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Production of hypotaurine from L-cysteinesulfinate by rat liver mitochondria

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Summary. Hypotaurine is the precursor of taurine production from L-cysteinesulfinate. It is recognized that hypotaurine production in the liver occurs in cytosol. In the present study, hypotaurine production from L-cysteinesulfinate in rat liver mitochondria was investigated. The mitochondrial preparation prepared according to the method of Hogeboom and washed repeatedly with 0.25 M sucrose solution was incubated with L-cysteinesulfinate. Products were derivatized with dabsyl chloride and dabsylated amino acids were analyzed by RP-HPLC. Presence of a peak corresponding to dabsyl-hypotaurine was confirmed. The peak of dabsyl-hypotaurine was converted quantitatively to dabsyl-taurine by the treatment with H_2O_2. Optimum pH of the reaction was shown to be broad between 6.0 and 7.8 and Km for L-cysteinesulfinate was 0.11 mM. Results indicate the presence of L-cysteinesulfinate decarboxylase activity in liver mitochondria. Mitochondrial cysteine metabolism was summarized and possible antioxidant roles of cysteine metabolites including hypotaurine in mitochondria are discussed.

Keywords: Amino acids – Hypotaurine – L-Cysteinesulfinate decarboxylase – Liver mitochondria – Cysteine metabolism – Antioxidant

Introduction

Taurine (2-aminoethylsulfonic acid, an organic sulfonic acid) is found in high concentrations in mammalian tissues, especially in excitable tissues such as heart and skeletal muscles (Jacobsen and Smith, 1968). In these tissues taurine is present as a free amino acid, but its biochemical functions are not fully understood except for bound taurine in conjugated bile acids in lipid metabolism. Recently, however, it has been found that taurine is contained in modified uridine (5-tauromethyluridine and 5-tauromethyl-2-thiouridine) of tRNAs in human and bovine mitochondria (Suzuki et al., 2002). This is the first report of taurine's involvement in biological macromolecules.

Major pathway of taurine formation in mammals is that via L-cysteinesulfinic acid (CSA) (another term L-alanine 3-sulfinic acid is also used; Roy and Trudinger, 1970; Trudinger and Loughlin, 1981; Ubuka et al., 1982), which is formed by the reaction catalyzed by cysteine dioxygenase (EC 1.13.11.20) (Griffith, 1987). CSA is converted to hypotaurine by the reaction catalyzed by CSA decarboxylase (EC 4.1.1.29) (Griffith, 1987). Hypotaurine is then oxidized to taurine in mammalian tissues by enzymatic and/or non-enzymatic reactions. Possible antioxidant role of hypotaurine has been studied (Fellman and Roth, 1985; Aruoma et al., 1988; Green et al., 1991), but the mechanism of hypotaurine oxidation (Kontro and Oja, 1985) and its role are not fully understood.

CSA decarboxylase is commonly found in mammalian liver and brain (Boeker and Snell, 1972). It has been recognized that hypotaurine formation in rat liver occurs in cytosol (Trudinger and Loughlin, 1981). The properties of the cytosolic CSA decarboxylase in rat liver has been studied (Jacobsen et al., 1964) and it was purified (Griffith, 1983; Weinstein and Griffith, 1987).

Mitochondrial cysteine metabolism is summarized in Figs. 1 and 2 based on our previous studies (Ubuka et al., 1990, 1992; Ubuka, 2002). On the mitochondrial metabolism of CSA, it has been recognized that CSA is taken up by mitochondria (Palmieri et al., 1979), in which CSA is transaminated by aspartate aminotransferase (EC 2.6.1.1) to form sulfinopyruvic acid. This compound decomposes nonenzymatically to form sulfinic acid and pyruvic acid. Sulfinic acid thus formed is oxidized to sulfuric acid by sulfite oxidase (EC 1.8.3.1) which confined in mitochondria (Cohen, 1972). These serial reactions occur in mitochondria because aspartate aminotransferase is

