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Metabolism of L-cysteine via transamination pathway (3-mercaptopyruvate pathway)

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Summary. We have studied the transamination pathway (3-mercaptopyruvate pathway) of L-cysteine metabolism in rats. Characterization of cysteine aminotransferase (EC 2.6.1.3) from liver indicated that the transamination, the first reaction of this pathway, was catalyzed by aspartate aminotransferase (EC 2.6.1.1). 3-Mercaptopyruvate, the product of the transamination, may be metabolized through two routes. The initial reactions of these routes are reduction and transsulfuration, and the final metabolites are 3-mercaptolactate-cysteine mixed disulfide [S-(2-hydroxy-2-carboxyethylthio)cysteine, HCETC] and inorganic sulfate, respectively. The study using anti-lactate dehydrogenase antiserum proved that the enzyme catalyzing the reduction of 3-mercaptopyruvate was lactate dehydrogenase (EC 1.1.1.27). Formation of HCETC was shown to depend on low 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) activity. Results were discussed in relation to HCETC excretion in normal human subjects and patients with 3-mercaptolactate-cysteine disulfiduria. Incubation of liver mitochondria with L-cysteine, 2-oxoglutarate and glutathione resulted in the formation of sulfate and thiosulfate, indicating that thiosulfate was formed by transsulfuration of 3-mercaptopyruvate and finally metabolized to sulfate.

Keywords: Amino acids – Cysteine metabolism – 3-Mercaptopyruvate pathway – Cysteine transamination – 3-Mercaptolactate-cysteine mixed disulfide – Sulfate formation

Introduction

Crawhall et al. reported that a mentally retarded patient excreted in the urine a large amount of a mixed disulfide, β-mercaptolactate-cysteine disulfide [1]. The

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same mixed disulfide and another one were found to be excreted in normal human urine [2], and these mixed disulfides were named as S-(2-hydroxy-2-carboxyethylthio)cysteine (HCETC, 3-mercaptolactate-cysteine mixed disulfide) and S-(carboxymethylthio)cysteine (CMTC, mercaptoacetate-cysteine mixed disulfide), respectively [2]. Until now, five cases excreting unusually large amounts of HCETC have been reported [3–5]. As the 3-mercaptolactate moiety of HCETC seemed to have derived from L-cysteine, we have studied the metabolism of L-cysteine via the transamination pathway (3-mercaptopyruvate pathway) and the biosynthesis of HCETC. Formation of inorganic sulfate, another end product of the 3-mercaptopyruvate pathway, from L-cysteine in rat liver mitochondria has also been studied.

Materials and methods

Animals

Male Wistar rats weighing 200–350 g were used. In some experiments, male Hartley guinea pigs weighing about 600 g were used. These animals were fed *ad libitum* on laboratory diets MF and RC4, respectively, of Oriental Yeast Co., Tokyo, Japan.

Enzyme reactions

For enzyme reactions to examine the activity of the 3-mercaptopyruvate pathway, homogenate, 105,000 × g supernatant, mitochondrial and microsomal fractions from rat liver [6, 7] or homogenates of other tissues [8] were used as the enzymes. Reactions were conducted in a reaction mixture containing L-cysteine, 2-oxoglutarate, pyruvate and potassium cyanide [6, 8]. Cyanide was included in the mixture as a sulfur acceptor of 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) reaction, and thiocyanate formed was determined.

Biosynthesis of HCETC was studied using homogenates of various rat tissues as enzymes and L-cysteine or 3-mercaptopyruvate as substrates [9–11].

Formation of sulfate was studied using rat liver mitochondria prepared according to Hogeboom [7]. Incubation mixture contained L-cysteine, 2-oxoglutarate, glutathione and mitochondrial fraction [12, 13].

Enzyme purification

Cysteine aminotransferase (EC 2.6.1.3) was purified from rat liver mitochondria [14] and cytosol [15]. Lactate dehydrogenase (EC 1.1.1.27) was purified from rat liver and its antiserum was raised in rabbits [16].

Analytical procedures

HCETC and cysteine-glutathione mixed disulfide were determined with an amino acid analyzer [10]. Free 3-mercaptolactate was determined after conversion to HCETC by a chemical reaction using S-(2-amino-2-carboxyethylsulfonyl)-L-cysteine (ACESC or Sacsylcysteine) [17]. By this reaction, cysteine portion of Sacsylcysteine reacted quantitatively with SH group of 3-mercaptolactate, and HCETC formed was determined by an amino acid analyzer [9].

