A Novel Mercaptopyruvate Sulfurtransferase Thioredoxin-Dependent Redox-Sensing Molecular Switch: A Mechanism for the Maintenance of Cellular Redox Equilibrium

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Abstract: An intermolecular disulfide bond serves as a thioredoxin-dependent redox-sensing switch for the regulation of the enzymatic activity of 3-mercaptopyruvate sulfurtransferase (MST, EC.2.8.1.2). A cysteine residue on the surface of each subunit was oxidized to form an intersubunit disulfide bond so as to decrease MST activity, and thioredoxin-specific conversion of a dimer to a monomer increased MST activity. Further, a low redox potential sulfenate was reversibly formed at a catalytic site cysteine so as to inhibit MST, and thioredoxin-dependent reduction of the sulfenate restored the MST activity. Concludingly, MST partly contributes to the maintenance of cellular redox homeostasis *via* exerting control over cysteine catabolism.

Key Words: Atmospheric oxygen, antioxidative stress, intermolecular disulfide bond, mercaptolactate-cysteine disulfiduria, mercaptopyruvate sulfurtransferase, molecular evolution, redox-sensing switch, thioredoxin.

INTRODUCTION

3-Mercaptopyruvate sulfurtransferase (MST, EC.2.8.1.2) is a 33 kDa simple protein enzyme which is widely distributed in prokaryotes and eukaryotes [1]. Eukaryotic MST is localized in the cytosol and mitochondria [2, 3], and catalyzes a transsulfuration from mercaptopyruvate to pyruvate in a step of degradation process of cysteine. MST possesses thiosulfate sulfurtransferase (TST, EC.2.8.1.1) activity (a transsulfuration from thiosulfate to sulfinate) [1, 4], and detoxifies environmental cyanide *via* a conversion of cyanide to thiocyanate [5-7].

Our previous study using protein engineering revealed that reciprocal conversion of catalytic properties between MST and TST was successful, which provided strong evidence that MST and TST were evolutionarily related enzymes [1, 4]. MST and TST consist of an N-terminal catalytically inactive domain and a C-terminal catalytically active domain; molecular evolution of the N-terminal domain has been extensively discussed [8]. A primitive TST molecule is a potential precursor of MST [9].

Recently we found that mammalian MST has a novel intermolecular disulfide bond between the dimer, which serves as a redox-sensing switch for the regulation of MST activity, and contributes to the maintenance of cellular redox equilibrium *via* control of cysteine catabolism [10]. Interestingly, mammalian TST has also evidently acquired the switch. From the point of view of molecular evolution and phylogemetics, the switch evolved in MST and TST during and after the increase of the oxygen concentration in the Earth's atmosphere [9]. Therefore, MST and TST serve as an antioxidant protein and/or an environmental adaptation protein [10-12].

Congenital insufficiency or deficiency of MST activity causes an inherited metabolic disorder, mercaptolactate-cysteine disulfiduria (MCDU), with symptoms of oversecretion of mercaptolactate-cysteine disulfide in urine, with or without mental retardation [13-19]. However, the specific pathogenesis of mental retardation has not been clarified. The results of our previous studies suggest the hypothesis that MST can effectively function as an antioxidant in the developing fetal organs, especially the brain, with its increasing oxygen concentrations in the course of development.

In this review, I focus on the intermolecular thioredoxin-dependent redox-sensing switch of MST in comparison with the classical redox-sensing switches which act *via* an intramolecular disulfide bond [20-28]. A redox-sensing molecular switch formed *via* an intermolecular disulfide bond

A REDOX-SENSING MOLECULAR SWITCH FORMED *VIA* AN INTERMOLECULAR DISULFIDE BOND

a) Behind the Discovery

Rat MST was activated with dithiothreitol (DTT) or reduced thioredoxin; DTT-treated MST was activated with reduced thioredoxin to approximately 2-fold the the activity level, but thioredoxin-treated MST was not activated with DTT [10]. The two different modes of the enzymatic activation indicate the presence of two different sites of action for thioredoxin. The first site is a catalytic site cysteine, which oxidants easily oxidize to inhibit MST *via* formation of a sulfenate, and thioredoxin reduces this to restore the activity [10] (described in the latter section). Furthermore, MST exhibits a monomer-dimer equilibrium *via* an intermolecular disulfide bond, and a change in the equilibrium depending on the redox status. Thus, we hypothesized that the second site was an intermolecular disulfide bond, which served as a redox-sensing switch for regulation of MST activity.

