Taurine as a constituent of mitochondrial tRNAs: new insights into the functions of taurine and human mitochondrial diseases

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Taurine (2-aminoethanesulphonic acid), a naturally occurring, sulfur-containing amino acid, is found at high concentrations in mammalian plasma and tissues. Although taurine is involved in a variety of processes in humans, it has never been found as a component of a protein or a nucleic acid, and its precise biochemical functions are not fully understood. Here, we report the identification of two novel taurine-containing modified uridines (5-taurinomethyluridine and 5-taurinomethyl-2-thiouridine) in human and bovine mitochondrial tRNAs. Our work further revealed that these nucleosides are synthesized by the direct incorporation of taurine supplied to the medium. This is the first reported evidence that taurine is a constituent of biological macromolecules, unveiling the prospect of obtaining new insights into the functions and subcellular localization of this abundant amino acid. Since modification of these taurine-containing uridines has been found to be lacking in mutant mitochondrial tRNAs for Leu(UUR) and Lys from pathogenic cells of the mitochondrial encephalomyopathies MELAS and MERRF, respectively, our findings will considerably deepen our understanding of the molecular pathogenesis of mitochondrial encephalomyopathic diseases.

Keywords: mitochondrial/mitochondrial disease/post-transcriptional modification/taurine/tRNA

Introduction

A characteristic structural feature of tRNAs is the presence of post-transcriptionally modified nucleosides at the anticodon first position (the ‘wobble’ position), which participate in codon–anticodon pairing (Curran, 1998). In mammalian mitochondrial (mt) tRNAs, uridine at the anticodon wobble position of the tRNA\(^{Leu(UUR)}\) and tRNA\(^{A339\text{A}}\) undergoes such post-transcriptional modification, which is responsible for precise codon recognition. However, we recently discovered that the normal uridine modification does not occur in mt tRNA\(^{Leu(UUR)}\) with either an A3243G or U3271C mutation, or in mt tRNA\(^{A339\text{A}}\) with an A8344G mutation obtained from human pathogenic cells of two mitochondrial encephalomyopathic diseases, MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; Yasukawa et al., 2000a) and MERRF (myoclonus epilepsy associated with ragged-red fibers; Yasukawa et al., 2000b), respectively. Since it seemed likely that an unmodified uridine would cause either a misreading (on the basis of the mitochondrial wobble rule; Watanabe and Osawa, 1995) or a decoding deficiency, we examined the translational abilities of these mutant tRNAs using an in vitro mitochondrial translation system developed in our laboratory (Takemoto et al., 1995, Hanada et al., 2001). The results showed that mt tRNA\(^{Leu(UUR)}\) with the MERRF A8344G mutation was incapable of translating cognate codons due to a complete loss of codon–anticodon pairing on the ribosome (Yasukawa et al., 2001), strongly implying that in addition to the pathogenic point mutation itself, deficient decoding arising from the modification defect is significantly involved in this mitochondrial dysfunction. This is the first known case of a human disease apparently caused by the loss of a post-transcriptional modification.

To understand the molecular mechanisms of MELAS and MERRF pathogenesis, which will hopefully lead to the development of appropriate therapeutic measures for these mitochondrial diseases, the chemical structures of the modified uridines in mammalian mt tRNAs for Leu(UUR) and Lys need to be ascertained. In this study, we describe the identification and determination of novel taurine-containing uridine derivatives from bovine and human mt tRNAs. Although taurine has never been found as a component of a protein or a nucleic acid, the unique chemical structures of the derivatives prompt us to speculate that dietary taurine is a direct substrate for them.

Taurine, one of the most abundant amino acids in mammalian plasma and tissues, is known to have pleiotropic effects including modulation of calcium fluxes, maintenance of photoreceptor cells, modulation of neuronal excitability, osmoregulation and cell proliferation (Huxtable, 1992). However, its precise biochemical functions are still not fully understood. We demonstrate here that the novel modified uridines in mt tRNAs are synthesized by direct incorporation of taurine supplied to the medium. This is the first evidence that taurine is a component of RNA, a finding that provides us with new clues to understanding its biological functions and subcellular localization, as well as the molecular pathogenesis of mitochondrial diseases.