Sulfate was determined by colorimetric method using barium chloranilate [12] and by ion chromatography [13].

Results

Formation of thiocyanate from L-cysteine and cyanide by rat liver

Formation of thiocyanate from L-cysteine and cyanide was studied in the presence of rat liver fractions [6]. Incubation of L-cysteine (40 mM), 2-oxoglutarate (5 mM), pyruvate (25 mM) and potassium cyanide (3 mM) at pH 9.0 resulted in the formation of 204, 161 and 267 nmol of thiocyanate/mg of protein per hour in the presence of rat liver homogenate, supernatant and mitochondrial fractions, respectively. Omission of 2-oxoglutarate or pyruvate halved the thiocyanate formation. By omission of L-cysteine or both 2-oxoglutarate and pyruvate, thiocyanate formation decreased to one tenth to one fourth of the complete reaction mixture. These results indicate that L-cysteine was transaminated and then 3-mercaptopyruvate formed was transsulfurated to form thiocyanate, and thus L-cysteine could be metabolized through the 3-mercaptopyruvate pathway.

Activities of 3-mercaptopyruvate pathway in rat tissues

Activity of thiocyanate formation in various rat tissues was examined using the same system as above [8]. Thiocyanate formed by dialyzed homogenates of liver, kidney and heart were 33.2, 15.9 and 6.9 μmol/g wet weight per hour, respectively. Cysteine-oxoglutarate aminotransferase activities in these dialyzed homogenates were 111.2, 44.1 and 192.7 μmol/g wet weight per hour, respectively. 3-Mercaptopyruvate sulfurtransferase activities in these homogenates were 1633.0, 390.7 and 146.1 μmol/g wet weight per hour, respectively. Thiocyanate formation by brain, spleen, skeletal muscle, lung, small intestine and blood was very low. Thus, the 3-mercaptopyruvate pathway was most active in the liver, followed by the kidney.

Purification and characterization of cysteine aminotransferase

Cysteine-oxoglutarate aminotransferase was purified from rat liver mitochondria [14] and cytosol [15]. Crude extract of mitochondria was prepared by sonication. The extract was subjected to heat treatment, ammonium sulfate fractionation, CM-Sephadex column chromatography, DEAE-cellulose column chromatography and isoelectric focusing. Purification of the enzyme from mitochondria was 324-fold. The purified enzyme was homogeneous as judged by disc electrophoresis, gel filtration on a Sephacryl S-200 column and isoelectric focusing using an electrofocusing column and 0.5% Ampholine (pH 9–11). The molecular weight was 88,000 and isoelectric point was 9.8. The enzyme catalyzed reversible transamination reaction between L-cysteine and 2-oxoglutarate, and its optimum pH was 9.7. Km values for L-cysteine and 2-oxoglutarate were 22.2 and 0.38 mM, respectively and those for 3-mercaptopyruvate and L-glutamate in the reverse reaction were 12.5 and 2.5 mM, respectively. The reaction was strongly inhibited by L-aspartate. The enzyme catalyzed transamination of, besides L-cysteine, L-cysteinesulfinic acid, L-aspartic acid and L-cysteic acid. Relative activities for these substrates when that for L-aspartic acid was taken as 100 were at pH 9.7 and 8.0, respectively: L-cysteinesulfinic acid, 420 and 837;

L-cysteic acid, 44.6 and 76.4; L-cysteine, 4.2 and 2.4. Km value for L-aspartic acid was 0.53 mM. The ratios of activities for L-cysteinesulfinic acid, L-aspartic acid and L-cysteine were constant during all purification steps. These results indicate that mitochondrial cysteine aminotransferase is identical with mitochondrial aspartate aminotransferase (EC 2.6.1.1).

