Nickel(II) Inhibits Tet-Mediated 5-Methylcytosine Oxidation by High Affinity Displacement of the Cofactor Iron(II)

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Supporting Information

Abstract: Ten-eleven translocation (Tet) family proteins are Fe(II)- and 2-oxoglutarate-dependent dioxygenases that regulate the dynamics of DNA methylation by catalyzing the oxidation of DNA 5-methylcytosine (5mC). To exert physiologically important functions, redox-active iron chelated in the catalytic center of Tet proteins directly involves the oxidation of the multiple substrates. To understand the function and interaction network of Tet dioxygenases, it is interesting to obtain high affinity and a specific inhibitor. Surprisingly, here we found that natural Ni(II) ion can bind to the Fe(II)-chelating motif (HXD) with an affinity of 7.5-fold as high as Fe(II). Consistently, we further found that Ni(II) ion can displace the cofactor Fe(II) of Tet dioxygenases and inhibit Tet-mediated SmC oxidation activity with an estimated IC₅₀ of 1.2 μM. Essentially, Ni(II) can be used as a high affinity and selective inhibitor to explore the function and dynamics of Tet proteins.

Ten-eleven translocation (Tet) family proteins comprise Tet1, Tet2, and Tet3 in mammals and display crucial functions in nuclear reprogramming, passive and active DNA demethylation, and regulation of gene expression, embryonic development, neurogenesis, and carcinogenesis.¹⁻³ Tet proteins with the participation of Fe(II) and 2-oxoglutarate (2-OG) trigger the oxidation of S-methylcytosine (SmC) to S-hydroxymethylcytosine (5hmC) by transferring one oxygenic atom from an oxygen molecule to C5-methyl of 5mC; meanwhile, 2-OG is transformed by the other oxygenic atom to CO₂ and succinate.⁴ Crystal structure analysis of the human Tet2–DNA complex indicates that Fe(II) and 2-OG are localized in the catalytic cavity of Tet proteins; and Fe(II) stabilized by residues H1382, D1384, H1881, and H1416; and 2-OG stabilized by R1896 and S1898. 5hmC oxidation occurs when the C5 methyl group of 5mC is orientated to the catalytic Fe(II) and 2-OG.⁶,⁷ Moreover, 5hmC can be iteratively oxidized by Tet to produce 5-formylmethylcytosine (5fC) and 5-carboxylmethylcytosine (5caC), which are intermediates of active DNA demethylation.⁸,⁹ Interestingly, some chemicals can alter DNA hydroxymethylation and demethylation by interacting with Tet dioxygenases. To understand the function and interaction network of Tet proteins, it is interesting to obtain high affinity and a specific inhibitor. Surprisingly, here we found that natural Ni(II) ion can bind to the Fe(II)-chelating motif (HXD) with an affinity of 7.5-fold as high as Fe(II). Consistently, we further found that Ni(II) ion can displace the cofactor Fe(II) of Tet dioxygenases and inhibit Tet-mediated SmC oxidation activity with an estimated IC₅₀ of 1.2 μM. Essentially, Ni(II) can be used as a high affinity and selective inhibitor to explore the function and dynamics of Tet proteins.

Received: March 25, 2017
Accepted: May 3, 2017
Published: May 3, 2017
It is known that Fe(II) binds to the catalytic center of Tet1; however, it is not known whether the bound Fe is stable enough to survive from SEC separation. By SEC-ICPMS analysis, we did observe the wild type Tet1CD-bound Fe(II) (left panel, wtTet1CD, Figure 1A). However, we did not observe the peak of free Fe(II), which would be eluted later than the Tet1CD-bound Fe (Figure 1A). These observations indicate that no bound Fe(II) dissociated from Tet1CD during SEC-ICPMS analysis. Therefore, the binding between Fe(II) and Tet1CD is quite stable. The substitution of an indispensable Fe-binding motif H(1620)XD(1622) by Y(1620)XA(1622) would significantly impair the Fe(II) binding (left panel, Tet1CD mutant, Figure 1A). The bound Fe(II) is reduced to 9.5% of that for wtTet1CD. The observation confirms the binding of Fe(II) to the catalytic center of Tet1.

As expected, Ni(II) can also stably bind to Tet1CD, as shown by SEC-ICPMS analysis (right panel, wtTet1CD, Figure 1A). Moreover, as revealed by the use of the Y(1620)-XA(1622)-substituted Tet1CD, Ni(II) also binds to the indispensable Fe-chelating motif H(1620)XD(1622) of Tet1CD (right panel, Tet1CD mutant, Figure 1A).

Figure 1. SEC-ICPMS analysis of Tet1CD-bound metal ions. (A) Determination of the binding site of Tet1CD with Ni(II). The Tet1CD mutant contains the substitution of HXD by YXA. (B) Replacement of Tet1CD-bound Fe(II) by Ni(II). (C) Peak areas of the bound Fe and Ni obtained from B. Fe(II) and Ni(II) were 100 μM and 20 μM, respectively. Tet1CD or mutant was about 1.5 μM. ND represents undetectable. Red arrow indicates protein-bound nickel. Notice that the unbound species were removed using ultrafiltration prior to SEC-ICPMS analysis.
ingly, by the use of Ni(II) at the 5-fold lower concentration (compared to that of Fe), we could observe the stable binding of nickel to Tet1CD (Figure 1B).

