Supporting Information

Reconsidering the *czcD* (NiCo) riboswitch as an iron riboswitch

Jiansong Xu and Joseph A. Cotruvo, Jr.*

Department of Chemistry and Center for RNA Molecular Biology, The Pennsylvania State University, University Park, PA 16802 USA

* Corresponding author. E-mail: juc96@psu.edu (J.A.C.).

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SUPPLEMENTARY TABLES

Table S1. Sequences of the DNA templates used for the riboswitch ITC studies and riboswitch-based sensors. The DNA template sequences for the *Eba*, *Lmo*, and *Cce czcD* riboswitches are derived from Furukawa et al.[1] Spinach2 sequences are shown in red.[2] In the sensor constructs, components of the appropriate truncated stem are shown in blue. Mutations present in the *Eba* variants are highlighted. Px-y denotes insertion of Spinach2 into stem x retaining y base pairs. Sensors **Lmo-1**, **Lmo-2**, and **Lmo-3** are the P1-1, P1-2, and P1-3 constructs.

Name	Sequence
Eba	5'-ccaagTAATACGACTCACTATAgAACTGAGCAGGCAAATGACCAGAGCGGTCATGCAGCCGGGCTGCGAAAGCGGCAACAGATGATTACACGCACATCTGTGGGACAGTT-3'
Eba-CG	5'-ccaagTAATACGACTCACTATAgAACTGAGCACGCAAATGACCAGAGCGGTCATGCAGCGGGCTGCGAAAGCGGCAACAGATGATTACACGCACATCTGTGGGACAGTT-3'
Eba-UA	5'-ccaagTAATACGACTCACTATAgAACTGAGCATGCAAATGACCAGAGCGGTCATGCAGCAGGGCTGCGAAAGCGGCAACAGATGATTACACGCACATCTGTGGGACAGTT-3'
Lmo	5'-ccaagTAATACGACTCACTATAgATCTGAACAGGCGGTGAACGTAACACGAGGTTCATGCAGCTGGGCTGCAATTATTTGCGGCAGCAGACTATGTATTCTAAGGGCATATCTGTGGGACAGTT-3'
Cce	5'-ccaagTAATACGACTCACTATAgAACTGAGCAGGCGATGGACCTTTCATAGAGGTACATGGGGCCGGGCCACCCAGTGAGTGGCAGCAGATTGCAATCATGCACATCTGTGGGACAGTA-3'
Sensei	5'-ccaagTAATACGACTCACTATAGGTGGACGAAGTCGGCAGAGGTCCATTAGTCGGGGCCGTAGTCACGGCAGCGGTTATTTTAGATCCACAACAGCCTATCCC-3'
Spinach2	5'-GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAGTAGGCTGCTTCGGCAGCCTACTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC-3'
Lmo-1	5'-ccaagTAATACGACTCACTATAgGATGTAACTGAATGAAATGGTGAAGGACGGGTCCAgaacaggcggtgaacgtaacacgaggttcatgcagctgggctgcaattatttgcggcagcagactatgtattctaagggcatatctgtgggacTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC-3'
Lmo-2	5'-ccaagTAATACGACTCACTATAgGATGTAACTGAATGAAATGGTGAAGGACGGGTCCAtgaacaggcggtgaacgtaacacgaggttcatgcagctgggctgcaattatttgcggcagcagactatgtattctaagggcatatctgtgggacaTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC-3'
Lmo-3	5'-ccaagTAATACGACTCACTATAgGATGTAACTGAATGAAATGGTGAAGGACGGGTCCActgaacaggcggtgaacgtaacacgaggttcatgcagctgggctgcaattatttgcggcagcagactatgtattctaagggcatatctgtgggacagTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC-3'
Sensei P1-2	5'-ccaagTAATACGACTCACTATAGATGTAACTGAATGAAATGGTGAAGGACGGGTCCAGGACGAAGTCGGCAGAGGTCCATTAGTCGGGGCCGTAGTCACGGCAGCGGTTATTTTAGATCCACAACAGCCTTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC-3'
Sensei P1-3	5'-ccaagTAATACGACTCACTATAGATGTAACTGAATGAAATGGTGAAGGACGGGTCCAtGGACGAAGTCGGCAGAGGTCCATTAGTCGGGGCCGTAGTCACGGCAGCGGTTATTTTAGATCCACAACAGCCTATTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC-3'
Sensei P1-4	5'-ccaagTAATACGACTCACTATAGATGTAACTGAATGAAATGGTGAAGGACGGGTCCAgtGGACGAAGTCGGCAGAGGTCCATTAGTCGGGGCCGTAGTCACGGCAGCGGTTATTTTAGATCCACAACAGCCTATTTGTTGAGTAGAGTGTGAGCTCCGTAACTAGTTACATC-3'