b) Structural and Functional Properties of the Redox-Sensing Molecular Switch

Mammalian MSTs and TSTs contain two or three exposed and two buried cysteines; one of the two or three ex-

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posed cysteines is a catalytic Cys^{247} (rat and human MST) in the active site (Figs. **1** and **2**). The exposed cysteine residue on the surface corresponding to the rat (human) Cys^{263} is

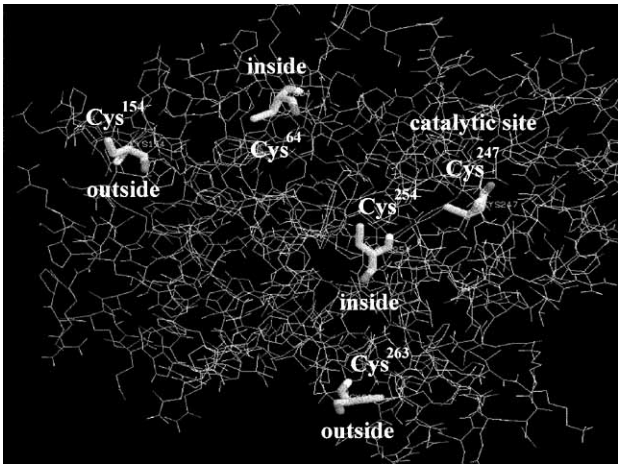

Fig. (1). Five cysteines in rat MST.

The ternary structure of rat MST was estimated with a computer, based on the nucleic acid sequence data of rat MST [1], and the x-ray structure of bovine rhodasene [39].

conserved among mammalian MSTs, but rat Cys^{154} is unique. Rat authentic hepatic and recombinant MSTs exhibit a monomer-dimer equilibrium, and the ratios of the monomer to the dimer were approximately 2 to 1, and 9 to 1, respectively, in 0.2 M potassium phosphate buffer, pH 7.0 , containing no reductants under air-saturated conditions [10, 29], which means that an unusual oxidant other than oxygen *per se* reacts with the exposed cysteine residue *in vivo*. HPLC analysis of mutant enzymes (C254S and C263S) strongly suggested that a symmetrical or asymmetrical dimer was formed *via* an intersubunit disulfide bond between Cys^{154} and Cys^{154}, Cys^{154} and Cys^{236}, or Cys^{236} and Cys^{236} [10] (Fig. **3**). Reduced thioredoxin or a reducing system containing thioredoxin, thioredoxin reductase, and NADPH cleaved the intersubunit disulfide bond to activate MST to approximately 2-fold the level of activity of DTT-treated MST (Fig. **4**). On the other hand, reduced glutathione and DTT affected the switch less. As the mid-redox potential of DTT is lower than that of reduced thioredoxin, the reaction specificity is determined by the structural properties of rat MST, in which the amino acid sequences around Cys^{154} and Cys^{263} are related or identical to parts of the rat and / or *E. coli* thioredoxin reductase sequence [10].

In the enzymatic activation experiment, I recognized that the ratio of the concentrations of MST, thioredoxin, thioredoxin reductase, and NDPH in the reducing system was critical for the enzymatic activation of MST (Fig. **5**). The ratio in the most effective reducing system was 1 : 1 : 0.05: 12.5 for [rat MST] : [rat thioredoxin] : [rat thioredoxin reductase] : [NADPH], and was 1 : 5 : 0.02 : 12.5 for [rat MST] : [*E. coli* thioredoxin] : [*E. coli* thioredoxin reductase] : [NADPH] [10]. The results suggest two questions.

First, why is the effective concentration of thioredoxin reductase for the activation of MST low? It is estimated that MST and thioredoxin reductase compete in the binding of thioredoxin, and the affinity of MST to thioredoxin is much lower than that of thioredoxin reductase. Therefore, the concentration of thioredoxin reductase is sufficiently low not to deprive MST of thioredoxin.

