{LH2}*K_a{}^{LH} / [H^+]^3) + (K_a{}^{LH3}*K_a{}^{LH2} / [H^+]^2) + (K_a{}^{LH3} / [H^+]) + ([H^+] / K_a{}^{LH4}) +1\} \quad \text{(I.31)}$$

$$L_T = [LH_4] * \{(K_a{}^{LH4}*K_a{}^{LH3}*K_a{}^{LH2}*K_a{}^{LH} / [H^+]^4) + (K_a{}^{LH4}*K_a{}^{LH3}*K_a{}^{LH2} / [H^+]^3) + (K_a{}^{LH3}*K_a{}^{LH4} / [H^+]^2) + (K_a{}^{LH4} / [H^+]) + 1\} \quad \text{(I.32)}$$

$$ML_T = [ML] * \{([H^+]^4 / K_a{}^{MLH4}*K_a{}^{MLH3}*K_a{}^{MLH2}*K_a{}^{MLH}) + ([H^+]^3 / K_a{}^{MLH3}*K_a{}^{MLH2}*K_a{}^{MLH}) + ([H^+]^2 / K_a{}^{MLH2}*K_a{}^{MLH}) + ([H^+] / K_a{}^{MLH}) +1\} \quad \text{(I.33)}$$

$$ML_T = [MLH] * \{([H^+]^3 / K_a{}^{MLH3}*K_a{}^{MLH2}*K_a{}^{MLH}) + ([H^+]^2 / K_a{}^{MLH3}*K_a{}^{MLH2}) + ([H^+] / K_a{}^{MLH3}) + (K_a{}^{MLH4} / [H^+]) + 1\} \quad \text{(I.34)}$$

$$ML_T = [MLH_2] * \{([H^+]^2 / K_a{}^{MLH3}*K_a{}^{MLH4}) + ([H^+] / K_a{}^{MLH3}) + (K_a{}^{MLH2} / [H^+]) + (K_a{}^{MLH}*K_a{}^{MLH2} / [H^+]^2) + 1\} \quad \text{(I.35)}$$

$$ML_T = [MLH_3] * \{(K_a{}^{MLH3}*K_a{}^{MLH2}*K_a{}^{MLH} / [H^+]^3) + (K_a{}^{MLH3}*K_a{}^{MLH2} / [H^+]^2) + (K_a{}^{MLH3} / [H^+]) + ([H^+] / K_a{}^{MLH4}) +1\} \quad \text{(I.36)}$$

$$ML_T = [MLH_4] * \{(K_a{}^{MLH4}*K_a{}^{MLH3}*K_a{}^{MLH2}*K_a{}^{MLH} / [H^+]^4) + (K_a{}^{MLH4}*K_a{}^{MLH3}*K_a{}^{MLH2} / [H^+]^3) + (K_a{}^{MLH3}*K_a{}^{MLH4} / [H^+]^2) + (K_a{}^{MLH4} / [H^+]) + 1\} \quad \text{(I.37)}$$

Plugging equation I.37 into I.13 gives:

$$\_MLH_4 = [H^+]^4 / \_ML \quad \text{(I.38)}$$

where, $\_ML = [H^+]^4 + [H^+]^3*K_a^{MLH_4} + [H^+]^2*K_a^{MLH_4}*K_a^{MLH_3} + [H^+]*K_a^{MLH_4}*K_a^{MLH_3}*K_a^{MLH_2} + K_a^{MLH_4}*K_a^{MLH_3}*K_a^{MLH_2}*K_a^{MLH}$

Plugging equation I.36 into I.14 gives:

$$\_MLH_3 = [H^+]^3*K_a^{MLH_4} / \_ML \quad \text{(I.39)}$$

Plugging equation I.35 into I.15 gives:

$$\_MLH_2 = [H^+]^2*K_a^{MLH_4}*K_a^{MLH_3} / \_ML \quad \text{(I.40)}$$

Plugging equation I.34 into I.16 gives:

$$\_MLH = [H^+]*K_a^{MLH_4}*K_a^{MLH_3}*K_a^{MLH_2} / \_ML \quad \text{(I.41)}$$

Plugging equation I.33 into I.17 gives:

$$\_ML = K_a^{MLH_4}*K_a^{MLH_3}*K_a^{MLH_2}*K_a^{MLH} / \_ML \quad \text{(I.42)}$$