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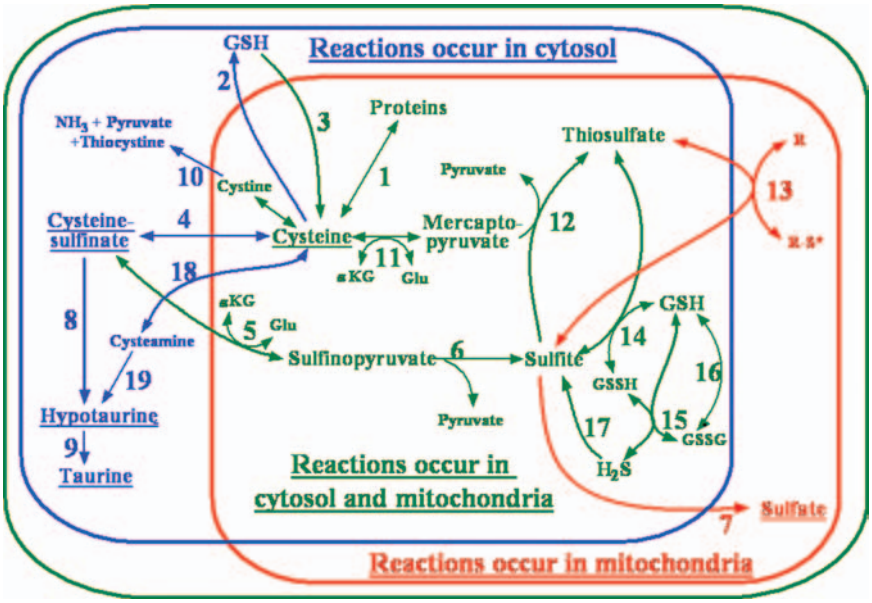

Fig. 1. Mammalian cysteine metabolism. Summarized from our previous publications (Ubuka, 2002; Ubuka et al., 1992, 1990). Numbers are reaction or enzyme which are shown in Fig. 2

No	Reaction or enzyme	Cytosol	Mitochondria
1.	proteolysis and protein synthesis	●	●
2.	glutathione synthesis	●	–
3.	glutathione degradation	●	●
4.	cysteine dioxygenase	●	–
5.	aspartate aminotransferase	●	●
6.	non-enzymatic	●	●
7.	sulfite oxidase	–	●
8.	cysteine sulfinate decarboxylase	●	●
9.	hypotaurine oxidation	●	?
10.	γ-cystathionase	●	–
11.	asparate amino transferase	●	●
12.	mercaptopyruvate sulfurtransferase	●	●
13.	thiosulfate sulfurtransferase	–	●
14.	glutathione-dependent thiosulfate reductase	●	●
15.	non-enzymatic	●	●
16.	glutathione reductase	●	●
17.	non-enzymatic	●	●
18.	biosynthesis and catabolism of pantetheine and coenzyme A	●	–
19.	cysteamine dioxygenase	●	–

Fig. 2. Reaction or enzymes of cysteine metabolism in cytosol (●) and mitochondria (●)

active in mitochondria and sulfite oxidase is localized in mitochondria. Thus it is reasonable to conclude that mitochondria are the main sites of sulfate formation in the cell.

We have studied in vivo production of hypotaurine and taurine in mice injected with CSA (Nakamura et al., 2006). Loading of CSA resulted in the increase in contents of hypotaurine and taurine in the liver, but hepatic taurine content did not increase by hypotaurine loading although hypotaurine increased greatly. From these results and our previous studies in cysteine metabolism in liver mitochondria (Ubuka et al., 1990, 1992), we assumed that CSA taken up by liver cells and transferred to mitochondria was decarboxylated to hypotaurine. The present study was undertaken to investigate this hypothesis and presents evidence to show that hypotaurine is formed when CSA is incubated with mitochondrial preparation obtained from rat liver.

Materials and methods

Animals

Male Wistar rats of 7–10 weeks of age were used. CSA, pyridoxal 5′-phosphate, hypotaurine, taurine and sucrose were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. 4-Dimetylaminoazobenzene-

4′-sulfonyl chloride (dabsyl chloride) was obtained from Tokyo Kasei Co. Ltd., Tokyo, Japan. A cation-exchange resin AG 50W (X8, 200–400 mesh) was obtained from Bio-Rad Co. Japan (Tokyo).