Second, why are the effective concentrations of thioredoxin and thioredoxin reductase different between the rat and *E. coli* reducing systems? A reducing system consisting of either a combination of rat thioredoxin and *E. coli* thiore-

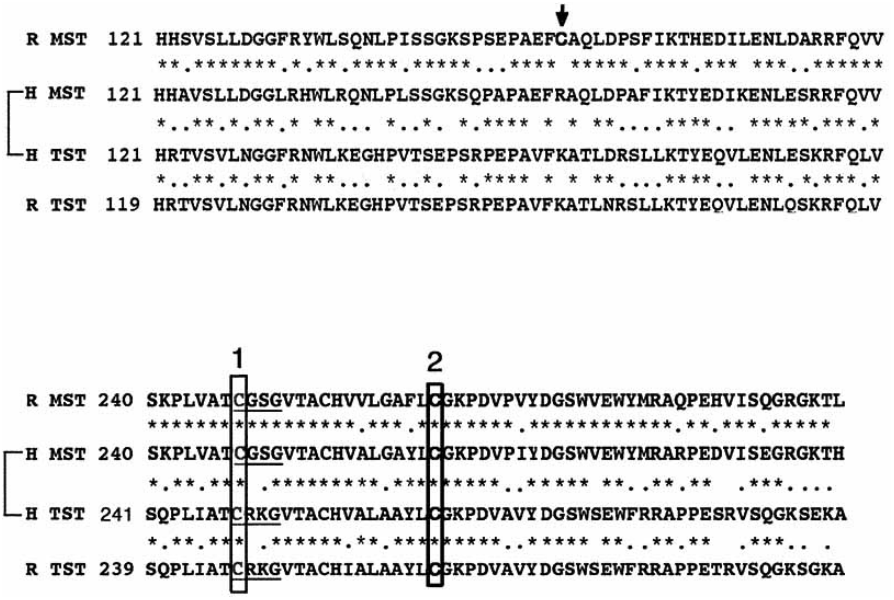

Fig. (2). The cysteine(s) composing a redox-sensing molecular switch.

Comparison of the amino acid sequences among the rat and human rhodanese family enzymes. H, *Homo sapiens* (BC009450 for MST, D87292 for TST); R, *Rattus norvegicus* (D50564 for MST, BC088449 for TST). Box1, the catalytic site; Box2 and arrow, the redox-sensing switch; , a related amino acid residue to that of human MST; *, an identical amino acid residue to that of human MST.

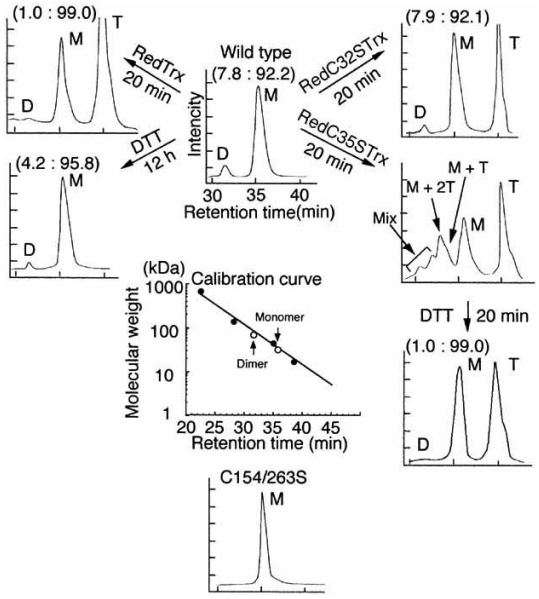

Fig. (3). HPLC data for untreated and treated wild-type MST with thioredoxin.

