Review

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3-Mercaptopyruvate sulfurtransferase: an enzyme at the crossroads of sulfane sulfur trafficking

https://doi.org/10.1515/hsz-2020-0249
Received July 10, 2020; accepted October 5, 2020;
published online October 21, 2020

Abstract: 3-Mercaptopyruvate sulfurtransferase (MPST) catalyzes the desulfuration of 3-mercaptopyruvate to generate an enzyme-bound hydropersulfide. Subsequently, MPST transfers the persulfide's outer sulfur atom to proteins or small molecule acceptors. MPST activity is known to be involved in hydrogen sulfide generation, tRNA thiolation, protein urmylation and cyanide detoxification. Tissue-specific changes in MPST expression correlate with ageing and the development of metabolic disease. Deletion and overexpression experiments suggest that MPST contributes to oxidative stress resistance, mitochondrial respiratory function and the regulation of fatty acid metabolism. However, the role and regulation of MPST in the larger physiological context remain to be understood.

Keywords: 3-mercaptopyruvate sulfur transferase; 3-mercaptopyruvate; hydrogen sulfide; persulfides; rhodanese superfamily; sulfane sulfur.

Introduction

In 1968, the analysis of urine from 2000 subjects (in the context of cystinuria screening) led to the discovery of a previously unknown sulfur-containing metabolite, 3-mercaptolactate (3ML), in one of the subjects (Crawhall et al. 1968). It turned out that the 3ML-excreting subject was lacking an enzymatic activity that normally converts cysteine-derived 3-mercaptopyruvate (3MP) into pyruvate (Figure 1 shows the structure of metabolites discussed in this review). The missing enzyme activity was that of 3-mercaptopyruvate sulfurtransferase (MPST) (Hannestad et al. 1981; Meister et al. 1954; Shih et al. 1977). In the absence of MPST activity, 3MP accumulates and is eventually converted to 3ML by lactate dehydrogenase (Ohta and Ubuka 1989).

Initially, and for a long time, MPST received relatively little attention. However, interest in MPST and its biological functions increased sharply in recent years, mainly for two reasons: Firstly, MPST has been recognized as one of the enzymes involved in the generation of hydrogen sulfide (H_2S), now established as a physiologically relevant signal transmitter (Shibuya et al. 2009; Wang 2012). Secondly, MPST activity has been connected to key cellular processes, including mitochondrial bioenergetics, fatty acid metabolism, and the protection against oxidative stress. Given the renewed and increasing interest in MPST and its functions, it appears that a dedicated review on this enzyme is timely and expedient. Here we summarize the current understanding of MPST, in both mechanistic and physiological terms.

Enzymology and structure of MPST

The catalytic cycle of MPST is shown in Figure 2A. Following binding into the active site pocket, 3MP is desulfurated, to generate a persulfidated active site cysteine and pyruvate as products. Pyruvate is then released from the active site, allowing acceptor molecules to enter. MPST facilitates transfer of the outer sulfur atom of the enzyme-bound persulfide to the acceptor molecule (a process also known as 'transpersulfidation'), after which the sulfurated acceptor is released. The individual steps of the ping-pong mechanism are separated by kinetic pauses (Hanaoka et al. 2017; Lec et al. 2018). 3MP desulfuration is very fast, with a rate constant in the 10^6 M^{-1} s^{-1} range (Lec et al. 2018). The release of pyruvate is thought to be the rate-limiting step of the MPST catalytic cycle, but sulfur transfer may also be rate-limiting, depending on the acceptor molecule (Lec et al. 2018). MPST can be inhibited by its product pyruvate, but only at very high concentrations

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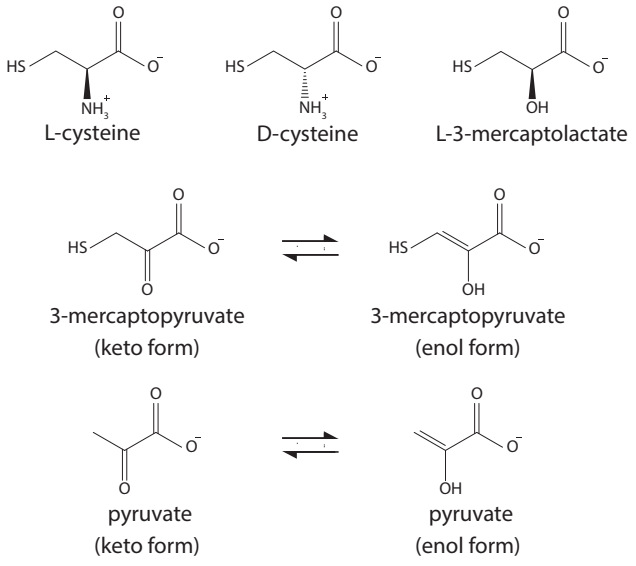

Figure 1: Metabolites discussed throughout this review. Structures of L-cysteine, D-cysteine, L-3-mercaptolactate, 3-mercaptopyruvate and pyruvate. For 3-mercaptopyruvate and pyruvate the keto-enol tautomerization is shown.

(K_i = 6.4 mM, IC_{50} = 13.1 mM) (Porter and Baskin 1996). Other biologically relevant keto acids, such as α-ketobutyrate and α-ketoglutarate, are also weak inhibitors of MPST (Porter and Baskin 1996).

MPST belongs to the rhodanese/Cdc25 phosphatase superfamily (Bordo and Bork 2002). All known MPSTs contain two rhodanese domains. The active site lies at the interface of these two domains, but most of the residues involved in binding and catalysis are located in the C-terminal domain. The catalytic cysteine is part of a conserved six amino acid motif (CG[S/T]GVT) that folds into a cradle-like loop and defines the active site pocket (Figure 2B and C). Variations on the active site motif appear to determine substrate specificity within the rhodanese family. The closely related thiosulfate sulfurtransferase (TST, also known as rhodanese) has a different motif (CRXGX[R/T]) and substrate preference. When the motif in MPST was mutated to match the motif of TST, MPST attained activity towards the typical TST substrate, thiosulfate (Nagahara and Nishino 1996).

Crystal structures of MPST bound to 3MP or pyruvate (PDB ID: 4jgt) further defined enzyme-substrate interactions (Yadav et al. 2013). Two conserved arginines (R188 and R197 in human MPST) position the substrate by forming hydrogen bonds with the carboxylate (R188 and R197) and carbonyl (R197) groups of 3MP (Figure 2B). R188 is essential for both desulfuration and transpersulfidation, while R197 has a more limited role for MPST activity (Kimura et al. 2015, 2017; Lec et al. 2018; Mikami et al. 2011; Nagahara and Nishino 1996; Shibuya et al. 2009). Active site loop residues S250 and T253 contribute to substrate binding: S250 by directly bonding to the 3MP carbonyl group and T253 by forming a water-mediated contact with the 3MP carboxylate (Figure 2B). The conserved Y108 also connects to the carboxylate through a water molecule (Figure 2B).