Results

Chemical structures of novel taurine-containing uridines

We succeeded in isolating mitochondrial disease-related mt tRNAs for Leu(UUR) and Lys from bovine liver by
means of an improved solid-phase DNA probe technique (Wakit & al., 1994; see Materials and methods). Subsequent liquid chromatography/mass spectrometry (LC/MS) analysis revealed the two previously unknown nucleosides to be a uridine derivative (U; molecular mass, 381 Da) in mt tRNA^{Leu(UUR)} and its 2-thio derivative (s^2U; 397 Da) in mt tRNA{Lys}, respectively (Figure 1A). The presence of the 2-thio derivative in mt tRNA{Lys} was demonstrated in two ways: (i) specific retardation of mt tRNA{Lys} migration on phenyl-mercuric gel electrophoresis (Iglo, 1988) due to the thioacylorn group (Figure 1B), and (ii) a prominent peak at 240 nm in the UV spectrum of the purified nucleoside under an alkaline condition (Figure 1C), which is known to be a characteristic feature of 2-thiouridine derivatives (Watanabe & et al., 1974). NMR analysis of the nucleoside revealed no H6 proton cross peak in the 1H-COSY spectrum, indicating the presence of a position at 5 in the uracil base. No modification was found in the ribose portion as all ribose protons and expected cross peaks were assigned (Figure 1D). The molecular weight of s^2U was determined with a high degree of precision by using a Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer with a 7-tesla magnet (Figure 1E). Its atomic composition was ascribed to C_{12}H_{19}N_{4}O_{4}S_{2}, also with excellent accuracy (0.03 p.p.m.). These findings indicate that the main modification occurs in the uracil base, the most plausible structure in both cases being a taurinomethyl possessing a sulfonic acid group derived from taurine (Figure 2A). The two nucleosides were thus named 5-taurinomethyluridine (tm^3U) and 5-taurinomethyl-2-thiouridine (tm^5s^2U). The former was chemically synthesized by the Mannich reaction (Jones & al., 1982) as described in Materials and methods. The novel nucleoside was determined to be tm^5s^2U by comparison with the synthetic product: LC/MS revealed that U* from mt tRNA^{Leu(UUR)} was co-eluted with synthetic tm^5s^2U at the same retention time (Figure 1F). In addition, their CID (collision-induced dissociation) fragment patterns and NMR spectra were clearly identical (data not shown).

We confirmed that these taurine-containing uridines are actually located at the anticodon wobble position of each tRNA by Donis-Keller’s RNA sequencing method (Donis-Keller, 1980), the post-labeling method (Kuchino & al., 1987) and mass spectrometric analysis. RNase T1-digested fragments containing anticodon wobble position, CA^3PAAACUA[tm^3s^2U]AAC^6P4UUAUACCCm^2CA^5Gp (mol. wt 8416) and CACPAACC[tm^3s^2U]UUt^6AA^5Gp (mol. wt 5059) were specifically detected from bovine mt tRNA^{Leu(UUR)} and mt tRNA{Lys}, respectively (data not shown). These two taurine-containing uridines were also found in human mt tRNAs for Leu(UUR) and Lys (Figure 2B).

**Direct incorporation of taurine into human mitochondrial tRNA**

Taurine, an abundant amino acid in mammalian plasma, is widely used as an ingredient of nutritional supplements. However, though taurine is clearly pleiotropic in its effects on the human body (Huxtable, 1992), its exact functions in biochemical terms are still to be elucidated. Since there has been no report so far of taurine being a component of any protein or nucleic acid, we attempted to determine whether it is a direct constituent of the two taurine-containing modified uridines that we identified in the mt tRNAs. For this purpose, we synthesized a stable isotopic taurine with [3H]oxazoline in the sulfonic acid group (Figure 3A); 80% of the synthetic taurine had two [3H]oxazolines, while the remaining 7% and 15% had one and three, respectively (Figure 3B). HeLa cells were grown for 48 h in a medium containing the [3H]taurines thus synthesized. The cells were then harvested and the mt tRNAs for Leu(UUR) and Lys were isolated (Figure 3C). When purified mt tRNA{Lys} was subjected to nucleoside analysis, tm^3s^2U incorporating [3H]taurine was clearly identified in the LC/MS mass chromatogram (Figure 3D); tm^5s^2U with a 4 Da increase in its mass (m/z 402) was detected at the same retention time as natural tm^5s^2U (m/z 398). Likewise, in mt tRNA^{Leu(UUR)}, tm^5s^2U incorporating [3H]taurine (m/z 386) was detected at the same retention time as natural tm^5s^2U (m/z 382) (see Figure 4A).