An arrow labeled "RedTrx 20 min" (the upper left) means that after *E. coli* reduced thioredoxin (1.5 nmol) was added to the wild-type MST (0.3 nmol), and the mixture was incubated on ice for 20 min. Another arrow labeled "RedC35STrx 20 min" (lower right) implies that *E. coli* reduced C35S thioredoxin instead was added. The arrow points to a seriously altered profile, suggesting formation of MST-thioredoxin adducts. An arrow proceeding downward labeled "DTT 20 min" implies that DTT (1.5 mM) instead was added, and the mixture was incubated in ice for 20 min. That arrow points to a profile indicating disappearance of the adducts. Another arrow labeled "RedC32STrx 20 min" (upper right) implies that *E. coli* reduced C32S thioredoxin instead was added. The profile was not changed. In another experiment, an arrow labeled "DTT 12 h" (lower left), implying that DTT (1.5 mM) was added, and the mixture was incubated on ice for 12 h. In the middle graph, a calibration curve shows that retention times of standard proteins [thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), and equine myoglobin (17 kDa)] for the calibration are 22.2, 28.2, 33.3, and 38.3 min, respectively. D, a dimer MST (66.6 kDa, 30.9 min); M, a monomer MST (32.8 kDa, 32.0 min); T, *E. coli* thioredoxin (11.8 kDa, 40.5 min); Mix, mixture of thioredoxin-MST complexes (D, D + T, D + 2T, D + 3T, D + 4T, and M + 2T). Elution profile for the C154/253S MST containing no dimer is shown at the bottom. (Reproduced Figure 3 from Nagahara, *et al*., *J. Biol. Chem*., **2007**, *282*, 1566).

doxin reductase, or *E. coli* thioredoxin and rat thioredoxin reductase , was not available for the activation of MST. Eukaryotic thioredoxin reductase effectively catalyzes the reduction of eukaryotic thioredoxin, but does not effectively catalyze the reduction of prokaryotic thioredoxin (Fig. **6**). On the other hand, rat MST well reacts with both the rat and *E. coli* thioredoxins.

It is concluded that redox status turns the redox-sensing switch on and off to regulate MST activity at the enzymatic level, and MST contributes to maintain the cellular redox homeostasis *via* regulation of cysteine catabolism [10, 13, 29].

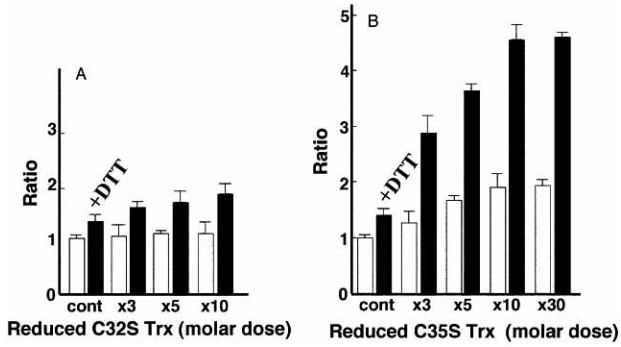

Fig. (4). Contribution of Cys^{32} of *E. coli* thioredoxin to MST activation.

Wild-type MST (12 μM) was incubated with (A) various concentration of *E. coli* reduced C32S thioredoxin (0, 36, 60, and 120 μM), or (B) various concentration of *E. coli* reduced S35S thioredoxin (0, 36, 60, 120, and 360 μM) in 10 μl of 20 mM potassium phosphate buffer, pH 7.4 on ice for 30 min, a 5-μl aliquot with or without 1.2 mM DTT was used for the rhodanese activity. Data are shown as the mean ± SE (bar), (n = 3). (Reproduced Figure 3 from Nagahara, *et al*., *J. Biol. Chem*., **2007**, *282*, 1567).

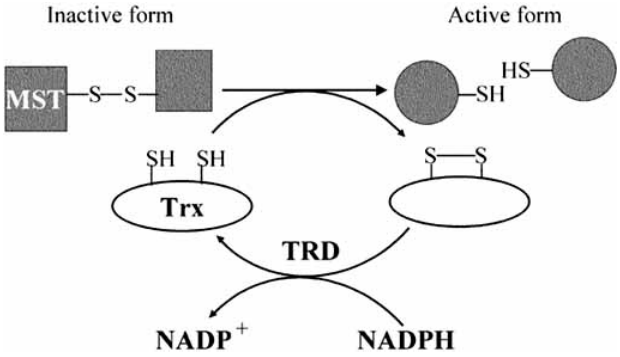

Fig. (5). Regulation of the redox-sensing molecular switch.

