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Classical Cys_2His_2 Zinc Finger Peptides Are Rapidly Oxidized by Either H_2O_2 or O_2 Irrespective of Metal Coordination

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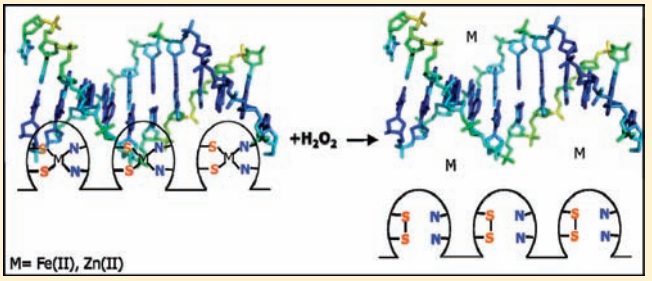
Supporting Information

ABSTRACT: ZIF268, a member of the classical zinc finger protein family, contains three Cys_2His_2 zinc binding domains that together recognize the DNA sequence 5′-AGCGTGGG-CGT-3′. These domains can be fused to an endonuclease to make a chimeric protein to target and cleave specific DNA sequences. A peptide corresponding to these domains, named ZIF268-3D, has been prepared to determine if the zinc finger domain itself can promote DNA cleavage when a redox active metal ion, Fe(II), is coordinated. The UV−vis absorption spectrum of Fe(II)-ZIF268-3D is indicative of Fe(II) coordination. Using fluorescence anisotropy, we demonstrate that Fe(II)-ZIF268-3D binds selectively to its target DNA in the same manner as Zn(II)-ZIF268-3D. In the presence of added oxidant, H_2O_2 or O_2, DNA cleavage is not observed by Fe(II)-ZIF268-3D. Instead, the peptide itself is rapidly oxidized. Similarly, Zn(II)-ZIF268-3D and apo-ZIF268-3D are rapidly oxidized by H_2O_2 or O_2, and we propose that ZIF268-3D is highly susceptible to oxidation.



INTRODUCTION

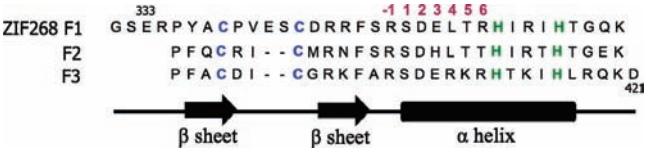
ZIF268 ("zinc interacting factor 268" or Egr1 "early growth response protein 1") is a zinc finger protein that is expressed during neuronal development to control expression of a wide range of genes including those involved in neural cell growth, neurotransmission, and neural differentiation.[1−4] ZIF268 belongs to the class of zinc finger proteins called "classical" zinc finger proteins (ZFs).[5−8] The salient feature of classical ZFs is the presence of modular domains with the sequence X_3-Cys-X_{2-4}-Cys-X_{12}-His-X_{3-4}-His-X_4.[6,9] The cysteine and histidine ligands within these domains coordinate zinc.[9,10] In the absence of zinc, these domains are unfolded and have a random coil conformation. In the presence of zinc, the coordinating ligands bind zinc in a tetrahedral geometry and the domain adopts a $\beta\beta\alpha$ fold (Scheme 1).[11] When folded, each domain can bind to target DNA via the α-helix.[6]

ZIF268 contains three sequential zinc binding domains which together preferentially bind to a target DNA sequence of 5′-AGCGTGGGCGT with each α-helix interacting with the major groove of the DNA (Figure 1).[5] Each ZF participates in three or four sequence specific base contacts utilizing amino acid residues from the α-helix in positions −1, 2, 3, and 6 (numbered with respect to the start of the α helix) (Scheme 1).[5,6] The ZF/DNA binding interaction is facilitated by hydrogen bonds between these amino acid side chains and DNA bases.[5,6,12]

The DNA binding properties of a range of classical ZFs are well understood, such that they have been successfully used for the design of novel sequence specific DNA binding proteins to control transcription.[13−16] Because each ZF functions as an independent module, ZF domains can be used as building blocks for the *de novo* design of peptides that recognize a given DNA sequence.[13,17] As such, classical ZFs have received significant attention as platforms for the development of artificial transcription factors.[13,18,19] One successful application of designed ZFs is in the area of novel zinc finger nucleases.[16] Here, the designed ZF is fused to a cleavage domain (e.g., an endonuclease) forming a chimeric protein. The ZF then binds to its target DNA and the cleavage domain cleaves the DNA as a first step in gene repair.[16,20−22]

Although ZFs preferentially bind zinc, which is thought to be the active metal ion *in vivo*, other metal ions can substitute for zinc.[23−33] One alternate metal ion is iron, which has been shown to bind to the ZF domains of several "nonclassical" ZFs, including tristetraprolin (TTP), neural zinc finger factor-1 (NZF-1), estrogen receptor (ER), and GATA (named for the

Scheme 1



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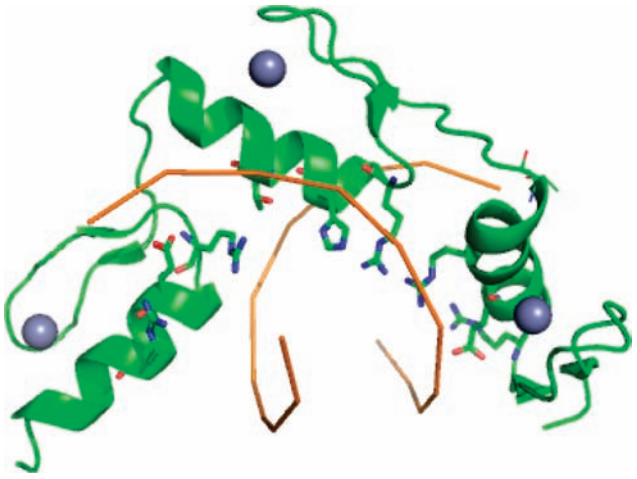

Figure 1. Ribbon diagram of three finger ZIF268, ZIF268-3D, bound to the DNA sequence GCGTGGGCG (PDB: 1AAY). Figure made in PyMol.

