Inorganic Chemistry
Article

Inorg. Chem. **2010**, *49*, 1211–1219 **1211**
DOI: 10.1021/ic9024298

Cysteine Oxidation Enhanced by Iron in Tristetraprolin, A Zinc Finger Peptide

Seung Jae Lee and Sarah L. J. Michel*

Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Maryland 21201-1180

Received December 7, 2009

Tristetraprolin (TTP or NUP475) is a non-classical zinc finger protein that is involved in inflammatory response. TTP regulates the production of cytokines by binding to specific mRNA sequences. TTP contains two Cys_3His metal binding domains that can coordinate zinc, cobalt, ferric and ferrous iron. When zinc, cobalt, ferric or ferrous iron are bound, TTP peptides can bind to their cognate RNA. During inflammation there are increased levels of reactive oxygen species and iron. It has been proposed that reactive oxygen species may play a role in regulating zinc finger protein function by oxidizing cysteine thiolates that bind zinc and inactivating the protein. To elucidate the effect of the reactive oxygen species H_2O_2 on the integrity of TTP and its ability to bind to target RNA, a simple and rapid assay using cobalt as a spectroscopic probe for zinc was developed. The oxidative susceptibility of peptides consisting of the zinc binding domains of a single zinc finger domain of TTP, TTP-D1 and the tandem zinc finger domains of TTP, TTP-2D was measured. Fe(II)-TTP-D1 and Fe(II)-TTP-2D were more rapidly oxidized by H_2O_2 than their Zn(II) bound counterparts. Electron paramagnetic resonance (EPR) spin trapping using 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide (EMPO) demonstrated that oxidation of ferrous iron substituted TTP-D1 and TTP-2D resulted in the formation of hydroxyl radicals via Fenton chemistry. The oxidized peptides exhibited a diminished affinity for target RNA compared to their unoxidized counterparts suggesting that oxidation of TTP inactivates the protein.

Introduction

Tristetraprolin (TTP), also known as NUP475 and Tis11, is a cytoplasmic metalloprotein that is involved in inflammatory response.[1] TTP regulates the stability of cytokines which are proteins that are expressed during inflammation by binding to adenine and uracil-rich (AU-rich) sequence elements that are present on the 3′ untranslated region (3′-UTR) of cytokine mRNA (mRNA).[1−3] When TTP binds to cytokine mRNA it forms an unstable complex that is degraded thereby attenuating the inflammatory response.[4−10] Tumor Necrosis Factor Alpha (TNFα) was the first cytokine identified to be regulated by TTP.[6] Numerous additional cytokines have since been shown to be under the control of TTP.[10] As such, TTP is emerging as a general regulator of inflammation.

TTP belongs to the class of proteins commonly referred to as zinc finger proteins.[11−18] These proteins utilize a combination of cysteine and histidine ligands to bind zinc and fold into the correct three-dimensional structure for function.[14,15,19] Peptides corresponding to the zinc binding domains can typically bind zinc and fold independently of the entire protein,[15] and these properties have been exploited to address fundamental questions regarding peptide and protein folding.[20] TTP is

*To whom correspondence should be addressed. E-mail: smichel@rx.umaryland.edu. Phone: (410) 706-7038. Fax: (410) 706-5017.

(1) Blackshear, P. J. *Biochem. Soc. Trans.* **2002**, *30*(Pt 6), 945–952.
(2) Sandler, H.; Stoecklin, G. *Biochem. Soc. Trans.* **2008**, *36*(Pt 3), 491–496.
(3) Cao, H. *Biochemistry* **2004**, *43*(43), 13724–38.
(4) Chen, C. Y.; Gherzi, R.; Ong, S. E.; Chan, E. L.; Raijmakers, R.; Pruijn, G. J.; Stoecklin, G.; Moroni, C.; Mann, M.; Karin, M. *Cell* **2001**, *107*(4), 451–464.
(5) Deleault, K. M.; Skinner, S. J.; Brooks, S. A. *Mol. Immunol.* **2008**, *45*(1), 13–24.
(6) Carballo, E.; Lai, W. S.; Blackshear, P. J. *Science* **1998**, *281*(5379), 1001–1005.
(7) Lai, W. S.; Blackshear, P. J. *J. Biol. Chem.* **2001**, *276*(25), 23144–23154.
(8) Lai, W. S.; Carballo, E.; Strum, J. R.; Kennington, E. A.; Phillips, R. S.; Blackshear, P. J. *Mol. Cell. Biol.* **1999**, *19*(6), 4311–4323.
(9) Lai, W. S.; Carballo, E.; Thorn, J. M.; Kennington, E. A.; Blackshear, P. J. *J. Biol. Chem.* **2000**, *275*(23), 17827–17837.
(10) Lai, W. S.; Parker, J. S.; Grissom, S. F.; Stumpo, D. J.; Blackshear, P. J. *Mol. Cell. Biol.* **2006**, *26*(24), 9196–9208.
(11) Anzellotti, A. I.; Farrell, N. P. *Chem. Soc.Rev.* **2008**, *37*(8), 1629–1651.
(12) Gamsjaeger, R.; Liew, C. K.; Loughlin, F. E.; Crossley, M.; Mackay, J. P. *Trends Biochem. Sci.* **2007**, *32*(2), 63–70.
(13) Maret, W. *J. Trace Elem. Med. Biol.* **2005**, *19*(1), 7–12.
(14) Jantz, D.; Amann, B. T.; Gatto, G. J., Jr.; Berg, J. M. *Chem. Rev.* **2004**, *104*(2), 789–799.
(15) Berg, J. M.; Godwin, H. A. *L. Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 357–371.
(16) Berg, J. M.; Shi, Y. *Science* **1996**, *271*(5252), 1081–1085.
(17) Klug, A.; Rhodes, D. *Cold Spring Harbor Symp. Quant. Biol.* **1987**, *52*, 473–482.
(18) Wolfe, S. A.; Nekludova, L.; Pabo, C. O. *Annu. Rev. Biophys. Biomol. Struct.* **2000**, *29*, 183–212.
(19) Giedroc, D. P.; Chen, X.; Apuy, J. L. *Antioxid. Redox Signaling* **2001**, *3*(4), 577–596.
(20) Reddi, A. R.; Guzman, T. R.; Breece, R. M.; Tierney, D. L.; Gibney, B. R. *J. Am. Chem. Soc.* **2007**, *129*(42), 12815–12827.

Published on Web 01/06/2010

a 34 kDa protein and contains two Cys_3His (or CCCH) zinc binding domains that must be metal bound and folded to bind to target mRNA sequences.[7−9,21−23] Zinc(II), cobalt(II), iron-(II), and iron(III) have all been shown to induce folding and RNA binding for a peptide (called TTP-2D) that is made up of the two zinc binding domains of TTP, suggesting that metal use by TTP is flexible.[21,23−26]