While the 3MP-coordinating side-chains are relatively well defined, less is known about the exact mechanism of catalysis and the role of side-chains therein. Quantum mechanics/molecular mechanics simulations indicated the feasibility of sulfur atom (S^0) transfer from 3MP to the active site thiolate (Huang and Yu 2016) (Figure 2C). While S^0 transfer is electrostatically unfavorable, the amide groups of the active site loop provide a hydrogen bonding network that stabilizes both thiolates and reduces their electrostatic repulsion by preventing the clash of lone pair orbitals (Huang and Yu 2016; Lec et al. 2018). Following S^0 transfer, the resulting pyruvate is generated as an enolate (Figure 2C) (Huang and Yu 2016; Lec et al. 2018). The nascent enolate carries two negative charges and needs to be protonated to tautomerize into the more stable keto form (Figure 2C, steps 2–4). The origin of the proton is not clear. Initially, it has been proposed that a putative Ser-His-Asp triad (S250/H74/D63), reminiscent of the catalytic motif of serine proteases, serves to donate the proton to the pyruvate enolate (Huang and Yu 2016; Yadav et al. 2013). However, mutagenesis of these residues had only minor effects on S^0 transfer rates (Lec et al. 2018; Nagahara and Nishino 1996; Yadav et al. 2020). Another suggestion is that the proton derives from one of the two active site resident H_2O molecules, the one in contact with T253 (W1 in Figure 2B and C) supposedly being the more likely candidate (Lec et al. 2018). However, neither of the two H_2O molecules is in H-bonding distance to the keto/enolate group (Yadav et al. 2013), and substitution of H_2O by D_2O reduced the maximal 3MP desulfuration rate only by half (Lec et al. 2018). From the crystal structure it seems more likely that the proton originates from R197, whose guanidinium group is in close contact with the 3MP keto group (Figure 2B and C).

It seems remarkable that MPST facilitates the breaking of a C-S bond without the involvement of any cofactors. However, 3MP appears to be a special (if not unique) thiol containing metabolite in that its sulfur is already 'labile' (Toohey and Cooper 2014). This inherent 'lability' of the C-S bond in 3MP seems to be connected to the presence of the β-keto group. Indeed, 3 MP is the only known β-keto thiol that is formed as part of normal metabolism. Due to the presence of the β-keto group, 3MP exists in a keto-enol equilibrium, like pyruvate, the product of 3MP

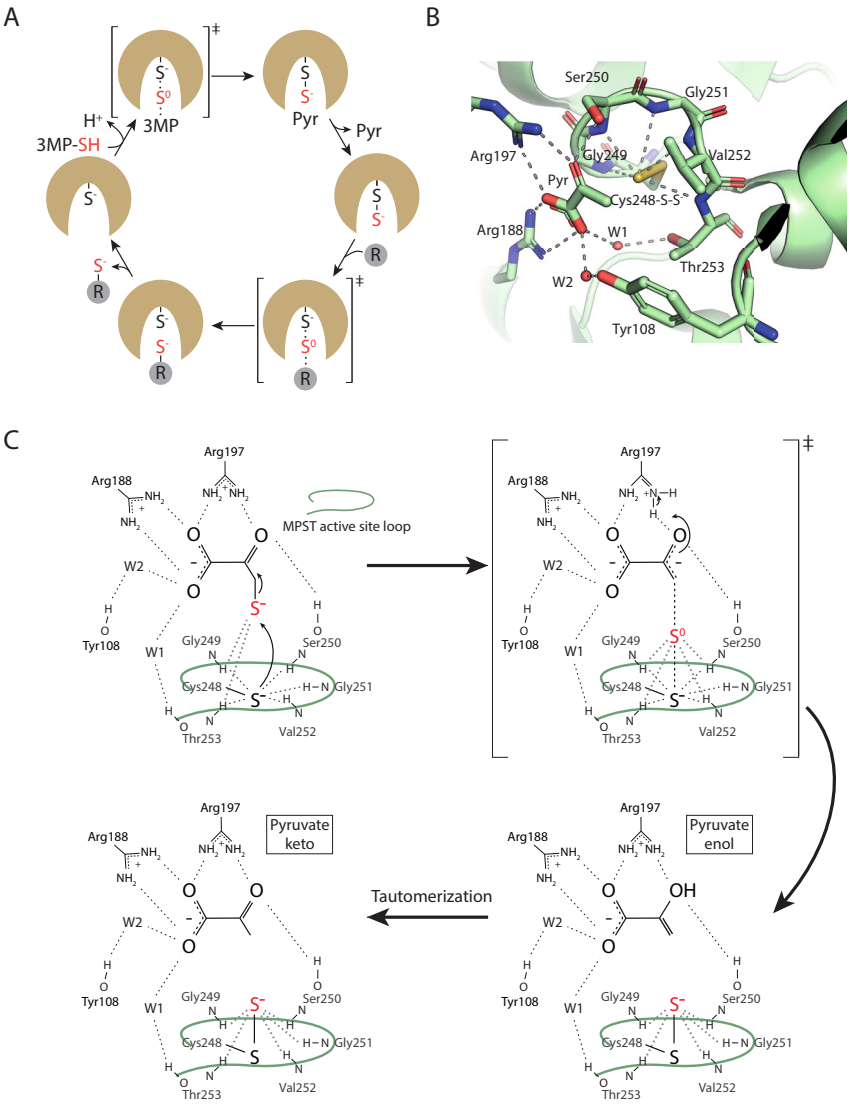

Figure 2: The MPST reaction mechanism. (A) The MPST reaction cycle: MPST catalyzes 3MP desulfuration to generate an enzyme-bound persulfide (-S-S$^-$) and pyruvate (Pyr). Subsequently, pyruvate leaves the active site, allowing for an acceptor molecule (R) to enter. The outer sulfur atom of the persulfide is then transferred to the acceptor molecule, regenerating the active site thiol. Finally, the sulfurated acceptor is released from the active site. The upper left and bottom right species represent transition state intermediates. (B) Crystal structure of human MPST with a persulfidated active site cysteine (C248-S-S$^-$) and pyruvate (Pyr) in the active site pocket (PDB ID: 4jgt). Residues involved in pyruvate binding and persulfide activation are highlighted. Dashed lines represent hydrogen bonds. The guanidinium groups of R188 and R197, the hydroxyl group of S250 and two water molecules (W1 and W2) are involved in pyruvate binding by forming hydrogen bonds with its carboxyl and keto groups. (C) Proposed mechanism for the catalysis of 3MP desulfuration based on sulfur atom (S^0) transfer. Amide groups in the active site loop (G249-T253, green line) form hydrogen bonds with both the active site thiolate and 3MP, drawing them together and promoting their activation. Following sulfane sulfur (S^0) transfer, pyruvate is released as an enolate and subsequently protonated to yield the enol. The origin of the proton is uncertain but, based on proximity, may be derived from the guanidinium group of R197. Finally, the pyruvate enol tautomerizes to generate the keto form.

desulfuration (Figure 1). How does the ability of 3MP and/or pyruvate to tautomerize make the C-S bond more 'labile'? Although 3MP certainly exists in a keto-enol equilibrium, the reactive species is the keto form (Figure 2C). When the active site thiolate of MPST attacks the sulfur atom of 3MP, the C-S bond breaks to form the enolate of pyruvate (Figure 2C), which is expected to be a good leaving group. Protonation of the enolate (as likely facilitated by the active site, see above) makes it an even better leaving group. In the absence of the β-keto group (like e.g. in 3ML or L-cysteine), the breaking of the C-S bond would create a carbanion, unable to delocalize its charge, which is a comparably bad leaving group. In other words, the β-keto group enables a mechanism of leaving group departure that promotes C-S bond cleavage. Interestingly, a similar mechanism of C-S bond 'lability' (based on keto-enol tautomerism) may apply to β-sulfinyl pyruvate (a β-keto sulfinic acid), which can be formed by transamination from cysteine sulfinic acid. Indeed, its C-S bond is reported to be so labile that it spontaneously decays into pyruvate and sulfite (Toohey and Cooper, 2014).