Plugging equation I.32 into I.18 gives:

$$\_LH_4 = [H^+]^4 / \_L \quad \text{(I.43)}$$

where, $\_L = [H^+]^4 + [H^+]^3*K_a^{LH_4} + [H^+]^2*K_a^{LH_4}*K_a^{LH_3} + [H^+]*K_a^{LH_4}*K_a^{LH_3}*K_a^{LH_2} + K_a^{LH_4}*K_a^{LH_3}*K_a^{LH_2}*K_a^{LH}$

Plugging equation I.31 into I.19 gives:

$$\_LH_3 = [H^+]^3*K_a^{LH_4} / \_L \quad \text{(I.44)}$$

Plugging equation I.30 into I.20 gives:

$$\_LH_2 = [H^+]^2*K_a^{LH_4}*K_a^{LH_3} / \_L \quad \text{(I.45)}$$

Plugging equation I.29 into I.21 gives:

$$\_LH = [H^+]*K_a^{LH_4}*K_a^{LH_3}*K_a^{LH_2} / \_L \quad \text{(I.46)}$$

Plugging equation I.28 into I.22 gives:

$$\_L = K_a^{LH4} * K_a^{LH3} * K_a^{LH2} * K_a^{LH} / \_L \quad \text{(I.47)}$$

An expression for the overall conditional formation constant may be written as:

$$\mathbf{K_f'} = K_f'^{MLH4} + K_f'^{MLH3} + K_f'^{MLH2} + K_f'^{MLH} + K_f'^{ML} \quad \text{(I.48)}$$

Substituting I.23-I.27 into I.48 yields the final equation used to fit the proton dependence of the conditional formation constant, where the various _ terms and formation constants, K_f, are defined above.

$$\mathbf{K_f'} = (\_LH_4 * K_f^{MLH4} / \_MLH_4) + (\_LH_3 * K_f^{MLH3} / \_MLH_3) + (\_LH_2 * K_f^{MLH2} / \_MLH_2) + (\_LH * K_f^{MLH} / \_MLH) + (\_L * K_f^{ML} / \_ML) \quad \text{(I.49)}$$

A simplification of the above equation may be made if one considers that it is found that a pH titration of ML reveals two protonation events, a one proton event at K_a^{MLH} and a three proton cooperative event at K_a^{MLH4}.

More fundamentally, this means that

$$ML_T = [ML] + [MLH] + [MLH_4] \quad \text{(I.50)}$$
$$L_T = [L] + [LH] + [LH_4] \quad \text{(I.51)}$$

With this in mind, I.49 simplifies to:

$$\mathbf{K_f'} = (\_LH_4 * K_f^{MLH4} / \_MLH_4) + (\_LH * K_f^{MLH} / \_MLH) + (\_L * K_f^{ML} / \_ML) \quad \text{(I.52)}$$

where
$$\_LH_4 = [H^+]^4 / \_Lcoop \quad \text{(I.53)}$$
$$\_LH = [H^+] * (K_a^{LH4})^3 / \_Lcoop \quad \text{(I.54)}$$
$$\_L = K_a^{LH} * (K_a^{LH4})^3 / \_Lcoop \quad \text{(I.55)}$$
$$\_MLH_4 = [H^+]^4 / \_MLcoop \quad \text{(I.56)}$$
$$\_MLH = [H^+] * (K_a^{MLH4})^3 / \_MLcoop \quad \text{(I.57)}$$
$$\_ML = K_a^{MLH} * (K_a^{MLH4})^3 / \_MLcoop \quad \text{(I.58)}$$

$$\_Lcoop = [H^+]^4 + [H^+] * (K_a^{LH4})^3 + K_a^{LH} * (K_a^{LH4})^3 \quad \text{(I.59)}$$
$$\_MLcoop = [H^+]^4 + [H^+] * (K_a^{MLH4})^3 + K_a^{MLH} * (K_a^{MLH4})^3 \quad \text{(I.60)}$$