Table S2. Primers used to generate or amplify DNA templates

Name	Sequence
Eba-top	5'-CCAAGTAATACGACTCACTATAGAACTGAGCAGGCAAATGACCAGAGCGGTCATGCAGCCGGGCTGCGAAA-3'
Eba-bottom	5'-AACTGTCCCACAGATGTGCGTGTAATCATCTGTTGCCGCTTTCGCAGCCCGGCTGCATGAC-3'
Eba-CG-top	5'-ccaagTAATACGACTCACTATAgAACTGAGCACGCAAATGACCAGAGCGGTCATGCAGCGGGGCTGCGAAA-3'
Eba-CG-bottom	5'-AACTGTCCCACAGATGTGCGTGTAATCATCTGTTGCCGCTTTCGCAGCCCCGCTGCATGAC-3'
Eba-UA-top	5'-ccaagTAATACGACTCACTATAgAACTGAGCATGCAAATGACCAGAGCGGTCATGCAGCAGGGCTGCGAAA-3'
Eba-UA-bottom	5'-AACTGTCCCACAGATGTGCGTGTAATCATCTGTTGCCGCTTTCGCAGCCCTGCTGCATGAC-3'
Lmo-top	5'-CCAAGTAATACGACTCACTATAGATCTGAACAGGCGGTGAACGTAACACGAGGTTCATGCAGCTGGGCTGCAATT-3'
Lmo-bottom	5'-AACTGTCCCACAGATATGCCCTTAGAATACATAGTCTGCTGCCGCAAATAATTGCAGCCCAGCTGC-3'
Cce-top	5'-CCAAGTAATACGACTCACTATAGAACTGAGCAGGCGATGGACCTTTCATAGAGGTACATGGGGCCGGGCCACCCAGTGAGT-3'
Cce-bottom	5'- TACTGTCCCACAGATGTGCATGATTGCAATCTGCTGCCACTCACTGGGTGGCCC -3'
Sensei-top	5'-CCAAGTAATACGACTCACTATAGGTGGACGAAGTCGGCAGAGGTCCATTAGTCGGGGCCGTAGTCA-3'
Sensei-bottom	5'-GGGATAGGCTGTTGTGGATCTAAAATAACCGCTGCCGTGACTACGGCCCCGACTAATGGA-3'
Lmo_P1-3_fwd	5'-GATGTAACTGAATGAAATGGTGAAGGACGGGTCCACTGAACAGGCGGTGAACG-3'
Lmo_P1-3_rev	5'-GATGTAACTAGTTACGGAGCTCACACTCTACTCAACAACTGTCCCACAGATATGCCCTTAG-3'
Lmo_P1-2_fwd	5'-GATGTAACTGAATGAAATGGTGAAGGACGGGTCCATGAACAGGCGGTGAACG-3'
Lmo_P1-2_rev	5'-GATGTAACTAGTTACGGAGCTCACACTCTACTCAACAATGTCCCACAGATATGCCCTTAG-3'
Lmo_P1-1_fwd	5'-GATGTAACTGAATGAAATGGTGAAGGACGGGTCCAGAACAGGCGGTGAACG-3'
Lmo_P1-1_rev	5'-GATGTAACTAGTTACGGAGCTCACACTCTACTCAACAAGTCCCACAGATATGCCCTTAG-3'
T7_Sp2_Amp_fwd	5'-CCAAGTAATACGACTCACTATAGGATGTAACTG-3'
Sp2_Amp_rev	5'-GATGTAACTAGTTACGGAGCTCACAC-3'