DNA consensus sequence "gata" to which it binds) with varying effects on function (i.e., ability to bind to target DNA or RNA).[23−26,34] Because iron is redox active, it has the potential to undergo oxidation chemistry.[35] There are two reports in which the oxidation chemistry of Fe(II)-bound "nonclassical" ZFs has been studied: we showed that Fe(II)-TTP is rapidly oxidized by H_2O_2 and Sarkar and co-workers showed that Fe(II)-ER cleaves target DNA in the presence of H_2O_2.[24,26] Given this precedence for iron substitution and oxidation in ZFs, we reasoned that we could utilize the redox properties of Fe(II)-ZIF268 as the basis for a novel nuclease. This assembly would yield a new platform for a ZF nuclease. Instead of requiring the preparation of a chimeric protein, the ZF itself would have nuclease activity. To test this hypothesis, a peptide composed of the ZF domains from the classical ZF, ZIF268, which we named ZIF268-3D, was prepared and tested for its ability to bind ferrous iron and selectively cleave DNA. As we report here, ZIF268-3D was capable of coordinating Fe(II) and Fe(II)-ZIF268-3D selectively bound to target DNA; however, in the presence of an oxidant (H_2O_2 or O_2), the protein did not cleave its DNA partner. This result led us to hypothesize that the ZF itself, ZIF268-3D, was being oxidized by H_2O_2 or O_2, preventing it from recognizing and oxidizing its partner DNA. We recently reported a novel spectroscopic assay to measure ZF oxidation that utilizes the well-understood Co(II) binding properties of ZFs.[26] This assay had been applied to the nonclassical ZF, TTP, which utilizes a Cys_3His ligand set to bind metal and which folds into a structure made up of loops and is described as "disklike."[36] We used this assay to measure the oxidation of ZIF268 by H_2O_2 as a function of metal coordination. The experiments for ZIF268 were performed under the same conditions as those we reported for TTP; however, we discovered that the rate of oxidation for ZIF268 was more rapid than for TTP.[26] Moreover, we did not observe a metal dependence of oxidation under these conditions. Therefore, we propose that the classical ZF motif, Cys_2His_2, is more sensitive to oxidation than the nonclassical ZF motif, Cys_3His.

EXPERIMENTAL SECTION

General Considerations. Electronic absorption measurements were performed on a Lambda 25 UV−vis spectrophotometer. All experiments were performed using metal free reagents and water that had been purified using a Milli-Q purification system, then passed over Sigma chelex-resin. Upon their preparation, buffers were purged with argon to degas and transferred into a Coy inert atmospheric chamber (97% N_2/3% H_2). The following metal salts or stocks, which were stored anaerobically, were used for metal binding studies: cobalt atomic absorption standard (Aldrich; 17 mM Co^{2+} in 1.0% HNO_3), zinc atomic absorption standard (Aldrich; 15.2 mM Zn^{2+} in 0.9% HCl), and $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ (Aldrich, ACS reagent, 99.9%). The following reagents were used for experiments and physical characterization: sodium ascorbate (≥98%), guanidine hydrochloride (99.9%), acetonitrile (HPLC grade), 2-thibarbituric acid (TBA), and 2-deoxy-D-ribose were from Sigma-Aldrich. Triton X-100 and bovine serum albumin (BSA) were from Bio Rad. T4 polynucleotide kinase from New England Biolabs and 5′[γ-^{32}P]-ATP from PerkinElmer, Inc., were used for labeling DNA. Trifluoroacetic acid (TFA), dithiothreitol (DTT), and H_2O_2 (ACS grade) were from VWR. NaOH (pellets, certified ACS), HCl (37% trace metal grade), and tris(hydroxymethyl)aminomethane (Tris) were from Fisher. Thrombin was from GE Healthcare.

All metal-binding titrations and oxidation studies were performed anaerobically with samples prepared and manipulated in a Coy inert atmospheric chamber. UV−vis measurements were taken using quartz cuvettes with screw caps and Teflon seals from Starna Cells. All titrations were repeated at least three times.

Cloning and Expression ZIF268-3D. A pGEX-2T plasmid containing the DNA sequence that encodes for a peptide that encompasses the 3-zinc fingers of ZIF268-3D (91 amino acids: GSERPYA**C**P-VES**C**DRRFSRSDELTR**H**IRI**H**TGQKPFQ**C**RI**C**MRNFSRSD**H**LT-T**H**IRTHTGEKPFA**C**DI**C**GRKFARSDERKR**H**TKI**H**LRQKD; metal binding ligands are in bold) was a generous gift from Dr. Scot Wolfe (University of Massachusetts Medical School). The pGEX-2T construct was transformed into *E. coli* BL21 (DE3) cells (Novagen) and grown in Luria−Bertani (LB) medium containing 100 μg/mL ampicillin at 37 °C until midlog phase. Typically, cell cultures were grown to 4 h post-induction with 1 mM IPTG before being harvested by centrifugation (7800*g* for 15 min at 4 °C). The cell pellets were resuspended in 30 mL of lysis buffer (1% (v/v) triton X-100, 10 mM DTT, 150 mM NaCl, 50 mM Tris) at pH 7.4 to which one-half of an EDTA-free protease inhibitor mini tablet (Roche) was added to prevent protease activity. Following lysis by French press at 1250 psi using a Thermo Spectronic French pressure cell at 4 °C, the cell debris was removed by centrifugation (12 100*g* for 15 min at 4 °C). The clarified supernatant was loaded onto an activated glutathione (GSH)-agarose column (Sigma) at 4 °C and allowed to equilibrate by shaking for 2 h. GST-ZIF268-3D was eluted from the column (10 mM reduced glutathione in 50 mM Tris-HCl, pH 9.0 at room temperature). The eluate was incubated with 25 units of thrombin (GE Healthcare) for 24 h at room temperature to cleave the GST protein from ZIF268-3D (Figure S5). To unfold the peptide and reduce the cysteine thiols of ZIF268-3D, the peptide was incubated with 30 mL of reduction buffer (6.4 M guanidine hydrochloride, 150 mM DTT, and 50 mM Tris at pH 7.4) for 4 h at 89 °C. The apo-ZIF268-3D was further purified using a nonmetallic HPLC system (Waters 626 LC system) with a Symmetry300-C18 reverse-phase HPLC column. The mobile phase consisted of an acetonitrile/water gradient both containing 0.1% TFA. The protein eluted from the column at 31% acetonitrile. The collected fractions were dried using a Savant SpeedVac concentrator housed in a Coy anaerobic chamber (97% N_2/3% H_2 atmosphere). All further protein manipulations were performed in this atmosphere to prevent cysteine oxidation. The mass of the peptide was confirmed using MALDI-TOF (calculated average mass, 10838.3 Da; detected, 10839.3 Da, $M + H^+$).