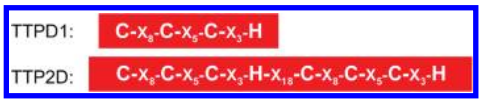
Zinc binds to zinc finger proteins with high affinity—nanomolar to femtomolar dissociation constants (K_d) for zinc have been reported.[27,28] Thus, once zinc has bound to a zinc finger protein it is difficult to remove, bringing up the question of how zinc fingers are inactivated in the cell. Oxidation of zinc finger proteins by reactive oxygen species in the cell has been proposed as a mechanism for controlling zinc finger protein activity.[29−33] Although zinc itself is redox inactive, coordinated sulfur atoms from cysteines are susceptible to oxidation.[32] Cysteine sulfurs can be oxidized to form disulfide bonds and higher order oxidized species (e.g., sulfenic acid, sulfinic acid, sulfonic acid, etc.).[34] Oxidation of cysteine residues results in zinc ion ejection causing the protein to lose function (e.g., ability to bind to DNA and regulate transcription).[29,32] The concept of zinc protein function being controlled by the redox state of the cell has been linked to a role of zinc finger sites in cellular signaling cascades. For example, the P53 tumor suppressor zinc finger protein senses stress as a result of redox change,[35] the RING zinc finger protein releases zinc in the presence of the reactive oxygen species, hydrogen peroxide, which then signals mitotic cell cycling,[36] and the zinc finger region of protein kinase C has been shown to be activated by oxidation.[37] Additionally, the ability of zinc fingers to be inactivated as a result of cysteine oxidation is being exploited for the design of novel drugs that target zinc fingers, for example, electrophilic molecules that oxidize specific cysteine residues that bind zinc in the HIV-nucleocapsid protein have been developed as anti-HIV agents.[38−41]

The molecular details of zinc finger oxidation for most types of zinc finger proteins are generally unresolved; however, there are a few reports that have successfully utilized mass spectrometry to characterize the oxidation state of cysteine sulfurs in zinc finger proteins both pre- and post-oxidation. For example, Baldwin and co-workers have identified oxidation sites on the Estrogen Receptor zinc finger protein using an alkylation/ESI-MS and an isotopic labeling/protease digestion/MALDI-MS approach.[42−44] Wilcox and co-workers have used an HPLC/MALDI-MS approach to identify oxidation products for the SP-1 zinc finger protein[34] while Hanas and co-workers have used an ESI-MS/Isotope Cluster Analysis approach.[30] Additionally, ESI-MS has also been used to quantify the number of protons associated with the zinc bound form of the HIV-nucleocapsid protein by correlating the measured molecular weight with the protein sequence,[45,46] although it has since been suggested that the ESI-MS technique may affect the protonation state of the cysteine sulfur atoms causing inaccurate measures of the cysteine oxidation states in some instances.[47] To perform mass spectrometry on an oxidized zinc finger peptide or proteins, chromatographic separation of the oxidized zinc finger followed by partial proteolysis of the oxidized zinc finger is typically required. Additionally, because oxidation of a cysteine thiol to a disulfide changes the mass by two units, it is sometimes difficult to discern whether a measured mass change of 2 is due to disulfide bond formation or just double protonation of the peptide/protein as a result of the experimental conditions. To overcome the difficulties inherent in accurately assigning a mass charge of 2 to a disulfide bond, alkylation or arylation of the remaining unoxidized thiol sulfur atoms can be performed so that a larger mass change due to oxidation is observed.[48] A drawback to this strategy; however, is that one does not always produce a complete alkylation/arylation reaction, and thus the strategy requires another purification step to separate fully alkylated/arylated products from partially alkylated/arylated products. Thus, mass spectrometry is a powerful tool to measure oxidation of zinc finger proteins with some limitations.

Another traditional approach that has widely been used to measure oxidation of cysteine thiols is via a DTNB assay or Ellman's reagent (5, 5′-dithiobis (2-nitrobenzoic acid)).[49−51]

(21) Worthington, M. T.; Amann, B. T.; Nathans, D.; Berg, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*(24), 13754–13759.
(22) Brewer, B. Y.; Malicka, J.; Blackshear, P. J.; Wilson, G. M. *J. Biol. Chem.* **2004**, *279*(27), 27870–27877.
(23) diTargiani, R. C.; Lee, S. J.; Wassink, S.; Michel, S. L. *Biochemistry* **2006**, *45*(45), 13641–13649.
(24) Michel, S. L.; Guerrerio, A. L.; Berg, J. M. *Biochemistry* **2003**, *42*(16), 4626–4630.
(25) Hudson, B. P.; Martinez-Yamout, M. A.; Dyson, H. J.; Wright, P. E. *Nat. Struct. Mol. Biol.* **2004**, *11*(3), 257–264.
(26) Lee, S. J.; Michel, S. L. J., unpublished results.
(27) Ghering, A. B.; Shokes, J. E.; Scott, R. A.; Omichinski, J. G.; Godwin, H. A. *Biochemistry* **2004**, *43*(26), 8346–8355.
(28) Petros, A. K.; Reddi, A. R.; Kennedy, M. L.; Hyslop, A. G.; Gibney, B. R. *Inorg. Chem.* **2006**, *45*(25), 9941–9958.
(29) Maret, W. *Antioxid. Redox. Signaling* **2006**, *8*(9&10), 1419–1441.
(30) Larabee, J. L.; Hocker, J. R.; Hanas, J. S. *Arch. Biochem. Biophys.* **2005**, *434*(1), 139–149.
(31) Wu, X.; Bishopric, N. H.; Discher, D. J.; Murphy, B. J.; Webster, K. A. *Mol. Cell. Biol.* **1996**, *16*(3), 1035–1046.
(32) Kroncke, K.; Klotz, L. *Antioxid. Redox. Signaling* **2009**, *11*(5), 1015–1027.
(33) Webster, K. A.; Prentice, H.; Bishopric, N. H. *Antioxid. Redox. Signaling* **2001**, *3*(4), 535–548.
(34) Xu, Y.; Wilcox, D. E. *J. Am. Chem. Soc.* **1998**, *120*, 7375–7576.
(35) Hainaut, P.; Mann, K. *Antioxid. Redox. Signaling* **2001**, *3*, 611–623.
(36) Chang, T. S.; Jeong, W. J.; Lee, D. Y.; Choo, C. S.; Rhee, S. G. *Free Radical Biol. Med.* **2004**, *37*, 521–530.
(37) Golapakrishna, R.; Anderson, W. B. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6758–6762.
(38) Schito, M. L.; Goel, A.; Song, Y.; Inman, J. K.; Fattah, R. J.; Rice, W. G.; Turpin, J. A.; Sher, A.; Appella, E. *AIDS Res. Hum. Retroviruses* **2003**, *19*(2), 91–101.
(39) Turpin, J. A.; Song, Y.; Inman, J. K.; Huang, M.; Wallqvist, A.; Maynard, A.; Covell, D. G.; Rice, W. G.; Appella, E. *J. Med. Chem.* **1999**, *42*(1), 67–86.
(40) Jenkins, L. M.; Byrd, J. C.; Hara, T.; Srivastava, P.; Mazur, S. J.; Stahl, S. J.; Inman, J. K.; Appella, E.; Omichinski, J. G.; Legault, P. *J. Med. Chem.* **2005**, *48*(8), 2847–2858.
(41) Jenkins, L. M.; Durell, S. R.; Maynard, A. T.; Stahl, S. J.; Inman, J. K.; Appella, E.; Legault, P.; Omichinski, J. G. *J. Am. Chem. Soc.* **2006**, *128*(36), 11964–11976.
(42) Atsriku, C.; Benz, C. C.; Scott, G. K.; Gibson, B. W.; Baldwin, M. A. *Anal. Chem.* **2007**, *79*(8), 3083–3090.
(43) Atsriku, C.; Scott, G. K.; Benz, C. C.; Baldwin, M. A. *J. Am. Soc. Mass. Spectrom.* **2005**, *16*(12), 2017–2026.
(44) Whittal, R. M.; Benz, C. C.; Scott, G.; Semyonov, J.; Burlingame, A. L.; Baldwin, M. A. *Biochemistry* **2000**, *39*(29), 8406–8417.
(45) Fabris, D.; Hathout, Y.; Fenselau, C. *Inorg. Chem.* **1999**, *38*(6), 1322–1325.
(46) Fabris, D.; Zaia, J.; Hathout, Y.; Fenselau, C. *J Am. Chem. Soc.* **1996**, *118*, 12242–12243.
(47) Magyar, J. S.; Godwin, H. A. *Anal. Biochem.* **2003**, *320*(1), 39–54.
(48) Meza, J. E.; Scott, G. K.; Benz, C. C.; Baldwin, M. A. *Anal. Biochem.* **2003**, *320*(1), 21–31.
(49) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.
(50) Zakowski, J. J.; Tappel, A. L. *Anal. Biochem.* **1978**, *89*(2), 430–436.
(51) Chauhan, R.; Mande, S. C. *Biochem. J.* **2001**, *354*(Pt 1), 209–215.