Table S3. Calculated $K_{d,M}$ values (effective K_d values for metal-citrate buffers) used for determination of apparent K_ds of *czcD* sensors. For full details, see the Supporting Information of Xu and Cotruvo.[3]

Chelator	Metal	log K_M	Adjusted $K_{d,M}$
Citric acid	Mn^{II}	4.15	7.30 x 10^{-5}
	Fe^{II}	4.40	4.10 x 10^{-5}
	Co^{II}	5.00	1.05 x 10^{-5}
	Ni^{II}	5.40	4.10 x 10^{-6}
	Zn^{II}	4.98	1.08 x 10^{-6}
	Mg^{II}	3.37	4.60 x 10^{-4}

Table S4. Fluorescence response of **Lmo-2** to first-row transition metal ions (see **Figure S1A**)

	Mn^{II}	Fe^{II}	Co^{II}	Ni^{II}	Zn^{II}
$K_{d,app}$ (nM)	5900 ± 200	270 ± 10	64 ± 2	52 ± 3	1969 ± 60
F_{max}/F_{min}	3.9	4.1	5.1	4.0	4.6
Hill coefficient (*n*)	2.1 ± 0.1	2.9 ± 0.4	1.9 ± 0.1	1.5 ± 0.1	2.2 ± 0.1

Conditions: 30 mM MOPS, 100 mM KCl, 3 mM $MgCl_2$, 1 mM citrate pH 7.2, 20 °C; 100 nM **Lmo-3**, 10 μM DFHBI-1T

Table S5. Fluorescence response of **Lmo-3** to first-row transition metal ions (see **Figure S1B**)

	Mn^{II}	Fe^{II}	Co^{II}	Ni^{II}	Zn^{II}
$K_{d,app}$ (nM)	N.R.[a]	2700 ± 200	820 ± 100	290 ± 30	N.R.
F_{max}/F_{min}	N.D.[b]	12.8	15.2	13.6	N.D.
Hill coefficient (*n*)	N.D.	2.5 ± 0.2	2.8 ± 0.5	2.2 ± 0.3	N.D.

[a] N.R.: No response detected. [b] N.D.: Not determined. Conditions: 30 mM MOPS, 100 mM KCl, 3 mM $MgCl_2$, 1 mM citrate pH 7.2, 20 °C; 100 nM **Lmo-3**, 10 μM DFHBI-1T

SUPPLEMENTARY FIGURES

Figure S1. Citrate-buffered fluorescence titrations of (A) **Lmo-2** and (B) **Lmo-3** with Mn^{II}, Fe^{II}, Co^{II}, Ni^{II}, and Zn^{II}. For each construct, fluorescence response was evaluated by titrating metal into 100 nM sensor and 10 µM DFHBI-1T, in 30 mM MOPS, 100 mM KCl, 3 mM $MgCl_2$, and 1 mM citrate, pH 7.2, at 20 °C. (C) Representation of the sensor constructs, illustrating the junction between the P1 stem and Spinach2, with **Lmo-1** and **Lmo-2** given as examples.

