A role for hydrogen bonding in DNA recognition by the non-classical CCHHC type zinc finger, NZF-1†

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SI Materials and Methods

Design of NZF-1-F2F3 Mutant Peptides

A single point mutation of histidine 515 and/or histidine 559 to glutamine was accomplished using a QuikChange Mutagenesis Kit (Agilent). The wild type (WT) NZF-1-F3F3 DNA corresponding to amino acids 487 – 584 of the full length protein from *Rattus norvegicus* ligated into a pET15b vector in which the hexahistidine tag had been removed, using the restriction sites *Nco*I and *Bam*HI, served as the template for the mutagenesis. The mutant sequences were confirmed via DNA sequencing performed at the Biopolymer/Genomic Core Facility housed at the University of Maryland School of Medicine.

Expression and Purification of NZF-1-F2F3 Mutant Peptides

All peptides were expressed and purified in a similar manner to WT NZF-1-F2F3. Briefly, the pET15b vector containing the gene of interest was transformed into BL21(DE3) *Escherichia coli* (Novagen) cells. Overnight cultures were grown in Luria – Bertani (LB) broth containing 100 µg/mL ampicillin at 37°C. These cells were used to inoculate a 1L culture of LB broth containing 100 µg/mL ampicillin. Cells were grown to mid-log phase at 37°C and expression was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Research Products International Corp). Cells were grown 4 hours post-induction and were harvested by centrifugation for 15 minutes at 7800*g* and 4°C. Cell pellets were resuspended in 25 mM Tris [tris(hydroxymethyl)-aminomethane] at pH 8.0, 100 µM $ZnCl_2$, and 5 mM dithiothreitol (DTT) containing a mini ethylenediaminetetraacetic acid (EDTA) free protease inhibitor tablet (Roche). Cells were then lysed via sonication on a Sonic Dismembrator Model 100 (Fisher) and cell debris was removed by centrifugation at 12,100*g* for 15 minutes at 4°C. The peptide of interest was purified from the bacterial supernatant using a stepwise salt gradient from 25 mM – 1 M sodium chloride on a SP Sepharaose Fast Flow column (Sigma). The cysteine thiols of the peptide were reduced by incubation with 10 mM tris(2-carboxyethyl)phosphine (TCEP) (Thermo) for 30 minutes at room temperature. The solution was then filtered and further purified using High Performance Liquid Chromatograph (HPLC) on a Waters 626 LC system and a Waters Symmetry Prep 300 C18 7 µm reverse phase column. An acetonitrile gradient was used containing 0.1% trifluoroacetic acid (TFA). The peptides eluted at 29% acetonitrile with 0.1% TFA. The purified peptides were lyopholized using a Thermo Savant SpeedVac concentrator housed in a Coy anaerobic chamber (97% N_2/3% H_2). Only peptides judged to be >95% pure via SDS PAGE were used in studies (Fig. S1). The yield of WT, H515Q, H559Q and H515/559Q of NZF-1-F2F3 as well as a single finger peptide, NZF-1-F2, were 2.6 mg/mL, 5.6 mg/mL, 4.7 mg/mL, 5.1 mg/mL, and 21 mg/ml, respectively.

Metal Binding Studies

Metal ion titrations were performed using a PerkinElmer Lambda 25 UV−visible Spectrometer. 20 – 25 µM apo-peptide was titrated with 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 1.9, 2.0, 5.0, 10.0, 15.0, and 20.0 equivalents of $CoCl_2$ (Fisher Scientific) and the appearance of the d-d transitions was monitored. $ZnCl_2$ (Fisher Scientific) was then titrated into the Co(II)-peptide and the disappearance of the d-d transitions was monitored as Zn(II) displaced Co(II) in the peptide. All titrations were performed in 200 mM HEPES, 100 mM NaCl, pH 7.5. A plot of A_{679} versus metal concentration was fit to a 1:1 binding model

using non-linear least squares analysis (KaleidaGraph, Synergy Software) and an upper limit dissociation constant (K_d) for the metals were determined.

