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Structural insights on mouse L-threonine dehydrogenase: A regulatory role of Arg180 in catalysis

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ABSTRACT

Mouse L-threonine dehydrogenase (mTDH), which belongs to the short-chain dehydrogenase/reductase (SDR) superfamily and mediates threonine catabolism, plays pivotal roles in both powerful biosynthesis and signaling in mouse stem cells and has a regulatory residue Arg180. Here we determined three crystal structures of mTDH: wild-type (WT) in the apo form; in complex with NAD^+ and a substrate analog, glycerol, or with only NAD^+; as well as the R180K variant with NAD^+. This is the first description of a structure for mammalian SDR-type TDH. Structural comparison revealed the structural basis for SDR-type TDH catalysis remains strictly conserved in bacteria and mammals. Kinetic enzyme assays, and isothermal titration calorimetry (ITC) measurements indicated the R180K mutation has little effect on NAD^+ binding affinity, whereas affects the substrate's affinity for the enzyme. The crystal structure of R180K with NAD^+, biochemical and spectroscopic studies suggested that the R180K mutant should bind NAD^+ in a similar way and have a similar folding to the WT. However, the R180K variant may have difficulty adopting the closed form due to reduced interaction of residue 180 with a loop which connects a key position for mTDH switching between the closed and open forms in mTDH catalysis, and thereby exhibited a significantly decreased k_{cat}/K_m value toward the substrate, L-Thr. In sum, our results suggest that activity of GalE-like TDH can be regulated by remote interaction, such as hydrogen bonding and hydrophobic interaction around the Arg180 of mTDH.

1. Introduction

L-Threonine 3-dehydrogenase (EC 1.1.1.103, TDH) is an oxidoreductase, that specifically acting on the >CHOH group of a donor molecule with NAD^+ or $NADP^+$ as the acceptor. TDH can catalyze the NAD^+-dependent dehydrogenation of the 3′ carbon atom of L-threonine (L-Thr). The product L-2-amino-3-ketobutyrate is nonenzymatically decomposes into aminoacetone and CO_2 (Elliott, 1958) or converted to glycine and acetyl-CoA in a CoA dependent reaction by 2-amino 3-ketobutyrate CoA ligase (Aoyama and Motokawa, 1981; McGilvray and Morris, 1969). Glycine then facilitates one-carbon metabolism via the glycine cleavage system to promote nucleotide synthesis and acetyl-CoA directly enters into the tricarboxylic acid cycle (Kaelin and McKnight, 2013). The reaction catalyzed by TDH is the first and rate-limiting step in the L-Thr degradation pathway. TDH is responsible for the bulk of threonine breakdown and is widely distributed in a broad range of species from bacteria to mammals (Aronson et al., 1989; Kazuoka et al., 2003; Wang et al., 2009).

Recent studies revealed that mouse TDH (mTDH) mediated-threonine catabolism plays a central role in the maintenance of mouse embryonic stem cells (mESCs) self-renewing and the regulation of mouse somatic cell reprogramming (Han et al., 2013; Kaelin and McKnight, 2013; Wang et al., 2009). It has been reported that mESCs depend exclusively on threonine and express exceptionally high levels of mTDH relative to differentiated cells (Wang et al., 2009). Specific chemical inhibitors of the mTDH

Abbreviations: TDH, L-threonine dehydrogenase; mTDH, L-threonine dehydrogenase from mouse; L-Thr, L-threonine; mESC, mouse embryonic stem cell; GalE, UDP-galactose 4-epimerase; SDR, short-chain dehydrogenase/reductase; MDR, medium-chain dehydrogenase/reductase; WT, wild-type; PRMT5, Protein arginine methyltransferase 5; ASU, asymmetric unit.

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enzyme selectively killed mouse ES cells but failed to affect the growth of differentiated cells (Alexander et al., 2011). Furthermore, mTDH-mediated threonine catabolism might influence the amount of S-adenosylmethionine, resulting in regulation of histone methylation in pluripotent stem cells (Shyh-Chang et al., 2013). It has also been indicated that mTDH induction also enhances reprogramming of mouse somatic cells into induced pluripotent stem cells. Protein arginine methyltransferase 5 (PRMT5) regulates mTDH enzyme activity through binding with mTDH and/or mediating specific arginine (Arg180) methylation of mTDH and functionally enhanced mTDH-facilitated reprogramming efficiency (Han et al., 2013). Moreover, in *Trypanosoma brucei*, TDH is one of enzymes bearing on threonine-derived acetate production for lipid biosynthesis (Millerioux et al., 2013). Inhibition of TDH and another protein participating in glucose-derived acetate production is lethal for the procyclic trypanosomes grown. Thus, TDH would be a target protein to design medicines of trypanosomiasis and mTDH is expected to be template to design the medicine which affects trypanosoma but not mammalian TDH in future. In spite of the pivotal role of mTDH, its three-dimensional structure has not been determined.

Previous reports mainly focused on the catalytic properties and mechanisms of TDH enzymes from lower prokaryotic organisms. Structures of two types of TDH enzymes have been reported, which are all from bacteria or archaea. One type is classified within the medium-chain dehydrogenase/reductase (MDR) superfamily. MDR-type TDHs are similar to Zn-dependent alcohol dehydrogenases with a Rossmann-fold domain in their C-terminal regions and have members mainly in bacteria (Bowyer et al., 2009; Hedlund et al., 2010; Ishikawa et al., 2007; Persson et al., 2008). The other type is classified within the short-chain dehydrogenase/reductase (SDR) superfamily. Most SDR enzymes are homodimers with a NAD(P)$^+$ binding Rossmann-fold domain in their N-terminal regions (Bray et al., 2009). To date, crystal structures of three SDR-type TDHs identified in the psychrophilic bacterium *Flavobacterium frigidimaris* KUC-1 (*Ff*TDH) (Yoneda et al., 2010), the thermophilic archaeon *Thermoplasma volcanium* (*Tv*TDH) (Yoneda et al., 2012) and *Cupriavidus necator* (*Cn*TDH) (Nakano et al., 2014) have been reported, which are similar to UDP-galactose 4-epimerases (GalEs). Mammalian TDHs belong to the SDR superfamily from sequence. However, structural description of mammalian SDR-type TDH is still lacking.

