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Structural Features of Zinc Coordination in Human MtnB Enzyme and its Implication on the Catalytic Mechanism

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Methionine salvage pathway plays a role of recycling 5-methylthioadenosine (MTA), which is a by-product from the polyamine biosynthesis process, back to the methionine, one of the essential amino acid.[1] The pathway consists of six enzymatic steps in eukaryotes as shown for human and yeast, whereas bacteria have some variations depending on the species.[2] Human MtnB enzyme, 5-methylthioribulose-1-phosphate dehydratase, catalyzes the third step of the pathway, converting 5-methylthioribulose-1-phosphate (MTRu-1-P) to 2,3-diketo-5-methylthiopentyl-1-phosphate (DK-KMP-1-P). The MtnB enzyme is a member of zinc-dependent class II aldolase family which includes FucA, RhuA and RibE.[3] It forms a tetramer in *C4* symmetry as other class II aldolase family members, and the active site is located at the subunit interface. The recent structural and enzymatic studies lead to the proposal of the catalytic mechanism employing Glu139* as an acid/base catalytic residue. In the active site, a zinc ion is octahedrally coordinated by three histidine residues (His115, His117 and His195) and three water molecules.[2]

Zinc ion is often incorporated in the active site of various enzymes with typical coordination number of 4 to 6 which is comparatively more flexible than other metal ions.[4] MtnB contains one zinc ion per subunit with six ligands in octahedral geometry.[2] Even though its crystal structure was reported recently, the geometry of the catalytic zinc ion was not fully analyzed because the previous study interpreted the crystal structure mainly with its implication in the enzymatic activity and cell death inhibitory role.[2] Thus here we analyzed the coordination geometry of the catalytic zinc ion in MtnB, which led us to better understanding of the catalytic mechanism. In order to assess the catalytic and structural roles of these three histidine residues coordinating to the catalytic zinc ion in the active site, we generated their alanine mutants and checked the consequence in the enzyme activity and the structural integrity.

Materials and Methods

Site-directed Mutagenesis. Three mutants of human MtnB (UniProtKB accession code Q96GX9) on its three catalytic histidine residues (His115, His117 and His195) were generated using QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA). The primers used are 5'- gca ggt gca gtg att GCT acc cac tct aaa gct -3' and 5'- agc ttt aga gtg ggt AGC aat cac tgc acc tgc -3' for H115A; 5'- gca gtg att cat acc GCC tct aaa gct gct gtg -3' and 5'- cac agc agc ttt aga GGC ggt atg aat cac tgc -3' for H117A; 5'- gta ctg gtc aga cgt GCT gga gta tat gtg tgg -3' and 5'- cca cac ata tac tcc AGC acg tct gac cag tac -3' for H195A; 5'- gca ggt gca gtg att GCT acc GCC tct aaa gct gct gtg -3' and 5'- cac agc agc ttt aga GGC ggt AGC aat cac tgc acc tgc -3' for H115A/H117A (the upper case denotes the codon for mutating residue). The nucleotide sequence was confirmed (Bionics, Korea).

Protein Expression and Purification. The wild type protein sample was over-expressed in Rosetta 2 (DE3) strain of *E. coli* as described previously,[5] and the protein samples for the mutants were also purified through the same protocol.

Circular Dichroism Spectroscopy. The concentration of the purified human MtnB wild type and mutants was adjusted to 0.9 mg/mL. CD spectra were acquired at 298 K on a Circular Dichroism System (Applied Photophysics, UK) with a 0.2 mm path-length quartz cuvette. Each spectrum was measured with 0.2 sec integration time at 1.0 nm wavelength increments. Three independent measurements were averaged.

Enzyme Assay. MtnB (5-methylthioribulose-1-phosphate dehydratase) enzyme activity was measured in a coupled reaction with *Bacillus* 2,3-diketo-5-methylthiopenyl-1-phosphate enolase (MtnW) as reported previously.[3] In detail, the enzyme activity of MtnB was assayed by measuring the increase of absorbance at 280 nm for HK-MTPenyl-1-P (2-hydroxy-3-keto-5-methylthiopentenyl-1-phosphate) produced in the coupled reaction. The substrate MTRu-1-P (5-methylthioribulose-1-phosphate) was produced in 100 μL of the reaction mixture containing 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 28 μg of *Bacillus* methylthioribose-1-phosphate isormerase (MtnA), and the concentration of MTRu-1-P in the reaction mixture was calculated from the equilibrium constant between MTRu-1-P and MTR-1-P ([MTRu-1-P]/[MTR-1-P] = 6.0). The reaction was started by adding the limiting amount (0.3 μg in 0.2 μL) of the APIP/MtnB protein to the reaction mixture, and was monitored at 280 nm with HP 8453 UV-Visible Spectrophotometer (Hewlett Packard, USA). The concentration of the product, HK-MTPenyl-1-P, was calculated from its molecular extinction