As shown in the LC/MS mass spectra obtained at the elution points of tm^5s^2U and tm^3s^2U (Figure 4A), 2–6 Da increases were observed in the molecular masses of tm^5s^2U and tm^3s^2U, respectively, obtained from purified mt tRNAs for Leu(UUR) and Lys, and the ratio of synthetic isotopic taurines with different numbers of [3H]oxazolines (Figure 3B) was exactly preserved both in tm^5s^2U and tm^3s^2U, demonstrating that the sulfonic acid groups of tm^5s^2U in mt tRNAs came directly from the taurine supplied in the medium. The CID spectra of tm^5s^2U and tm^3s^2U possessing two [3H]oxazolines confirmed that fragment ions containing the sulfonic acid group specifically exhibited a 4 Da increase in mass due to the presence.
of the two [$^{18}$O]oxgens (Figure 4B and C). These results provided direct evidence that taurine in the medium was incorporated into mitochondria without decomposition to become a component of the taurine-containing uridines in the mt tRNAs.

Judging from the relative abundances in the spectra of the natural (black) and isotopic (red) taurine-containing uridines (Figure 4A, lower panels), $\text{tm}^3\text{U}$ from mt tRNA$\text{Leu}(\text{UUR})$ and $\text{tm}^3\text{s}^2\text{U}$ from mt tRNA$\text{Lys}$ consisted of 96 and 91% [$^{18}$O]taurine, respectively. Although a small quantity of taurine is known to be synthesized from cysteine de novo in human cells, our experiment clearly proved that taurine supplied to the medium was efficiently incorporated into the mt tRNAs in spite of the presence of medium cysteine, suggesting that plasma taurine is a major constituent of the taurine-containing uridines, even in the human body.
**Taurine uptake by mitochondria**

Our findings revealed a new pathway for the transport of cytoplasmic taurine to mitochondria, implying the existence of a putative mitochondrial taurine transporter responsible for cytoplasmic taurine uptake by mitochondria, where it is used to synthesize \( \text{tm}^{3}\text{s}^{3}\text{U} \). Although there has been no report of taurine transport into mitochondria, when we examined mitochondrial taurine uptake \textit{in vitro} using the standard method for taurine uptake by intact cells (Uchida et al., 1992; Qian et al., 2000), we found that taurine was time-dependently incorporated into isolated bovine mitochondria (Figure 5). This suggests active transport of cytoplasmic taurine into mitochondria, because it is known that lipophobic taurine does not penetrate the lipid bilayer by diffusion (Huxtable, 1992).

**Decoding activity of mitochondrial tRNA with taurine-containing wobble uridine**

In previous studies, we found that taurine modification was lacking at the wobble uridine of mutant tRNAs from pathogenic cells obtained from patients with MELAS and MERRF (Yasukawa et al., 2000a, b, 2001). To estimate the effect of the C5 taurine modification at the wobble position of mt tRNA\textsuperscript{Leu(UUR)} on the decoding activity and to clarify whether it does contribute significantly to the defective tRNA function observed in mitochondrial diseases, we carried out a ribosome-binding experiment with modified wild-type tRNA missing the \( s^{3}\text{U} \) modification but still containing the C5 taurine modification. A small amount of
succeeded in purifying from wild-type mt tRNA^{lys} with \( \text{tm}^{35}\text{S} \) by phenyl-mercuric gel electrophoresis (Figure 6A). We used this in the ribosome-binding experiment, which was carried out with the AAA codon-programmed small subunit ribosome as described previously (Yasukawa et al., 2001). The results showed that while the absence of the \( \text{s} \)U modification considerably reduced AAA codon binding, mt tRNA^{lys} with \( \text{tm}^{35}\text{S} \) still retained a high level of efficiency (63% activity of \( \text{tm}^{35}\text{S} \)U) compared with its transcript (Figure 6B). We thus concluded that in addition to the \( \text{s} \)U modification, the C5 taurine modification confers codon-binding efficiency.