(1) $$P + Co \rightleftharpoons PCo$$

(2) $$K_d = \frac{[P][Co]}{[PCo]}$$

(3) $$[PCo] = \frac{[P]_{total} + [Co]_{total} + K_d - \sqrt{([P]_{total} + [Co]_{total} + K_d)^2 - 4[P]_{total}[Co]_{total}}}{2[Co]_{total}}$$

Electron Paramagnetic Resonance (EPR) Spectroscopy

Electron paramagnetic resonance (EPR) spectra were collected on a Bruker EMX EPR Spectrometer controlled with a Bruker ER 041 XG microwave bridge at 12 K. Temperature was maintained with a continuous-flow liquid He cryostat and an ITC503 temperature controller (Oxford Instruments, Inc). The spectrometer parameters were microwave frequency, 9.47 GHz; microwave power, 2 mW; magnetic field amplitude, 10 G. Samples were prepared anaerobically in a Coy inert atmospheric chamber (97% N_2; 3% H_2). 400 – 500 μM Co(II)-NZF-1-F2F3 WT and mutant peptides were prepared in 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 100 mM NaCl, pH 7.5. The final sample was mixed with glycerol (1:1, by volume). Samples were transferred to a Quartz 4 mm OD EPR cell with a rubber septum and the samples were frozen in liquid nitrogen for storage prior to the experiment.

Oligonucleotide Probes

Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA), in the HPLC purified formed with a 5' end labeled fluorescein. The end labeled strand of the β-retinoic acid receptor (βRAR), CACCGAAAGTTCACTC, was purchased along with its anti-sense unlabeled complement. A random DNA strand, TGTTTCTGCCTCTGT, was purchased in a similar matter. The oligonucleotides were annealed by mixing a ratio of 1.25:1 unlabeled:labeled in 10 mM Tris, 10 mM NaCl, pH 8.0. The solution is placed in a water bath set to 10°C higher than the melting temperature of the DNA strands. The annealing reaction was allowed to proceed for 5 minutes prior to the water bath being turned off and subsequently allowed to cool overnight. The resultant double stranded DNA were then quantified and stored at -20°C until use.

Fluorescence Anisotropy (FA)

Fluorescence Anisotropy assays were performed on an ISS PC-1 spectrofluorometer configured in the L format. A wavelength/band pass of 495 nm /2 nm for excitation and 517 nm /1 nm for emission were used. Titrations were performed in 50 mM HEPES, 100 mM NaCl, pH 7.5 in the presence of 0.05 mg/mL of bovine serum albumin (BSA) to prevent adherence of the DNA or peptide to the cuvette walls. All experiments were performed using Spectrosil far-UV quartz window fluorescence cuvettes (Starna Cells). The anisotropy, r, was monitored as peptide was titrated into 10 nM fluorescently labeled DNA. Each data point represents the average of 60 readings taken over the period of 115 seconds. The change in anisotropy was plotted versus peptide concentration and the data was fit to a one-site binding model:

$$P + D \rightleftharpoons PD$$

$$K_d = \frac{[P][D]}{[PD]}$$

$$PD = \frac{P_{total} + D_{total} + K_d - \sqrt{(P_{total} + D_{total} + K_d)^2 - 4P_{total}D_{total}}}{2D_{total}}$$

Generating PyMOL Models

Models of NZF-1-F3, H515Q, and H559Q were generated in PyMOL using the NZF-1-F2 solution structure (PDBID: 1PXE) as a scaffold. The peptide was displayed as a "cartoon" and the metal coordinating side chains along with H515 and Y520 were displayed as "sticks." To create NZF-1-F3, the "mutagenesis" tool was employed and Y520 was chosen as the amino acid to mutate. The most likely backbone dependent rotamer of arginine was chose to replace the tyrosine in order to mimic the wild type NZF-1-F3. H515Q was created in a similar manner, using the mutagenesis tool to mutated H515 into the most likely backbone dependent rotamer of glutamine. The NZF-1-F3 model was used as the scaffold to create the H559Q mutant in which the non-metal coordinating histidine was mutated to the most likely backbone dependent rotamer of glutamine. The distance between two non-hydrogen atoms were measured using the "measurement" tool.