Scheme 1. Bar Figure of TTP-D1 and TTP-2D



Ellman's reagent reacts with cysteine thiols to form a thionitrobenzoate-peptide complex. Thionitrobenzoate anion which gives an optical absorption profile with a maximum at 412 nm is then released. This assay is straightforward to perform and is a very accessible laboratory method, but can have limited sensitivity. One drawback is that noninteger values for free thiols are sometimes recorded, and the range of range of error can be broad. Moreover, the assay can be pH sensitive, and there is controversy regarding the chemistry of DTNB itself.[52,53]

During inflammatory response, when TTP is activated, the internal redox potential of the cell is elevated leading to increased levels of reactive oxygen species.[54−57] Additionally, there is evidence that intracellular iron levels can be elevated.[58,59] The role of the cellular redox potential and/or reactive oxygen species in modulating TTP activity is not known; although both factors are involved in the inflammatory response.[54−57] In the work reported here, we sought to determine whether TTP could be oxidized. Because iron is redox active, we predicted that iron substituted TTP would be more susceptible to oxidation than zinc substituted TTP. We sought to measure the oxidative susceptibility of TTP to test this hypothesis. We have developed a quick and simple assay to survey oxidative susceptibility of TTP that can easily be applied to other classes of zinc finger proteins/zinc binding domains. The assay that we developed relies on the well-understood cobalt binding properties of zinc finger proteins, requires only a UV−visible spectrometer to perform, and is quantitative. Additionally, it does not require any purification, proteolysis, or modification of oxidation products making it an alternative to the mass spectrometry or DTNB based assays when they are not suitable. Berg and co-workers were the first to utilize cobalt binding to newly isolated zinc finger proteins and peptides to assess their oxidation states.[15,60,61] Our assay utilizes this cobalt binding approach to rapidly and reproducibly monitor and quantify the oxidation of TTP-D1 and TTP-2D (Scheme 1) as a function of time and/or metal content. Using this assay, we determined that zinc protects TTP peptides from oxidation while ferrous iron promotes oxidation. We demonstrate that iron promoted oxidation is a result of the generation of hydroxyl radicals via a Fenton chemistry reaction. Moreover, we show that a small amount of oxidation has a profound effect on TTP's ability to bind to RNA leading us to speculate on a role for iron and oxidation in promoting RNA degradation by TTP. In addition, we propose that this assay may be easily applied to study oxidation of other zinc finger protein families with either zinc or iron bound. Such an approach may be useful for in vitro studies aimed at addressing the emerging question of how the function of specific types of zinc finger proteins are modulated by their local cellular redox potential.

Experimental Section

General Considerations. Electronic absorption measurements were performed on a Lambda 25 UV−visible scanning spectrophotometer. Electron Paramagnetic Resonance (EPR) measurements were performed on a Bruker EMX EPR spectrometer controlled with a Bruker ER 041 X G microwave bridge at room temperature. All experiments were performed using metal free reagents and water that had been purified using a Milli-Q purification system and passed over Sigma chelex-resin. Upon their preparation, buffers were purged with helium to degas and transferred into a Coy inert atmospheric chamber (95% N_2, 5% He). 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: EMPO (2-Ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide) was purchased from Alexis Biochemicals, 2-thiobarbituric acid, 2-Deoxy-D-ribose, dithiothreitol (DTT), and Diethylenetriaminepentaacetic acid (DTPA) were from Sigma-Aldrich, and H_2O_2 (ACS grade) was from VWR. All metal-binding titrations and oxidation studies were performed anaerobically with samples prepared and manipulated in a Coy inert atmospheric container. UV−visible measurements were taken using quartz cuvettes with screw caps and Teflon seals from Starna Cells, and EPR samples were prepared in a Coy anaerobic chamber in a capillary tube that had been placed in a Wildmad Quartz EPR tube. All titrations were performed with high-precision, well-calibrated pipetters.

Preparation of TTP Peptides. A peptide corresponding to the first zinc finger domain of TTP, TTP-D1, was purchased from Biosynthesis, Inc. (Lewisville, Texas) as "crude" and purified as described previously.[24] The peptide corresponding to the double finger domain of TTP, TTP-2D, was expressed and purified as previously described.[23] The masses of the peptides were confirmed using MALDI−TOF Mass Spectrometry [TTP-D1 - expected: 4143.7 observed: 4143.8; TTP-2D- expected: 8582.8 observed: 8582.1]. After purification, the peptides are in their reduced apo-forms and are >95% pure as evidenced by a single HPLC peak and SDS PAGE.

Oxidation Studies of TTP-D1: Measurement of Initial Protein Concentrations. To determine the amount of "active" or fully reduced TTP-D1 prior to oxidation studies, a 250 μM solution of the peptide was prepared in 20 mM phosphate, 10 mM NaCl at pH 7.4. The total concentration of peptide was determined by measuring the absorption at 276 nm and using a previously determined extinction coefficient of 4260 M^{-1} cm^{-1}.[24] One equivalent of Co^{2+} was then added to the peptide, and the concentration of "active" protein determined based upon the extinction coefficient of 670 M^{-1} cm^{-1} at 655 nM because of d-d transitions arising from tetrahedral coordination of cobalt to the cysteine/histidine metal binding sites of the peptide.[60] Co(II) binds to TTP-D1 with a K_d of 2.0×10^{-6} M.[24] Typically more than 95% of the protein bound cobalt. The pH of TTP-D1 did not change upon addition of cobalt.

Oxidation Assay for Zn(II)TTP-D1, Fe(II)-TTP-D1, apo-TTP-D1. Using the calculated amount of "active" peptide as the starting concentration, stoichiometric Zn(II) was added to a

(52) Ming, D.; Markley, J. L.; Hellekant, G. *Biotechniques* **1995**, *18*(5), 808–810.
(53) Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Methods Enzymol.* **1983**, *91*, 49–60.
(54) Winterbourn, C. C. *Nat. Chem. Biol.* **2008**, *4*(5), 278–286.
(55) Finkel, T. *Curr. Opin. Cell Biol.* **2003**, *15*(2), 247–254.
(56) Mates, J. M.; Segura, J. A.; Alonso, F. J.; Marquez, J. *Arch. Toxicol.* **2008**, *82*(5), 273–299.
(57) Stadtman, E. R.; Berlett, B. S. *Chem. Res. Toxicol.* **1997**, *10*(5), 485–494.
(58) Nanami, M.; Ookawara, T.; Otaki, Y.; Ito, K.; Moriguchi, R.; Miyagawa, K.; Hasuike, Y.; Izumi, M.; Eguchi, H.; Suzuki, K.; Nakanishi, T. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*(12), 2495–2501.
(59) Morris, C. J.; Earl, J. R.; Trenam, C. W.; Blake, D. R. *Int. J. Biochem. Cell Biol.* **1995**, *27*(2), 109–122.
(60) Berg, J. M.; Merkle, D. L. *J. Am. Chem. Soc.* **1989**, *111*, 3759–3761.
(61) Krizek, B. A.; Merkle, D. L.; Berg, J. M. *Inorg. Chem.* **1993**, *32*, 937–940.