Sulfur acceptors for MPST

Generally, a persulfide can act as a nucleophile (using the outer sulfur atom) or an electrophile (using either the inner or outer sulfur atom) (Huang and Yu, 2016). When a sterically unconstrained persulfide acts as an electrophile, the incoming nucleophile may either attack the inner or the

outer sulfur atom. However, given the choice, it is much more likely to attack the inner sulfur atom, to release the outer sulfur atom as a sulfide anion (HS^-), which is a very good leaving group (Filipovic et al. 2018). If the nucleophile is a thiolate, the result of this reaction is the formation of a disulfide bond between the two reactants.

Importantly, this is not what happens when the MPST-bound persulfide reacts with an incoming nucleophile, which is a thiolate in most cases. The active site loop sterically prevents any nucleophilic attack on the inner sulfur while at the same time it increases electrophilicity on the outer sulfur (Huang and Yu 2016). Hence, any nucleophilic attack can only occur on the outer sulfur atom. This transfers the outer sulfur from the MPST active site persulfide to the receiving acceptor thiol. This process is also called 'transpersulfidation' as it effectively transfers a persulfide group from one thiol to another. Following 'transpersulfidation', the active site thiolate is regenerated in its unmodified form, allowing the enzyme to enter a new cycle. In principle, and in addition to steric constraints, 'transpersulfidation' can be promoted by stabilizing the persulfide in its protonated state (Saund et al. 2015). However, this does not seem to apply to the MPST 'transpersulfidation' mechanism which appears to operate on a deprotonated persulfide, although evidence in support of this notion is still limited (Huang and Yu 2016) (Figure 2B and C).

Known acceptors of the outer sulfur are either small molecule thiols, protein thiols or cyanide (Figure 3). Small molecule monothiol clients include L-cysteine (L-Cys) and glutathione (GSH). Sulfur transfer to L-Cys or GSH generates the respective persulfides, L-CysSSH and GSSH (Figure 3) (Kimura et al. 2015, 2017). It has been proposed that MPST can also catalyze the formation of corresponding polysulfides (L-CysSS_nSH and GSS_nSH, respectively) (Kimura et al. 2017). It is however not obvious how this can happen. In principle, there are two possibilities, both of which appear to be rather unlikely. Firstly, it may be postulated that MPST is catalyzing polysulfidation of its active site cysteine and then transfers the polysulfide chain in one step to the accepting thiol. However, the known geometry of the active site is unlikely to allow additional steps of 3 MP desulfuration when the active site cysteine is already persulfidated. Secondly, it may be postulated that MPST catalyzes repeated sulfur transfers to one and the same substrate molecule, e.g. GSH being sulfurated to GSSH, being in turn sulfurated to GSSSH and so on. Mechanistically this seems possible. However, the large excess of free GSH competing for access to the active site would make this process highly inefficient, unless GSSH possesses a very substantial kinetic advantage over the much more abundant GSH. In any case, the postulated 'polysulfidase' activity of MPST remains a matter of debate, as it has been observed in cell lysates but not in intact cells (Kimura et al. 2017).

Unlike L-Cys or GSH, dihydrolipoic acid (DHLA) is a dithiol, much like the protein client thioredoxin (Trx), both of which harbor a close pair of thiols. In these cases, one of the two thiols forms a persulfide, which is then rapidly attacked at the inner sulfur by the vicinal thiol, thus generating an intramolecular disulfide and H_2S (Figure 3) (Yadav et al. 2013; Mikami et al. 2011; Westrop et al. 2009; Williams et al. 2003). This mechanism explains the involvement of MPST in H_2S generation.

Unlike the MPST active site persulfide, the small molecule and Trx persulfides are not shielded against nucleophilic attack at the inner sulfur atom. In other words, these thiol-containing molecules are not expected to transfer their persulfides to other molecules. Instead, they form intra- or intermolecular disulfide bonds and release H_2S. However, in the specific case of the MPST client protein Mocs3 the situation is different. The persulfidated Mocs3 does not release H_2S, but instead transfers sulfur to the AMP-activated Urm1 (ubiquitin-related modifier 1) protein, generating a thiocarboxylate (COSH) at its C-terminus (Figure 3). Thiocarboxylated Urm1 has two known fates: First, it is a substrate for the thiolation of wobble uridines in $tRNA^{Gln,Glu,Lys}$ anticodons, as catalyzed by the tRNA-thiolating proteins Ncs6 and Ncs2 (Huang et al. 2008; Jüdes et al. 2016; Noma et al. 2009). tRNA thiolation is crucial to avoid codon-specific pauses during translation, which can cause protein aggregation (Nedialkova and Leidel 2015). Second, thiocarboxylated Urm1 is covalently attached to other proteins in a process known as urmylation (Jüdes et al. 2016). However, the exact mechanism and *in vivo* relevance of protein urmylation remain to be defined (Brachmann et al. 2020).

It remains unclear which of the described MPST sulfur acceptors are the physiologically most relevant ones. The interaction between MPST and Mocs3 has been detected in intact cells (Fräsdorf et al. 2014). Apart from Mocs3, kinetic considerations suggest that Trx and L-Cys are the preferred sulfur acceptors (Yadav et al. 2020). MPST can also transfer sulfur to cyanide (CN^-), to generate thiocyanate (SCN^-), which is much less toxic and readily excreted by the kidney. The MPST-catalyzed sulfuration of cyanide is relatively slow (k_{cat}/K_M = 4 x 10^2 M^{-1} s^{-1}), and apparently outcompeted by TST (k_{cat}/K_M = 3 x 10^3 $M^{-1}s^{-1}$) (Libiad et al. 2015; Yadav et al. 2013). Nonetheless, a 3MP prodrug, sulfanegen, has been shown to prevent the lethal effects of cyanide poisoning, suggesting a significant contribution of MPST to cyanide detoxification (Chan et al. 2011). Possible reasons are the presence of MPST in erythrocytes (which