Redox change turns the switch on and off. The thioredoxin-dependent MST activation was regulated by a reducing system containing thioredoxin, thioredoxin reductase and NADPH. In the reaction mixture, MST and thioredoxin reductase may compete in binding thioredoxin. Trx. thioredoxin; TRD, thioredoxin reductase.

c) Regulatory Mechanism of the Switch by Thioredoxin

Thioredoxin contains two redox-active cysteine residues (Cys^{32} and Cys^{35} in *E. coli* thioredoxin, and Cys^{31} and Cys^{34} in rat thioredoxin), which are involved in the reduction of the oxidized counterparts. *E. coli* reduced C32S thioredoxin did not activate MST [10] (Fig. **4**). On the other hand, *E. coli* reduced C35S thioredoxin dose-dependently activated MST [10] (Figs. **4** and **7**). HPLC analysis revealed that *E. coli* reduced C32S thioredoxin did not affect the dimer, but *E. coli* reduced C35S thioredoxin did [10] (Fig. **3**). An MST-C35S thioredoxin complex (44.6 kD), an MST-2xC35S thioredoxin complex (56.4 kDa), an MST-3xC35S thioredoxin complex (68.2 kDa), a 2xMST-C35S thioredoxin complex (77.4 kDa), a 2xMST-2xC35S thioredoxin complex (89.2 kDa, overlapped), and a 2xMST-3xC35S thioredoxin complex (101 kDa) were formed [10] (Fig. **3**), although the

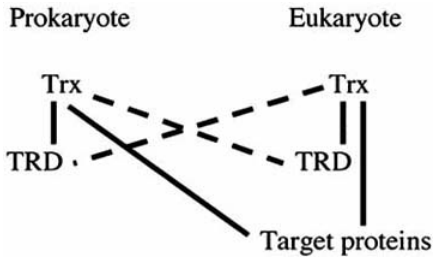

Fig. (6). Species specificity for a combination between thioredoxin and thioredoxin reductase

A solid line, a good combination between the two molecules; a dotted line, an incompatible combination. Trx. thioredoxin; TRD, thioredoxin reductase.

chromatogram did not clearly distinguish all of these MST-thioredoxin complexes. These findings suggest that Cys^{32} of thioredoxin reacts with an intersubunit disulfide bond to form thioredoxin -MST complexes as intermediates.

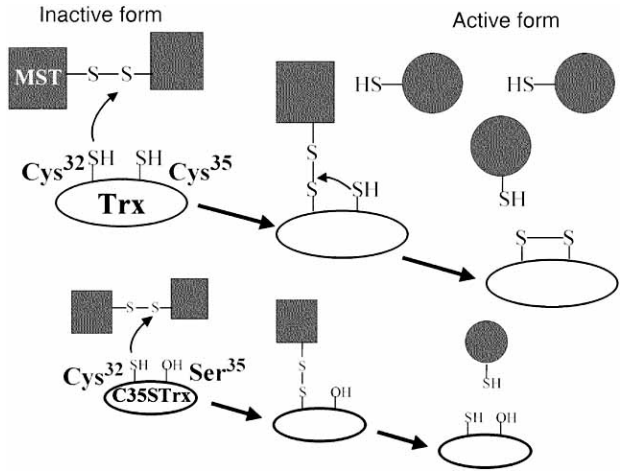

Fig. (7). Attack mode of thioredoxin on MST.

Trx. thioredoxin. Details are provided in the text.

d) Other Intermolecular Redox-Sensing Switches

In a recent report, plant malate dehydogenese also was found to contain an intermolecular disulfide bond, which either thioredoxin-*h*1 or DTT cleaves to activate the enzymatic activity [30]. This machinary is similar to that of MST. Among the other reported proteins possessing an intermolecular redox-sensing switch, three transcriptional regulators; CprK (H_2O_2 / DTT) (effective treatment with an "oxidant / reductant" for this experiment) [31], ArcB sensor kinase (H_2O_2 / DTT or glutathione) [32], and PpsR1 (H_2O_2 / DTT or ferricyanide) [33] are well characterized. In an exceptional case, the hetero-oligomer ATP synthase is inhibited *via* the formation of an intersubunit disulfide bond between the *bc*' and γ subunits due to a mechanical standstill of the molecular motor ($CuCl_2$ / DTT) [34].

Interestingly, human thioredoxin forms a homo-dimer *via* an intermolecular disulfide bond between the Cys^{72} of each molecule [35, 36], but the physiological significance remains unclear. The fact that glutathionylation of Cys^{72} of human thioredoxin abolished the thioredoxin activity [37] is consistent with the fact that the dimer is an inactive form [36] (Fig. **8**).