SI Figures

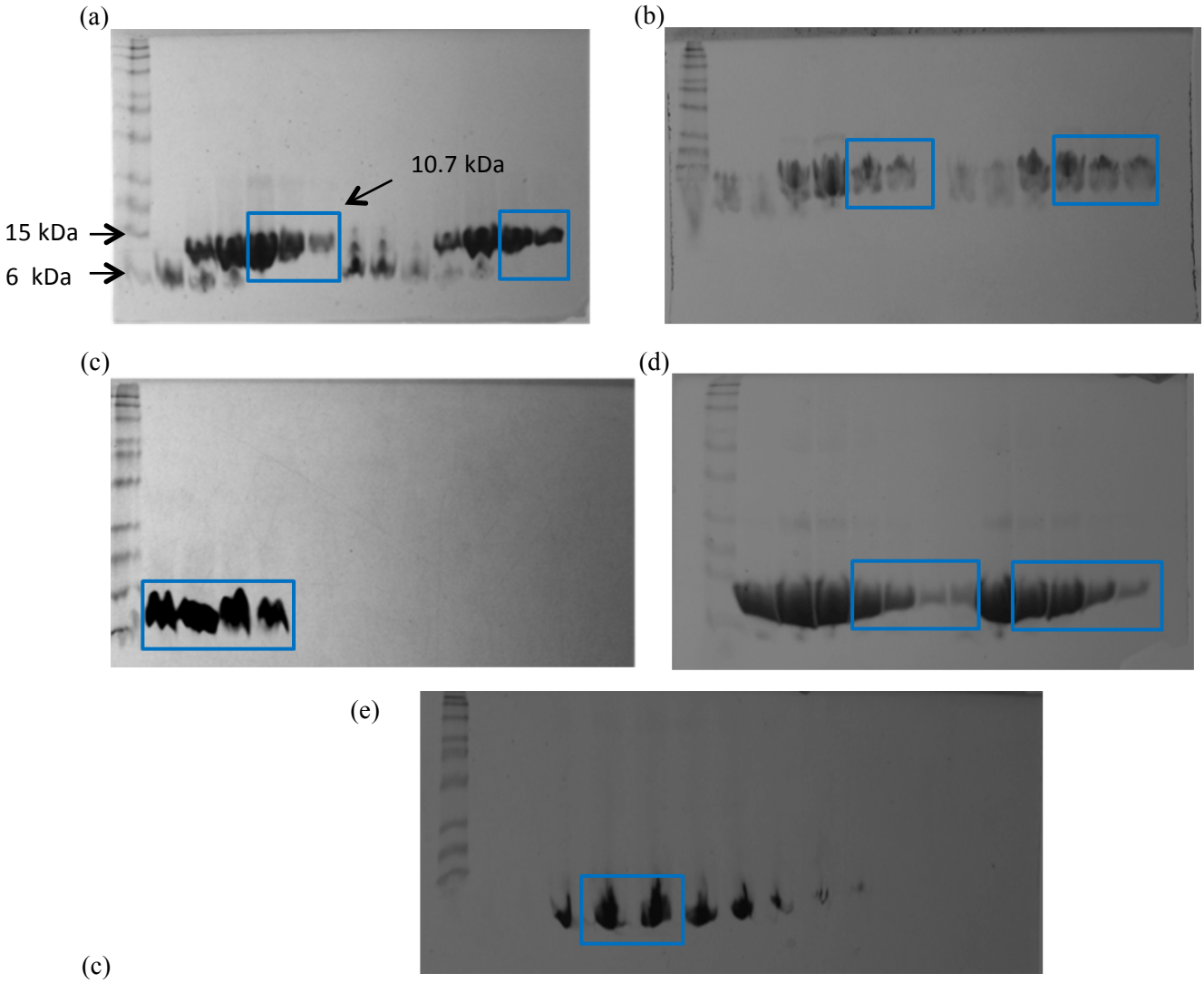

Fig. S1 HPLC purified NZF-1-F2F3. Pure peptide used for studies boxed in blue. (a) WT NZF-1-F2F3 (b) H515Q (c) H559Q (d) H515/559Q (e) NZF-1-F2.