We further mixed 100 μM Fe(II) and 20 μM Ni(II) together with Tet1CD (1.5 μM), and found that about 60% of the Tet1CD-bound Fe(II) was replaced by Ni(II) (Figure 1B and C). The results proved that Ni(II) can displace the critical cofactor Fe(II) from the catalytic center. Since the concentration of the involved Tet1CD (~1.5 μM) is much lower than that of the metal ions (20–100 μM), eq 1 was applied to estimate relative affinity of Ni(II) to Fe(II) binding to Tet1CD.

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R_{\text{Ni}/\text{Fe}} = \frac{C_{\text{Ni}-\text{Tet}}/C_{\text{Ni}}}{C_{\text{Fe}-\text{Tet}}/C_{\text{Fe}}}
\]

(1)

In this equation, \(R_{\text{Ni}/\text{Fe}}\) represents the relative affinity of nickel binding with Tet1CD versus that of iron; \(C_{\text{Ni}-\text{Tet}}\) and \(C_{\text{Fe}-\text{Tet}}\) are the concentrations of Tet1CD-bound Ni(II) and Fe(II) in the reaction solutions, respectively. \(C_{\text{Ni}}\) and \(C_{\text{Fe}}\) indicate the concentrations of the unbound Ni(II) and the unbound Fe(II) (approximately to their total concentrations), respectively.

Surprisingly, according to eq 1, the estimated affinity of natural Ni(II) is 7.5 folds higher than that of the cofactor Fe(II), suggesting that Ni(II) displays a much higher affinity than the native cofactor of Tet dioxygenases Fe(II).

Next, we examined the in vitro catalytic activity of Tet dioxygenases in the presence of Ni(II) using the developed ultrahigh performance liquid chromatography–tandem mass spectrometry approach (UHPLC-MS/MS, see Supporting Methods). An enzymatically methylated lambda DNA (mDNA) was used as a substrate. The level of 5mC was determined to be estimated about 20 5mC per 100 dC using UHPLC-MS/MS. Both 5hmC (retention time: 2.6 min) and 5fC (retention time: 5.2 min) cannot be detected in the mDNA substrate (Figure 2A). Consistent with our previous work, the observation suggests that artificial 5mC oxidation in the process of enzymatic digestion of DNA is very minor or negligible. Upon the addition of purified Tet1CD (0.5 μg per sample, 50 nM), the level of 5hmC is enormously increased from the undetectable level to 4.0 (±0.2) per 100 dC, and huge amounts of 5fC (1.9 (±0.01) per 100 dC) are also detected in mDNA (Figure 2A and B), suggesting that the purified Tet1CD has an excellent enzymatic activity. Expectedly, Ni(II) inhibits Tet1CD-mediated oxidation of 5mC and 5fC in the mDNA substrate, as manifested by the decrease in the levels of 5hmC and 5fC in mDNA. Compared with that of 10 μM Fe(II)-stimulated Tet1CD (no Ni(II)), 2.0 μM Ni(II) induces a dramatic decrease in the frequency of 5hmC by 84%, and 50–100 μM Ni(II) can completely inhibit the 5hmC formation (Figure 2B). The nickel inhibition is dose-dependent (Figure 2B and Supporting Figure S2). Assuming that 5hmC formation is linearly inhibited by Ni(II) at concentrations between 0 and 2 μM, the 50% inhibitory concentration (IC50) of nickel is estimated to be about 1.2 μM. Even increasing the cofactor Fe(II) to 20 μM, Ni(II) of 2.0 μM can also efficiently decrease the level of 5hmC by 75% (Supporting Figure S3). The inhibition of Ni(II) ion on 5fC formation is even much more dramatic. The addition of 2.0 μM Ni(II) largely abolishes 5fC (about 96%), and no 5fC can be detected in mDNA substrate when 10–100 μM Ni(II) was added (Figure 2B). Our data provide compelling evidence that Ni(II) ion can directly inhibit the catalytic activity of Tet dioxygenases. The results also clearly suggest that Tet dioxygenases are highly sensitive to Ni(II).

The formation of ShmC and 5fC, which is catalyzed by Tet1CD, is accompanied by the global 5mC frequency reducing by 30% (Supporting Figure S4). In the presence of inhibitory Ni(II), while ShmC and 5fC are inhibited, 5mC recovers in a Ni(II)-dose dependent manner (2.0–100 μM Ni(II); Supporting Figure S4).

Last, we examined a metal ion with a much larger ionic radius, cadmium (Cd(II) (0.095 nm) > Ni(II) (0.069 nm) > Fe(II) (0.061 nm)). Compared with Tet1CD-mediated mDNA oxidation, 20 μM Cd(II) decreased the levels of ShmC and 5fC by 77% and 90%, respectively (Supporting Figure S5). The observed inhibition caused by 20 μM Cd(II) is equal to that of 2 μM Ni(II) (Figure 2), suggesting Tet dioxygenases are 10 times more sensitive to Ni(II) than cadmium(II).