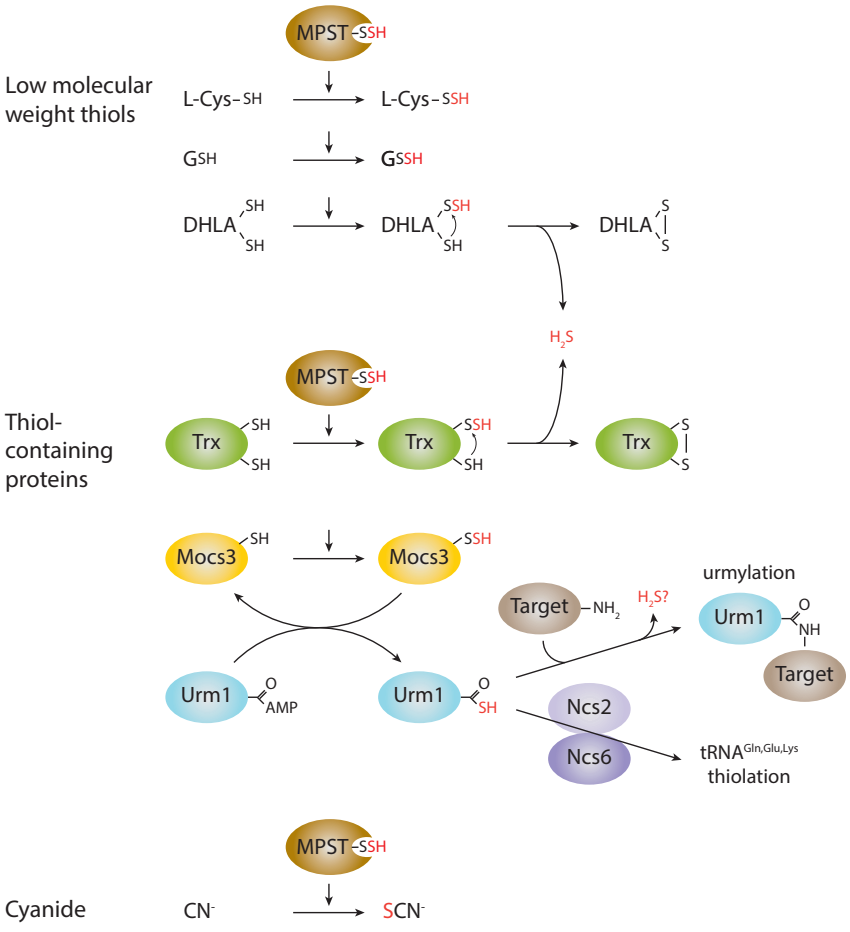

Figure 3: Sulfur acceptors for MPST. L-Cysteine (L-Cys), glutathione (GSH) and dihydrolipoic acid (DHLA) are the most common low-molecular-weight thiol-containing sulfur acceptors. Amongst thiol-containing proteins, thioredoxin (Trx) and the sulfurtransferase Mocs3 are the only known acceptors. In the case of DHLA and Trx, the persulfide is rapidly attacked by the neighboring thiol, leading to H_2S release and the formation of an intramolecular disulfide. In the case of Mocs3, the persulfide outer sulfur is transferred to the Urm1 (ubiquitin-related modifier 1) protein, forming a thiocarboxylate (COSH) at its C-terminus. Thiocarboxylated Urm1 can either participate in protein urmylation or transfer its sulfur to tRNAs. Cyanide (CN^-) is also an acceptor of the MPST sulfur, forming the less toxic thiocyanate (SCN^-).

lack TST), and in the cytosol of most other cells (TST being confined to the mitochondrial matrix).

In sum, experiments and kinetic simulations support a role for MPST-mediated sulfur transfer in tRNA thiolation, protein urmylation, cysteine persulfidation, H_2S generation (via Trx persulfidation), and cyanide detoxification, although it is not clear which acceptor is preferred under which conditions.

Sources of 3MP

The main source of 3MP is the transamination of L-Cys, as catalyzed by glutamate-oxaloacetate aminotransferase (GOT), also called aspartate aminotransferase (AST) or cysteine aminotransferase (CAT) (Figure 4). GOT is a pyridoxal phosphate (PLP)-dependent enzyme that catalyzes the transfer of the L-Cys amino group to α-ketoglutarate, generating 3MP and glutamate as products. Two paralogues are present in all eukaryotes, one cytosolic (GOT1) and one mitochondrial (GOT2) (Figure 4). Both paralogues prefer L-aspartate (L-Asp) over L-Cys (the K_M for L-Asp is ~14x lower for GOT1 and ~42x lower for GOT2) (Akagi 1982; Ubuka et al. 1978). Indeed, it has been observed that L-Asp competes GOT- and MPST-dependent H_2S production (Flannigan et al. 2013; Shatalin et al. 2011; Shibuya et al. 2009). General inhibitors of PLP-dependent enzymes, such as aminooxyacetic acid, also inhibit GOT-dependent 3MP production (Miyamoto et al. 2014). In an alternative pathway, operating in kidney and cerebellum, 3MP can also be generated by the oxidative deamination of D-cysteine, as catalyzed by D-amino acid oxidase (DAO), a peroxisomal protein (Figure 4) (Shibuya et al. 2013).

One of several open questions relates to intracellular 3MP trafficking. Three subcellular compartments (cytosol, mitochondria and peroxisome) can generate 3MP from either L- or D-cysteine, but it is not clear if and how 3MP can move between compartments (Figure 4). Peroxisomes do not contain MPST, which implies that 3MP must leave the peroxisome to be desulfurated by either cytosolic or mitochondrial MPST (Figure 4). Since peroxisomes and mitochondria exchange metabolites (Schrader et al. 2015), 3 MP trafficking between these organelles seems conceivable.

Another key question relates to the amount of 3MP produced in the cell. The inefficient transamination of

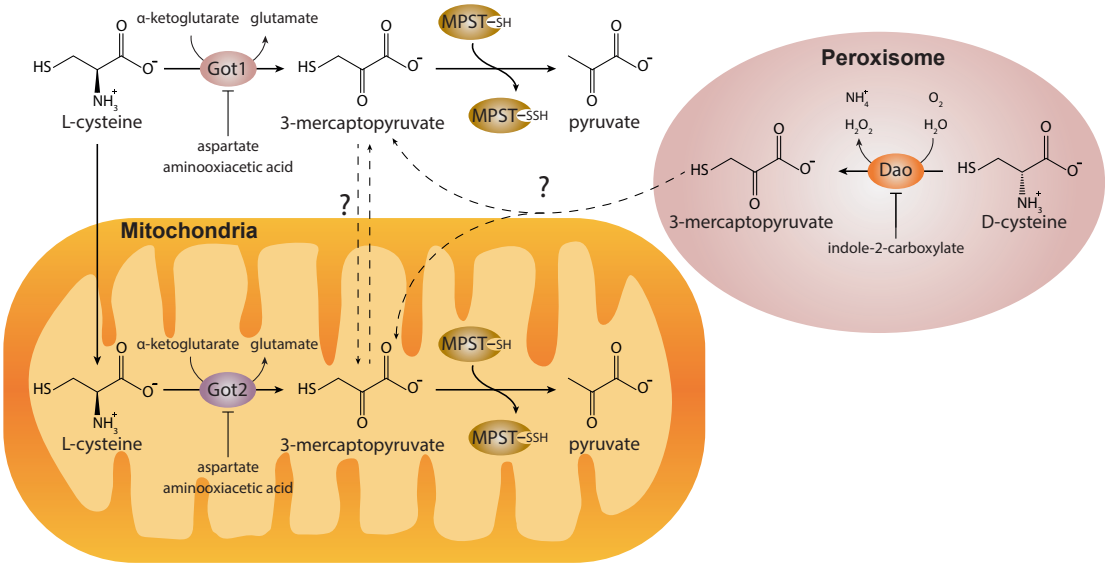

Figure 4: Sources of 3MP and their subcellular location. 3MP can be generated by the transamination of L-cysteine as catalyzed by the cytosolic or mitochondrial glutamate-oxaloacetate aminotransferases (GOT1 and GOT2, respectively). 3MP can also be generated through the oxidative deamination of D-cysteine in the peroxisome, catalyzed by D-amino acid oxidase (DAO). It is unknown whether 3MP moves between subcellular compartments and, if so, by which mechanisms.