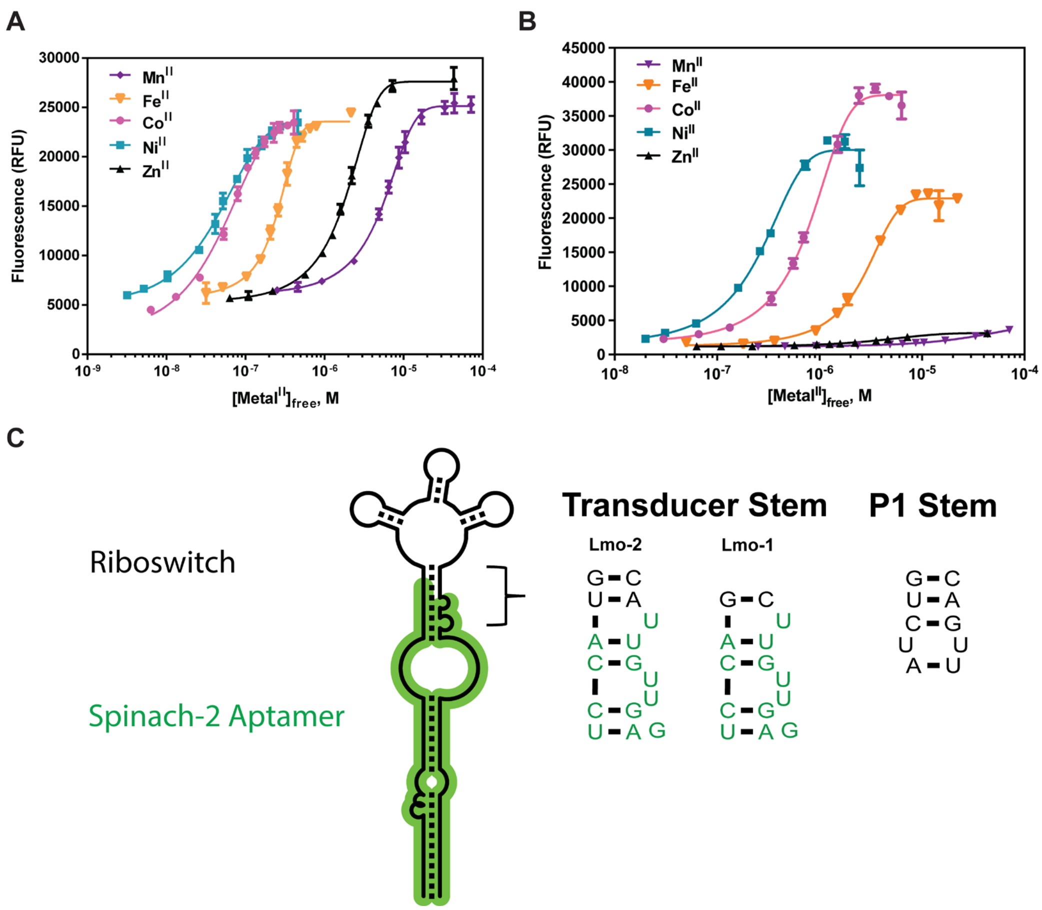


Figure S2. Representative thermograms from ITC studies of the *Eba* riboswitch (9.4-11μM RNA) with Ni^{II}, Zn^{II}, and Mn^{II}. The data are fitted to a model with one set of equivalent binding sites. Experimental conditions and fitted parameters are provided in **Table 2**.