Interestingly, Ni(II) can also inhibit the activities of HIF-prolyl hydroxylase PHD2, histone demethylase JHDM2A/JMJD1A, and ABH2/3, all of which belong to Fe(II)- and 2-OG-dependent dioxygenases and contain similar catalytic domains to Tet proteins. In the presence of 100 μM Fe(II), IC50 values of nickel were 22 μM, 25 μM, and 75 μM for PHD2, JHDM2A, and ABH3, respectively. According to eq 1, the estimated affinities of Ni(II) relative to iron(II) are 4.5 for PHD2, 4.0 for JHDM2A, and 1.3 for ABH3. As we showed in this study, the estimated affinity of nickel(II) relative to iron(II) for Tet1CD is about 7.5. Evidently, among these Fe(II)- and 2-OG-dependent epigenetic dioxygenases, Ni(II) displays the highest affinity against Tet1 proteins.

Previously, a number of chemicals can affect the regulation and function of Tet dioxygenases, including vitamin C, arsenite, redox-active quinones, and 2-OG analogs (DMOG...
and 2-HG). Among these small bioactive molecules, only DMOG and 2-HG can directly bind to the catalytic center of Tet dioxygenases but with weak affinity, thus inhibiting the oxidation activity at the concentration of millimolar.

In this work, we demonstrated Ni(II) and Cd(II) can repress Tet-mediated DNA hydroxymethylation. Interestingly, nickel can function as a high affinity inhibitor of Tet dioxygenase. This study also provides a novel chemical mechanism to nickel-caused DNA methylation change. Mechanistically, the Ni(II) ion displaces the Fe(II) cofactor to directly bind with the Fe binding motif of Tet dioxygenases (HXD) at micromolar concentrations, causing a dysfunction of Tet dioxygenases in SmC oxidation. By our findings, Ni(II) will display promising applications in exploring the function of Tet dioxygenases in vitro an in vivo.

**EXPERIMENTAL SECTION**

**SEC-ICPMS Assay.** We developed a SEC-ICPMS assay to monitor Tet1CD-bound nickel and iron ions. Purified Tet1CD proteins (about 5 μg, a final concentration of 1.5 μM) were incubated with 100 μM FeCl₂ or 20 μM NiCl₂, and 1 mM 2-OG in a 30 μL buffer (50 mM HEPES, pH 8.0, and 1 mM DTT). The proteins and protein-bound fractions were collected by centrifugation at 6000 × g for 10 min using ultrafiltration tubes (MW cut off 3 kDa, Pall) and washed three times with 200 μL of 50 mM HEPES buffer to remove the excessive unbound metals. The protein fractions retained on a Pall Ultra-Centrifugal membrane were recovered with 30 μL of 50 mM HEPES buffer. The collected proteins were analyzed by SEC-ICPMS. SEC was performed on The HiTrap Desalting column (5 mL, GE Healthcare) to separate protein-bound metal ions from the unbound ones. The SEC was performed on The HiTrap Desalting column (5 mL, GE Healthcare) to separate protein-bound metal ions from the unbound ones. The mobile phase (20 mM ammonium acetate, pH 7.0) was applied at a flow-rate of 2.0 mL/min. The column was connected to Agilent 8800 ICPMS for iron element analysis. m/z 56 and 60 were used to quantify iron and nickel ions, respectively. The injection volume for each sample was 20 μL.

**In Vitro Methylation DNA Oxidation Reactions.** In vitro oxidation reactions were performed as described previously. The methylated Lambda DNA (0.5 μg) was incubated with purified Tet1CD or mutant Tet1CD proteins (0.5 μg, a final concentration of 50 nM) in a 100 μL HEPES buffer (50 mM HEPES, pH 8.0, 50 mM NaCl, 1 mM 2-OG, 10 μM FeCl₂, 1 mM ATP, 1 mM DTT, and 1 mM vitamin C) for 2 h at 37 °C. Before initiating oxidation reactions, a series of concentrations of NiCl₂ or CdCl₂ (0.2, 5, 10, 20, 50, and 100 μM) were added into the solutions. Tet1CD proteins were inactivated by heating for 5 min at 95 °C. Then, the reacted DNA samples were digested with 0.5 units of DNase I, 1.0 unit of CIP, and 0.002 units of SVP at 37 °C overnight. The enzymatic digests were filtered by ultrafiltration tubes (MW cutoff: 3 kDa, Pall) to remove the enzymes and then analyzed by UHPLC-MS/MS.

**ASSOCIATED CONTENT**

1. Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00261.

Figures S1–S5 and Supporting Materials and Methods (PDF)

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work is supported by the National Natural Science Foundation of China (21327006, 214353008, and 21621064), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB14030200), and the Key Research Program of Frontier Sciences, CAS.

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