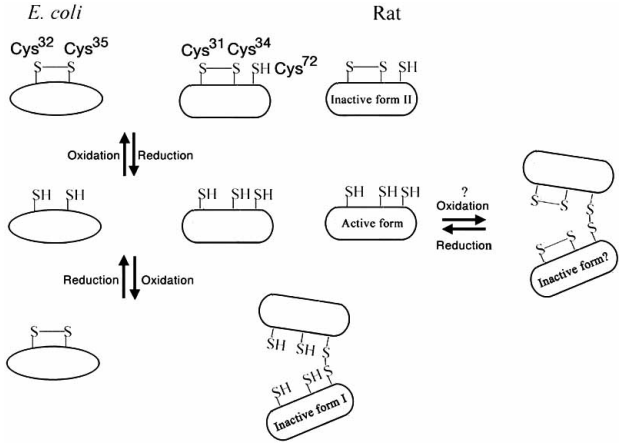

Fig. (8). A possible rat thiorodoxin activation induced by redox change.

In E. coli thioredoxin (NP_418228), exposed cysteines are redox-active Cys^{32} and Cys^{35} alone. In rat thioredoxin (NP_446252), redox active Cys^{61} and Cys^{68} are omitted. Active form, a reduced thioredoxin with full activity; Inactive form I, a dimer form *via* a disulfide bond between Cys^{72} of each monomer without thioredoxin activity due to a steric hindrance; Inactive form II, an oxidized thioredoxin without the activity.

e) Intramolecular Redox-Sensing Switches

The physiological function of a intramolecular disulfide bond serving as a redox-sensing switch includes regulation of enzymatic activity (phosphatase Cdc25B [20], fructose-1,6-bisphosphatase [21, 23], AhpF (NADH:peroxiredoxin oxidoreductase) [35], and acetyl-CoA carboxylase [25]), or translational regulation (transcription factor OxyR [27] and NPH1(protein kinase) [28]).

LOW REDOX POTENTIAL SULFENATE FORMATION AT A CATALYTIC SITE CYSTEINE: ANOTHER MECHANISM BY WHICH MST SERVES AS AN ANTI-OXIDANT PROTEIN

In the C-terminal catalytically active domain, a catalytic site cysteine is conserved in the rhodanese family (Fig. **2**). A catalytic site Cys^{247} in rat MST is a target of the oxidants, which is supported by the results of MALDI-TOF mass spectrometric analysis, and also protein chemical study using iodoacetate [11, 29]. A stoichiometric concentration of hydrogen peroxide is easily oxidized to inhibit the enzyme [11, 38]. The activity of MST can be completely restored by DTT, reduced thioredoxin or thioredoxin with a reducing system containing thioredoxin reductase and NADPH, but reduced glutathione does not restore the activity [11].

In the reduction process, thioredoxin peroxidase activity was detected [11]. Thus, mild oxidation of rat MST resulted in the formation of a sulfenate (SO^-) at Cys^{247} *via* a donation of one electron to the oxidants, i.e., an antioxidant function. It is noteworthy that the redox potential of the sulfenate is lower than that of reduced glutathione. On the other hand, an

excess molar dose of hydrogen peroxide oxidized MST to form a sulfinate or a sulfonate at the catalytic site cysteine, resulting in inactivation of MST.

CONCLUSIONS

1) Oxidants inhibit MST *via* oxidation of a catalytic site cysteine to form a low redox potential sulfenate.

2) At the same time, an inactive form, a dimer is formed *via* an intermolecular disulfide bond.

3) Oxidative stress also inhibits methionine synthase (EC 2.1.1.13) [40, 41], and activates cystathionine β-synthase (EC 4.2.1.22) [42, 43] and glutamate-cysteine ligase (EC 6.3.2.2) [44]. Thus, oxidative stress decreases cysteine degradation, and increases of cellular reductants such as glutathione and thioredoxin.

4) Then, thioredoxin reduces the sulfenate and the disulfide in MST(s) to restore the MST activity.

5) In this process, MST can partly contribute the maintenance of cellular redox equilibrium and the intermolecular disulfide serves as a redox-sensing switch for the regulation of MST activity.

6) These facts suggest that mental retardation in MCDU can be caused by redox imbalance due to congenital defect of MST.

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