L-Cys relative to L-Asp, and the highly restricted tissue expression of DAO would suggest that 3MP is generated in rather small amounts. However, in the absence of MPST activity, cells generate considerable amounts of 3ML, leading to the 3ML-cysteine disulfiduria phenotype found in MPST-deficient patients (Ohta and Ubuka 1989; Shih et al. 1977). This observation indicates that 3MP generation is a significant process in most cells. It is furthermore possible that 3MP production is favored under certain metabolic conditions. For example, transamination of L-Cys should be favored when L-Asp concentrations are low. Cells have a special need for L-Asp to support adenine monophosphate synthesis. However, L-Asp synthesis requires electron acceptors (NAD^+, FAD), which can become limiting when mitochondrial respiration is compromised, thus leading to a shortage of L-Asp (Sullivan et al. 2015). Under these conditions, L-Cys may take over as the main substrate of GOT1/GOT2. In addition to a general shortage of L-Asp, a compartment-specific shortage may also occur. GOT1 and GOT2 are also part of the malate-aspartate shuttle system that translocates electrons between the cytosol and the mitochondrial matrix. Perturbations of the shuttle may lead to compartment-specific L-Asp shortage, thus promoting compartment-specific 3MP production.

Another hypothetical possibility is that GOT1 and/or GOT2 are posttranslationally or allosterically regulated to favor L-Cys as a substrate under certain conditions. It also is conceivable that GOT1 and GOT2 are not the only enzymes catalyzing L-Cys transamination into 3MP. Perhaps L-serine and L-alanine aminotransferases can also catalyze the reaction, given that L-Cys is structurally more similar to these amino acids than to L-Asp. There is also the question if 3MP could be formed by oxidative deamination of L-Cys. However, for mammalian cells there is currently no evidence in support of this notion.

Subcellular localization of MPST

Mammalian cells express two MPST splicing isoforms. The shorter isoform shows a dual cytosolic-mitochondrial localization, apparently with a preference for mitochondria, which contain 2.5–3 times more specific MPST activity than the cytosol (Fräsdorf et al. 2014; Koj et al. 1975; Nagahara et al. 1998). The dual localization seems to be caused by an inefficient N-terminal mitochondrial matrix signal peptide. It is not yet clear if the MPST signal peptide is cleaved upon mitochondrial import (Miyamoto et al. 2014). Interestingly, the signal peptide of the closely related TST is not cleaved upon mitochondrial import (Miller et al. 1991). The longer MPST splicing isoform contains 20 additional amino acids at the N-terminus. This protein is predicted to be exclusively cytosolic and to have the same enzymatic properties as the shorter isoform (Fräsdorf et al. 2014; Yadav et al. 2020). The distribution of MPST between cytosol and mitochondria may be variable and regulated.

For example, it has been reported that certain stresses, such as smoking or endurance exercise training, or genetic defects, such as Down syndrome, increase mitochondrial MPST content and/or activity (Moeller et al. 2017; Panagaki et al. 2020; Sollanek et al. 2017). The mechanisms regulating mitochondrial import of MPST are not clear, but may have important physiological consequences, as a shift in subcellular distribution is expected to affect compartment-specific MPST functions. For instance, tRNA thiolation only occurs in the cytosol, while the maintenance of respiration depends on mitochondrially located MPST (Jüdes et al. 2016; Módis et al. 2013a; Noma et al. 2009).

Posttranslational modifications of MPST

Little is known about posttranslational modifications that may regulate MPST activity, stability or localization. Two serine residues close to the N-terminus (S2 and S35 in the shorter splicing variant) were found to be phosphorylated (Bian et al. 2014), and this may serve the regulation of mitochondrial targeting, as negative charges interfere with mitochondrial import (Martin et al. 1991). So far, the only other reported posttranslational modification is the oxidation of the active site cysteine by hydrogen peroxide (H_2O_2), which abolishes MPST activity (Módis et al. 2013b; Nagahara and Katayama 2005). However, the kinetics of MPST thiol oxidation by H_2O_2 is rather modest (k_i = 0.055 s^{-1}, K_i = 120.5 μM, k_i/K_i = 4.56 × 10^2 $M^{-1}s^{-1}$ for rat MPST) (Nagahara and Katayama 2005). *In vitro*, at low H_2O_2 concentrations, a sulfenate is formed at the catalytic cysteine, while at high H_2O_2 concentrations, a non-reversible sulfonate is formed (Nagahara and Katayama 2005). The physiological significance of MPST inactivation by H_2O_2 is questionable. In principle, it may play a role in the regulation of mitochondrial bioenergetics. MPST stimulates mitochondrial respiration and ATP production because H_2S produced from MPST-derived persulfides feeds electrons into the electron transport chain through the action of sulfide quinone oxidoreductase (SQR) (Módis et al. 2013a). Externally applied H_2O_2 was observed to abrogate the mitochondrial respiratory activity afforded by 3MP (Módis et al. 2013b). Recombinant rat MPST has been observed to form intermolecular disulfide conjugates (C154-C154, C154-C263 and C263-C263) with diminished MPST activity (Nagahara et al. 2007). It remains unclear if such crosslinks are also formed *in cellulo* and under physiologically relevant conditions. Of note, one of the cysteines found to form intermolecular disulfide bonds (C154) is specific to rat MPST and human MPST has not been observed to form disulfide-linked dimers (Fräsdorf et al. 2014; Yadav et al. 2013).

Tissue distribution of mammalian MPST

MPST is a widely expressed protein in human tissues, unlike the H_2S-producing enzymes cystathionine-beta-synthase (CBS) and cystathionine-gamma-lyase (CSE), which are more restricted in terms of tissue distribution. Furthermore, MPST seems to be the only facilitator of H_2S production and cyanide detoxification in the bloodstream, since erythrocytes do not contain CBS, CSE or TST (Vitvitsky et al. 2015). The highest MPST protein levels have been detected in liver, kidney, and large intestine, i.e. in organs with intense cysteine metabolism and markedly higher sulfide production (Akahoshi et al. 2020; Nagahara et al. 1998; Shibuya et al. 2013; Tomita et al. 2016). Lower MPST levels are found in brain, heart, lung, testis, small intestine and secretory glands, including the pancreas (Akahoshi et al. 2020; Nagahara et al. 1998; Tomita et al. 2016). The highest tissue-specific MPST activities were detected in liver and kidney, followed by the heart (Nagahara et al. 1998; Yadav et al. 2020). Activity can also be detected in the lung, brain and testis (Nagahara et al. 1998). Immunohistochemistry reveals cell type specific differences within organs. While the liver exhibits MPST expression across different cell types, the kidney shows marked heterogeneity, as glomeruli completely lack MPST expression (Tomita et al. 2016). For the brain, some studies report MPST expression to be limited to either neurons or astrocytes, while others observe expression in both (Shibuya et al. 2009; Tomita et al. 2016; Zhang et al. 2017; Zhao et al. 2013). In the colon, absorptive colonocytes show MPST expression, while goblet cells do not (Ramasamy et al. 2006). There is a strong polarization of MPST expression within the colonic tissue, with the mucosal surface expressing strongly, and cells at the crypt base lacking expression (Ramasamy et al. 2006). This apparently reflects the exposure to H_2S-producing colon-resident bacteria, and indeed MPST protein levels in colonocytes can be increased by treatment with non-toxic levels of H_2S donors (Ramasamy et al. 2006; Zuhra et al. 2019). A decrease of tissue-specific MPST expression was found to occur under certain pathological conditions, such as colorectal cancer and osteoarthritis (Nasi et al. 2020; Ramasamy et al. 2006).