250 μM solution of apo-TTP-D1 in 20 mM phosphate, 10 mM NaCl buffer at pH 7.4. After allowing the solution to equilibrate for 10 min, 1 equiv of H_2O_2 was added, and the solution was placed on a shaker at 400 rpm. Each reaction was allowed to proceed for a set time period: 3 min, 6 min, 15 min, 30 min, 1 h, 1.5 h. After the prescribed time period, the pH of reaction was reduced to ~2 by addition of 1 M HCl (typically 30 μL) to unfold the peptide and dissociate the zinc. The apo peptide was then isolated by size exclusion filtration using a YM-3 microcon centrifugal filter (Millipore) which separates the apo peptide from metal ions and reagants. The peptide was then lyophilized using a speed vacuum concentrator under anaerobic conditions. The concentration of "active" peptide was then determined by measuring the total peptide concentration by measuring the absorbance at 276 nm followed by the amount of peptide that could bind cobalt by measuring the absorbance at 655 nm. The amount of oxidized peptide was determined using the equation: % oxidized = { $[\text{peptide}_{\text{total}}] - [\text{peptide}_{\text{reduced}}]$}/$[\text{protein}_{\text{total}}]$. Oxidation reactions of Fe(II)-TTP-D1 and apo-TTP-D1 with H_2O_2 were performed analogously. The average rate of oxidation was determined by dividing the percent of the peptide that was oxidized at 15 min by the reaction time. All experiments were performed in triplicate.

Oxidation Studies of TTP-2D. Oxidation studies of Zn(II)-TTP-2D, Fe(II)-TTP-2D, and apo-TTP-2D were performed using the protocol described above for TTP-D1, except that 125 μM of peptide was used with 20 mM Hepes, 10 mM NaCl at pH 7.4 as the buffering system. TTP-2D exhibits better solubility under these conditions. Co(II) binds to TTP-2D with a K_d of 3.3×10^{-6} M.[23] The average rate of oxidation was determined by dividing the percent of the peptide that was oxidized at 60 min by the time. All experiments were performed in triplicate.

Detection of Hydroxyl Radicals. To determine if Fe(II)-TTPD1 and Fe(II)-TTP-2D produce hydroxyl radicals in the presence of H_2O_2 via the Fenton Reaction, two methods to identify hydroxyl radicals were used: the deoxyribose assay[62] and EPR spin trapping.[63,64] In the deoxyribose assay, 2-deoxy-D-ribose (5.6 mM, 20 equivs) was added to a solution of either Fe(II)-TTP-D1 (277 μM in 28 mM phosphate buffer, pH 7.4) or Fe(II)-TTP2D (277 μM in 28 mM Hepes buffer, pH 7.4) followed by addition of 1 equiv (277 μM) H_2O_2 to Fe(II)-TTP-D1 or 2 equiv (554 μM) H_2O_2 to Fe(II)-TTP-2D. The solution was then incubated at room temperature for 5 min upon which 28 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 adduct which shows a strong visible absorbance at 532 nm.

To detect the formation of hydroxyl radicals using EPR spectroscopy, EMPO was added to a solution of 125 μM Fe(II)-TTP-2D (or 250 Fe(II)-TTP-D1) in 12.5 mM phosphate buffer at pH 7.4. 250 μM H_2O_2 was then added to the solution, and the EPR spectrum of EMPO−OH was measured. Controls for this experiment included substituting Fe(II)-TTP-2D with either 250 μM $(NH_4)_2Fe(II)(SO_4)_2$ or 250 μM $(NH_4)_2Fe(II)(SO_4)_2$ plus 50 μM DTPA which are known generators of hydroxyl radicals[65] as well as omitting H_2O_2 from the experiment with Fe(II)-TTP-2D and using apo-TTP-2D. Spectrometer parameters for the EPR experiments were as follows: microwave power 20.12 mW, modulation amplitude 0.8 G, time constant 327 ms, scan time 320 s.

RNA Binding of Oxidized and Reduced Zn(II)-TTP-2D and Fe(II)-TTP-2D by Fluorescence Anisotropy (FA). The binding of Zn(II)-TTP-2D and Fe(II)-TTP-2D preoxidation and postoxidation (time = 6 min) to a fluorescently labeled RNA oligonucleotide of the sequence UUUAUUUAUUU-F was studied by fluorescence anisotropy (FA).[66] Measurements were taken with an ISS PC-1 spectrofluorometer configured in the L format. Initially, a full excitation/emission spectrum was run to determine the optimum excitations/emission wavelengths for the experiment. The excitation wavelength/band-pass used in the experiments was 492 nm/2 nm and the emission wavelength/bandpass was 523 nm/1 nm. In a typical experiment, a 10 nM solution of fluorescently labeled-RNA in 200 mM HEPES, 100 mM NaCl, 2 mM DTT with 0.05 mg/mL bovine serum albumin at pH 7.4 was added to a Spectrosil far-UV quartz window fluorescence cuvette (Starna Cells). The anisotropy, r, of the free RNA oligonucleotide was then measured. M-TTP-2D (M = Zn, Fe (II)) was then titrated into the cuvette from a stock solution (2.0 equiv of metal, 200 mM HEPES, 100 mM NaCl at pH 7.4) in a stepwise fashion, and the resultant change in anisotropy was recorded. 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 TTP-2D bound to RNA at a given RNA concentration), using the equation

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

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

$$\text{P} + \text{R} \rightleftharpoons \text{PR}$$

$$K_d = \frac{[\text{P}][\text{R}]}{[\text{PR}]}$$

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

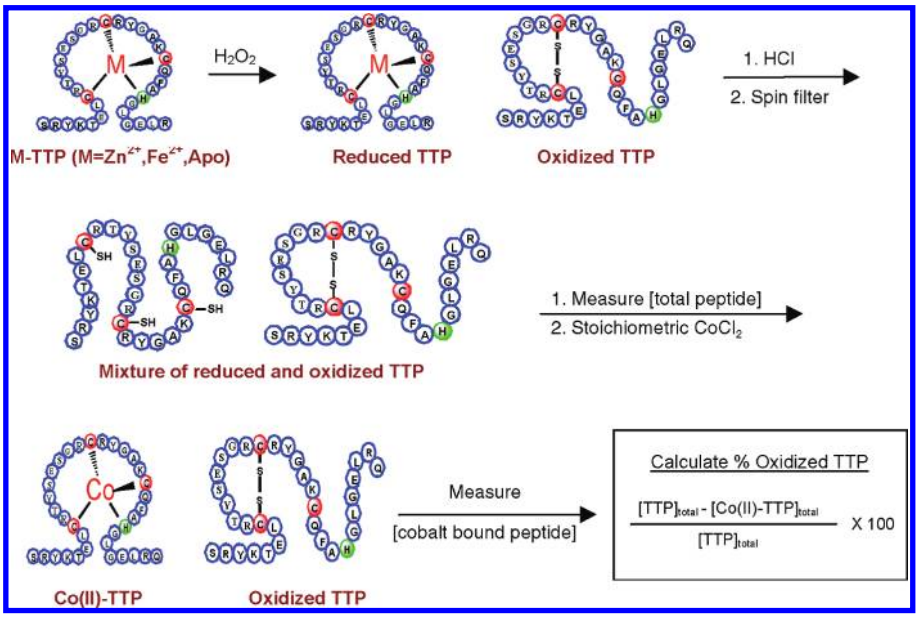
where P is the protein (TTP-2D) concentration and R is the RNA concentration. Each data point from the fluorescence anisotropy assay represents the average of 31 readings taken over a time course of 100 s. Each titration was carried out in triplicate.