Co(II) and Zn(II) Binding. The affinity of ZIF268-3D for Co(II) was measured by monitoring the UV−vis spectrum of a solution of apo-ZIF268-3D (20 μM in 200 mM Tris, 100 mM NaCl, pH 7.4) as cobalt

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was titrated. The data was fit to a 1:1 binding equilibrium using KaleidaGraph software (Synergy software), and an upper limit dissociation constant (K_d) was determined.[25,26] The relative affinity of Zn(II) for the ZIF268-3D peptide was determined by monitoring the displacement of Co(II) by Zn(II).[25,37] The pH of the ZIF268-3D solutions did not change during either titration. All experiments were done in triplicate.

Fe(II) Binding. The UV−vis spectrum of Fe(II)-ZIF268-3D was obtained by adding 3 equiv of Fe(II) (to fill the three Cys_2His_2 domains) to 20 μM ZIF268-3D peptide (in 200 mM Tris, 100 mM NaCl, 10 μM sodium dithionite, pH 7.4). Sodium dithionite was present to ensure that Fe(II) was completely reduced. A complete titration of ZIF268-3D was not performed because, upon addition of >3 equiv of Fe(II), a new band around 500 nm formed. This band is likely attributable to Fe(III)-ZIF268-3D.

Oligonucleotide Probes. The following HPLC-purified oligonucleotides were purchased from Operon Biotechnologies, Inc. (Huntsville, AL) either labeled with fluorescein (**F**) or unlabeled: consensus DNA sequence 5′-AGCAGCTGAGCGTGGGCGT-[**F**]AGTGAGCT-3′ and 5′-AGCTCACTACGCCCACGCTCAGC-TGCT-3′ and nonspecific DNA sequences 5′-AGCAGCTGACAG-TGGGACT[**F**]AGTGAGCT and 5′-AGCTCACTAGTCCCACTG TCAGCTGCT. Upon receipt, the oligonucleotides were resuspended in DNase-free water and quantified. For annealing, each oligonucleotide was mixed such that there was a 1.25:1 ratio of unlabeled to labeled oligonucleotide, in 10 mM Tris, 10 mM NaCl annealing buffer (pH 8.0). The annealing reaction mixtures were placed in a water bath set to a temperature 90 °C. The water bath was then immediately turned off, and the annealing reaction was allowed to cool overnight. Double-stranded oligonucleotides were quantified and stored at −20 °C.

Fluorescence Anisotropy (FA) To Measure DNA Binding. The affinities of Zn(II)-ZIF268-3D, Fe(II)-ZIF268-3D, and apo-ZIF268-3D for a target consensus DNA sequence and a nonspecific DNA sequence were measured under nonoxidizing conditions using fluorescence anisotropy (FA).[38] Studies were performed on an ISS PC-1 spectrofluorometer configured in the L format. Initially, a full excitation/emission spectrum was run to determine the optimum excitation/emission wavelengths for the experiment. The excitation wavelength/band-pass used in the experiments was 497 nm/2 nm, and the emission wavelength/bandpass was 522 nm/1 nm.

In a typical titration, a 5 nM solution of fluorescently labeled-dsDNA in 200 mM Tris, 100 mM NaCl with 0.08 mg/mL bovine serum albumin (BSA) at pH 7.4 was added to a Spectrosil far-UV quartz window fluorescence cuvette (Starna Cells). The BSA was added to prevent the protein from adhering to the cuvette's walls. The anisotropy (r) of the free DNA oligonucleotide was measured. M(II)-ZIF268-3D (M = Fe, Zn and apo) was then titrated into the cuvette from a stock solution (3.0 equiv of M(II), 200 mM Tris, 100 mM NaCl at pH 7.4) in a stepwise fashion, and the resultant change in anisotropy was recorded. For titrations with Fe(II)-ZIF268-3D, 10 μM sodium ascorbate at pH 7.4 was included in the buffer as a reductant. The protein was added until the anisotropy values reached saturation. The data was analyzed by converting the anisotropy (r) to fraction bound (F_{bound}) [the fraction of ZIF-268 bound to DNA at a given DNA concentration], using the equation

$$F_{bound} = \frac{r - r_{free}}{r_{bound} - r_{free}}$$

where r_{free} is the anisotropy of the fluorescein-labeled oligonucleotide and r_{bound} is the anisotropy of the DNA-ZIF268-3D complex at saturation. In all cases, F_{bound} was plotted against the protein concentration and fit using a one-site binding model:

$$P + D \rightleftharpoons PD$$

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

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

Here, P is the protein (ZIF268-3D) concentration and D is the DNA concentration. Each data point from the fluorescence anisotropy assay represents the average of 31 readings taken over a time course of 100 s. Each titration was carried out in triplicate. Over the course of a titration, there was minimal change in the measured total fluorescence intensity ($\leq$3.0%). Therefore, a correction for quantum yield changes was not necessary.

Detection of Hydroxyl Radicals: Deoxyribose Assay. To determine if ZIF268-3D produced hydroxyl radicals in the presence of H_2O_2, the deoxyribose assay was used.[39,40] In this assay, 2-deoxy-D-ribose (5.0 mM, 20 equiv) was added to a solution of Fe(II)-ZIF268-3D (250 μM in 25 mM Tris, 12.5 mM NaCl, pH 7.4) followed by addition of 3 equiv (750 μM) of H_2O_2 to Fe(II)-ZIF268-3D. The solution was then incubated at room temperature for 5 min upon which 25 mM 2-thiobarbituric acid in 0.05 M NaOH (100 equiv) was added followed by an addition of 1 M HCl (30 μL). The tube was heated for 8 min at 100 °C. Hydroxyl radical formation was characterized by the generation of an MDA−TBA (malondialdehyde thiobarbituric acid) adduct which shows a strong visible absorbance at 532 nm.