Methods

Mitochondrial preparation

Mitochondrial preparation was prepared from rat liver as reported previously (Ubuka et al., 1990, 1992) according to the method of Hogeboom (1955) using 0.25 and 0.34 M sucrose solutions containing 10 mM Tris · HCl (pH 7.4) and 0.1 mM EDTA. The mitochondrial fraction obtained by the centrifugation at $8000 \times g$ was washed 2–5 times with 0.25 M sucrose solution containing 10 mM Tris · HCl (pH 7.4). The final pellet was suspended in the same solution at a concentration of mitochondrial fraction from 2 grams of fresh liver per ml. The mitochondrial suspension was divided into 2 parts, one of which was sonicated at 20 kH for 5 min under cooling in an ice-water bath using a sonicator (Ultrasonic processor VC-130PB, Sonics & Materials, Inc., Newtown, CT, U.S.A.). Intact and sonicated mitochondrial suspensions were used for enzyme reactions. Protein concentration was determined by the biuret reaction using bovine serum albumin as the standard.

Enzyme reaction

Reaction mixture contained, in a final volume of 1.0 ml placed in a test tube (15 mm × 110 mm), 100 μmol of potassium phosphate buffer (pH 7.8), 0.1 μmol of pyridoxal 5′-phosphate, 20 μmol of CSA (neutralized with potassium hydroxide solution) and 0.5 ml of mitochondrial preparation. The mixture was incubated at 37 °C for 30 min with shaking at 100 strokes per min. The reaction was terminated by the addition of 1.0 ml of 1 M perchloric acid and resulting precipitate was centrifuged off. Perchloric acid in the resulting supernatant was eliminated by the neutralization with 20% potassium hydroxide and centrifugation. The resulting supernatant (PCA extract) was used for the identification and determination of reaction products.

Identification and determination of hypotaurine and taurine

To 0.5 ml of the PCA extract placed in a test tube with a Teflon-lined screw cap, 50 μl (50 nmol) of L-asparagine (internal standard), 0.5 ml of 0.1 M sodium bicarbonate buffer (pH 9.0), and 1.0 ml of dabsyl chloride solution (3.3 mg per 5 ml of acetone) were added. The mixture was heated at 70 °C for 10 min, and then evaporated to dryness using a flash evaporator at 50 °C. The resulting residue was dissolved in 1.0 ml of 70% ethanol and filtered (dabsylated sample).

The dabsylated sample was subjected to reversed-phase HPLC (RP-HPLC) according to the previously reported method (Futani et al., 1994; Nakamura and Ubuka, 2003) with some modifications. The column used was ODS-80Ts (4.6 mm ID × 150 mm L, particle size 5 μm) with a guard column of ODS-80Ts (3.2 mm ID × 15 mm L). Solvents used were: A, 50 mM sodium acetate (pH 4.033) and B, acetonitrile. Elution program was as follows: 0–3 min, 28% B; 3–30 min, linear gradient of B from 28 to 29%; 30–40 min, linear gradient of B from 29 to 30%. Chromatography was performed at a flow rate of 0.7 ml per min at 20 °C. L-Asparagine was used as an internal standard, because its content in mitochondria was negligibly low.

Treatment of dabsylated samples with H_2O_2 was performed according to our previous experiments (Futani et al., 1994). To 200 μl of the dabsylated sample, 10 μl of 4% H_2O_2 solution was added (final concentration, 0.2%). After the incubation at 40 °C for 2 h, the mixture was subjected to RP-HPLC as above.

Direct H_2O_2 treatment of PCA extract was performed as follows. To 0.5 ml of the PCA extract obtained above, 10 μl of 30% H_2O_2 solution was added (final concentration, 0.6%), and the mixture was incubated at room temperature (about 28 °C) for 2 h. The mixture was applied to AG50W column (H^+ form, 7 mm ID × 52 mm L, bed volume 2.0 ml) and the column was washed with 10 ml of water. The initial effluent and the washing were combined and evaporated to dryness at 60 °C with a flash evaporator. Complete evaporation was necessary because H_2O_2 deteriorated dabsyl chloride and inhibited dabsylation. The dry residue was dissolved in 0.5 ml of water and dabsylation reaction and RP-HPLC were performed as above.