Cytosolic cysteine aminotransferase was purified 408-fold from 54,000 × g supernatant of rat liver by ammonium sulfate fractionation, heat treatment, DEAE-cellulose column chromatography, CM-cellulose column chromatography, Sephacryl S-200 column chromatography and isoelectric focusing (pH range 3–10) [15]. Yield was 29%. The purified enzyme was homogeneous as judged by gel filtration, isoelectric focusing and disc electrophoresis. The molecular weight was 74,000 and isoelectric point was 6.2. The enzyme catalyzed the reversible reaction of cysteine transamination. When enzyme assay was performed at pH 8.0, the best substrate of the purified enzyme was L-cysteinesulfinic acid, followed by L-aspartic acid and L-cysteic acid. Activity toward L-cysteine was 5.3% of that toward L-aspartic acid. When assayed at pH 9.7, the enzyme was active toward L-aspartic acid and L-cysteinesulfinic acid at a comparable degree. Activity toward L-cysteine was 6.3% of that toward L-aspartic acid. Transamination reaction of cysteine was strongly inhibited by L-aspartic acid. Km values were 22.2, 1.6 and 0.06 mM for L-cysteine, L-aspartic acid and 2-oxoglutarate, respectively. The ratio of activities toward L-cysteine and L-aspartic acid were constant during the all purification steps. These results indicate that cytosolic cysteine aminotransferase is identical with cytosolic aspartate aminotransferase.

Reduction of 3-mercaptopyruvate by lactate dehydrogenase

It has long been assumed with no definitive evidence that the *in vivo* reduction of 3-mercaptopyruvate formed by cysteine transamination is catalyzed by lactate dehydrogenase. In order to clarify this question, the reduction of 3-mercaptopyruvate and its inhibition were studied using purified lactate dehydrogenase from rat liver and its antiserum [16]. The purification steps of lactate dehydrogenase consisted of ethanol fractionation, ammonium sulfate fractionation, affinity elution from CM-Sephadex column and DEAE-Sephadex column chromatography. Final purification was 234-fold from the homogenate with a yield of 28%. SDS-polyacrylamide gel electrophoresis exhibited that the purified enzyme was homogeneous and molecular weight of the subunit was 36,500. Polyacrylamide gel electrophoresis and activity staining revealed that the purified enzyme was isozyme M_4. The enzyme catalyzed the reduction of pyruvate and 3-mercaptopyruvate as shown in Table 1.

Antiserum against the purified lactate dehydrogenase was raised in rabbits. Specificity of the antiserum was examined by Ouchterlony double diffusion analysis and immunoelectrophoresis [16]. The antiserum formed one precipitin line with the purified enzyme and with rat liver homogenate, indicating that the antiserum was specific for the purified lactate dehydrogenase preparation.

Inhibition of the reduction of 3-mercaptopyruvate by the antiserum against lactate dehydrogenase was examined [16]. As shown in Table 1, the antiserum

Table 1. Inhibition by anti-lactate dehydrogenase (LDH) antiserum of the reduction of pyruvate and 3-mercaptopyruvate by purified lactate dehydrogenase and rat liver homogenate[a]

Enzyme	Incubation with	Remaining activity μmol/ml, min (inhibition, %)	
		Substrate	
		3-Mercaptopyruvate	Pyruvate
Purified LDH	Saline	0.80 ± 0.02 (0)	3.34 ± 0.03 (0)
	Control serum	0.80 ± 0.20 (0)	3.28 ± 0.00 (1.8)
	Anti-LDH antiserum	0.00 ± 0.01 (100.0)	0.01 ± 0.06 (99.7)
Rat liver homogenate	Saline	3.94 ± 0.01 (0)	16.57 ± 0.08 (0)
	Control serum	3.99 ± 0.06 (0)	16.22 ± 0.09 (2.1)
	Anti-LDH antiserum	0.04 ± 0.01 (99.1)	0.06 ± 0.01 (99.6)

[a] Purified rat liver lactate dehydrogenase or rat liver homogenate was incubated with rabbit antiserum raised against the purified enzyme, with saline or with control rabbit serum. After centrifugation, enzyme activities in the supernatant were determined with 3-mercaptopyruvate (16 mM) or pyruvate (1.6 mM) as substrates. Results are expressed as mean ± SD of at least two separate experiments.

inhibited completely the reduction of 3-mercaptopyruvate as well as that of pyruvate by the purified lactate dehydrogenase. The antiserum also inhibited completely the reduction of these substrates by rat liver homogenate. These results show that the reduction of 3-mercaptopyruvate in rat liver is catalyzed by lactate dehydrogenase, and not by other enzymes.