**Discussion**

Our findings are the first reported instance of any modified nucleoside having a sulfonic acid group in a side chain, and it will be of great interest to clarify the decoding properties of these nucleosides in relation to the strong electrostatic of the sulfonic acid group. Since we have also found these two taurine-containing uridines in ascidian mt tRNA counterparts (Kondow et al., 1999; A.Kondow, T.Suzuki and K.Watanabe, unpublished observations), it is likely they are common to vertebrate and protocordate mitochondria. Meanwhile, modified uridine at the wobble position of yeast mt tRNA was
reported as 5-carboxymethylaminomethyluridine (cmmm$^5$U; Osawa, 1995). In addition, we found that Caenorhabditis elegans mt tRNAs have cmmm$^5$U at the wobble position (M.Sakurai, T.Ohtsuki, T.Suzuki and K.Watanabe, in preparation). Since glycine is attached through a methylene group at position 5 in the uracil base of cmmm$^5$U instead of taurine, cmmm$^5$U and $^5$m$^3$U both have negatively charged side chains. Further, by analogy with the findings of the present study, glycine is presumably a direct substrate for cmmm$^5$U synthesis. Thus, there appears to be a relationship between the mitochondrial decoding property and the evolutionarily conserved chemical character of modified uridines in mt tRNAs.

In previous studies (Yasukawa et al., 2000a,b), we found a lack of taurine modification at the wobble uridine of mt tRNA$^{Leu(UUR)}$ and tRNA$^{Lys}$ from pathogenic cells of MELAS and MERRF, respectively. We also demonstrated that the modification deficiency of mutant tRNA$^{Lys}$ with the MERRF 8344 mutation causes defective translation of both AAA and AAG codons due to weak codon–anticodon interaction on the ribosomal small subunit (Yasukawa et al., 2001). Since the 2-thio group of wobble uridine is known to confer stable codon–anticodon interaction (Ashraf et al., 1999), loss of the 2-thio group of mt tRNA$^{Lys}$ with the MERRF 8344 mutation is assumed to be one of the main reasons for the weak binding to cognate codons. In the case of MELAS, mt tRNA$^{Leu(UUR)}$ has been shown to have $^5$m$^3$U at the wobble position, indicating that a decoding disorder arising from lack of the C5 taurine modification is also a causative factor of mitochondrial diseases. Yarian et al. (2002) reported that the C5 modification (mnm$^3$U34) in the anticodon stem loop derived from Escherichia coli tRNA clearly stabilizes codon–anticodon interaction at both the ribosomal A and P sites. The fact that misreading of asparagine codons by tRNA$^{Lys}$ was greatly reduced in $^{tm}mU$ and $^{tm}E$ mutants, respectively, containing hypomodified mnm$^3$U34 and s$^5$U34 instead of the fully modified mnm$^3$5$^3$U34 (Hagervall et al., 1998), indicates that both the 2-thio group and C5 modification confer efficient codon recognition. In the present work, we observed that absence of the s$^5$U modification considerably reduced AAA codon binding, but mt tRNA$^{Lys}$ with $^5$m$^3$U retained relatively high efficiency compared with its transcript (Figure 6B). Also, we previously reported that the binding efficiency of MERRF tRNA lacking both the 2-thio and C5 taurine
modifications was reduced 10-fold (Yasukawa et al., 2001). The binding efficiency of the MERRF tRNA was at the same level as that of its human transcript (our unpublished observations). Thus, our findings demonstrate the apparent contribution of the C5 taurine modification in efficient codon recognition.