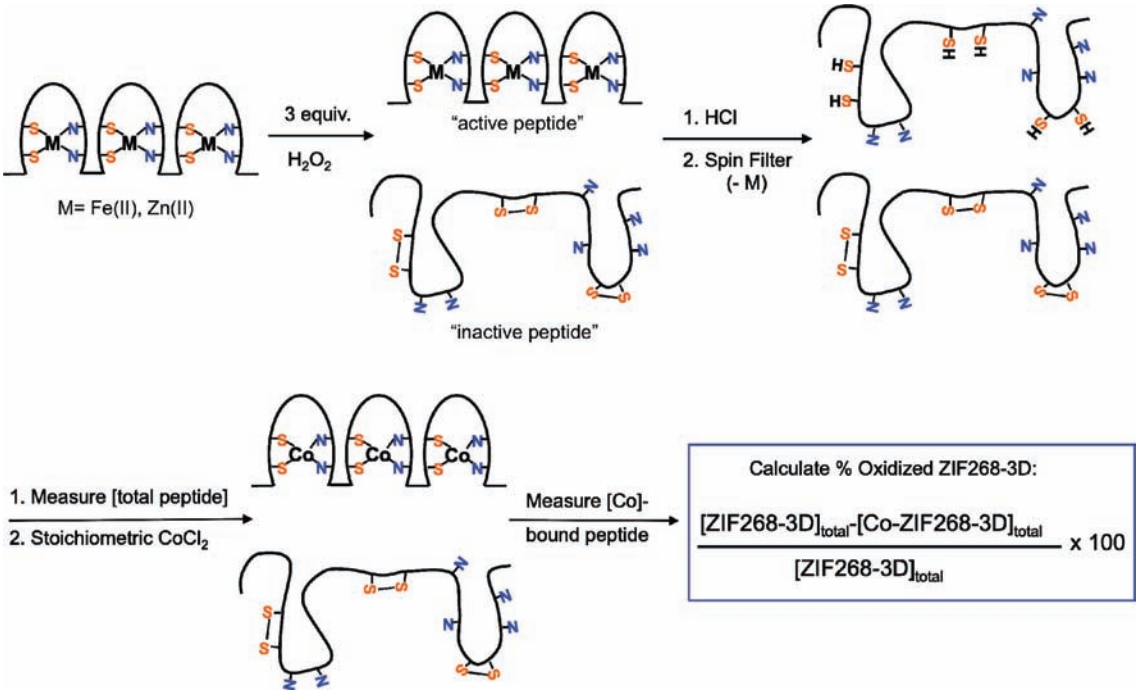
Labeling of DNA for DNA Cleavage Studies. HPLC purified oligonucleotides, 5′-AGCAGCTGAGCGTGGGCGTAGTGAGC T-3′ and 5′-AGCTCACTACGCCCACGCTCAGCTGCT-3′, which correspond to the consensus DNA sequence (without any fluorescent tags), were purchased from Integrated DNA Technologies (IDT) and used without further purification. Oligonucleotides were radiolabeled at their 5′[γ-^{32}P]-ATP with T4 kinase purchased from New England Biolabs Inc. The 5′[^{32}P]-labeled DNA was isolated after passage over a Micro-BioSpin P-6 column (Bio-Rad). 1.0 equiv of the 5′[^{32}P] radiolabeled oligonucleotide and 1.1 equiv of its unlabeled complementary strand were annealed in 10 mM sodium phosphate, pH 7.4 by heating in a 90 °C water bath for 1 min followed by cooling to room temperature over 6 h to afford a 1 μM ^{32}P-labeled duplex DNA.

Maxam−Gilbert A + G Reaction. An A + G sequencing ladder was generated by treating 1 μL of DNA in 10% formic acid (10 μL) at 37 °C for 30 min followed by lyophilization.[41] Piperidine (20% in H_2O) was then added, and the reaction was incubated at 90 °C for 30 min followed by lyophIlization. Water (30 μL) was then added to the tube, and the reaction was lyophilized. This step was repeated three times to remove all traces of residual piperidine.

Reaction with M-ZIF268-3D (M = Fe^{2+}, Zn^{2+} and Apo) and H_2O_2. Zn(II)-ZIF268-3D was prepared by adding stoichiometric Zn-(II) (3 equiv Zn(II)/1 equiv ZIF268-3D peptide) to a 100 nM solution of apo-ZIF268−3D in 10 mM sodium phosphate at pH 7.4. Similarly, Fe(II)-ZIF268-3D was prepared by adding stoichiometric Fe(II) (3 equiv Fe(II)/1 equiv ZIF268-3D peptide) to a 100 nM solution of apo-ZIF268-3D in 10 mM sodium phosphate, 1 mM sodium ascorbate at pH 7.4. Apo-ZIF268-3D was prepared in 10 mM sodium phosphate at pH 7.4. To each peptide, 50 nM ^{32}P radiolabeled dsDNA was added. A 10 mM H_2O_2 solution was then allowed to react for 2 min in a 37 °C water bath before being quenched with 10 mM thiourea.[42] The reaction mixture was lyophilized followed by addition of H_2O (30 μL), and then lyophilized twice again. The reaction products were analyzed as described below in the subsection "Analysis of DNA".

Reaction with Fe(II)-ZIF268-3D and Fe(II)-Bleomycin under O_2. Fe(II)-ZIF268-3D and Fe(II)-bleomycin were prepared by adding stoichiometric Fe(II) to 200 nM solutions of either apo-ZIF268-3D or bleomycin in 10 mM sodium phosphate, 1 mM ascorbate at

Scheme 2



pH 7.4. Radiolabeled dsDNA (50 nM) was then added to each solution. The whole mixture was exposed to O_2 for 3, 10, 30, 120 min in a 37 °C water bath followed by addition of 10 mM thiourea to quench the reaction.[41] The reaction mixture was lyophilized for 2 h followed by addition of H_2O (30 μL), and then lyophilized twice again. The reaction products were analyzed as described in the subsection "Analysis of DNA".

Analysis of DNA. DNA oxidation was analyzed by running a 20% polyacrylamide (19:1 acrylamide/bis-acrylamide) gel under denaturing conditions (7 M urea).[42] The resultant gels were analyzed by phosphor-imaging with a Molecular Dynamics phosphor screen and phosphorimager.

Oxidation Studies of ZIF268-3D: Measurement of "Active" Protein. These experiments follow our previously published protocol.[26] To quantify the amount of "active" or fully reduced ZIF268-3D peptide prior to oxidation studies, a 75 μM solution of the peptide was prepared in 20 mM Tris, 10 mM NaCl at pH 7.4. The total concentration of ZIF268-3D was measured by measuring the absorption at 280 nm and using a calculated extinction coefficient of 2000 M^{-1} cm^{-1}. Co(II) (3 equiv) was then added to ZIF268-3D. The concentration of the reduced protein was determined by measuring the absorbance at 643 nm due to d−d transitions from Co(II) coordination and determining the concentration using the extinction coefficient of 1950 M^{-1} cm^{-1}. Typically, greater than 95% of ZIF268-3D was in its reduced form.