Biosynthesis of HCETC by rat tissues

Biosynthesis of HCETC has been studied using homogenates of various rat tissues, and the mixed disulfide formed was determined with an amino acid analyzer [9–11]. As shown in Table 2, HCETC was formed when 3-mercaptopyruvate was incubated with heart homogenate, but it was not produced by liver and kidney homogenates [9, 11]. It was confirmed that free 3-mercaptolactate was present in the reaction mixture with heart homogenate after the incubation [9]. In the same reaction mixture, cysteine-glutathione mixed disulfide was detected [10, 11]. Table 2 also shows that, when only L-cysteine was incubated with heart homogenate, no HCETC was produced. However, the addition of 2-oxoglutarate to the reaction mixture resulted in the formation of this mixed disulfide. These findings indicate that L-cysteine was transaminated to form 3-mercaptopyruvate, which was reduced to 3-mercaptolactate. 3-Mercaptolactate thus formed was utilized for the formation of HCETC.

Sulfate formation through 3-mercaptopyruvate pathway in rat liver mitochondria

Sulfate formation from L-cysteine in rat liver mitochondria was studied as reported [12, 13]. As shown in Table 3, the incubation of 10 μmol each of

Table 2. Formation of 3-mercaptolactate-cysteine mixed disulfide [S-(2-hydroxy-2-carboxyethylthio)cysteine (HCETC)] from L-cysteine or 3-mercaptopyruvate by rat heart homogenate[a]

Exp. No.	Substrates (mM)	Products (μmol/reaction flask)		
		HCETC	Cys	3-ML
1A	3-MP (18)	0.41 ± 0.12	0.56 ± 0.03	0.68
1B	3-MP (60)	2.10	2.26	3.64
2A	Cys (40)	0.00 ± 0.00	b	b
2B	Cys (40) OG (10)	0.21 ± 0.02	b	b

[a] Experiment 1 was performed with 6 ml per flask of reaction mixture containing 4 ml of 30% rat heart homogenate and substrates, and incubated at pH 7.4 and 37°C for 60 min. Experiment 2 was performed with 3 ml of reaction mixture containing 2 ml of 30% rat heart homogenate and substrates, and incubated at pH 8.4 and 37°C for 60 min. Reactions were terminated with 3% sulfosalicylic acid (final concentration), and products were determined with an amino acid analyzer. 3-Mercaptolactate (3-ML) formed in experiment 1 was converted to HCETC by reacting with S-(2-amino-2-hydroxyethylsulfonyl)systeine, and HCETC formed was determined with an amino acid analyzer. Values are means ± SD of 2 or 3 separate experiments except for 3-ML (1A) and 1B. Abbreviations: *3-MP* 3-mercaptopyruvate; *Cys* L-cysteine; *OG* 2-oxoglutarate, *b* Not determined.

Table 3. Sulfate formation from L-cysteine in rat liver mitochondria[a]

Substrates (mM)	Products (μmol/h, mitochondria from 1 g of liver)		
	Sulfate (A)	Thiosulfate (B)	A + 2B
Cys (10) OG (10) GSH (10)	4.68 ± 1.36	1.50 ± 0.34	7.68 (6.00)[b]
Cys (10) OG (10)	2.88 ± 1.25	1.94 ± 1.15	6.76 (5.28)
Cys (10) GSH (10)	1.69 ± 0.57	0.00 ± 0.00	1.69 (1.32)
Cys (10)	1.29 ± 0.51	0.00 ± 0.00	1.29 (1.00)

[a] Substrates and rat liver mitochondria from 0.5 of liver were incubated at pH 8.4 and 37°C for 30 min. Sulfate and thiosulfate formed were determined by ion chromatography. Values are means ± SD of 3 separate experiments. Abbreviations: *Cys* L-cysteine; *OG* 2-oxoglutarate; *GSH* glutathione;

[b] Ratios of A + 2B are shown in parentheses.

L-cysteine , 2-oxoglutarate and glutathione with mitochondrial suspension resulted in the formation of 4.68 μmol of sulfate per hour per mitochondria from 1 g of liver. The addition of 2-oxoglutarate strongly accelerated the sulfate formation compared with the sulfate formation in the reaction with L-cysteine alone, indicating that this mitochondrial sulfate-forming pathway proceeded through the 3-mercaptopyruvate pathway. The addition of GSH further accelerated the sulfate-forming activity in mitochondria. This seems to indicate that thiosulfate reductase (EC 2.8.1.3) which requires glutathione as a co-substrate [18] participates in the mitochondrial sulfate-forming system [12, 13].