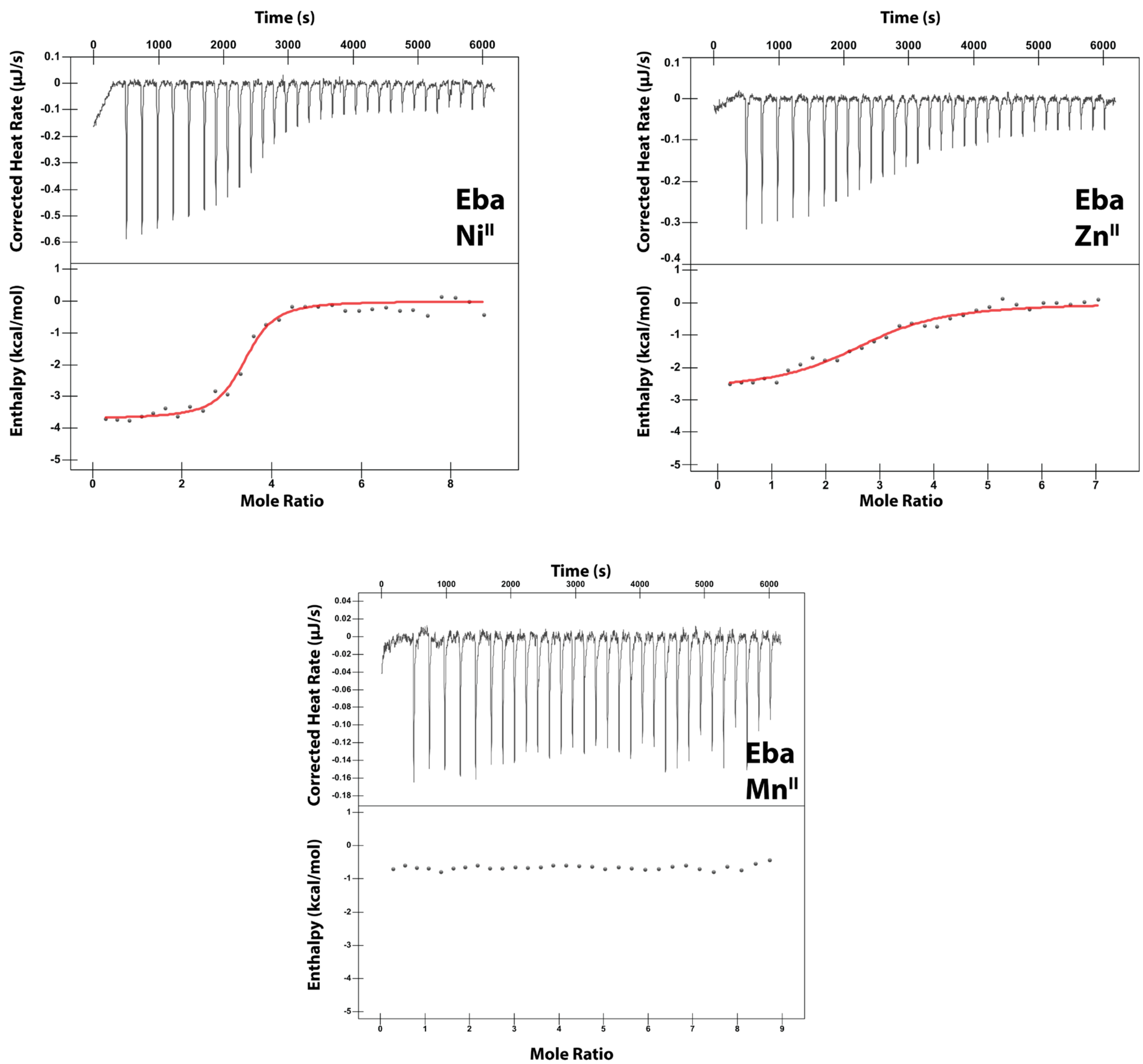


Figure S3. Representative thermogram from ITC studies of the M3 variant of the *Eba* riboswitch (8 µM RNA) with Co^{II}. The data are fitted to a model with one set of equivalent binding sites. Conditions: 30 mM MOPS, 100 mM KCl, 3 mM $MgCl_2$, pH 7.2, 20 °C.