Results

Determination of "Active" Peptide. TTP contains two zinc binding domains. Each domain is unfolded in the absence of metal and will fold in the presence of Zn(II), Co(II), Fe(II), or Fe(III).[23,24] Two peptides were prepared for these studies: TTP-D1 which is composed of just the first zinc binding domain of TTP and TTP-2D which is composed of both zinc binding domains (Scheme 1). TTP-D1 and TTP-2D were purified in their apo, unfolded states and were stored and manipulated anaerobically. Upon purification, the peptides were quantified by first determining the concentration of the unfolded peptide by measuring the peptide's absorbance at 276 nm and using the calculated extinction coefficients of 4260 M^{-1} cm^{-1} for TTP-D1 and 8520 M^{-1} cm^{-1} for TTP-2D.[23,24] Each peptide binds stoichiometric cobalt

(62) Gutteridge, J. M.; Halliwell, B. *Biochem. J.* **1988**, *253*(3), 932–933.
(63) Zhao, G.; Arosio, P.; Chasteen, N. D. *Biochemistry* **2006**, *45*(10), 3429–3436.
(64) Bou-Abdallah, F.; Lewin, A. C.; Le Brun, N. E.; Moore, G. R.; Chasteen, N. D. *J. Biol. Chem.* **2002**, *277*(40), 37064–37069.
(65) Halliwell, B.; Gutteridge, J. M. *Methods Enzymol.* **1990**, *186*, 1–85.
(66) Lakowicz, J. *Principles of Fluorescence Spectroscopy*, 2nd ed.; Kluwer Academic/Plenum Publishers: New York, 1999.

Scheme 2. Diagram of Oxidation Assay



with micromolar affinity, and extinction coefficients of Co(II)-TTP-D1 and Co(II)-TTP-2D had been previously reported.[23,24] Upon measuring the concentration of apo-TTP-D1 and apo-TTP-2D as purified, stoichiometric Co(II) was added to each peptide. This resulted in the appearance of d-d transitions between 500 and 700 nm because of cobalt coordination in a tetrahedral coordination geometry. By measuring the absorbance at a wavelength within the d-d transition region (655 nm) and using the previously determined extinction coefficients at this wavelength, the amount of peptide that bound Co(II) was ascertained.[23,24] Zinc finger peptides only bind cobalt when the cysteine residues are fully reduced; therefore this assay provides a measurement of the proportion of "active" peptide.[67] We note that we also observe charge transfer bands in the region of 250−400 nm upon Co(II) binding to TTP-D1 and TTP-2D that can be attributed to sulfur to metal transitions (Supporting Information, Figure S1).[60,67] If we measure bands in this region to determine the amount of cobalt that is binding to the peptide, the proportion of "active" peptide matches that determined by measuring the d-d bands. We chose to use the d-d bands for these studies because they appear in a unique region of the UV−visible spectrum and can be measured directly. In contrast, the charge transfer bands appear at the same region as the peptide backbone which necessitates subtracting the absorbance for the peptide backbone to measure the charge transfer bands directly. Typically, TTP-D1 and TTP-2D exhibited greater than 90% activity, and only peptides that were at least 90% active were used for oxidation studies.

M-TTP-D1 or M-TTP-2D (M = Zn(II), Fe(II), or apo) + H_2O_2. To determine how the identity of the metal ion coordinated to the peptides TTP-D1 or TTP-2D affects their reactivity with H_2O_2, experiments in which peptides with either Fe(II), Zn(II), or no metal (apo) bound were reacted with stoichiometric H_2O_2 for specific time periods (3 to 90 min) were performed. After the prescribed reaction time was completed, the pH of the solution lowered by adding HCl to unfold the peptide and release any remaining bound metal ions. The apo-peptide was then separated from the metal ions and reagents using a centrifugal filtration device and resuspended in buffer. The total peptide concentration was measured using the absorbance at 276 nm and known extinction coefficient. Typically, 90% or greater peptide was recovered after the reaction. Stoichiometric cobalt was then added to the peptide, and the amount of peptide that bound cobalt was determined. Scheme 2 outlines this protocol. Figure 1 and Figure 2 show the Co-binding data in the d-d region for TTP-D1 and TTP-2D as a function of reaction time and metal ion. When ferrous iron was bound to either TTP-D1 or TTP-2D, the measured oxidation was rapid. In contrast, the zinc bound peptides were slower to oxidize. When the peptides were reacted with H_2O_2 in the absence of metal, the average rate of oxidation was between that observed for the Fe(II) bound peptides and the Zn(II) bound peptides.

To calculate the proportion of each peptide that was oxidized under each condition, the amount of oxidized peptide was divided by the total peptide concentration. By multiplying this number by 100, the percent oxidized peptide at a given time point was determined. Figure 3 plots this data for TTP-D1 and TTP-2D as a function of both metal ion and time. When zinc was bound to TTP-D1 the oxidation was slow—after 1.5 h only 37% of the peptide was oxidized. Similarly, when zinc was bound to TTP-2D, the maximum oxidation measured was 35% after 1.5 h. In contrast, when Fe(II) was bound to either TTP-D1 or TTP-2D, oxidation was rapid. Fe(II)-TTP-D1 was fully oxidized after 15 min, and Fe(II)-TTP-2D was fully oxidized after 60 min. The average rates of oxidation of apo-TTP-D1 and apo-TTP-2D fell between the average rates of oxidation for Fe(II)-TTP-D1 and Zn(II)-TTP-D1 and for Fe(II)-TTP-2D and Fe(II)-TTP-2D, respectively. Because Fe(II)-TTP-D1 was fully oxidized after 15 min, the average rate of oxidation for TTP-D1 as a function of metal ion was

(67) Frankel, A. D.; Berg, J. M.; Pabo, C. O. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*(14), 4841–4845.