Discussion

Metabolic pathways of L-cysteine may be classified into two types [19, 20]. One is the cysteinesulfinate pathway (oxidation pathway) and the other is the desulfuration metabolism. In the former pathway, the L-cysteine sulfur is first oxidized by cysteine dioxygenase (EC 1.13.11.20) before being split from the carbon skeleton, and cysteinesulfinate is formed. Cysteinesulfinate is finally metabolized to sulfate or taurine. In the desulfuration metabolism, L-cysteine is metabolized without initial oxidation of sulfur atom.

The 3-mercaptopyruvate pathway (Fig. 1) described in this paper belongs to the latter type. The presence of this pathway was originally suggested by Meister et al. in 1954 as an alternative pathway of cysteine metabolism [21]. The discovery of HCETC in normal human urine [2] and in the urine of patients with β-mercaptolactate-cysteine disulfiduria [1, 3–5] attracted our attention to the L-cysteine metabolism which leads to the biosynthesis of HCETC. The formation of thiocyanate from L-cysteine in the presence of 2-oxo acids, cyanide and rat tissue homogenates clearly indicates that L-cysteine was transaminated and that the sulfur atom of 3-mercaptopyruvate formed was transferred to cyanide, namely, L-cysteine could be metabolized through the 3-mercaptopyruvate pathway.

Rat tissues contain high activities of 3-mercaptopyruvate sulfurtransferase [8, 22, 23]. Incubation of L-cysteine or 3-mercaptopyruvate with rat liver or

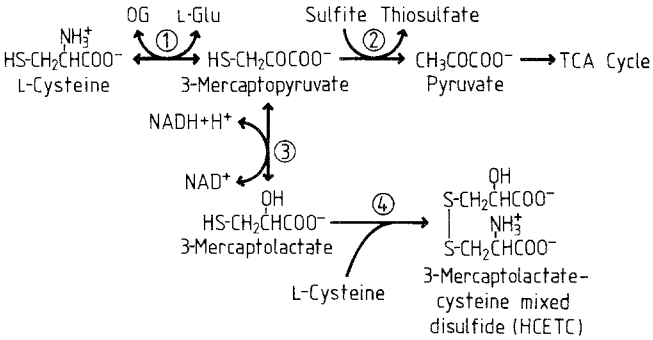

Fig. 1. Scheme of cysteine metabolism through transamination pathway (3-mercaptopyruvate pathway) and biosynthesis of 3-mercaptolactate-cysteine mixed disulfide [S-(2-amino-2-carboxyethylthio)cysteine, HCETC]. Enzymes are: *1* aspartate aminotransferase; *2* 3-mercaptopyruvate sulfurtransferase; *3* lactate dehydrogenase; *4* unknown

kidney homogenate failed to form HCETC. However, HCETC was formed when these substrates were incubated with rat heart homogenate as shown in Table 2. 3-Mercaptopyruvate sulfurtransferase activity in the liver and kidney is very high compared to the activity of cysteine transamination [6, 8]. Thus, in these tissues, 3-mercaptopyruvate formed seems to be metabolized preferentially by 3-mercaptopyruvate sulfurtransferase reaction.

In the heart, on the other hand, 3-mercaptopyruvate sulfurtransferase activity is relatively low and cysteine transaminase activity is relatively high compared to the liver and kidney. As shown in experiments with rat liver (Table 1), 3-mercaptopyruvate is reduced to 3-mercaptolactate by lactate dehydrogenase and not by other enzymes. Heart tissue contains high activity of this enzyme [24]. It seems that these conditions together favored HCETC formation when the heart homogenate was used as the enzyme source.

It has been shown that 3-mercaptopyruvate sulfurtransferase activity in guinea pig tissues [23] are very low compared to those in rat tissues [23] and in human erythrocytes [22]. Amino acid analysis revealed that guinea pig liver contained 0.180 ± 0.080 μmol/g wet weight of HCETC and 0.45 ± 0.05 μmol/g wet weight of cysteine [25]. Incubation of 3-mercaptopyruvate or L-cysteine and 2-oxo acids with guinea pig liver and kidney homogenates resulted in the formation of substantial amount of HCETC [26].