Oxidation Assay for Zn(II)-ZIF268-3D, Fe(II)-ZIF268-3D, and Apo-ZIF268-3D. Oxidation studies were performed following our recently published method as shown in Scheme 2.[26] Solutions of 75 μM Zn(II)-ZIF268-3D, Fe(II)-ZIF268-3D, or apo-ZIF268-3D in 40 mM Tris, 20 mM NaCl at pH 7.4 were allowed to equilibrate for 5 min. H_2O_2 (3 equiv, 250 μM) was then added, and the solution was placed on a shaker at 400 rpm. Each reaction was allowed to proceed for a set time period: 1, 3, 6, 9, 15, 30 min after which the pH of the reaction was reduced to ~2 by addition of 1 M HCl. The addition of acid unfolded the peptide and released the metal ions. The apo peptide was then isolated from the reaction using size exclusion filtration (YM-3 microcon). After lyopholization of the peptide, the concentration of "active" ZIF268-3D peptide was determined by measuring the total ZIF268-3D peptide concentration (absorbance at 280 nm). The amount of reduced ZIF268-3D was quantified by adding stoichiometric cobalt and measuring the concentration of Co(II)-ZIF268-3D. The amount of oxidized ZIF268-3D was calculated using the following equation:

$$\%\ \text{oxidized} = [[ZIF268\text{-}3D_{total}]\text{-}[ZIF268\text{-}3D_{reduced}]]/[ZIF268\text{-}3D_{total}]$$

The average rate of oxidation was determined by dividing the percent of the peptide that was oxidized by the reaction time. A second set of experiments, in which 0.03 equiv of H_2O_2 (2.25 μM) was added to the peptides (at 75 μM concentration), were performed following the same protocol. All experiments were performed in triplicate.

Fluorescence Anisotropy (FA) To Measure DNA Binding with Oxidized ZIF268-3D. The affinities of Zn(II)-ZIF268-3D, Fe(II)-ZIF268-3D, and apo-ZIF268−3D postoxidation (time =3 min, oxidation conditions: 75 μM peptides, 250 μM H_2O_2) for its target consensus DNA sequence and a nonspecific DNA sequence were measured using fluorescence anisotropy (FA) following the procedure described above in the subsection "Fluorescence Anisotropy (FA) To Measure DNA Binding". All experiments were performed in triplicate.

RESULTS AND DISCUSSION

ZIF268 is the prototypical classical zinc finger protein.[8,43] It contains three sequential Cys_2His_2 domains that bind to a specific DNA sequence when zinc is coordinated.[13] ZIF268 has been extensively used in protein design to prepare chimeric proteins with endonucleases.[14−16,19,44−49] The chimeras recognize DNA via the zinc finger and cleave DNA via the endonuclease.[16,22,50,51] On the basis of our recent discovery that a nonclassical ZF, TTP, with Cys_3His domains can bind to ferrous iron and promote oxidation chemistry[26] as well as the work of Sarkar and co-workers that demonstrated iron bound to

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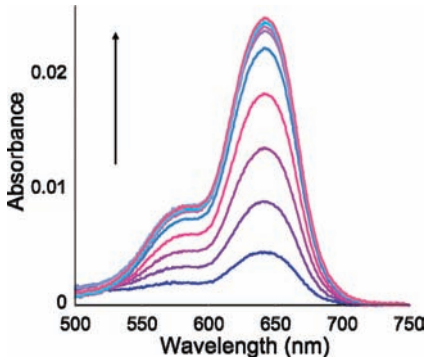

Figure 2. Plot of the change in the absorption spectrum between 500 and 750 nm as apo-ZIF268-3D is titrated with Co(II). All spectrophotometric experiments were performed in a 200 mM Tris, 100 mM NaCl at pH 7.4.

another nonclassical Cys_4 type zinc finger (ER) cleaved DNA,[24] we hypothesized that Fe(II)-ZIF268 would cleave target DNA under oxidizing conditions.

Metal Binding Studies. In order to prepare Fe(II)-ZIF268-3D, we first sought to verify that the peptide bound Zn(II). We measured the affinity of Zn(II) for ZIF268-3D using a competitive titration in which first cobalt was titrated with the protein followed by zinc.[25,52] This is a common approach used to study zinc binding to zinc finger proteins. Zinc has a d^{10} electron configuration making it spectroscopically silent while Co(II) has a d^7 electronic configuration making it spectroscopically active.[37,52−55] A titration with Co(II) results in distinct d−d transitions in the visible region of the optical spectrum that are indicative of tetrahedral geometry which can be fit using an appropriate equilibrium expression.[25,37,56] Following this titration, titration of zinc into the cobalt(II)-ZF complex results in diminution of the Co(II)-ZF spectrum as a result of zinc replacing cobalt. This data can be fit to yield an upper limit dissociation constant (K_d) for zinc.[25,37]

Co(II) Direct Titration. A titration of Co(II) with ZIF268-3D resulted in an absorbance spectrum with peaks centered at 578 and 643 nm which is indicative of tetrahedral coordination (Figure 2).[53] In addition, peaks at 284, 313, and 346 nm which are likely ligand to metal charge transfer (LMCT) bands between cysteine sulfur atoms and cobalt are also observed.[57−60] The shape of the absorbance spectrum matches that reported for Co(II) binding to other classical zinc proteins and peptides such as TFIIIA, CP-1, and Sp1 and is indicative of a Cys_2His_2 site.[52,53,59] The intensity of the d−d transition bands stopped increasing after the addition of 3 equiv of Co(II), indicating that cobalt binds to the three Cys_2His_2 metal-binding domains of ZIF268-3D in a 3:1 cobalt/ZIF268-3D ratio. The shape of the spectrum did not change which implies that Co(II) [and Zn(II), by inference] binds in the same manner to all three sites. This observation allowed us to treat the binding interaction as a 1:1 equilibrium. Using nonlinear least-squares analysis, an upper limit dissociation constant $K_d \geq$ of $3.2 \pm 0.6 \times 10^{-7}$ M for Co(II) binding and an extinction coefficient of 1950 M^{-1} cm^{-1} (650 M^{-1} cm^{-1} per cobalt site) at 643 nm were determined. A plot of concentration of cobalt titrated versus absorbance, fit as described above, is shown in Figure 3. The measured upper limit K_d of 320 nM for cobalt binding to ZIF268 fits the trend of binding affinities reported for other zinc finger proteins.[61]