The final equation used to fit the data, I.52, is put in terms of pH and pKa's. Additionally, the following substitutions, which were derived from the thermodynamic scheme in Figure 1, were made:

$$K_f^{MLH} = (K_a^{LH} / K_a^{MLH}) * K_f^{ML} \quad \text{(I.61)}$$

$$K_f^{MLH_4} = (K_a^{LH} / K_a^{MLH}) * ((K_a^{LH4})^3 / (K_a^{MLH4})^3) * K_f^{ML} \quad \text{(I.62)}$$

The resulting equation that is used to fit the conditional formation constants as a function of pH is:

$$\mathbf{K_f'} = \{((\_LH_4 * (10^{(-pK_a^{LH} + pK_a^{MLH})}) * ((10^{(-pK_a^{LH}4*3 + pK_a^{MLH}4*3)}) * K_f^{ML}) / \_MLH_4) + ((\_LH * (10^{(-pK_a^{LH} + pK_a^{MLH})}) * K_f^{ML}) / \_MLH) + (\_L * K_f^{ML} / \_ML)\} \quad \text{(I.63)}$$

where $\_LH_4 = 10^{(-4*pH)} / \_Lcoop$

$\_LH = 10^{(-pH - 3*pK_a^{LH})} / \_Lcoop$

$\_L = 10^{(-pK_a^{LH}4* 3 - pK_a^{LH})} / \_Lcoop$

$\_Lcoop = 10^{(-4*pH)} + 10^{(-pH - pK_a^{LH}4*3)} + 10^{(-pK_a^{LH}4*3 - pK_a^{LH})}$

$\_MLH_4 = 10^{(-4*pH)} / \_MLcoop$

$\_MLH = 10^{(-pH - 3*pK_a^{MLH}4)} / \_MLcoop$

$\_ML = 10^{(-pK_a^{MLH}4*3 - pK_a^{MLH})} / \_MLcoop$

$\_MLcoop = 10^{(-4*pH)} + 10^{(-pH - pK_a^{MLH}4*3)} + 10^{(-pK_a^{MLH}4*3 - pK_a^{MLH})}$

II. Fitting ML pH Titrations

A general expression for the change in total signal, S_T, as a function of pH for the metal substituted ferredoxin maquettes may be written as follows:

$$S_T = (S_{ML} * \_ML) + (S_{MLH} * \_MLH) + (S_{MLH_2} * \_MLH_2) + (S_{MLH_3} * \_MLH_3) + (S_{MLH_4} * \_MLH_4) \quad \text{(II.1)}$$

Where $S_{ML(H)_x}$ is the intrinsic signal of $ML(H)_x$ and $\_ML(H)_x$ is the fraction of $ML(H)_x$ present over ML_T .

In the case of the Fe/Co/Zn Ferredoxin Maquettes, the pH titration is best fit to the following model:

$$S_T = (S_{ML} * \_ML) + (S_{MLH} * \_MLH) + (S_{MLH_4} * \_MLH_4) \quad \text{(II.2)}$$

where $\_ML$, $\_MLH$, and $\_MLH_4$ are defined in equations I.56-I.58, I.60.