The presence of hypotaurine in the reaction products was also examined using an amino acid analyzer. The PCA extract obtained above was 20 times diluted with 2.5% trichloroacetic acid solution. Twenty microlitre of the diluted solution was applied to a Hitachi automatic amino acid analyzer L-9800 BF equipped with a column #2622sc (PF) (4.6 mm ID × 60 mm L) designed for the analysis of physiological fluids. Analyzer was operated by EZ Chrom Elite software for Hitachi using custom-made buffer solutions and ninhydrin reagents.

Results and discussion

Figure 3A shows a chromatogram of RP-HPLC of the dabsylated standard amino acid mixture. Hypotaurine and taurine are clearly separated from other dabsyl-amino acids.

Figure 3B is a chromatogram of dabsylated sample of PCA extract of the reaction mixture, showing that a peak corresponding to dabsyl-hypotaurine is present. A small peak corresponding to dabsyl-taurine is also present.

When the same sample as that of Fig. 3B was treated with 0.2% H_2O_2 at 40 °C for 2 h, a peak corresponding to dabsyl-hypotaurine in Fig. 3B disappeared completely and the peak corresponding to dabsyl-taurine increased as shown in Fig. 3C. The increase of the peak calculated as taurine in Fig. 3C was 96% of the peak in Fig. 3B calculated as hypotaurine. In other experiments of RP-HPLC, the peaks of dabsylated authentic hypotaurine and taurine added to dabsylated reaction products overlapped completely with these peaks which were regarded as dabsyl-hypotaurine and dabsyl-taurine, respectively.

Figure 3D is a chromatogram of a sample, of which PCA extract before dabsylation was treated with H_2O_2 and AG50W column as described under Methods section. It has been reported that hypotaurine is oxidized to taurine by H_2O_2 (Awapara, 1953; Sturman, 1980; Futani et al., 1994). It is well known that taurine is not retained in the column of H^+-form of strongly acidic cation-exchange resin such as Dowex 50 and AG50W. As shown in the figure, a peak corresponding to taurine was detected when the effluent from the AG50W column was subjected to dabsylation and RP-HPLC.

When the PC extract was analyzed by amino acid analyzer, a tiny but distinct peak of hypotaurine was detected on the chromatogram (data not shown).

The results described here indicate that hypotaurine was formed when CSA was incubated with sonicated