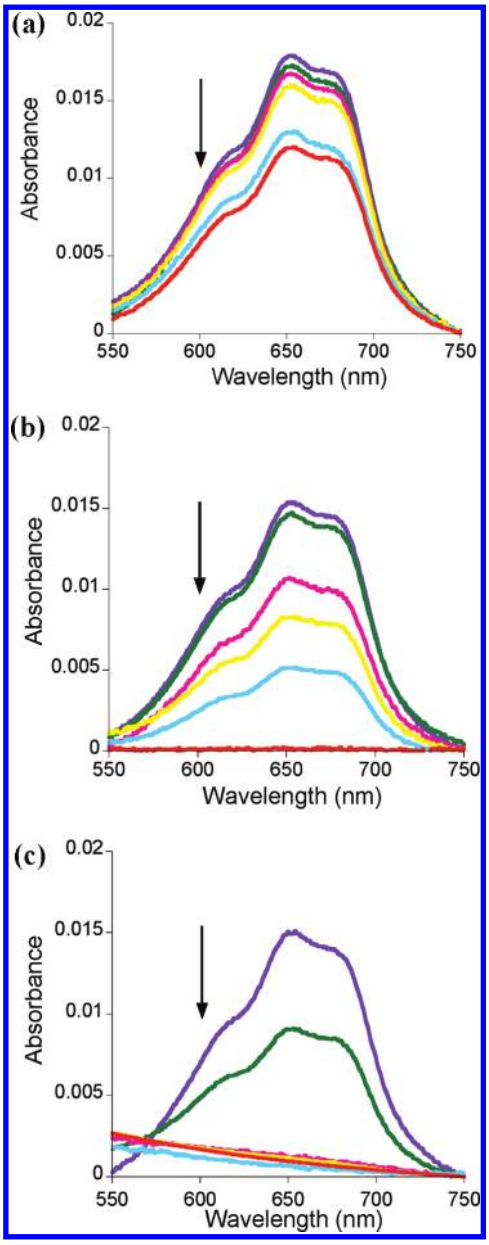


Figure 1. Plots of the absorbance spectra of Co(II)-TTP-D1 between 550 and 750 nm. The spectra were generated by adding stoichiometric $CoCl_2$ to apo-TTPD1 that was isolated from oxidation reactions with H_2O_2; (a) Co(II)-TTP-D1 from the Zn(II)-TTPD1 oxidation reaction at times (3, 6, 15, 30, 60, 90 min); (b) Co(II)-TTP-D1 from the apo-TTPD1 oxidation reaction at times (3, 6, 15, 30, 60, 90 min); (c) Co(II)-TTP-D1 from the Fe(II)-TTP-D1 oxidation reaction at times (3, 6, 15, 30, 60, 90 min). 250 μM of peptide in 20 mM Phosphate/10 mM NaCl at pH 7.4 was used for all reactions.

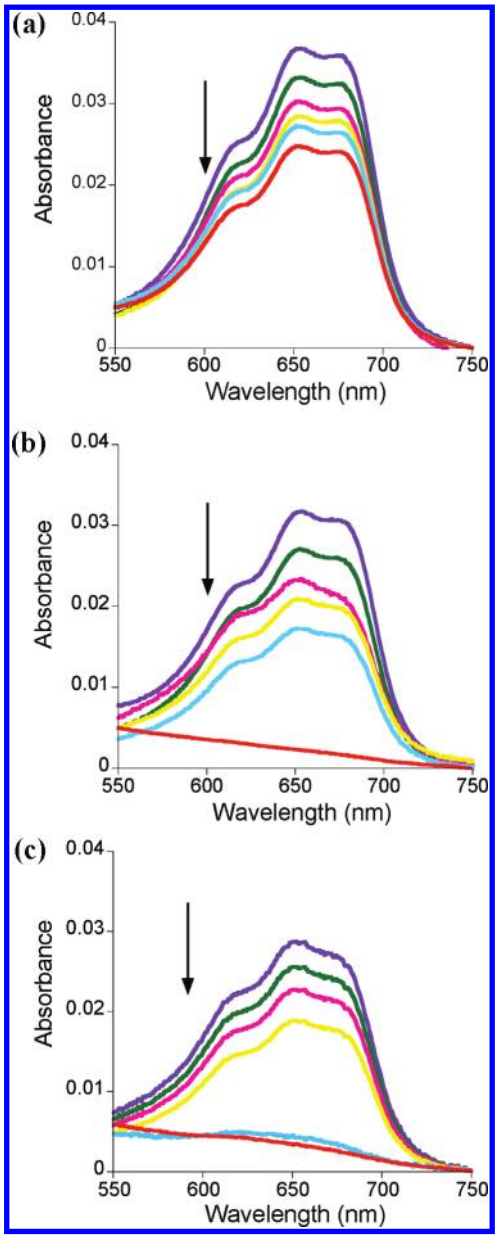


Figure 2. Plots of the absorbance spectra of Co(II)-TTP-2D between 550 and 750 nm. The spectra were generated by adding stoichiometric $CoCl_2$ to apo-TTP2D that was isolated from oxidation reactions with H_2O_2; (a) Co(II)-TTP-D1 from the Zn(II)-TTP-2D oxidation reaction at times (3, 6, 15, 30, 60, 90 min); (b) Co(II)-TTP-2D from the apo-TTP-2D oxidation reaction at times (3, 6, 15, 30, 60, 90 min); (c) Co(II)-TTP-2D from the Fe(II)-TTP-2D oxidation reaction at times (3, 6, 15, 30, 60, 90 min). 125 μM of peptide in 20 mM Hepes/10 mM NaCl at pH 7.4. was used for all reactions.

calculated at this time point. Fe(II)-TTP-D1 was oxidized at an average rate of 6.7% per minute, apo-TTP-D1 at 3.0% per minute and Zn(II)-TTP-D1 at 0.79% per minute. Fe(II)-TTP-2D was not fully oxidized until 60 min, so average rates of oxidation were calculated at this time point. Fe(II)-TTP-2D was oxidized at an average rate of 1.6% per minute, apo-TTP-2D at 0.91% per minute and Zn(II)-TTP-2D at 0.48% per minute.

The reaction of Fe(II) with H_2O_2 often occurs via Fenton chemistry which generates Fe(III), hydroxide, and hydroxyl radical.[68−71] The hydroxyl radical has been implicated in oxidative damage of zinc finger proteins, and we hypothesized that Fe(II)-TTP peptides would generate hydroxyl radicals. Two methodologies were used to confirm hydroxyl radical formation: trapping the radical with an EPR active spin-trap, EMPO, and observing the hydroxyl radical spectrophotometrically via the deoxyribose assay. Both experiments showed the

(68) Haber, F.; Weiss, J. *Proc. R. Soc. London, Ser. A* **1935**, *147*, 332–351.
(69) Halliwell, B.; Gutteridge, J. M. *FEBS Lett.* **1992**, *307*(1), 108–112.
(70) Walling, C. *Acc. Chem. Res.* **1998**, *31*, 155–157.
(71) Dixon, W. J.; Hayes, J. J.; Levin, J. R.; Weidner, M. F.; Dombroski, B. A.; Tullius, T. D. *Methods Enzymol.* **1991**, *208*, 380–413.

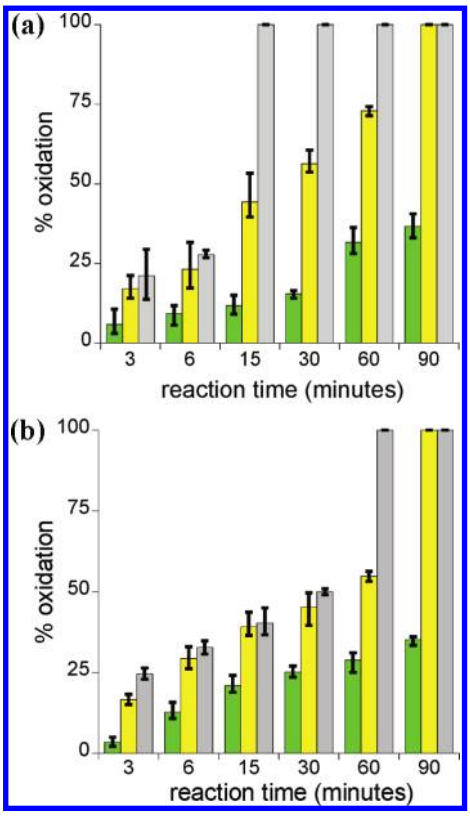


Figure 3. Plot of the percent oxidation of (a) M-TTP-D1 [M = Zn(II), apo, Fe(II)] and (b) M-TTP-2D [M = Zn(II), apo, Fe(II)] as a function of reaction time. The Zn(II) data is colored green, the apo data is colored yellow, and the Fe(II) data is colored gray.

formation of a hydroxyl radical when Fe(II)-TTP-2D or Fe(II)-TTP-D1 were reacted with H_2O_2 (Fe(II)-TTP-2D in Figure 4 and Supporting Information, Figure S2 Fe(II)-TTP-D1, data not shown).