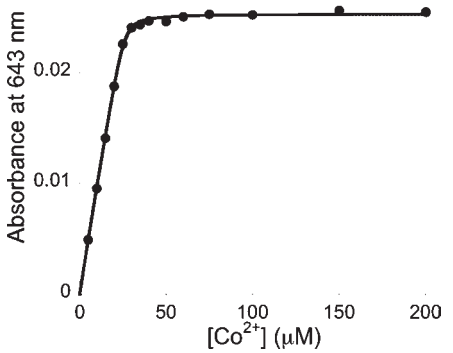

Figure 3. Plot of the absorption spectrum at 643 nm as a function of concentration as Co(II) is added to apo-ZIF268-3D. A solution of 20 μM peptide was used for this experiment, and saturation occurred upon addition of 3 equiv of Co(II). The data was fit to a 1:1 binding equilibrium, and an upper limit dissociation constant K_d of $3.2 \pm 0.6 \times 10^{-7}$ M was determined. The solid lines represent the nonlinear least-squares fit to the 1:1 binding model.

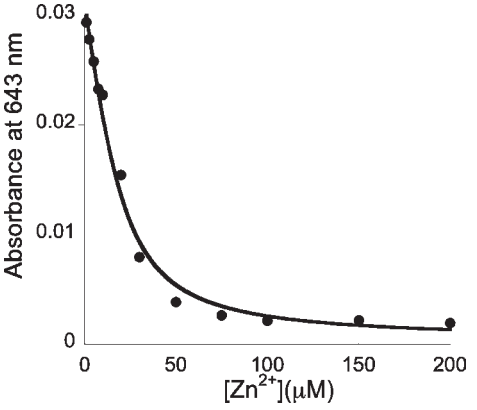

Figure 4. Plot of the absorption spectrum at 643 nm as a function of concentration as Zn(II) is added to to Co(II)-ZIF268-3D. A solution of 20 μM Co(II)-ZIF268-3D was used for this experiment. The data could be fit to a 1:1 binding equilibrium to yield K_d of $3.9 \pm 0.2 \times 10^{-8}$ M.

This is the first direct measurement of the affinity of Co(II) for ZIF268-3D.

Zn(II) Back-Titration. To measure the affinity of zinc for ZIF268-3D, a solution of ZIF268-3D with 100-fold excess Co(II) was titrated with Zn(II).[25,37] The decrease in the Co(II)-ZIF268-3D absorbances was monitored. By plotting [Zn] versus absorbance at 643 nM, the data could be fit to a competitive binding equilibrium to yield an upper limit K_d for the Zn(II)-ZIF268-3D of $3.9 \pm 0.2 \times 10^{-8}$ M (Figure 4). This affinity fits well within the range of affinities reported for zinc binding to other classical zinc fingers (K_d between nanomolar to femtomolar).[61]

UV−Vis Spectrum of Fe(II)-ZIF268-3D. To prepare Fe(II)-ZIF268-3D, 3 equiv of $(NH_4)_2Fe(SO_4)_2$ was added to apo-ZIF268-3D. This resulted in the appearance of bands around 287 and 342 nm (Figure 5). We assigned these bands as charge-transfer bands on the basis of their similarity to bands observed for Fe(II) binding to three other zinc finger peptides, consensus peptide-1 (CP-1), tristetraprolin (TTP), and neural zinc finger factor-1 (NZF-1) (Table 1).[23,25,26,34] In addition, the spectrum is similar to that reported for reduced rubredoxin and peptide

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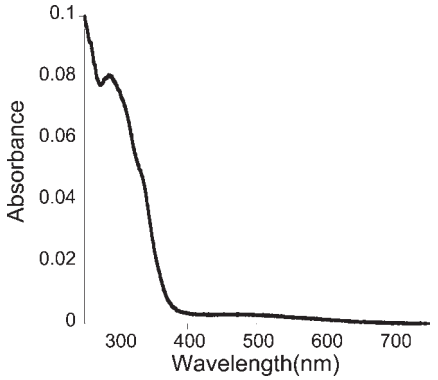

Figure 5. Absorption spectrum of Fe(II)-ZIF268-3D (200 mM Tris, 100 mM NaCl, 10 μM sodium dithionite). The spectrum was generated by subtracting the apo-ZIF268 spectrum from the Fe(II) bound ZIF268-3D spectrum.

Table 1. Optical Spectra for Fe(II)-Peptides

Fe(II)-peptides	absorbance (nm)
ZIF268-3D	342, 280
CP-1	340, 280[34]
TTP-2D	338, 292[25]
NZF-1	247, 312[23]
rubredoxin	338, 311[65,66]
METP	332, 310[62]

models of rubredoxin which utilize four cysteines to bind Fe(II) in a tetrahedral geometry.[62−66]

DNA Binding Studies of Fe(II)-ZIF268-3D and Zn(II)-ZIF268-3D. To determine if Fe(II)-ZIF268-3D binds DNA in the same manner as Zn(II)-ZIF268-3D, a fluorescence anisotropy assay (FA) was utilized.[38] For these studies, the affinity of Fe(II)-ZIF268-3D for a DNA molecule that contained the target sequence **AGCGTGGGCG**T (bases for which a direct amino acid/base interaction occurs are bolded) was measured and compared to the affinity of Zn(II)-ZIF268-3D for the same DNA sequence.[67] The DNA was labeled with fluorescein on a central thymine (5′-AGCAGCTGAGCGTGGGCGT[F]AGTGAGCT-3′) that had been shown by Jantz and Berg to be innocuous for binding of ZIF268-3D derivatives.[68] The FA experiment revealed that Fe(II)-ZIF268-3D bound DNA with an affinity (K_d) of $5.8 \pm 0.4 \times 10^{-9}$ M (Figure 6). In comparison, Zn(II)-ZIF268-3D bound to this same sequence with an affinity (K_d) of $1.7 \pm 0.2 \times 10^{-8}$ M (Figure 7). Thus, Fe(II) substitution for Zn(II) in ZIF268-3D does not significantly affect DNA binding affinity. Apo-ZIF268-3D did not show any affinity for this DNA target sequence as evidenced by no change in anisotropy during early stages of the titration followed by precipitation upon addition of excess apo-ZIF268-3D. Apo-ZIF268-3D is unfolded; therefore, it is not expected to bind to DNA.