Moreover, it was reported that Arg180 of mTDH plays a regulatory role in catalysis. The enzyme activity decreased when Arg180 was mutated to a lysine and might be increased when Arg180 was methylated by PRMT5 (Han et al., 2013). Interestingly, this residue is not conserved in prokaryotic bacteria or archaea, but conserved in some eukaryotic organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster*, zebrafish. It might represent a distinction between higher eukaryotic and lower prokaryotic SDR-type TDH enzymes. How Arg180 plays a role in catalysis remains uncertain.

In the present study, we solved three crystal structures of the mTDH enzyme: mTDH wild-type (WT) in complex with NAD$^+$ and a substrate analog, glycerol (mTDH/NAD$^+$/glycerol), or only NAD$^+$ (mTDH/NAD$^+$); WT without NAD$^+$ or any substrate (mTDH apo); and mTDH R180K mutant bound with NAD$^+$ (mTDH-R180K/NAD$^+$). This is the first description of a structure for mammalian SDR-type TDH. Our structures showed that the structural basis for the catalytic mechanism of SDR-type TDHs is highly conserved in bacteria and mammals and mTDH also undergoes stepwise dynamical structural changes during catalysis. Moreover, Arg180 of mTDH, which is not conserved in prokaryotic genera, contributes to the stability of its surrounding region and is located distal to the active center. Kinetic enzyme assay and ITC, in combination with the NAD$^+$-complexed R180K crystal structure and biochemical and spectroscopic methods indicated the R180K mutant should bind NAD$^+$ in a similar way and have a similar folding to the WT. However, the R180K mutant exhibited a decreased k_{cat}/K_m value toward the substrate, L-Thr. It is possibly because the R180K variant may have difficulty adopting the closed form upon the substrate binding due to reduced interaction of this residue with its surrounding atoms. In summary, our observations suggest the Arg180 works as a remote switch to convert the closed and open forms during mTDH catalysis.

2. Materials and methods

2.1. Cloning, expression and purification

The gene encoding mTDH without the predicted N-terminal 46-amino-acid signal sequence was amplified from the mouse cDNA library by PCR and cloned into modified pET-28a (+) (Novagen) vectors, in which the thrombin protease sites were substituted for tobacco etch virus (TEV) cleavage sites. All of the mutants were generated by the Mutant Best kit (Takara). All clones were verified by DNA sequencing. The recombinant proteins were produced in *Escherichia coli* BL21(DE3) cells (Novagen) grown at 16 °C for 20 h. Protein samples for crystallization were purified using a Ni-chelating column (Qiagen), treated with TEV to remove His_6 tag, and further purified on a Ni-chelating column before size-exclusion chromatography using a Hiload 16/60 Superdex 200 column (GE healthcare) equilibrated with protein buffer A (20 mM Tris–HCl [pH 8.0], 0.5 M NaCl and 1 mM EDTA).

2.2. Crystallization, data collection and structure determination

All the crystals were grown using the sitting drop vapor diffusion method at 10 °C. The mTDH WT enzyme in the apo form was crystallized by mixing equal volumes of 7.5 mg/ml protein and crystallization buffer 1 (0.1 M HEPES [pH 7.0], 10% w/v PEG4000, 10% v/v 2-propanol). Crystals of mTDH WT in complex with NAD$^+$ were obtained by mixing an equal volume of crystallization buffer 2 (0.2 M NaCl, 0.1 M HEPES [pH 7.5], 25% w/v PEG3350) and 7 mg/ml protein pre-incubated with NAD$^+$ at 1:2.5 M ratio. Crystals of mTDH R180K with NAD$^+$ were prepared by incubating 7 mg/ml protein with NAD$^+$ at 1:2.5 M ratio, followed by mixing an equal volume of crystallization buffer 3 (0.2 M trimethylamine N-oxide dihydrate, 0.1 M Tris–HCl [pH 8.5], 20% w/v PEGMME2000). The final concentration of NAD$^+$ was 0.5 mM. All the crystals used for diffraction data collection were quickly soaked in reservoir solution containing 25% glycerol (v/v) as cryoprotectant and cooled to 100 K in a stream of nitrogen gas before they were mounted.

Data sets of all the crystals were collected on beam line 17U (BL17U) at Shanghai Synchrotron Radiation Facility (SSRF). The data sets were integrated and scaled with HKL2000/HKL3000 (Minor et al., 2006) or iMosflm (Battye et al., 2011) and SCALA (Evans, 2006) in the CCP4 program suite (Winn et al., 2011). Initial phases of NAD$^+$-complexed mTDH WT were solved by molecular replacement using the program Phaser (McCoy et al., 2007), employing the structure of *Ff*TDH (PDB code: 2YY7) as a template. The structure of mTDH/NAD$^+$ was used as the search model for the other two structures with Phaser. The models were further built and refined using Refmac5 (Murshudov et al., 1997) and COOT (Emsley et al., 2010) by manual model correction. Crystal diffraction data and refinement statistics are presented in Table 1.

On the basis of the structure of glycerol, we modeled the L-Thr molecule into the active site of mTDH/NAD$^+$ and then minimized the energy of the complex using SWISS-PDB VIEWER (http://www.expasy.ch/spdbv/).