Phylogenetic distribution and gene organization of MPST

The MPST gene does not seem to be universally conserved. Apparently, it is absent in Archaea. Within Eubacteria its distribution is patchy: it can be found in some, but not all branches of the eubacterial phylogenetic tree. Within Eukaryota, MPST is present in most phyla, including animals, fungi and plants. Within Animalia, MPST seems to have been lost in tunicates and most insects. However, the MPST gene is present in all vertebrates, with homologues present in fish, amphibia, reptiles, birds and mammals. Within the terrestrial vertebrates (tetrapods), an interesting pattern, consistently conserved from Amphibia to Mammalia, is apparent: the MPST gene is always located right next to the paralogous TST gene (Figure 5A). The distribution of chromatin features such as CpG islands, RNA polymerase II binding sites and histone acetylation/methylation marks typically associated with transcriptional activity (Figure 5B) strongly suggests that the MPST-TST intergenic region contains a bidirectional promoter and that the two genes are co-regulated.

The proximity between the MPST and TST genes may have important implications for the generation of transgenic animals. Genetic disruption of either the MPST or TST gene may potentially affect the regulation of the respective other gene, dysregulating its expression. Indeed, the first $MPST^{-/-}$ mouse line that was generated showed altered TST protein levels in the liver and heart (Nagahara et al. 2019; Peleli et al. 2020). Likewise, a recent report describes altered MPST mRNA and protein levels in a $TST^{-/-}$ mouse line (Carter et al. 2020). A more recently generated $MPST^{-/-}$ mouse line, with a targeted deletion restricted to exon 2 (more distant from the promoter region), did not show alterations in TST protein levels (Akahoshi et al. 2020).

How and why the vertebrate MPST-TST gene cluster was formed and maintained remains to be understood. The original gene duplication event that led to the first MPST and TST genes from a common precursor gene probably occurred very early in evolution, based on the wide distribution of both genes within the eubacterial and eukaryotic domains (Bordo and Bork 2002). It therefore appears that a gene reorganization event that occurred in the common ancestor of tetrapods brought the MPST and TST genes in conjunction, to be maintained in all tetrapod lineages to the present day, presumably because it provided some adaptive advantage. Proximity and a shared promoter region may play a role in mutual antagonistic regulation, given the potentially opposing roles of the two enzymes in H_2S and persulfide metabolism: While MPST contributes to persulfide and H_2S generation, TST is part of the mitochondrial H_2S/persulfide removal pathway. The regulation of the MPST-TST gene pair and the potential antagonism of the two enzymatic functions certainly deserves further investigation.

Regulation of MPST expression and its connection to physiology

MPST mRNA or protein levels are observed to change or fluctuate. MPST protein levels in mammals are reported to increase during development, and then to decrease with increasing age (Nagahara et al. 2015; Shibuya et al. 2013; Tomita et al. 2016; Zivanovic et al. 2020). Plants also show an age-dependent decline in MPST protein levels (Papenbrock and Schmidt 2000; Rajjou et al. 2008).

Changes in MPST expression have also been connected to metabolic disease, in particular to obesity and diabetes. MPST protein expression is elevated in the liver of obese adults and a positive correlation between liver fatty acid (FA) content and MPST protein expression has been noted (Caira et al. 2017; Li et al. 2018). In contrast, a negative correlation between FA content and MPST protein expression is observed for white adipose tissue (Katsouda et al. 2018). Animal experiments suggest a causal role of MPST in the regulation of liver FA content, because overexpression of liver MPST increased liver FA levels and depletion of liver MPST lowered liver FA levels (Li et al. 2018).

Correlations between MPST protein levels and diabetic symptoms have also been found, again in a tissue-dependent manner. Diabetogenic conditions led to a decrease of MPST levels in cardiac and smooth muscles, and to an increase in skeletal muscles (Jin et al. 2015; Mullen and Ohlendieck 2010; Zhang et al. 2016). Furthermore, a decrease in MPST expression was linked to erectile dysfunction, which is common in men who have diabetes. Rats with erectile dysfunction showed a decrease of MPST protein levels in penile tissue, which could be partially countermanded by tadalafil, a drug used to treat erectile dysfunction (Yilmaz-Oral et al. 2020; Zhang et al. 2016).

Increased MPST expression in the brain was linked to schizophrenia. Postmortem brain samples of schizophrenic subjects exhibited elevated MPST mRNA and protein levels (Ide et al. 2019). Mice overexpressing MPST developed behavioral traits presumably related to schizophrenia (Ide et al. 2019).

The exposure of cells to sulfide (as for example produced by gut commensal bacteria) affects the expression level of MPST in a concentration-dependent manner: low