Fe(II)-ZIF268 Undergoes Fenton Chemistry with H_2O_2. As a nuclease, Fe(II)-ZIF268-3D needs to generate reactive oxygen species, such as hydroxyl radicals, in order to cleave DNA. Fe(II) is known to undergo Fenton chemistry in the presence of H_2O_2. In addition, the nonclassical ZF peptide, and Fe(II)-TTP has been reported to promote Fenton chemistry.[26] By reacting Fe(II)-ZIF268-3D with H_2O_2 and monitoring hydroxyl radical

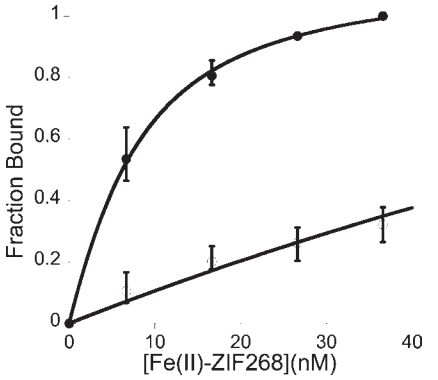

Figure 6. Comparison of the change in anisotropy (as fraction bound) upon the addition of Fe(II)-ZIF268-3D (75 μM) prior to oxidation (●) and Fe(II)-ZIF268-3D upon exposure to H_2O_2 (250 μM) for 3 min (○) to the 5′-AGCAGCTGAGCGTGGGCGT[F]AGTGAGCT-3′ DNA. The solid lines represent the nonlinear least-squares fit to the 1:1 binding model. Fluorescence anisotropy experiments were performed in 200 mM Tris, 100 mM NaCl, 10 μM sodium dithionite at pH 7.4.

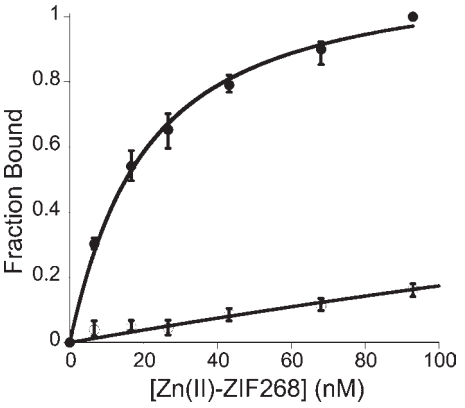

Figure 7. Comparison of the change in anisotropy (as fraction bound) upon the addition of Zn(II)-ZIF268-3D (75 μM) prior to oxidation (●) and Zn(II)-ZIF268-3D upon exposure to H_2O_2 (250 μM) for 3 min (○) to 5′-AGCAGCTGAGCGTGGGCGT[F]AGTGAGCT-3′ DNA. The solid lines represent the nonlinear least-squares fit to the 1:1 binding model. Fluorescence anisotropy experiments were performed in a 200 mM Tris, 100 mM NaCl at pH 7.4.

formation with the deoxyribose assay, we determined that Fe(II)-ZIF268-3D undergoes Fenton chemistry in the presence of H_2O_2 (Figure S1).[39,40] This experiment showed that an Fe(II)-coordinated classical ZF can be oxidized by H_2O_2. This finding is similar to that reported by Sarkar and co-workers who demonstrated that Fe(II)-ER (estrogen receptor, nonclassical zinc finger) generated hydroxyl radicals in the presence of H_2O_2[24] as well as our work on Fe(II)-TTP (tristetraprolin zinc finger, another nonclassical ZF).[26]

DNA Cleavage of M-ZIF268-3D [M = Zn(II) and Fe(II)]. To determine whether the hydroxyl radicals generated when Fe(II)-ZIF268-3D is reacted with H_2O_2 result in DNA cleavage, an experiment in which Fe(II)-ZIF268-3D/DNA(^{32}P) was exposed to H_2O_2 for an indicated period of time, and the ^{32}P labeled DNA was analyzed by gel electrophoresis, (Figure S2). We expected to see specific cleavage near Fe(II)-ZIF268-3D/DNA binding region (5′-GCGTGGGCG) if the hydroxyl radicals were cleaving DNA. However, we were unable to detect any cleavage.

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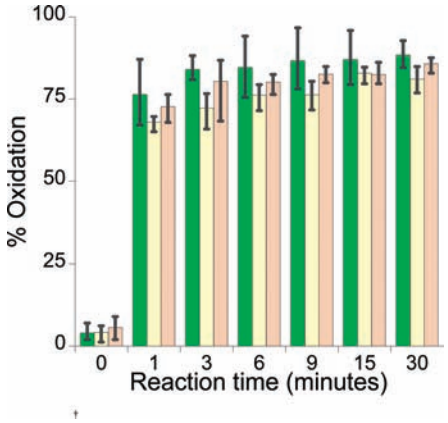

Figure 8. Plot of the percent oxidation of M(II)-ZIF268-3D (M = Zn(II), Fe(II) and apo) as a function of reaction time. The Zn(II) data is colored green, the apo data is colored yellow, and that of the Fe(II) is colored light red.

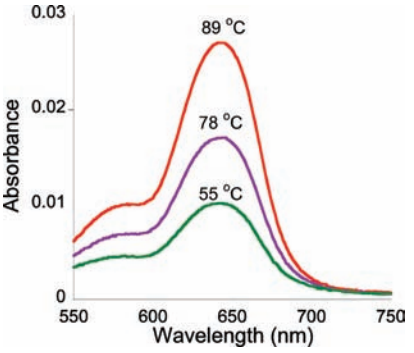

Figure 9. Comparison of the UV−vis spectrum of ZIF268-3D with stoichiometric cobalt added as a function of temperature (55 °C colored green, 78 °C colored purple, and 89 °C colored red) between 550 and 750 nm. All spectrophotometric experiments were performed in a 200 mM Tris, 100 mM NaCl buffer at pH 7.4.