In human body fluids, including plasma, taurine concentrations range from 10 to 100 μM, while intracellular concentrations can rise to several hundred times as great (Huxtable, 1992). The taurine concentration gradient across the cell membrane is maintained by a high-affinity taurine transporter (Uchida et al., 1992; Figure 7A). Most cytoplasmic taurine is known to be excreted as such, or in bile salts that are taurine-conjugated metabolites of cholesterol such as taurocholate (Huxtable, 1992). Our results strongly suggest a new pathway for the transport of cytoplasmic taurine into mitochondria (Figure 7A). Time-dependent taurine uptake by isolated bovine mitochondria suggests active transport of taurine across the mitochondrial membrane. In practice, since cytosolic taurine is known to accumulate at concentrations up to 40 mM (Huxtable, 1992), mitochondrial taurine uptake is considered to be more efficient in cells. In this regard, taurine has been shown to be localized in most subcellular compartments, including mitochondria (Lobo et al., 2000).

The biosynthesis of tm3(s)U is considered to involve several modification enzymes, including a putative taurine transferase (Figure 7B). The first step of tm3(s)U biosynthesis is likely to be similar to an xmp3-U-type modification with a methane group at the root of position 5, such as mmmp3(s)U (5-methylaminomethyl-2-thio-uridine) in bacterial tRNAs or ccmmp3U in yeast mt tRNAs (Björk, 1995). In E. coli, the tm3E gene is known to be responsible for the initial step of this type of modification (Elseviers et al., 1984). It can be assumed that the pathogenic point mutations of MELAS or MERRF abolish the tRNA recognition by the mitochondrial tm3E homolog (tm3E+) (Figure 7B). Thus, each of the point mutations (3243, 3271 or 8344) is considered to be selected as a negative determinant in the initial step of taurinomethyl synthesis.

It is of interest that taurine appears to be an essential nutrient for cats and possibly for primates, including humans (Hayes et al., 1975; Geggel et al., 1985; Hayes, 1985). As noted earlier, taurine is involved in a variety of important processes, including synthesis of bile salts, modulation of calcium fluxes, maintenance of photoreceptor cells, modulation of neuronal excitability, antioxidation, osmoregulation and cell proliferation (Huxtable, 1992). Although taurine-containing small peptides such as glutamyl-taurine (Marmela et al., 1985) have been isolated from brain, taurine has thus far been found as a component of a protein or a nucleic acid, and its precise biochemical functions are still obscure. In the case of humans, infants and young children biosynthesize very little taurine, so dietary taurine is essential for normal human development (Sturman, 1993). The cat and the fox have no biosynthetic pathway for this amino acid, and, in both of these animals, a deficiency of dietary taurine has been shown to cause cardiomypathy (Pion et al., 1987; Moise et al., 1991), which significantly is a major manifestation of human mitochondrial encephalomyopathies (Wallace, 2000). Although taurine plays crucial roles in myocardial functions such as calcium flux modulation and cardiac contractility, our evidence strongly suggests that incomplete modification of tm3(s)U in mt tRNAs due to a low plasma taurine level is likely to be one of the main causative factors of cardiomypathy in cats, in a similar way to mitochondrial encephalomyopathies in humans (Figure 8). Further study of taurine import into mitochondria and the biosynthetic pathway(s) of tm3(s)U will shed new light on the biochemical role(s) of taurine.

Materials and methods

Purification of individual mitochondrial tRNAs from bovine liver

The crude RNA fraction was extracted from bovine liver according to the literature (Nishimura, 1971). The RNA fraction (3.4 g) was obtained by anion-exchange column chromatography using DEAE-Sepharose Fast Flow (8 × 73 cm; Amersham Biosciences) with a linear gradient of NaCl and MgCl2, 25 l of elution buffer A (20 mM Tris–HCl pH 7.5, 200 mM NaCl and 8 mM MgCl2) and elution buffer B (20 mM Tris–HCl pH 7.5, 450 mM NaCl and 16 mM MgCl2) with gravitational flow, and a flow rate of ~6.5 mL/min. The fractions containing mt tRNAs for Leu(UUR) and Lys were determined by dot hybridization (Yokogawa et al., 1989) using the following synthetic DNA probes: B1L for bovine RNAi(UUR), 5'-GTTAAGGAGGATTTGACTTGGATA-3' and B1K for bovine RNAi(UUR), 5'-GTTAATGCTATATGCTTCTTAGT-3'.

