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Cadmium coordination to the zinc binding domains of the non-classical zinc finger protein Tristetraprolin affects RNA binding selectivity

Jamie L. Michalek, Seung Jae Lee, Sarah L.J. Michel *

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

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ABSTRACT

Tristetraprolin [(TTP), also known as NUP475 and TIS11] is a non-classical zinc finger protein that regulates inflammatory response via a protein-RNA interaction. Specifically, TTP recognizes AU-rich RNA sequences located on the 3′ untranslated region of messenger RNA associated with cytokines. Recently, TTP was shown to be upregulated when cells were exposed to cadmium. Other types of zinc finger proteins have been shown to bind Cd(II), thus the Cd(II) binding properties of TTP were pursued. Metal binding titrations using Co(II) as a spectroscopic probe for Cd(II) were performed. Cd(II) was found to coordinate to the two Cys_3His structural zinc sites of TTP (TTP-2D) with an upper-limit dissociation constant, K_d, of 3.5 ± 0.1 nM. Upon reconstitution of TTP-2D with Cd(II), the protein recognized target RNA, UUUAUUUAUUU, with a dissociation constant, K_d, of 2.4 ± 0.2 nM. The Cd(II)TTP-2D/RNA binding event was more sensitive to base mutations than the Zn(II)TTP-2D/RNA binding event. A single base mutation within the UUUAUUUAUUU oligomer decreased the Cd(II)TTP-2D binding affinity 50-fold and a double mutation decreased the affinity 1000–2000 fold. In comparison, only 2-fold and 15–25 fold changes for Zn(II)TTP-2D binding to the identical RNA sequences are measured.

1. Introduction

Zinc finger proteins (ZFs) are a large family of metalloproteins that utilize zinc ions for structural integrity: in the absence of Zn(II) these proteins are unfolded while in the presence of Zn(II), a functional structure is adopted [1–8]. Zn(II) is generally thought to be the *in vivo* metal cofactor for ZFs; however, there is a growing body of literature that implicates these proteins as targets for toxic metal ions [9–29]. In many instances, these toxic metal ions are proposed to coordinate to the ZF in place of Zn(II) and alter the protein's structure and function [10–13,15–18,20–34].

Cadmium is a toxic metal and environmental pollutant in which humans are exposed to daily from a wide range of sources including cigarette smoke, car exhaust and waste from industrial production of plastics and batteries [35–37]. Cadmium has a biological half-life of around 20 years in humans and is classified as a potent carcinogen that is known to target major organs in the body (e.g. kidney, liver, lungs, bones, pancreas) [34–38]. Cadmium toxicity is associated with oxidative stress and DNA damage [34–36,39–41]. The mechanism(s) of cadmium toxicity is not well understood, although cadmium is known to target thiol groups in proteins including metallothionein [18,34,36,42–46]. Furthermore, because cadmium has similar electronic properties as zinc, and is found directly below zinc on the periodic table, cadmium has been implicated in targeting ZFs [12,13,15,17,30,31,34,47].

The reports of Cd(II) binding to ZFs, include those involved in transcriptional activation and DNA repair [10,12,13,16–18,20,21,30,33,47,48]. The functional consequences of Cd(II) coordination to ZFs can vary. For example, when Cd(II) is bound to classical ZFs, such as transcription factor IIIA (TFIIIA) and specificity protein-1 (Sp-1), which utilize a Cys_2His_2 ligand set to coordinate metal ions, the proteins are no longer able to recognize and bind to their target DNA [12,13,18,20,33,49,50]. When Cd(II) is coordinated to the DNA repair ZF, XPA, which uses a Cys_4 ligand set to bind metal ions, the protein still binds to target DNA, albeit with decreased affinity compared to the Zn(II) bound form [16]. In contrast, when Cd(II) is coordinated to the estrogen receptor protein (ER), which also utilizes a Cys_4 ligand set to bind metals, there is no effect on DNA recognition compared to the Zn(II) bound form [10,30,51]. Thus, Cd(II) coordination appears to have different functional effects on different types of ZFs.