M-TTP-2D + RNA. Fluorescence anisotropy experiments were performed to assess the effect of oxidation on RNA binding (Figure 5). We had previously used this technique to quantify TTP-2D/RNA binding.[23] TTP-2D binds to RNA with nanomolar affinity when either Zn(II) or Fe(II) is coordinated, but apo-TTP-2D does not bind RNA because the peptide is unfolded.[23] Zn(II)-TTP-D1 also binds RNA, but with a diminished affinity (micromolar); thus, the studies here focused on TTP-2D.[24] We hypothesized that weaker binding would be observed for the oxidized Zn(II)-TTP-2D or Fe(II)-TTP-2D. By titrating samples of Zn(II)-TTP-2D and Fe(II)-TTP-2D both as isolated (unoxidized) and after 6 min of oxidation with a segment of RNA of the sequence UUUAUUAUUU-F that was fluorescently labeled with fluorescein at the 3′ end, we determined the effect of oxidation on binding affinity. For both Zn(II) and Fe(II)-TTP-2D, oxidation resulted in a diminution in affinity. The Zn(II)-TTP-2D that had been oxidized for 6 min exhibited a DNA binding affinity of 570 ± 110 nM, compared to 16 ± 1 nM for the unoxidized protein (Figure 5a). Similarly, the Fe(II)-TTP-2D that had been oxidized for 6 min exhibited a RNA binding affinity of 3.3 ± 0.5 μM compared to 12 ± 1 nM for the unoxidized protein (Figure 5b). The larger decrease in DNA binding affinity of the oxidized Fe(II)-TTP-2D compared to that of the Zn(II)-TTP-2D was consistent with our oxidation results that showed

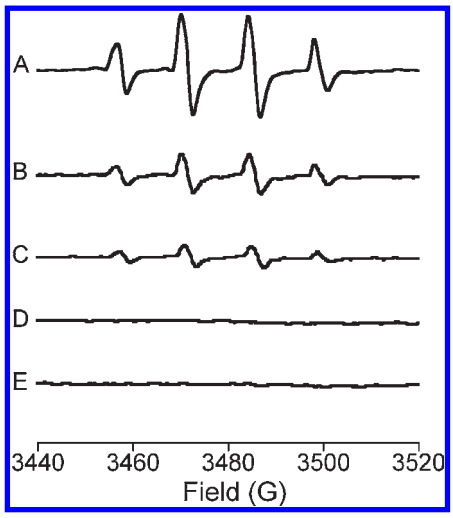


Figure 4. X-band EPR spectra of EMPO−OH; (A) 75 mM EMPO was added to 250 μM $(NH_4)_2Fe(II)(SO_4)_2$ and 50 μM DTPA in 12.5 mM Phosphate at pH 7.4 followed by addition of 250 μM H_2O_2; (B) 75 mM EMPO was added to 250 μM $(NH_4)_2Fe(II)(SO_4)_2$ in 12.5 mM Phosphate at pH 7.4 followed by addition of 250 μM H_2O_2; (C) 75 mM EMPO was added to 125 μM Fe(II)-TTP-2D in 12.5 mM Phosphate at pH 7.4 followed by addition of 250 μM H_2O_2; (D) 75 mM EMPO was added to 125 μM Fe(II)-TTP in 12.5 mM Phosphate at pH 7.4; (E) 75 mM EMPO was added to 125 μM apo-TTP-2D in 12.5 mM Phosphate at pH 7.4 followed by addition of 250 μM H_2O_2.

that at 6 min, 32.8% of Fe(II)-TTP-2D was oxidized compared to 12.8% of Zn(II)-TTP-2D peptide.

Discussion

Tristetraprolin (TTP) is a Cys_3His (or CCCH)-type zinc finger protein that is expressed during inflammatory response.[1] The CCCH metal binding domains can coordinate to either zinc or iron.[21,23,24] Upon metal ion coordination, the protein folds and functions (i.e., binds RNA). During inflammation the redox state of the cell changes leading to an elevation of intracellular levels of reactive oxygen species such as hydrogen peroxide.[32,72] The role of hydrogen peroxide in biological systems is diverse—elevated levels have been implicated in disease progression and aging, but this molecule has also been shown to be a secondary messenger involved in signaling pathways including inflammation.[54,57,73−75] Here we sought to understand how the presence of hydrogen peroxide affected the integrity and function of the zinc finger protein TTP. Studies were performed with the zinc bound, ferrous iron bound, and apo forms of peptides corresponding to the zinc binding domains of TTP.

There are scattered reports of oxidation of other types of zinc finger proteins; however, the methodologies used to monitor oxidation have usually utilized a mass spectroscopic approach.[30,34,42−44,76,77] The benefit of this approach is that one is able to identify the chemical composition of oxidized products; however, a disadvantage lies in the inability of mass spectrometry to be used as a simple tool for quantification.

(72) Maret, W. *Biometals* **2009**, *22*(1), 149–157.
(73) Balaban, R. S.; Nemoto, S.; Finkel, T. *Cell* **2005**, *120*(4), 483–495.
(74) Berlett, B. S.; Stadtman, E. R. *J. Biol. Chem.* **1997**, *272*(33), 20313–20316.
(75) Closa, D.; Folch-Puy, E. *IUBMB Life* **2004**, *56*(4), 185–191.
(76) Posewitz, M. C.; Wilcox, D. E. *Chem. Res. Toxicol.* **1995**, *8*(8), 1020–1028.
(77) Wilcox, D. E.; Schenk, A. D.; Feldman, B. M.; Xu, Y. *Antioxid. Redox Signaling* **2001**, *3*(4), 549–564.

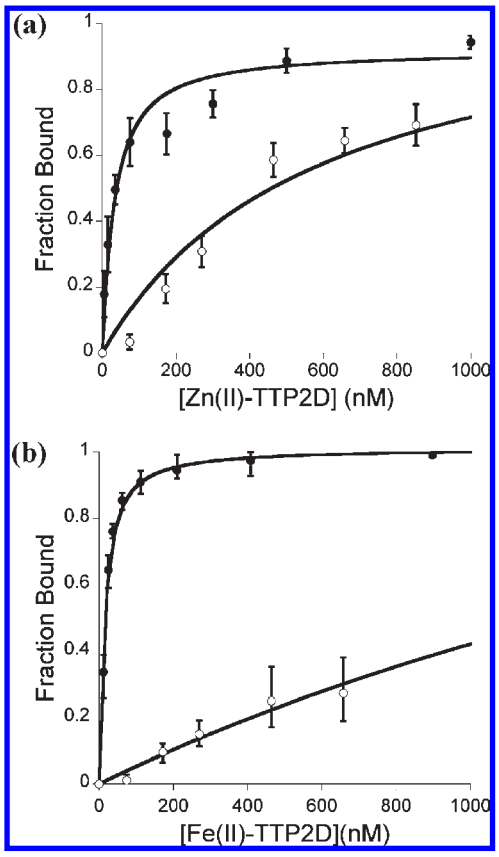


Figure 5. Comparison of the change in anisotropy (as fraction bound) upon the addition of (A) Zn(II)-TTP-2D prior to oxidation (closed circle) and Zn(II)-TTP-2D upon exposure to H_2O_2 for 6 min (open circle) to the oligonucleotide UUUAUUUAUUU-F (B) Fe(II)-TTP-2D prior to oxidation (closed circle) and Fe(II)-TTP-2D upon exposure to H_2O_2 for 6 min (open circle) to the oligonucleotide UUUAUUUAUUU-F. The solid lines represent nonlinear least-squares fits to a 1:1 binding model. All fluorescence anisotropy experiments were performed in a 200 mM HEPES/100 mM NaCl/2 mM DTT buffer at pH 7.4.