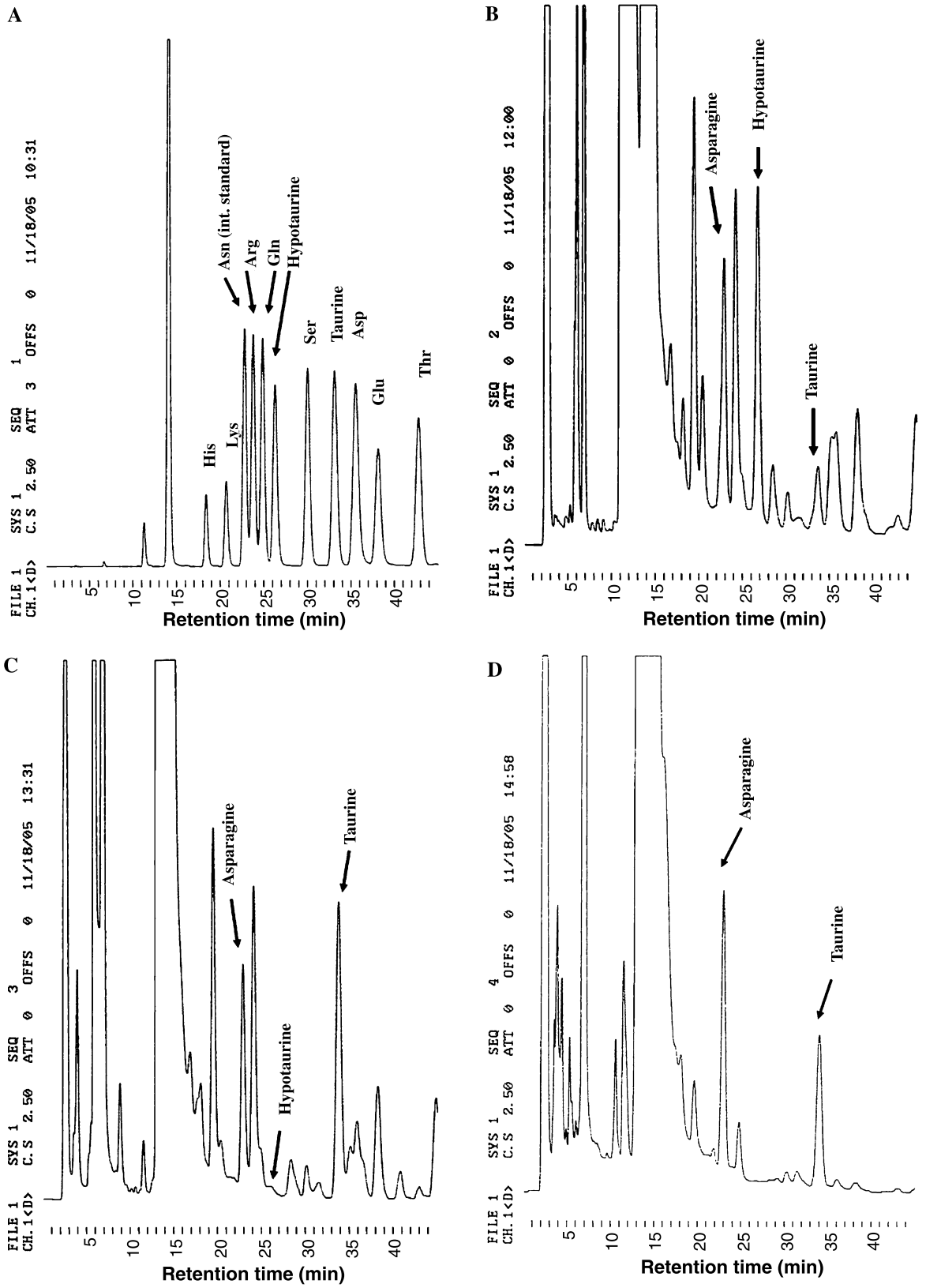

Fig. 3. RP-HPLC of dabsyl derivatives of authentic amino acids (**A**) and of products of reaction mixture containing L-cysteinesulfinate, pyridoxal 5′-phosphate and rat liver mitochondrial preparation (**B**). Upon the treatment of dabsylated products (**B**) with H_2O_2, dabsyl-hypotaurine peak disappeared and dabsyl-taurine peak increased (**C**). When PCA-extract of the enzyme reaction products was treated with H_2O_2, a cation-exchange resin column, and with dabsyl chloride, dabsyl-taurine was detected (**D**). Asparagine in **B**–**D** is an internal standard added to each sample before dabsylation. See Methods for experimental details

mitochondrial preparation. In a typical experiment with sonicated mitochondrial preparation, the amount of hypotaurine plus taurine formed was 8.94 nmol per mg of protein per 60 min. When a mitochondrial preparation without sonication was used, the products (hypotaurine plus taurine, nmol per mg of protein per 60 min) was 5.48 (61% of the yield with the sonicated), and when pyridoxal 5′-phosphate was avoided from the reaction mixture, the product was 7.52 (84% of the reaction with pyridoxal 5′-phosphate). When the wash of mitochondrial preparation with 0.25% sucrose solution was repeated 5 times, the yield of mitochondrial preparation decreased, but hypotaurine-forming activity per mg of protein was retained at a similar level. These results indicate that hypotaurine-forming activity, namely, CSA decarboxylase activity is associated with mitochondria, and that pyridoxal-5′-phosphate is required in the reaction. Studies on the effect of pH and CSA concentration in the present system have shown that optimum pH was broad between 6.0 and 7.8, and that Km for CSA was 0.11 mM.

In the reaction lacking CSA, a small peak of taurine (less than 0.1 nmol per mitochondrial fraction obtained from 1 g of liver) was detected. The peak was much smaller than the taurine peak in Fig. 1B. This result seems to show that a small amount of taurine may be present in mitochondria.