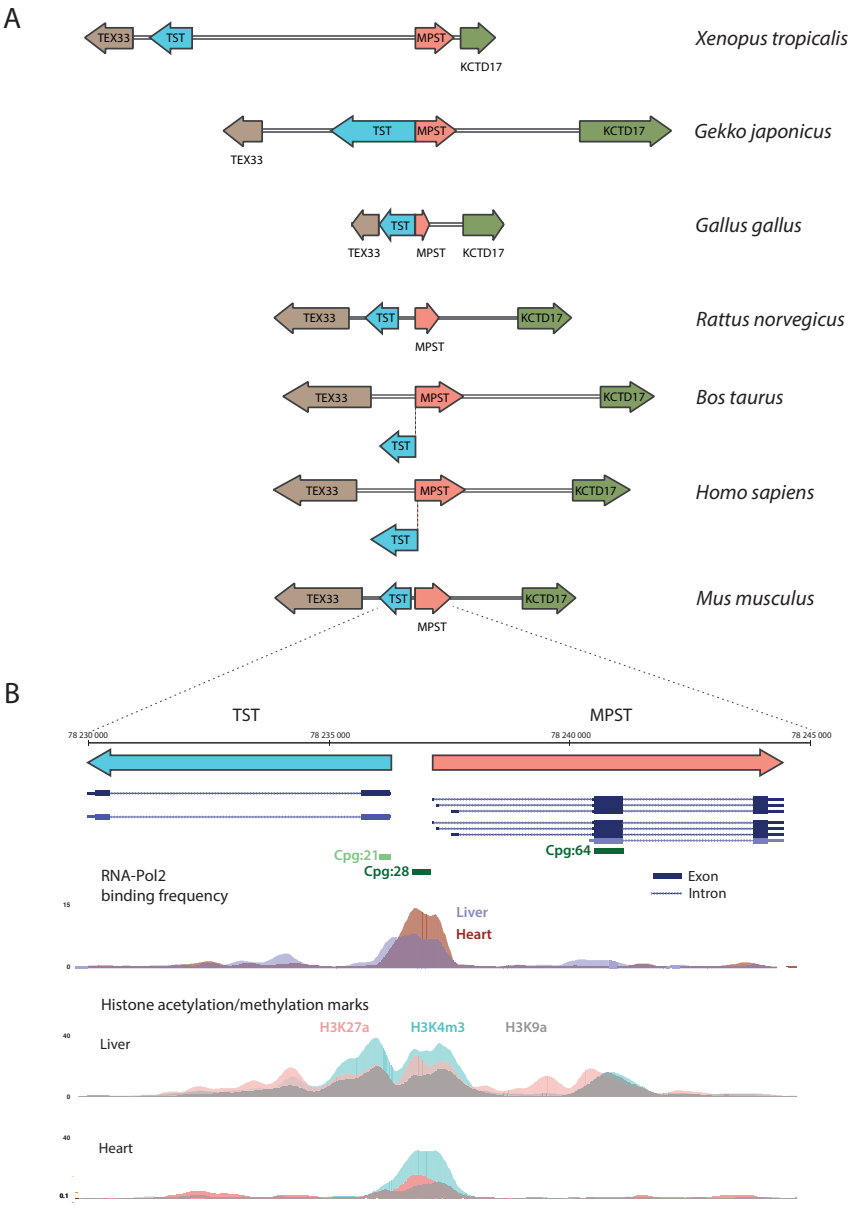

Figure 5: Organization of the vertebrate MPST gene locus. (A) Conserved organization of the genes surrounding the vertebrate MPST gene locus, from amphibians to mammals. TEX33: "testis expressed 33"; KCTD17: "potassium channel tetramerization domain containing 17". The TST gene is always located next to the MPST gene but transcribed in the opposite direction. The dashed line indicates a partial overlap of the MPST and TST genes in *Bos taurus* and *Homo sapiens*. (B) The murine TST-MPST gene tandem reveals features of a bidirectional promoter. UCSC genome browser representation of the TST-MPST locus in the mm9 mouse genome version (https://genome.ucsc.edu). Chromatin signatures that suggest the presence of a bidirectional promoter in the TST-MPST intergenic area include: CpG islands (the color intensity is proportional to the density of CpG islands), RNA polymerase II (RNA-Pol2) binding, as well as histone acetylation/methylation marks typically associated with transcriptional activation (H3K27a, H3K4m3, H3K9a).

levels of H_2S stimulate MPST expression, while cytotoxic H_2S levels caused MPST degradation and a corresponding decrease in MPST protein expression (Perna et al. 2013; Ramasamy et al. 2006; Zuhra et al. 2019). The effect of sulfide on MPST expression seems to depend on the cell's intrinsic tolerance to sulfide, as the same H_2S dosage elevated MPST expression in colorectal cells but depleted MPST in endothelial cells (Perna et al. 2013; Ramasamy et al. 2006). Oxygen availability is another factor that seems to influence MPST expression. Conditions of mild hypoxia induced MPST expression at both the mRNA and protein level (Li et al. 2013; Tao et al. 2017), and MPST expression seems to be required for the migration of vascular cells subjected to hypoxia (Tao et al. 2017).

While many studies report a change of MPST mRNA or protein levels in response to certain treatments or conditions, the underlying regulatory mechanisms remain unclear. The human MPST gene possesses features typical of a housekeeping gene (Nagahara et al. 2004). However, MPST gene expression also seems to be highly regulated. NF-kB is a positive regulator of MPST mRNA expression (Li et al. 2018). A range of proteins bind to the 5′-untranslated region (UTR) of the MPST mRNA, and some to the 3′-UTR (Li et al. 2018; Tao et al. 2017). Hypoxic conditions reduce the number of proteins that bind to the 5′-UTR, and this is associated with an increase of MPST mRNA levels (Tao et al. 2017). In addition, there seems to be an interplay between some MPST mRNA-binding proteins and MPST

itself. Sterol regulatory element-binding protein 1 (SREBP-1), a transcription factor involved in FA metabolism, not only interacts physically with the 5′-UTR of MPST mRNA but also with the MPST protein (Li et al. 2018; Tao et al. 2017). In addition, MPST influences SREBP-1 protein levels, as an increase or decrease of MPST levels leads to a proportional increase or decrease of SREBP-1 levels (Li et al. 2018). Finally, as discussed above, the likely presence of a bidirectional promoter between the MPST and TST genes indicates their coordinated (potentially antagonistic) regulation.

Pharmacological inhibitors of MPST

Initial studies aiming at the development of pharmacological MPST inhibitors focused on pyruvate derivatives (chloropyruvate and phenylpyruvate), since pyruvate was known to act as a competitive inhibitor at high concentrations. Chloropyruvate was only evaluated in enzymatic assays, while phenylpyruvate was studied both in enzymatic assays and in cell culture (Nagahara et al. 2004; Ostrakhovitch et al. 2019; Wing and Baskin 1992). As both compounds are poor inhibitors of MPST ($K_i \approx 4$ mM for chloropyruvate and $IC_{50} \approx 6$ mM for phenylpyruvate), and probably affect many other pyruvate-dependent enzymes, their use as MPST inhibitors is highly questionable (Nagahara et al. 2004; Wing and Baskin 1992).

More recently, a highly selective MPST inhibitor has been reported, I3-MT-3 (Hanaoka et al. 2017). Biochemical assays and the X-ray structure of I3-MT-3 in the MPST active site pocket show that this compound is an uncompetitive inhibitor. It binds to the active site after the catalytic cysteine has been persulfidated, thus blocking sulfur transfer to acceptor molecules (Hanaoka et al. 2017). The estimated dissociation constant (K_d) of I3-MT-3 is 0.5 μM, and the estimated IC_{50} is 13.6 μM (Augsburger et al. 2020; Hanaoka et al. 2017). In cell culture, the concentration at which it blocked MPST-dependent H_2S production varied within the 1–100 μM range, depending on the cell type (Augsburger et al. 2020; Hanaoka et al. 2017; Nasi et al. 2020; Panagaki et al. 2020). Aside from lowering H_2S production, the use of I3-MT-3 identified physiological processes in which MPST appears to be involved. For example, the inhibitor indicated a role for MPST in preventing calcification of chondrocytes and in the formation of endothelial networks (Abdollahi Govar et al. 2020; Nasi et al. 2020).

Several studies showed an antiproliferative and antimigratory effect of I3-MT-3, along with a decrease of cellular reductive capacity (Abdollahi Govar et al. 2020; Augsburger et al. 2020; Oláh et al. 2018; Panagaki et al. 2020). In addition, blockage of MPST activity seems to have profound metabolic effects. Depending on cell type and dose, I3-MT-3 can either promote or suppress mitochondrial respiration and ATP production. On the one hand, it promoted mitochondrial activity in colon cancer cells and in Down syndrome fibroblasts, both of which express higher than normal levels of mitochondrially localized MPST (Augsburger et al. 2020; Panagaki et al. 2020). On the other hand, I3-MT-3 lowered mitochondrial respiration and ATP production in endothelial cells (Abdollahi Govar et al. 2020). A global metabolomic analysis indicated that MPST inhibition by I3-MT-3 perturbs various metabolic pathways. In particular, it caused an increase of metabolites involved in sulfur metabolism, including methionine, methionine sulfoxide, cystathionine, cysteine, lanthionine, cysteine sulfinic acid, hypotaurine and taurine (Abdollahi Govar et al. 2020). This suggests that MPST activity influences the cellular content of numerous sulfur-containing metabolites. The use of I3-MT-3 offers further potential to delineate the role of MPST in biochemical and physiological processes.