The pooled fractions were combined, precipitated with ethanol and dissolved in a binding buffer (1.2 M NaCl, 30 mM HEPES–KOH pH 7.5 and 15 mM EDTA). To isolate individual mt tRNAs with the highest efficiency, we devised and successfully improved an original solid-phase DNA probe method (Wakita et al., 1994), which we have named ‘chapel’ column chromatography. A biotinylated DNA probe, as described above, complementary to each tRNA was immobilized on avidin Sepharose. Two columns, one for each individual mitochondrial tRNA, were connected in tandem and the DEAE RNA fraction was circulated through the chapel column by a peristaltic pump at a temperature of 65°C to entrap the target mt tRNAs. After washing out non-specific RNA with a wash buffer (0.6 M NaCl, 15 mM HEPES–KOH pH 7.5 and 7.5 mM EDTA), each individual tRNA was eluted separately from its respective column with a low-salt buffer (20 mM NaCl, 0.5 mM HEPES–KOH pH 7.5 and 0.25 mM EDTA) at 65°C. The individual tRNAs were purified homogeneously. A detailed description of this method will be reported in a specialized journal.
Purification of individual mitochondrial tRNAs from HeLa cells
The crude RNA fraction was extracted from HeLa cells according to the literature (Nishimura, 1971; Chomczynski and Sacchi, 1987). The RNA fraction was obtained by anion-exchange column chromatography using DEAE–Sepharose Fast Flow. The subsequent procedures were the same as described above. Synthetic DNA probes with 3’ biotin complementary to each tRNA were biotinylated for human mt tRNA(s) (UUR), 5’-GCAGATT-ACCCGGGCTGCTGCAATCTAC-3’ and kH1 for human mt tRNA(s), 5’-TACCTGTAAGAAGTTGGTGG-3’.

Liquid chromatography mass spectrometry
An LCQ ion trap (IT) mass spectrometer (ThermoFinnigan) equipped with an electrospray ionization (ESI) source and a MALDI 2002 liquid chromatography system (Microm BioResources) was used to analyze nucleosides from the mitochondrial tRNAs. Purified tRNAs (0.01–0.05 A260 units) were digested into nucleosides at 37°C for 1 h in 10 μl of a reaction mixture containing 20 mM HEPES–KOH pH 7.5, 10 μg/ml nuclease P1, and 0.5 U/ml bacterial alkaline phosphatase. The hydrolysates were analyzed by LC/MS as follows. An ODS reversed-phase column with a 3 x 10 mm pre-column cartridge (Inertsil ODS-3, 2.1 x 250 mm; GL Sciences) was connected online to the electrospray interface. The conditions for the chromatography were determined as described by Pomerantz and McCloskey (1990). Collision-induced dissociation (CID) spectra were obtained by an LC/MS/MS experiment using a data-dependent scan. The MS/MS setting was as follows: isolation width, m/z 1.0; activation amplitude, 15%; activation time, 30 msec.

NMR analysis
1H-COSY spectrum of purified 53U in D2O was obtained using a Bruker Daltonics AMX-500 at 298 K.

FT-ICR mass spectrometry
Purified 53U (5 pmol/μl) from mt tRNA(s) was subjected to ESI/FT-ICR mass spectrometry by infusion at a flow rate of 5 μl/min. The data were obtained using a Bruker Daltonics Bio APEX II 70e FT mass spectrometer with the kind help of Dr. K. Ueda (Bruker Daltonics).

Phenyl-mercuric gel electrophoresis
The presence of the 2-thio derivative in mt RNA for Lys was verified by electrophoresis in acrylamide gel immobilized with a phenyl-mercuric compound developed by Igloi (1988). To analyze purified mt tRNAs, we used 10% acrylamide gel containing 7 M urea and 0.05 mg/ml (N-acryloylamino) phenyl-mercuric chloride, which was kindly provided by Mr. N. Shiga of our laboratory.