The actual equation used to fit the data is put in terms of pH and pKa's, giving the final expression below.

$$S_T = (S_{ML} * (\__{ML})) + (S_{MLH} * (\__{MLH})) + (S_{MLH4} * (\__{MLH4})) \quad \text{(II.3)}$$

$$\__{MLH4} = 10^{(-4*pH)} / \__{MLcoop}$$
$$\__{MLH} = 10^{(-pH - 3*pK_a^{MLH}{}_4)} / \__{MLcoop}$$
$$\__{ML} = 10^{(-pK_a^{MLH}{}_4*3 - pK_a^{MLH})} / \__{MLcoop}$$
$$\__{MLcoop} = 10^{(-4*pH)} + 10^{(-pH - pK_a^{MLH}{}_4*3)} + 10^{(-pK_a^{MLH}{}_4*3 pK_a^{MLH})}$$

The above model is one in which there are two protonation events, a one proton event at K_a^{MLH} and a three proton cooperative event at K_a^{MLH4}.
Thus, $K_a^{MLH4} = K_a^{MLH3} = K_a^{MLH2}$.

III. 1:1 Binding Fdm Maquettes (Fluorescence)

$$K_d = [M] * [L] / [ML] \quad \text{(III.1)}$$

where, K_d is the equilibrium dissociation constant, [M] is the concentration of free metal, [L] is the concentration of free ligand, and [ML] is the concentration of a 1:1 Metal-Ligand Complex.

Consideration of mass balance gives the following expressions:

$$L_T = [ML] + [L] \quad \text{(III.2)}$$
$$M_T = [ML] + [M] \quad \text{(III.3)}$$

Where L_T and M_T are total concentrations of all forms of ligand and metal in solution, respectively.

Substitution of (III.2) and (III.3) into (III.1) results in the following expression,

$$[ML]^2 + -(M_T + L_T + K_d)*[ML] + (M_T * L_T) = 0 \quad \text{(III.4)}$$

Solving the quadratic equation leads to the following expression:

$$[ML] = \{(M_T + L_T + K_d) - \{(M_T + L_T + K_d)^2 - (4* M_T L_T)\}^{.5}\}/ 2 \quad \text{(III.5)}$$

The total fluorescence intensity, F, is governed by the sum of the fluorescence intensities of L and ML, where “a” and “b” are constants proportional to the fluorescence quantum yields of L and ML, respectively.

$$F = a*[L] + b*[ML] \quad \text{(III.6)}$$

The initial fluorescence, F_o, is governed solely by the ligand, L. The final limiting fluorescence, F_{lim}, is governed solely by the metal-ligand complex, ML. Thus, $F_o = F_L$, where F_L is the intrinsic fluorescence of L, and $F_{lim} = F_{ML}$, where F_{ML} is the intrinsic fluorescence of ML.

$$F_o = a^* L_T \quad \text{(III.7)}$$

$$F_{lim} = b^* L_T \quad \text{(III.8)}$$

Solving (III.7) and (III.8) for "a" and "b", and plugging into (III.6) gives:

$$F = (([L]^*F_o) / [L_T]) + (([ML]^*F_{lim}) / [L_T]) \quad \text{(III.9)}$$

Expressing "[L]" as "L_T – [ML]" (III.2), and further simplifying (III.9) leads to:

$$F = F_o + \{[ML]^*(F_{lim} - F_o) / L_T\} \quad \text{(III.10)}$$

Substitution of III.5 into [ML] gives:

$$F = F_o + \{(F_{lim} - F_o) / (2^*L_T)\} * \{(M_T + L_T + K_d) - \{(M_T + L_T + K_d)^2 - (4^*M_TL_T)\}^{.5}\} \quad \text{(III.11)}$$

The number of equivalents of metal added relative to peptide, the independent variable, x, is related to M_T according to:

$$M_T / L_T = x \rightarrow \quad M_T = L_T * x \quad \text{(III.12)}$$

Substitution of (III.12) into (III.11) gives the final expression used to fit the 1:1 binding isotherm:

$$F = F_o + \{\{(F_{lim} - F_o) / (2^* L_T)\} * \{(x^* L_T + L_T + K_d) - \{(x^* L_T + L_T + K_d)^2 - (4^*x^*(L_T)^2)\}^{.5}\}\} \quad \text{(III.13)}$$