Phenotypes of MPST deficiency

In humans, MPST deficiency causes a specific form of disulfiduria, i.e. the appearance of 3ML (in a mixed disulfide with cysteine) in the urine (Crawhall et al. 1968, 1971; Hannestad et al. 1981). This observation revealed that 3MP is constantly produced from dietary cysteine and cannot be desulfurated in the absence of MPST. Instead, it is converted to 3ML by lactate dehydrogenase (Ohta and Ubuka 1989). Initially, the 3ML-Cys disulfiduria was thought to be associated with mental retardation, as 3ML-Cys disulfide conjugates were found in the urine of mentally retarded patients (Crawhall et al. 1968; Law and Fowler 1976). However, other studies discovered 3ML-Cys disulfiduria in patients with no signs of mental retardation (Hannestad et al. 1981; Niederwiesler et al. 1973). While information about the phenotypes of MPST-deficient humans is very limited and somewhat inconsistent, the study of MPST deficiency in model organisms and cell lines has pinpointed a number of phenotypes that may also be relevant to humans.

To study the phenotypes of MPST deficiency in mammals, MPST knock-out mice were generated. The first $MPST^{-/-}$ mice generated were reported to exhibit anxiety-like traits and dysregulation of brain serotonin levels (Nagahara

et al. 2013). Cardiovascular profiling revealed higher superoxide and H_2O_2 levels as well as lower NADPH/$NADP^+$ and GSH/GSSG ratios, and aged mice developed hypertension and cardiac hypertrophy (Peleli et al. 2020). However, these mice also showed perturbed TST levels in the liver and heart (Nagahara et al. 2019; Peleli et al. 2020). As explained above, it is possible that the deletion in the MPST gene (covering exons 1 and 2) influenced the regulation of the associated TST gene, making it difficult to interpret the phenotypes. Recently, two new $MPST^{-/-}$ mouse lines have been generated by deleting exon 2 only (Akahoshi et al. 2020; Ide et al. 2019). The $MPST^{-/-}$ mice of Akahoshi et al. do not show changes in liver TST protein levels, suggesting that a deletion restricted to exon 2 does not perturb TST expression (Akahoshi et al. 2020). Serum and urine from these mice exhibited increased 3ML levels, as observed in MPST-deficient humans. Levels of L-Cys and other cysteine-derived metabolites were unchanged (Akahoshi et al. 2020). The mice did not show anxiety-like traits, but exhibited phenotypes suggesting abnormal vasodilation and thermoregulation (Akahoshi et al. 2020). The $MPST^{-/-}$ mice generated by Ide and colleagues were only studied in relation to a possible connection between MPST activity and schizophrenia (Ide et al. 2019). While MPST overexpressing mice showed increased schizophrenia-like behavioral traits, $MPST^{-/-}$ mice reportedly showed less such traits than their wild type littermates (Ide et al. 2019).

Apart from mice, the MPST gene has been knocked out in *Escherichia coli*, yeast, zebrafish, *Arabidopsis thaliana* and various mammalian cell lines. Despite the diversity of models, MPST knock-outs generally appear to exhibit an increased sensitivity to oxidative stress, i.e. decreased fitness in response to either externally applied or endogenously generated oxidants (Katsouda et al. 2020; Li et al. 2019; Mironov et al. 2017; Peleli et al. 2020; Shatalin et al. 2011; Toliver-Kinsky et al. 2019). This suggests that MPST has a protective function against oxidants. While MPST is capable of directly reacting with H_2O_2 (see above), the kinetics are poor and there is no evidence implicating MPST as a direct H_2O_2 scavenger. More likely, MPST-mediated persulfide and/or H_2S production may have a cytoprotective function (Ezeriņa et al. 2018; Wen et al. 2013). The impact of MPST deficiency on endogenous H_2S levels seems to be variable amongst the different knock-out model systems: while most exhibit decreased H_2S levels, some show unchanged or even increased H_2S levels (Ide et al. 2019; Li et al. 2019, 2018; Peleli et al. 2020). Arguably, the cytoprotective effect of MPST expression is more directly linked to sulfane sulfur (per/polysulfide) content, which is found to be decreased in MPST knock-out cells (Kimura et al. 2017; Li et al. 2019).

MPST activity is known to increase intracellular sulfane sulfur content in two ways. Firstly, by directly generating and transferring persulfides, as described above. Secondly, by promoting the generation of H_2S (through persulfidation of Trx, as discussed above) which is then oxidized by SQR to again yield persulfides. How persulfides actually protect cells against oxidants is not yet fully understood. Two mechanistic explanations for the antioxidative effect of persulfides have been proposed: Firstly, persulfides react more readily with one- or two-electron oxidants than the corresponding thiols (Cuevasanta et al. 2015; Ida et al. 2014). This is explained by the α-effect and the decreased pK_a of persulfides (Cuevasanta et al. 2015, 2017; Edwards and Pearson 1962). Secondly, persulfidation seems to protect protein thiols against irreversible hyperoxidation. Persulfides on proteins become oxidized only on the outer sulfur atom, allowing subsequent reduction of the S-S bond to regenerate the original thiol in its unmodified form (Dóka et al. 2020; Millikin et al. 2016; Zivanovic et al. 2020). However, if MPST is involved in widespread protein persulfidation (beyond its known targets Mocs3 and Trx) is currently unknown.

On a final note, the study of MPST knock-out organisms revealed further phenotypes, possibly, but not necessarily, related to increased oxidative stress sensitivity, and deserving further study. For example, *E. coli* lacking MPST are less resistant to antibiotics (Shatalin et al. 2011) and *Arabidopsis thaliana* lacking str1, one of its two MPST isoforms, show defects in embryo development and maturation (Höfler et al. 2016; Mao et al. 2011).

Future perspectives

MPST is a versatile protein, serving a range of sulfur acceptors and various physiological processes. Many open questions remain to be answered. How and under which circumstances is 3MP generated and exchanged between subcellular compartments? What are the physiologically relevant acceptors of the MPST persulfide? How is the subcellular distribution of MPST regulated? How is the tandem MPST-TST gene regulated and what is the physiological function of such coordinated regulation? How exactly is MPST activity linked to oxidative stress resistance, fatty acid metabolism and mitochondrial activity? How exactly does it relate to ageing and metabolic disease?

Acknowledgments: We thank Dr. Aubry Miller (German Cancer Research Center, Heidelberg) and Dr. Sara Cruz-Molina (Max Planck Institute for Molecular Biomedicine,

Münster) for valuable discussions regarding the chemistry of MPST catalysis and the MPST-TST gene tandem, respectively. We acknowledge support by the Deutsche Forschungsgemeinschaft (SPP1710 to T.P.D.).

Author contribution: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: The study was supported by the Deutsche Forschungsgemeinschaft (SPP1710 to T.P.D.).

Conflict of interest statement: The authors declare no conflicts of interest regarding this article.

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