Chemical synthesis of 5-taurinomethyluridine
2’-O-Isopropylideneuridine (142 mg, 0.33 mmol), taurine (313 mg, 2.54 mmol), paraformaldehyde (75 mg, 2.5 mmol) and triethylamine (0.35 ml, 2.5 mmol) were suspended in water (5 ml). The mixture was then heated and stirred at 110°C for 24 h. The cooled products were evaporated under reduced pressure. The residue was dissolved in formic acid/water (98.2 v/v; 5 ml) and the solution was allowed to stay at room temperature for 1 h. It was then concentrated under reduced pressure and the residue was co-evaporated with ethanol. The product was purified by reversed-phase column chromatography (Sep-Pak tC18, Waters; 10 μg). The appropriate fractions eluted from the column with water/acetonitrile (95:5 v/v) were collected and evaporated under reduced pressure. The residue was lyophilized from water to give mt5U (triethylammonium salt; 0.24 g, 25%).

Chemical synthesis of 55Otaurine
The synthesis scheme was determined as previously described in the literature (Hornung, 1955). Cystamine–2HCi (300 μmol) was dissolved in 20 mmol 55OH2 (95.1 atom% of 55O; Isotec). Br2 (1.8 mmol) was slowly dropped into the solution, which was then stirred for 60 min to oxidize the cystamine. The product was dried in vacuo and dissolved in 500 μl normal water (155OH2). The 55Oxygen contents in the synthetic taurine were determined by mass spectrometric analysis (Figure 2B); synthetic taurine with one, two or three 55Oxynegs comprised 7, 80 and 13% of the total, respectively. The differences would have arisen from dissolved oxygen in the reaction mixture and 2H3O impurity in 55O2H2O.

Pulse labeling with 55Otaurine
HeLa cells (3 x 106 cells) grown until semi-confluence in a rich medium (DMEM/F12; Gibco) with 10% FBS were cultured for 48 h in the presence of the synthetic 55Otaurine in a medium containing DMEM (Gibco), 15 mM HEPES–KOH, 0.2 mM 55Otaurine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco) and 10% dialyzed FBS (Gibco) (intrinsinc taurine removal). Total RNA was extracted from the cells using Isogen (Nippon Gene). mt tRNAs were subsequently isolated as described above.

Taurine uptake by isolated bovine mitochondria
The conditions were determined as described in the literature (Uchida et al., 1992; Quan et al., 2000) with modifications. Mitochondria were prepared from fresh bovine liver according to the standard methods (Schwartzbach et al., 1996). Isolated mitochondria (3 mg, protein weight) were incubated at 37°C in 10 mM HEPES–KOH pH 7.5, 150 mM KCl, 5 mM MgCl2, 0.44 mM mannitol, 0.2 mM DTT and 10 μM 55Otaurine (New England Nuclear). After washing three times with the buffer without taurine, mitochondria were dissolved in 1.5% Triton X-100 to measure the taurine incorporated by liquid scintillation counting.

Ribosome binding
Unmodified mt tRNA(s) was transcribed by T7 RNA polymerase (Milligan and Uhlenbeck, 1989) from template DNA synthesized by Klenow enzyme using two oligo DNAs: 5’-CCGGGTATACGACT- CACTATACACTAAGAGGTATAGTACTACCTTTTAAAGTG- TAGAGA-3’ and 5’-TGGTCACACAAAGGAGGATATGCTCT- AACCTCTAATTAAAAAGGTAGTGCTATA-3’. Purified bovine mt tRNA(s) and its transcript were dephosphorylated by bacterial alkaline phosphatase (Takara Bio) and labeled at the 5’ end with 32P according to the literature (Midgeley and Murray, 1985). 5532P-labeled mt tRNA(s) with 32P was separated from that with 32P by phenyl-mercuric gel electrophoresis as described above. The labeled tRNAs were eluted from the gel and quantified by measuring the absorbance at 260 nm. Ribosomal P-site binding was performed as described previously (Asahara et al., 1999; Yasukawa et al., 2001) with a slight modification. Binding of labeled tRNAs to the cognate codon on the ribosomal 30S subunit was carried out at 0°C for 1 h in 5 μl of a mixture consisting of 50 mM Tris–HCl pH 7.5, 30 mM MgCl2, 60 mM KCl, 1 mM DTT, 2 mM spermine, 0.2 μM E.coli 30S subunit, 0.1 μM 5532P-labeled tRNA and 0.1 μg/ml poly A (Sigma). The mixture was passed through a nitrocellulose filter (0.45 μm, Advantec) to quantify the bound tRNA as described previously (Asahara et al., 1999).

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References


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