Cadmium coordination is expected to have an effect on the fold of the ZF. Zinc coordination to a ZF is a defining factor for the protein's overall fold, and because Cd(II) has a larger atomic radius than Zn(II) (0.95 Å versus 0.74 Å) [52], it is likely to alter the protein's structure [13,14,17,21,23,27,34,47]. To date, only one structure of a Cd(II)-ZF has been reported [27]. In this recent structure, Cd(II) was bound to the classical ZF, SUPERMAN. The overall protein fold is maintained when Cd(II) binds, but changes in the orientation of specific side chains are observed [27]. Cd(II)-SUPERMAN shows a decreased binding affinity for its target DNA and this is attributed to

* Corresponding author. Tel.: +1 410 706 7038; fax: +1 410 706 5017.
E-mail address: smichel@rx.umaryland.edu (S.L.J. Michel).

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the disruption of one of the protein's side chains, Ser17, that is involved in direct contact with the DNA [27].

Recently, cadmium was shown to affect the expression of the ZF Tristetraprolin (TTP) [53]. In these studies, TTP expression was up-regulated in hepatocytes, perhaps in a direct connection with cadmium toxicity [53]. TTP is a 'non-classical' ZF that is localized in the cytoplasm where it regulates inflammatory response at the mRNA level [54–56]. TTP contains two Cys_3His zinc-binding domains (or 'zinc fingers') that selectively recognize RNA associated with cytokines in order to promote the deadenylation and degradation of the cytokine mRNA [54–60]. The zinc fingers bind to the RNA sequence UUUAUUUAUUU, which is part of the 3′ untranslated region (3′ UTR) of cytokine mRNA [28,55,56,59–61]. Cytokines are proteins that are expressed during inflammation, thus TTP regulates inflammation by regulating cytokine expression at the mRNA level [54–56,58–62].

We sought to determine if cadmium substitution could occur within the zinc finger domains of TTP and to assess the effects of cadmium coordination on RNA recognition and selectivity. A protein construct of TTP that contains the two zinc-binding domains, named 'TTP-2D' that we have previously shown to be soluble and capable of folding around Zn(II) was prepared and isolated [28]. The cadmium binding properties of TTP-2D were assessed and the effects on RNA recognition by Cd(II)TTP-2D were measured. We determined that cadmium can bind to apo-TTP-2D in a manner similar to zinc, yet Cd(II)TTP-2D is more selective for its RNA targets than Zn(II)TTP-2D. We discuss these findings in the context of a biological role.

2. Materials and methods

2.1. Cloning, expression and purification of a murine two-domain TTP construct (TTP-2D)

We have previously reported the cloning, expression and purification of a construct of TTP that just contains the two zinc-binding domains [28]. The sequence of TTP-2D is: MSRYKTEL**C**RTYSESGR**C**RYGAK**C**QFA**H**GLG ELRQANRHPKYKTEL**C**HKFYLQGR**C**PYGSR**C**HFI**H**NPTEDLAL. The general cloning protocol involved strategically ligating the gene fragment of interest into the pET-15b vector to avoid the incorporation of a hexahistidine tag, followed by transformation of the vector into BL21-(DE3) competent cells (Novagen). The transformed cells were grown in Luria–Bertani (LB) medium containing 100 μg/mL ampicillin at 37 °C until mid-log phase. Protein expression was induced using 1 mM IPTG and cultures were allowed to grow 4 h post-induction before harvesting by centrifugation at 7800×*g* for 15 min at 4 °C. Cell pellets were resuspended in 8 M urea and 10 mM MES pH 6 containing one EDTA-free protease inhibitor mini tablet (Roche). Resuspended cells were lysed using a Thermo Spectronic French Pressure Cell at 1250 psi and 4 °C followed by centrifugation (12,100 rpm for 15 min at 4 °C) to remove cellular debris. The clarified supernatant was incubated on a SP-Sepharose gravity column at room temperature with rocking for 1 h. After drainage of "flow through," a step gradient from 0 to 2 M NaCl in 4 M urea and 10 mM MES pH 6 was used. TTP-2D eluted from the column in 600 mM NaCl. SDS-PAGE was used to confirm the purification. TTP-2D was then immediately incubated with 10 equivalents dithiothreitol (DTT) per cysteine residue at 56 °C for 3 h to reduce any disulfide bonds. The protein was then acidified using 1 M hydrochloric acid and further purified using reverse-phase HPLC with a Symmetry-C18 column and non-metallic Waters 626 LC system. A gradient of acetonitrile and water, both containing 0.1% trifluoroacetic acid, was used and TTP-2D eluted at 32% acetonitrile. Fractions from the HPLC purification were dried using a Savant SpeedVac concentrator housed in a Coy anaerobic chamber (97% nitrogen/3%hydrogen atmosphere). All protein manipulations beyond this point take place in the anaerobic chamber to prevent cysteine oxidation.