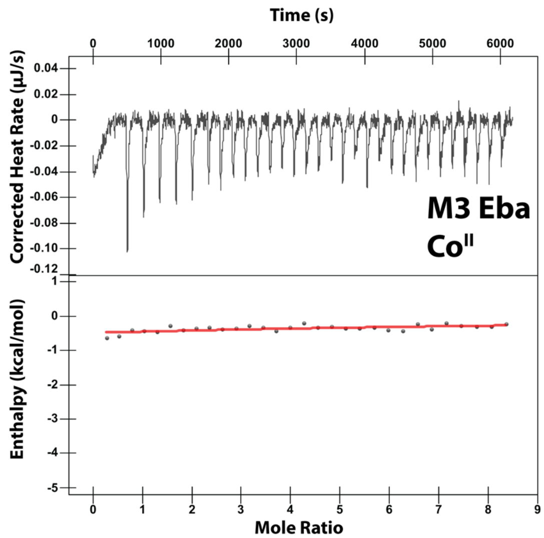


Figure S4. Simulated ITC thermograms for metal ion binding to the *Eba* riboswitch, to estimate $K_{d,app}$ for Mn^{II}-*Eba*. Simulation parameters are based on the experimental parameters for other metal ions (10 µM RNA in cell, 600 µM metal ion in syringe, 30 × 1.2 µL injections, 20 °C) and n = 3 and ΔH = -4.5 kcal/mol. Comparing to the experimental data in **Figure S2**, we suggest a $K_{d,app}$ between 0.1 and 1 mM.

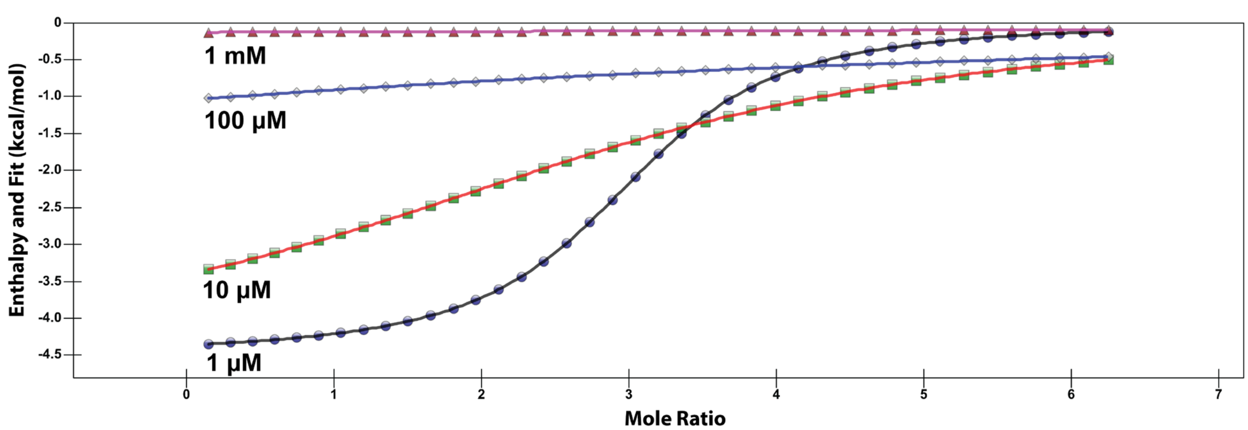


Figure S5. Representative thermograms from ITC studies of the CG and UA variants of the *Eba* riboswitch (8.5-11 μM) with Co^{II}. The data are fitted to a model with one set of equivalent binding sites. Experimental conditions and fitted parameters are provided in **Table 2**.