Our observation that Fe(II)-ZIF268-3D/DNA with H_2O_2 does not cleave DNA led us to speculate that the oxidation conditions were too harsh and that the hydroxyl radical was oxidizing the peptide and disrupting the peptide/DNA interaction. To test this hypothesis, we repeated the oxidation experiment with dioxygen (O_2) which is a milder oxidant and generates hydroxyl radicals via the Haber−Weiss reaction.[69] As a control, we used Fe(II)-bleomycin which is well-known to cleave DNA when oxidized by O_2.[70] The results of Fe(II)-ZIF268-3D/DNA with O_2 versus Fe(II)-bleomycin with O_2 are given in Figure S3. Although Fe(II)-bleomycin clearly cleaves DNA when O_2 is added, Fe(II)-ZIF268-3D does not.

Oxidation Studies of M-ZIF268-3D [M = Zn(II), Fe(II)] + H_2O_2. Given that Fe(II)-ZIF268-3D does not cleave DNA even though it generates hydroxyl radicals, we hypothesized that the peptide itself was being oxidized. Such oxidation would disrupt peptide/DNA binding and explain the lack of DNA cleavage that we observed. We sought to identify the rate of protein oxidization to test this hypothesis. Following a protocol that we developed to measure oxidation of Fe(II)-TTP,[26] we measured the rate of oxidation of ZIF268 when either Fe(II) or Zn(II) was bound in the presence of H_2O_2. Scheme 2 outlines the general protocol, which involves reacting the protein with stoichiometric H_2O_2 for specific time periods (1−30 min), isolating the peptide and measuring the amount of reduced peptide that remains after the reaction.[26] Using this assay and the same conditions used for TTP, we discovered that ZIF268 is rapidly oxidized by H_2O_2 when Fe(II) or Zn(II) is bound as well as in the absence of metal (apo-ZIF268-3D) (Figures 8 and S4). Within 3 min, 86% of Zn(II)-ZIF268-3D, 71% of Fe(II)-ZIF268-3D, and 72% of apo-ZIF268-3D are oxidized. This is in striking contrast to TTP, a nonclassical ZF with two Cys_3His zinc binding domains, which is oxidized much more slowly and which shows preferential oxidation of the Fe(II) bound form. For example, after 3 min only 4% of Zn(II)-TTP, 25% of Fe(II)-TTP, and 17% of apo-TTP are oxidized.[26] This rapid oxidation helps explain why ZIF268 does not cleave its DNA binding partner. We speculate that these differences in the rates of oxidation for ZIF268 and TTP are related to their structures. ZIF268 forms a highly structured α-helical and β-sheet fold,[6] whereas TTP does not contain significant α-helical and β-sheet content when folded.[36] It appears that the highly structured, folded form of ZIF268 is more susceptible to oxidation by H_2O_2 than the less structured, folded form of TTP. We then performed the oxidation reactions for ZIF268-3D under milder conditions (0.03 equiv of H_2O_2, instead of 3 equiv) to determine if we could see a metal dependence on oxidation under mild oxidation conditions. As shown in Figure S5, we did see a metal dependence of oxidation under these conditions. After 30 min, 27% of Zn(II)-ZIF268-3D was oxidized compared with 84% of Fe(II)-ZIF268-3D. Thus, the oxidation of ZIF268-3D by H_2O_2 is more rapid for Fe(II)-ZIF268-3D than for Zn(II)-ZIF268-3D under mild oxidation conditions; however, this difference is abrogated under stronger oxidation conditions.

ZIF268-3D Is Oxidized upon Purification. In order to prepare apo-ZIF268-3D for metal binding studies, we had to use much harsher reduction conditions than are typical for preparing apo-zinc finger peptides. We overexpressed ZIF268-3D as a GST fusion, purified it using glutathione agarose, and cleaved the GST from ZIF268-3D using thrombin protease (Figure S6). Typically, zinc finger peptides are isolated in the apo-form by first reducing any possible cysteine disulfide bonds by addition of dithiothreitol (DTT) and heating at 55 °C for 2 h, followed by acidification and purification by reverse phase HPLC.[71] This produces the fully reduced, metal-free (apo) form of the peptide. However, we found that this temperature resulted in incomplete reduction of the ZIF268-3D. As Figure 9 shows, only 44% of ZIF268-3D was reduced under these conditions as measured by the peptide's ability to bind Co(II). Similarly, heating at 78 °C for 2 h results in 61% reduced ZIF268-3D. In order to obtain fully apo-ZIF268-3D, we discovered that addition of 6.4 M guanadinium hydrocholoride to unfold the peptide and 150 mM DTT to reduce cysteine thiols, followed by heating for 4 h at 89 °C, was required. This result is consistent with our observation that ZIF268-3D is more susceptible to oxidation than TTP, which does not require such stringent purification protocols.

Binding Affinities (K_ds) of Oxidized and Reduced M-ZIF268-3D [M = Zn(II), Fe(II)] to DNA. The likely consequence of rapid oxidation of ZIF268-3D is disruption of DNA binding. We measured the affinity of the oxidized peptides for target DNA. After 3 min of oxidation under the conditions of 75 μM peptide and 250 μM H_2O_2, Zn(II)-ZIF268-3D bound to target DNA with a dissociation constant, K_d, of $7.3 \pm 0.8 \times 10^{-7}$ M (Figure 7). Similarly, for Fe(II)-ZIF268-3D, a dissociation constant of $K_d = 2.2 \pm 0.3 \times 10^{-7}$ M (Figure 6) was measured for oxidized peptide.

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Therefore, oxidation causes ZIF268-3D to bind to its target DNA with weaker affinity confirming that oxidation disrupts DNA binding.

CONCLUSIONS

The impetus for the studies reported here was to develop a novel nuclease based on the ZIF268 protein by coordinating Fe(II) to the protein and using oxidation chemistry to cleave DNA. Instead, we discovered that ZIF268 is easily oxidized, irrespective of metal coordination. This observation suggests that Fenton chemistry is not the only oxidation reaction that occurs for Fe(II)-ZIF268-3D in the presence of H_2O_2. There is precedent for oxidation of zinc fingers occurring directly at the thiol sulfurs that coordinate the metal ions, and this has been proposed to be a general mechanism for regulating zinc finger function *in vivo*.[72−74] We speculate that this type of oxidation is operative for ZIF268. Although ZIF268 does not appear to be a suitable platform to develop redox-active nucleases, other types of zinc finger proteins may be potential alternatives.

ASSOCIATED CONTENT

Supporting Information. Deoxyribose assay, DNA cleavage gels, co-oxidation assays, and SDS PAGE of ZIF268-3D. This material is available free of charge via the Internet at http://pubs.acs.org.

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