2.2. Metal binding titrations

Metal binding titrations were performed in screw-capped quartz cuvettes (Starna Cells) using a Perkin-Elmer Lambda 25 spectrometer. Titrations were carried out in 200 mM HEPES, 100 mM NaCl, pH 7.5 using metal-free reagents and water that had been purified using a Milli Q purification system and passed over Sigma chelex-resin. Upon preparation, buffers were degassed with argon then transferred into a Coy inert atmospheric chamber (97% Nitrogen and 3% Hydrogen). The following metal salts or stocks, which were stored anaerobically, were used for the titrations: cobalt chloride (EM Science), zinc atomic absorption standard (Aldrich 15.2 mM Zn^{2+} in 0.9% HCl), cadmium chloride (Aldrich). Titrations were carried out in triplicate.

2.3. Preparation of Cd(II)TTP-2D

Cd(II)TTP-2D was prepared by the addition of two molar equivalents of Cd(II) to apo-TTP-2D (typically 25 μM) in the presence of 200 mM HEPES, 100 mM NaCl, pH 7.5. The UV visible spectrum was then measured and a band centered around 240 nm was observed (in the protein subtracted spectrum).

2.4. Co(II) and Cd(II) binding

The affinity of TTP-2D for cobalt was measured by monitoring a titration of apo-TTP-2D (typically 25 μM in 750 μL of 200 mM HEPES, 100 mM NaCl pH 7.5) with $CoCl_2$ in a step-wise fashion. $CoCl_2$ was titrated in excess (20 molar equivalents) in order to ensure that all TTP-2D was present in the Co(II) bound species. The data at 655 nm (where the d-d envelope maximum is observed) was fit to a 1:1 binding equilibrium using Kaliedagraph software (Synergy software) and linear, least squares analysis. The relative affinity of TTP-2D for Cd(II) was then determined by monitoring the displacement of Co(II) by Cd(II) following the method developed by Berg and Merkle [63]. The data were then fit to a competitive binding equilibrium.

$$\mathrm{Co(II)TTP{-}2D} + \mathrm{Cd(II)} \underset{K_d^{Cd}}{\rightleftharpoons} \mathrm{Cd(II)TTP{-}2D} + \mathrm{Co(II)}$$

2.5. NMR spectroscopy

To measure the stoichiometry of cadmium binding to the two-domain peptide of TTP, Cd(II) was titrated with apo-TTP-2D and monitored by 1-D NMR spectroscopy using a a Varian 400 MHz spectrophotometer. All experiments were performed in ^2H_2O (Cambridge Isotope Laboratories, Inc.) at 25.0 °C with a relaxation time of 5 s and averaging of 128 scans. A solution of cadmium chloride (dissolved in ^2H_2O) was titrated with apo-TTP-2D in the following molar equivalents: 0, 1, 1.5, 1.75, 2 and 3. NMR sample preparation of apo-TTP-2D consisted of dissolving 250 nmol of dried peptide into 500 μL ^2H_2O and slowly bringing the pH of the solution to 6.7 using deuterated Tris (tris(hydroxymethyl)aminomethane). The apo-TTP-2D sample had a final volume of 685 μL giving a concentration of 365 μM. Each addition of $CdCl_2$ to the TTP-2D sample was carried out under anaerobic conditions to prevent cysteine oxidation. All data was processed using Spinworks software [64].

2.6. Circular Dichroism (CD) studies

Changes in secondary structure due to Zn(II) versus Cd(II) coordination to TTP-2D were determined by analyzing far-UV CD spectra acquired on a Jasco-810 spectropolarimeter. CD spectra were recorded from 180 to 260 nm at a scan rate of 100 nm/min, with each spectrum representing the average of 3 accumulations. Data were acquired at