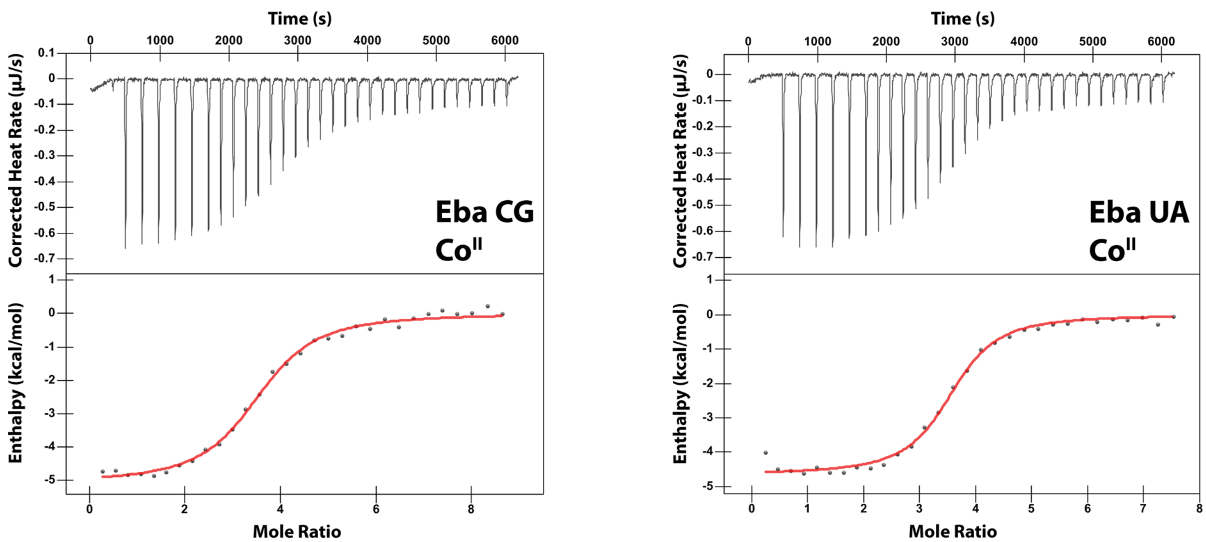


Figure S6. Citrate-buffered fluorescence titrations of (A) Co^{II} and (B) Fe^{II} into Sensei sensors. For each construct, fluorescence response was evaluated by titrating metal into 100 nM sensor and 10 μM DFHBI-1T, in 30 mM MOPS, 100 mM KCl, 3 mM $MgCl_2$, and 1 mM citrate, pH 7.2, at 20 °C. The sensors did not show any fluorescence activation with the metals.