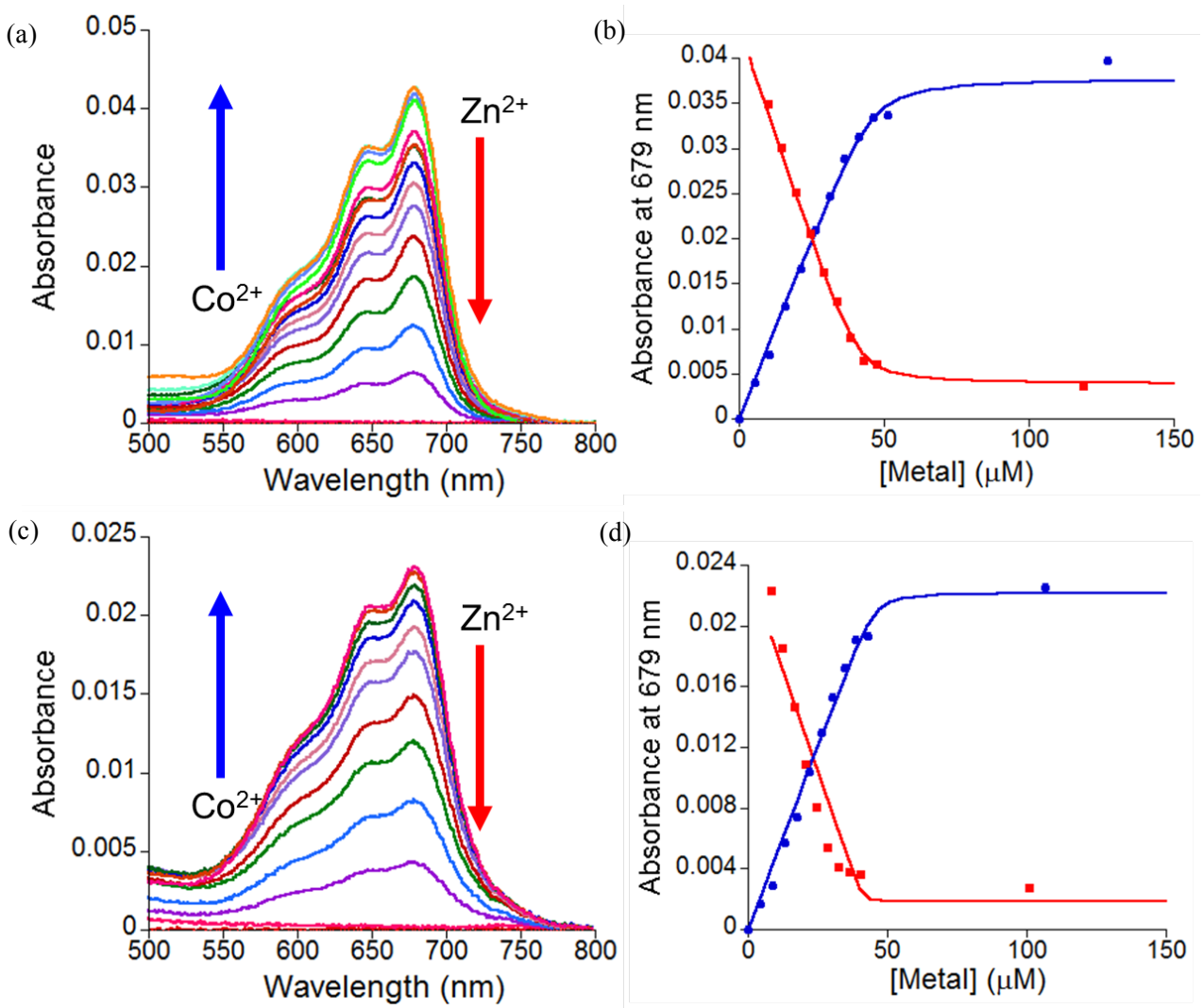

Fig. S2 (a) Plot of the change in the absorption spectrum between 500-800 nm as Co(II) and Zn(II) are titrated with 25 μM H559Q. (b) Plot of the absorbance at 679 nm as a function of added Co(II) (blue) or Zn(II) (red) to H559Q. The solid line represents non-linear least squares fits to 1:1 and competitive binding equilibria, respectively. (c) Plot of the change in the absorption spectrum between 500-800 nm as Co(II) and Zn(II) are titrated with 21 μM H515/559Q. (d) Plot of the absorbance at 679 nm as a function of added Co(II) (blue) or Zn(II) (red) to H515/559Q. The solid line represents non-linear least squares fits to 1:1 and competitive binding equilibria, respectively.