Kaczmarek et al., (1970) reported that a crude mitochondrial fraction prepared from rat brain contained CSA decarboxylase activity and Urban and Reichert reported the purification of CSA decarboxylase from rat brain (Urban and Reichert, 1981). As mentioned above, however, in liver, it has been recognized that CSA decarboxylase is a cytosolic enzyme (Jacobsen et al., 1964; Trudinger and Loughlin, 1981; Griffith, 1983; Weinstein and Griffith, 1987). Present results indicate that CSA decarboxylase activity is also present in liver mitochondria.

It has been found that taurine is contained in modified uridines (5-tauromethyluridine and 5-tauromethyl-2-thiouridine) of tRNAs in human and bovine mitochondria and these modified uridines are located at anticodon wobble position of mitochondrial tRNA (Suzuki et al., 2002). This means that taurine is an important constituent in mitochondrial function.

It has been found that mitochondria are the source of intracellular active oxygen species and it is postulated that these species are responsible for aging and carcinogenesis (Singh, 2004). In mitochondria, antioxidant systems such as glutathione peroxidase, superoxide dismutase, catalase and antioxidant vitamins are regarded to function for the elimination of active oxygen species. As summarized in Figs. 1 and 4, cysteine is metabolized through various pathways. In these pathways the sulfur atom of cysteine is successively oxidized from −2 to +6 of oxidation state as shown in Fig. 4. Therefore, in addition to above-mentioned agents, cysteine metabolites such as hydrogen sulfide, sulfite, thiosulfate and hypotaurine might be candidates for chemical antioxidants in mitochondria. These compounds are all intermediates of cysteine metabolism leading to the final metabolites, sulfate and taurine.

The possible antioxidant action of hypotaurine has been studied (Fellman and Roth, 1985; Aruoma et al., 1988; Green et al., 1991), but the mechanism of hypotaurine

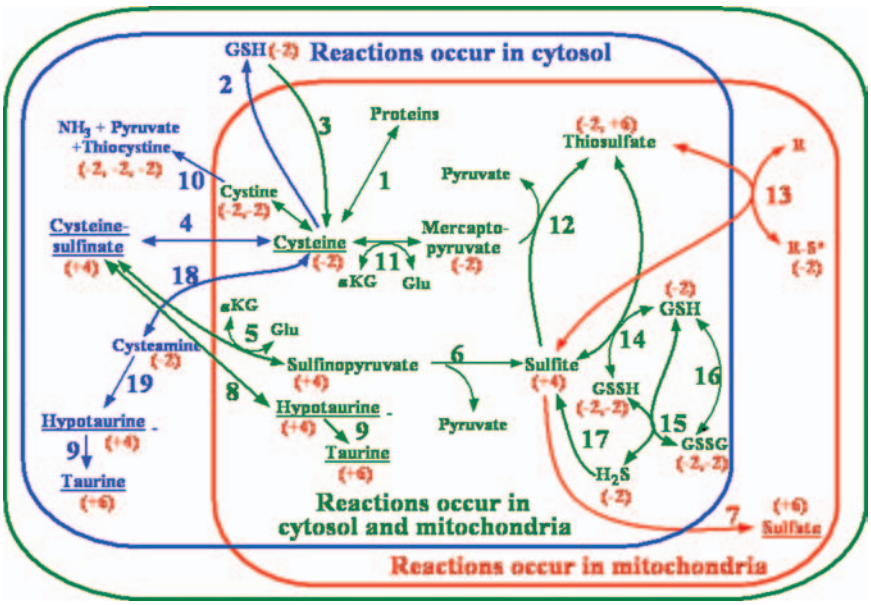

Fig. 4. Mammalian cysteine metabolism. Hypotaurine formation in mitochondria is included in the figure. Oxidation states of sulfur atoms of cysteine metabolites are shown in parentheses

oxidation (Kontro and Oja, 1985) is not fully understood. Further studies on the characterization of liver mitochondrial cysteinesulfinate decarboxylase and the significance of cysteine metabolism including hypotaurine formation in liver mitochondria are under investigation.

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