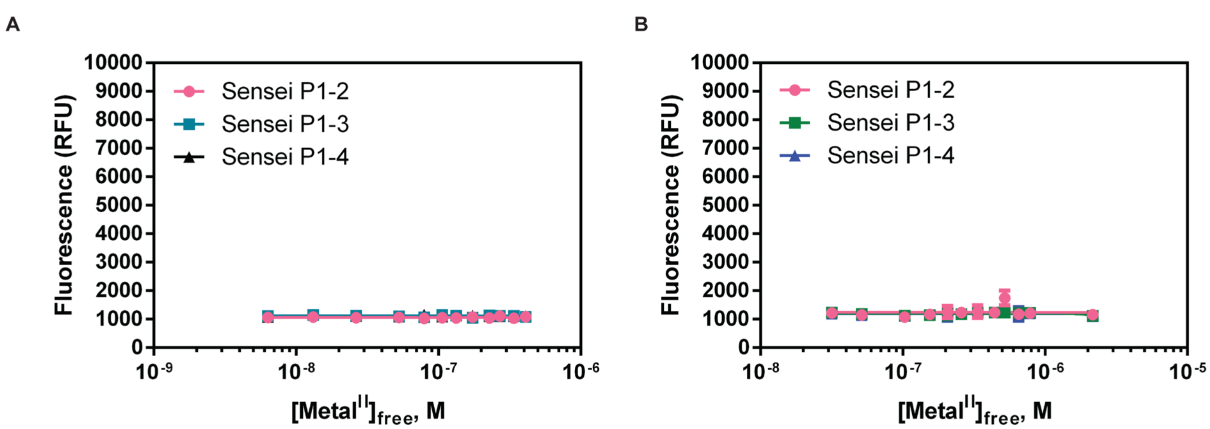
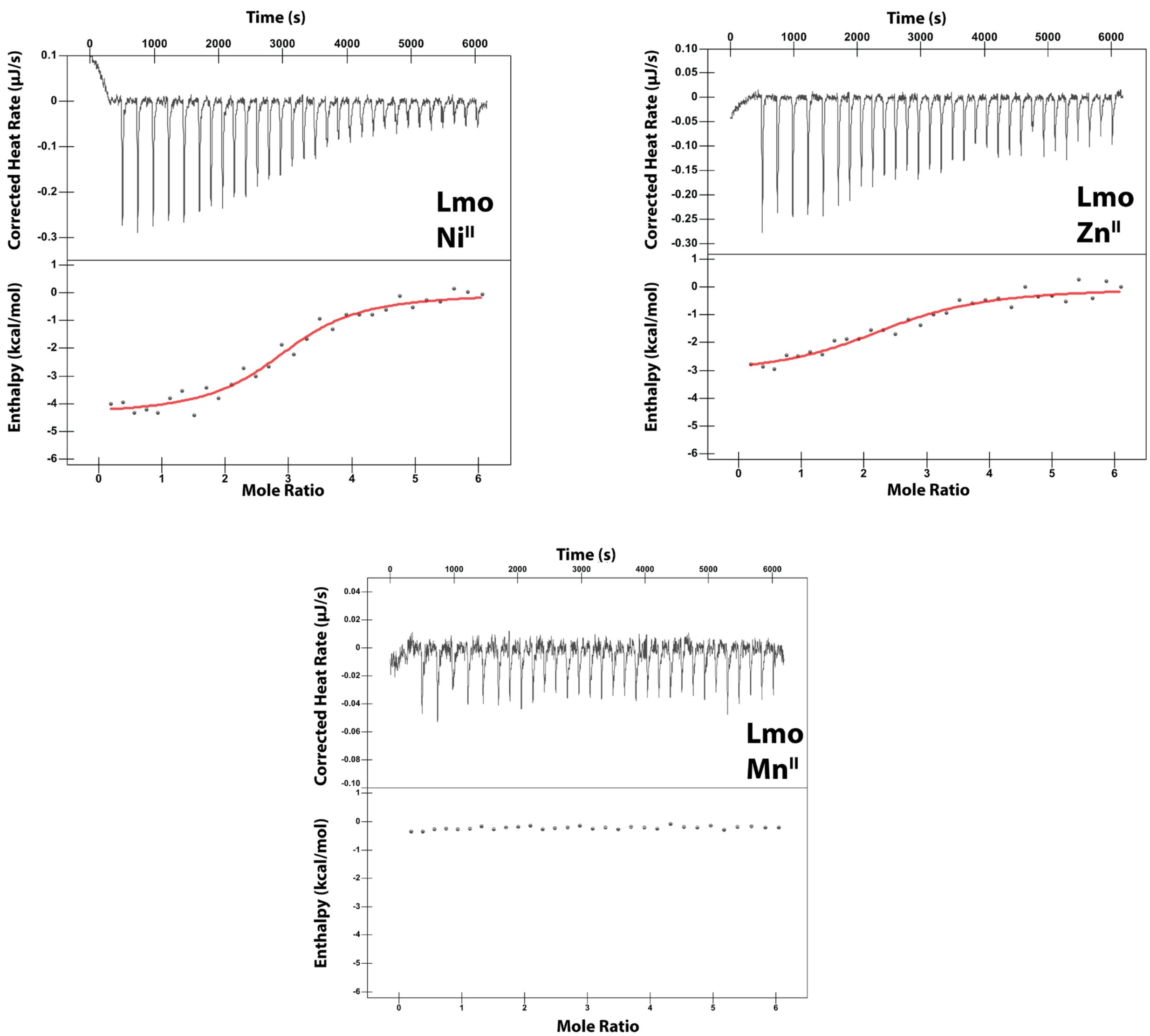


Figure S7. Representative thermograms from ITC studies of the *Lmo* riboswitch (8-10 μM RNA) with Ni^{II}, Zn^{II}, and Mn^{II}. The data are fitted to a model with one set of equivalent binding sites. Experimental conditions and fitted parameters are provided in **Table 2**.



REFERENCES

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2. Strack, R. L.; Disney, M. D.; Jaffrey, S. R., A superfolding Spinach2 reveals the dynamic nature of trinucleotide repeat-containing RNA. *Nat. Methods* **2013,** *10*, 1219-1224.
3. Xu, J.; Cotruvo, J. A. J., The *czcD* (NiCo) riboswitch responds to iron(II). *Biochemistry* **2020,** *59*, 1508-1516.