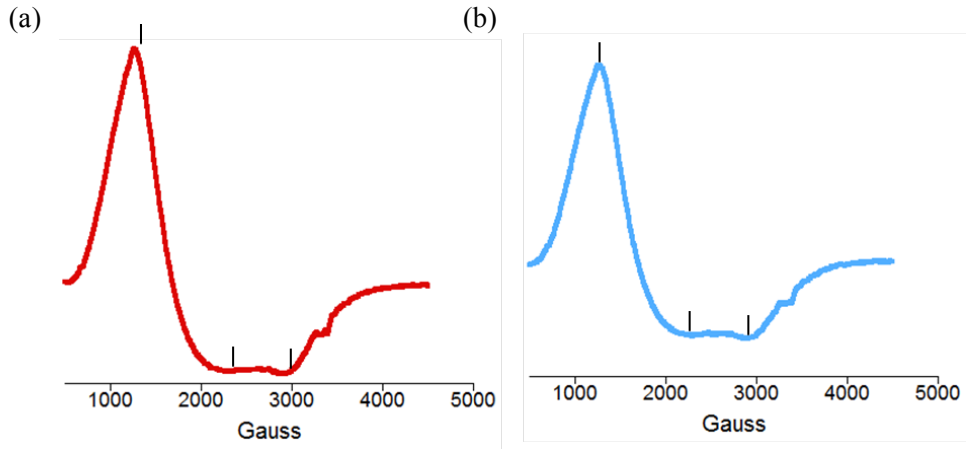

Fig. S3 (a) EPR spectra of Co(II)-H515/559Q. (b) EPR spectra of Co(II)-H523/567F.

Table S1 (a) Upper limit dissociation constants for Co(II) and Zn(II) binding to NZF-1-F2F3 WT and mutant peptides along with their dissociation constants for binding to βRAR.

Peptide	K_d Co(II) (M)	K_d Zn (II) (M)	K_d to βRARE (M)
WT	$7.0 \pm 0.4 \times 10^{-8}$	$1.2 \pm 0.7 \times 10^{-10}$	$1.4 \pm 0.2 \times 10^{-8}$
H515Q	$6.9 \pm 0.3 \times 10^{-7}$	$3.0 \pm 0.9 \times 10^{-9}$	$3.0 \pm 0.2 \times 10^{-6}$
H559Q	$5.2 \pm 1.2 \times 10^{-7}$	$5.1 \pm 2.1 \times 10^{-10}$	$3.7 \pm 0.3 \times 10^{-6}$
H515/559Q	$3.9 \pm 1.0 \times 10^{-7}$	$1.2 \pm 0.6 \times 10^{-11}$	n.b.