coefficient, 9.5×10^3 M^{-1} cm^{-1} at 280 nm. Three independent measurements were performed. The substrate for the coupled reaction, MTR-1-P (5-methylthioribose 1-phosphate), was synthesized through the enzyme reaction of MTAP, 5-methylthioadenine phosphoyrylase, as described.[6]

Results and Discussion

We recently reported the crystal structure of human MtnB enzyme (Protein Data Bank ID 4M6R).[2] As described therein, the MtnB enzyme forms a tetramer and each subunit contains a zinc ion in the active site.[2] Despite the essential role of the zinc ion in the catalysis, the coordination geometry and its functional implications were not addressed in the previous study. So we initiated the relevant studies here by analyzing the geometric features of the zinc coordination. As summarized in Table 1, the bond distances from the zinc ion to three histidine residues are 2.11 Å for His115, 2.12 Å for His117, and 2.18 Å for His195 in average of the four subunits, which are in the typical range of zinc coordination distance found in protein structures.[4] The mean distances to water molecules are 2.14 Å for Wat115, 2.47 Å for Wat117, and 2.23 Å for Wat195, which are a little longer than the distance to histidine residues. Notably, one of the three water molecules, Wat117, has an appreciably longer distance than other five ligands and concurrently the zinc ion is positioned closer to His117, which results in deviation from the planarity of zinc coordination, as shown in Figure 1. The deviation from the planarity can be described by the bond angles of His117, zinc and other ligands; the angles are 100.3° for His117-zinc-His195, 98.9° for His117-zinc-Wat195, whereas 79.4° for Wat117-zinc-Wat195 and 81.9° for Wat117-zinc-His195 (Fig. 1). These features suggest that the geometry of zinc coordination in MtnB can be considered as in between six-liganded octahedral and five-liganded pyramidal geometry, and presumably that the coordination number could alternate between six and five depending upon the circumstances. This point seems to be important regarding the proposed catalytic mechanism, in which the coordination number changes as the reaction proceeds. Specifically, the substrate-free structure observed in the crystal structure has the formal coordination number of six, whereas the substrate-bound model from *in silico* docking adopts a five-liganded square pyramidal geometry in which two oxygen atoms, O2 and O3, of the substrate, MTRu-1-P or 5-methylthioribulose-1-phosphate, are liganded. This zinc coordination geometry of the substrate-bound MtnB structure is similar to the other class II aldolase family members that bind to the common substrate-analog (PGH, phosphoglycolohydroxamate) also in the five-liganded square pyramidal geometry in which two oxygen atoms of the substrate-analog are liganded. In the proposed catalytic mechanism of MtnB enzyme, the reaction proceeds via enol intermediate and free hydroxide ion. The intermediate hydroxide ion could be stabilized by coordinating to zinc ion. This intermediate state involving an enol and a hydroxide would adopt the six-liganded zinc geometry. Conclusively, the zinc coordination geometry in the active site of MtnB seems to be flexible as implied by its intermediary geometry between six- and five-liganded states observed in the crystal structure, and this flexibility of the zinc would be essential to accommodate the substrate and the intermediate as the catalysis proceeds.

In order to assess the structural and functional significance of three zinc-coordinating histidine residues, we generated their single mutants, *i.e.* H115A, H117A and H195A, and one additional double mutant, H115A/H117A. The four mutants were investigated for the structural integrity and the enzymatic activity. Firstly, we performed the circular dichroism (CD) spectroscopy analysis for the mutants. The

Table 1. Bond distance between zinc and the ligand, Å

	A	B	C	D	Mean ± S.D.
His115	2.10	2.08	2.15	2.12	2.11 ± 0.03
His117	2.13	2.08	2.16	2.10	2.12 ± 0.04
His195	2.25	2.13	2.20	2.14	2.18 ± 0.06
Wat115	2.09	2.22	2.08	2.15	2.14 ± 0.06
Wat117	2.69	2.49	2.40	2.30	2.47 ± 0.17
Wat195	2.40	2.16	2.18	2.17	2.23 ± 0.12