IV. Determining Competition Constants between Fe/Co/Zn substituted Fdm Maquettes (Fluorescence)

Consider the following equilibria, where M_A is a metal displaced by another metal, M_B.

$$M_AL + M_B \rightleftharpoons M_BL + M_A \quad \text{(IV.1)}$$

The equilibrium competition constant, K_{comp}^{AB}, may be defined as:

$$K_{comp}^{A/B} = [M_BL]*[M_A] / [M_AL]*[M_B] \quad \text{(IV.2)}$$

Consideration of mass balance gives the following expressions:

$$L_T = [M_BL] + [M_AL] \quad \text{(IV.3)}$$
$$M_{AT} = [M_AL] + [M_A] \quad \text{(IV.4)}$$
$$M_{BT} = [M_BL] + [M_B] \quad \text{(IV.5)}$$

Where L_T, M_{AT}, and M_{BT} are the total concentrations of all forms of ligand metal A, and metal B in solution, respectively.

Substitution of (IV.3)-(IV.5) into (IV.2), and algebraic rearrangement results in the following expression:

$$A*[M_BL]^2 + B*[M_BL] - K_{comp}^{A/B}*M_{BT}*L_T = 0 \quad \text{(IV.6)}$$

Where, $A = 1 - K_{comp}^{A/B}$

$$B = M_{AT} - L_T + (K_{comp}^{A/B})(L_T) + (K_{comp}^{A/B})(M_{BT})$$

Solving the quadratic equation leads to the following expression:

$$[M_BL] = \{-B + \{B^2 + (4*A*K_{comp}^{A/B}*M_{BT}*L_T)\}^{.5}\}/ 2A \quad \text{(IV.7)}$$

The total fluorescence intensity, F, is governed by the sum of the fluorescence intensities of M_AL and M_BL, where "a" and "b" are constants proportional to the fluorescence quantum yields of M_AL and M_BL, respectively.

$$F = a*[M_AL] + b*[M_BL] \quad \text{(IV.8)}$$

The initial fluorescence, F_o, is governed solely by M_AL. The final limiting fluorescence, F_{lim}, is governed solely by M_BL.

$$F_o = a^* [L_T] \quad \text{(IV.9)}$$
$$F_{lim} = b^* [L_T] \quad \text{(IV.10)}$$

Solving (IV.9) and (IV.10) for "a" and "b", and plugging into (IV.8) gives:

$$F = (([M_AL]*F_o) / [L_T]) + (([M_BL]*F_{lim}) / [L_T]) \quad \text{(IV.11)}$$

Expressing "$[M_AL]$" as "$L_T - [M_BL]$" (IV.3), and further simplifying (IV.11) leads to:

$$F = F_o + \{[M_BL]*(F_{lim} - F_o) / L_T\} \quad \text{(IV.12)}$$

Substitution of IV.7 into $[M_BL]$ gives:

$$F = F_o + \{\{-B + \{B^2 + (4*A*K_{comp}^{A/B}*M_{BT}*L_T)\}^{.5}\}/ 2A*L_T\}*(F_{lim} - F_o) \quad \text{(IV.13)}$$

The number of equivalents of metal B added relative to peptide, the independent variable, x, is related to M_{BT} according to:

$$M_{BT} / L_T = x \rightarrow \quad M_{BT} = L_T * x \quad \text{(IV.14)}$$

Substitution of (IV.14) into (IV.13) gives the final expression used to fit the competition equilibrium:

$$F = F_o + \{\{-B + \{B^2 + (4*A*K_{comp}^{A/B}*x*L_T^2)\}^{.5}\}/ 2A*L_T\}*(F_{lim} - F_o) \quad \text{(IV.15)}$$

$$A = 1 - K_{comp}^{A/B}$$
$$B = M_{AT} - L_T + (K_{comp}^{A/B})(L_T) + (K_{comp}^{A/B})(x*L_T)$$

V. Determining Competition Constants between Fe/Co/Zn substituted Fdm Maquettes (Absorbance)

Consider the following equilibria, where M_A is a metal displaced by another metal, M_B.