These facts indicate that 3-mercaptopyruvate sulfurtransferase activity is the critical factor for the formation of HCETC.

It should be added that the intraperitoneal injection of 500 μmol of 3-mercaptolactate into rats resulted in the excretion of 1.3 ± 0.4 μmol/animal of HCETC in the 20-hour urine following the injection (Ubuka et al., unpublished results).

It has been shown that 3-mercaptopyruvate sulfurtransferase is deficient in patients with 3-mercaptolactate-cysteine disulfiduria who excrete about 500 μmol/day of HCETC [1] or 58.7 mmol of 3-mercaptolactate/mole of creatinine [5] in their urine. It was also reported that 3-mercaptolactate was almost entirely excreted as mixed disulfide with cysteine [27]. These facts indicates that 3-mercaptopyruvate is formed and reduced to 3-mercaptolactate, and the latter is excreted as HCETC. Thus, the presence of such patients supports the 3-mercaptopyruvate pathway is functioning in the human body. Excretion of HCETC by normal subjects ranged from trace amounts to 10 μmol/day [28]. These values increased only two-fold after the oral administration of 0.8 mmol of L-cysteine/kg of body weight [29]. These results indicate that 3-mercaptopyruvate is mainly metabolized via 3-mercaptopyruvate sulfurtransferase reaction in normal humans.

As shown in Table 3, sulfate and thiosulfate were formed when L-cysteine, 2-oxoglutarate and glutathione were incubated with rat liver mitochondria. Incubation of L-cysteine alone resulted in the formation of only about one μmol of sulfate per hour per mitochondria from 1 g of liver. By the addition of 2-oxoglutarate, sulfate formation was doubled and thiosulfate was also formed. As thiosulfate contains two sulfur atoms in the molecule, the sum of sulfate (A) and thiosulfate (B), calculated as A + 2B, was about 5 times the amount of sulfate formed when L-cysteine alone was used as the substrate. Addition of glutathione

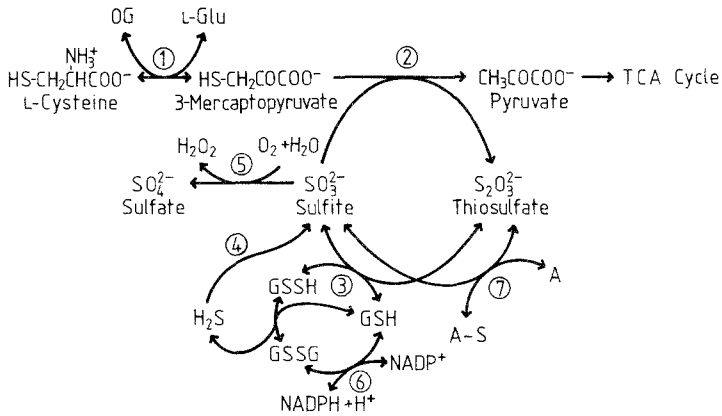

Fig. 2. Scheme of mitochondrial sulfate-forming pathway. Enzymes are: *1* aspartate aminotransferase; *2*,3-mercaptopyruvate sulfurtransferase; *3* thiosulfate reductase; *4* unknown; *5* sulfite oxidase; *6* glutathione reductase; *7* thiosulfate sulfurtransferase

further accelerated the sulfate formation from L-cysteine and 2-oxoglutarate. Wainer reported that sulfate was formed from L-cysteine by a pathway different from the cysteinesulfinate pathway [30, 31]. Our results indicate that, in mitochondria, sulfate is formed from L-cysteine via the 3-mercaptopyruvate pathway as shown in Fig. 2 [12, 13].

It has been proposed that the physiological sulfur acceptor of 3-mercaptopyruvate sulfurtransferase is sulfite [32]. The product, thiosulfate, is the substrate of thiosulfate sulfurtransferase (EC 2.8.1.1), a mitochondrial enzyme [32]. Therefore, it is likely that the function of the 3-mercaptopyruvate pathway is to supply divalent sulfur for the production of thiosulfate, which may be utilized for the formation of iron-sulfur clusters [33] and excess thiosulfate is oxidized to sulfate. Major sulfate in the cell is produced through the cysteinesulfinate pathway. Therefore, the main role of the 3-mercaptopyruvate pathway seems not to be sulfate formation, but the supply of divalent sulfur atom to iron-sulfur clusters.

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