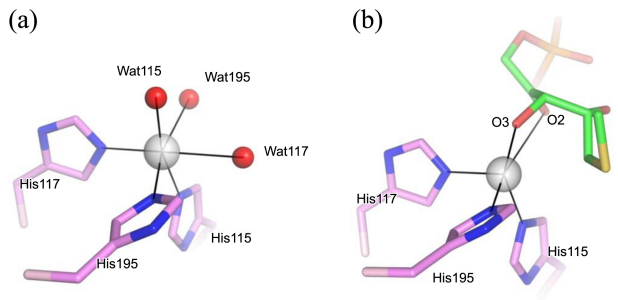

Figure 1. Coordination geometry of the catalytic zinc. The ball in gray color represents the catalytic zinc ion. (a) Apo structure with a six-liganded octahedral geometry. (b) Substrate-bound structure with a five-liganded pyramidal geometry. O2 and O3 of the substrate, MTRu-1-P, occupy the positions of Wat195 and Wat115 respectively.

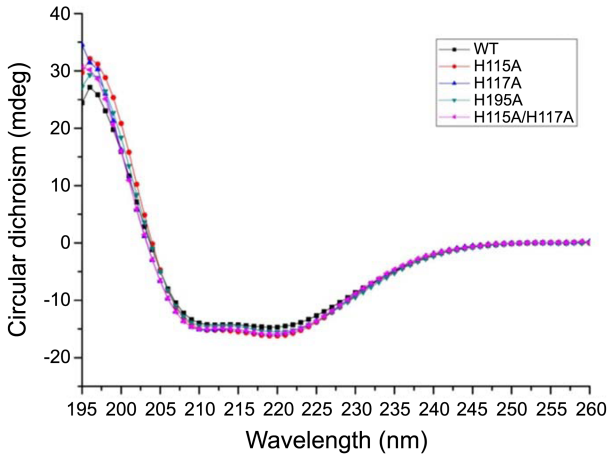

Figure 2. Circular dichroism spectrum of MtnB wild type and the mutants.

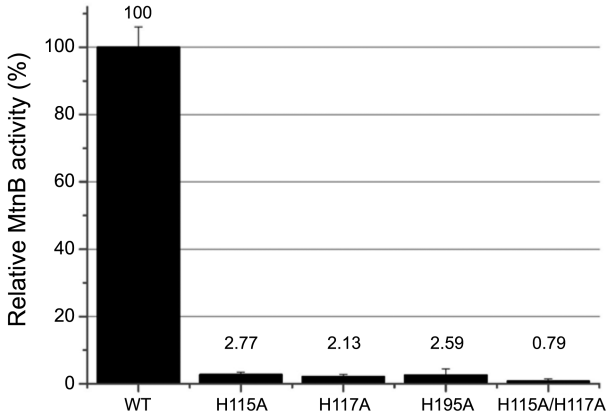

Figure 3. Mutation effects of three histidine residues on the catalytic activity. Data represent mean relative specific activities of mutants with the standard error of three independent measurements. The wild type's specific activity is set to 100%.

spectra are so characteristic of the α/β-fold protein,[7] which are well consistent with its crystal structure (Fig. 2). Importantly, the spectra of all the mutants overlap so well with the wild type in all range of scanned wavelength, which strongly implies that there is no significant structural disturbance induced by the mutations (Fig. 2). Given that the loss of one or two ligands, histidine residues in this case, must diminish the zinc binding to any extent, this CD spectroscopy results indicate that the global fold stability may not be significantly dependent upon the zinc-histidine coordination. In other words, it may imply that the existence of zinc ion in the active site is not detrimental for the stability of the global fold. Secondly, we measured the relative specific activity of the mutants. All the tested mutants showed drastic decrease in the catalytic activity: the three single mutants retains only 2.13% to 2.77% of the wild type's activity, and the double mutant, H115A/H117A, showed 0.79%, reflecting that the zinc binding in the active is critical for the catalysis.

In summary, we analyzed the geometric features of zinc coordination in the active site of MtnB and checked the mutational effects of three histidine residues coordinating to the zinc on the global fold and catalytic activity. The results indicate that the catalytic zinc ion seems to have variability in the coordination number from 5 to 6 and this variability must be important to accommodate the substrate and the intermediate as the reaction proceeds. In addition, the coordination of histidine residues to the zinc is crucial for the catalysis of MtnB, but is not essential for maintaining its global fold.

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