$$M_AL + M_B \rightleftharpoons M_BL + M_A \quad \text{(V.1)}$$

The equilibrium competition constant, $K_{comp}^{A/B}$, expressed as an equilibrium formation constant, may be defined as:

$$K_{comp}^{A/B} = [M_BL]*[M_A] / [M_AL]*[M_B] \quad \text{(V.2)}$$

Consideration of mass balance gives the following expressions:

$$L_T = [M_BL] + [M_AL] \quad (V.3)$$
$$M_{AT} = [M_AL] + [M_A] \quad (V.4)$$
$$M_{BT} = [M_BL] + [M_B] \quad (V.5)$$

Where L_T, M_{AT}, and M_{BT} are the total concentrations of all forms of ligand metal A, and metal B in solution, respectively.

Substitution of (V.3)-(V.5) into (V.2), and algebraic rearrangement results in the following expression:

$$A*[M_BL]^2 + B*[M_BL] - K_{comp}^{A/B}*M_{BT}*L_T = 0 \quad (V.6)$$

Where, $A = 1 - K_{comp}^{A/B}$

$B = M_{AT} - L_T + (K_{comp}^{A/B})(L_T) + (K_{comp}^{A/B})(M_{BT})$

Solving the quadratic equation leads to the following expression:

$$[M_BL] = \{-B + \{B^2 + (4*A*K_{comp}^{A/B}*M_{BT}*L_T)\}^{.5}\}/\ 2A \quad (V.7)$$

The total absorbance, Abs, is governed by the sum of an initial absorbance, Abs_o, and the absorbencies of M_AL and M_BL, where "ε_{M_AL}" and "ε_{M_BL}" are the extinction coefficients of M_AL and M_BL, respectively. "b" is the path length of the cuvette.

$$Abs = Abs_o + \varepsilon_{M_AL}*b*[M_AL] + \varepsilon_{M_BL}*b*[\ M_BL] \quad (V.8)$$

Expressing "$[M_AL]$" as "$L_T - [M_BL]$" (V.3), leads to

$$Abs = Abs_o + \varepsilon_{M_AL}*b*[L_T - [M_BL]] + \varepsilon_{M_BL}*b*[M_BL] \quad (V.9)$$

Simplification of (V.9) gives (V.10). Substitution of V.7 into $[M_BL]$ gives (V.11).

$$Abs = Abs_o + (\varepsilon_{M_AL}*b*L_T + (\varepsilon_{M_BL}*b - \varepsilon_{M_AL}*b)*[M_BL]) \quad (V.10)$$

$$Abs = Abs_o + (\varepsilon_{M_AL}*b*L_T + (\varepsilon_{M_BL}*b - \varepsilon_{M_AL}*b)* \{\{-B + \{B^2 + (4*A*K_{comp}^{A/B}*M_{BT}*L_T)\}^{.5}\}/\ 2A\} \quad (V.11)$$

The number of equivalents of metal B added relative to peptide, the independent variable, x, is related to M_{BT} according to:

$$M_{BT} / L_T = x \rightarrow \quad M_{BT} = L_T * x \qquad \text{(V.12)}$$

Substitution of (V.12) into (V.11) gives the final expression used to fit the competition equilibrium:

$$Abs = Abso + (\varepsilon_{M_AL} * b * L_T + (\varepsilon_{M_BL} * b - \varepsilon_{M_AL} * b) * \{\{-B + \{B^2 + (4 * A * K_{comp}^{A/B} * x * L_T^2)\}^{.5}\} / 2A\} \qquad \text{(V.13)}$$

$$A = 1 - K_{comp}^{A/B}$$
$$B = M_{AT} - L_T + (K_{comp}^{A/B})(L_T) + (K_{comp}^{A/B})(x * L_T) \qquad \text{(V.14)}$$

References

A.E Martell and R.M Smith, *Critical Stability Constants*, Vol. 1 (New York: Plenum Press, 1974)