As we were interested in comparing the relative rates of oxidation of TTP as a function of metal ion bound, we sought to develop a simple method to quantify the oxidation products. We developed a spectroscopic assay that relies on the intrinsic cobalt binding properties of the zinc finger peptides to probe TTP oxidation. Cobalt is often used as a spectroscopic probe for zinc in zinc finger proteins. Cobalt(II) is a good probe of zinc(II) sites because it has a similar atomic radius [0.58 Å for Co(II) versus 0.60 Å for Zn (II)] and polarizability; but has the advantage of having a partially filled d-shell, d^7, which allows for d-d transitions to be observed optically in a region of the UV−visible spectrum that is not obscured by absorbances from the peptide.[61] Moreover, the cobalt binding properties of TTP-D1 and TTP-2D were well understood with micromolar dissociation constants being reported for both constructs.[23,24] The assay requires relatively small amounts of peptide—volumes of 1 mL or less at micromolar concentrations, and it is straightforward to vary the reaction time and conditions. Many types of zinc finger proteins have been studied by making small peptides that correspond to the zinc binding domains of the protein because these domains fold and function only in the presence of zinc.[19,21,27,67,78−82] Additionally, the cobalt binding properties of these zinc binding domain peptides are either well understood or are easily ascertainable. As such, we propose that it will be facile to apply our assay to study oxidation of the wide range of zinc finger proteins that are known.

Using our assay, we demonstrated that ferrous iron substituted TTP-2D peptides are more rapidly oxidized by H_2O_2 than the unfolded metal-free peptides and zinc bound peptides. Moreover, while we observed complete oxidation of the ferrous substituted peptides, we never observed complete oxidation of the zinc substituted peptides. Rather, the zinc substituted peptides remained less than 40% oxidized even after 3 h. We ascribe this elevated oxidation rate for the ferrous peptides to the generation of hydroxyl radicals from the reaction of ferrous bound TTP with H_2O_2 via Fenton chemistry. As Figure 4 shows, iron must be coordinated to TTP for hydroxyl radicals to be generated. There is one other report of Fenton chemistry occurring in a zinc finger protein—Sarkar and co-workers demonstrated that iron substituted Estrogen Receptor zinc finger protein produces free radicals via Fenton chemistry.[83] Zinc is redox inactive, so it will not promote Fenton-type chemistry. We also note that oxidation of TTP could be promoted by other oxidants such as dioxygen or superoxide.

A comparison of the average rates of oxidation between the single zinc finger peptide TTP-D1 and the double zinc finger peptide, TTP-2D revealed that oxidation was more rapid for the single zinc finger peptide. TTP-2D includes a linker region between the two zinc finger domains that is absent in the single zinc finger domain. In other types of zinc finger proteins, such as TFIIIA, the linker region contributes to the protein stability by promoting folding,[84] and we suggest that the presence of the linker in TTP-2D may protect the peptide from oxidation by stabilizing the protein fold. TTP-D1 can fold independently of the other domain; however, the two domain construct exhibits significantly tighter RNA binding than the single domain.[22−24] This suggests cooperativity between the domains, and the increased susceptibility to oxidation of the single zinc finger domain may also be related to the loss of cooperative effects.

Oxidation of TTP-2D has a large effect on RNA binding. FA data demonstrated that when Zn(II)-TTP-2D had been oxidized for 6 min, which translates to 12.8% oxidized protein, the affinity for target RNA was 36 times weaker than the unoxidized Zn(II)-TTP-2D. Similarly, Fe(II)-TTP-2D that had been oxidized for 6 min, which translates to 32.8% oxidation, exhibited 275 times weaker affinity for RNA. We suggest that the oxidized peptides impede the unoxidized peptide/RNA binding event.

We had previously shown that iron (ferric and ferrous) can bind to TTP-2D and retain function.[23] The dissociation constant for Fe(III) binding to TTP-2D is in the micromolar regime and in the nanomolar regime for Zn(II). Given that the estimates of "free" metal ions and available metal ions

(78) Thickman, K. R.; Davis, A.; Berg, J. M. *Inorg. Chem.* **2004**, *43*(25), 7897–7901.
(79) Guerrerio, A. L.; Berg, J. M. *Biochemistry* **2004**, *43*(18), 5437–5444.
(80) Blasie, C. A.; Berg, J. M. *Inorg. Chem.* **2000**, *39*(2), 348–351.
(81) Roehm, P. C.; Berg, J. M. *Biochemistry* **1997**, *36*(33), 10240–10245.
(82) Michael, S. F.; Kilfoil, V. J.; Schmidt, M. H.; Amann, B. T.; Berg, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*(11), 4796–4800.
(83) Conte, D.; Narindrasorasak, S.; Sarkar, B. *J. Biol. Chem.* **1996**, *271*(9), 5125–5130.
(84) Laity, J. H.; Dyson, H. J.; Wright, P. E. *J. Mol. Biol.* **2000**, *295*(4), 719–727.

in the cell correlate with these dissociation constants, we proposed that both metal ions would be bound to TTP-2D under physiological conditions.[23] Here, we found that Fe(II)-TTP-2D is more susceptible to oxidation than Zn(II)-TTP-2D. It will be of interest to understand how this increased sensitivity fits into the biological context of TTP. TTP binding to cytokine RNA molecules promotes their degradation, so the effect of iron in increasing oxidation of TTP may be beneficial. During inflammation, when TTP is activated, iron levels are elevated, and we speculate that this will increase the fraction of TTP that is occupied by iron relative to zinc. An increase in iron occupancy will increase levels of protein oxidation and therefore decrease the protein's activity. Thus, we speculate that there may be a link between iron and oxidative stress for TTP.

The concept of redox control of zinc finger proteins is emerging as a possible general explanation for how zinc finger protein function is controlled intracellularly. Zinc binding affinities for zinc finger peptides are typically in the nanomolar to picomolar regime which implies that once zinc is bound to a zinc finger protein,[15] it is thermodynamically prohibitive to release the zinc ion. The cellular availability of zinc in a given cell is very low, with femtomolar to picomolar quantities being reported.[85,86] Together, this suggests that upon zinc coordination the zinc will remain bound unless some exogenous agent can modify the binding site to release the zinc ion. Oxidation of zinc finger proteins has been invoked as a mechanism by which zinc finger function is controlled in vivo with the additional suggestion that zinc may play a role in cell signaling in response to inflammation accompanied by oxidative and/or nitrosative stress.[29,32,33] Here, we have determined that zinc bound TTP peptides can be oxidized and that RNA recognition is diminished as a consequence. These findings support the hypothesis that oxidation leads to inactivation of zinc finger protein function. We speculate, therefore, that oxidation may be a mechanism for controlling TTP function when zinc is bound. By quantifying the relative rates of oxidation for other types of zinc finger proteins, as we have done for TTP, we can begin to address how easy it is to oxidize specific types of zinc finger peptides/proteins and relate these findings to the proteins' functions.

Acknowledgment. We are grateful to the NSF CAREER Award CHE-0747863 for support of this research. We would like to thank Mr. Yunbo Jiang, Dept. of Chemistry, Johns Hopkins University for assistance with EPR experiments.

Supporting Information Available: Representative full UV−visible spectra of Co(II)-TTP-2D and the deoxyribose assay. This material is available free of charge via the Internet at http://pubs.acs.org.

(85) Outten, C. E.; O'Halloran, T. V. *Science* **2001**, *292*(5526), 2488–2492.
(86) Bozym, R. A.; Thompson, R. B.; Stoddard, A. K.; Fierke, C. A. *ACS Chem. Biol* **2006**, *1*(2), 103–111.