Molecular and Biochemical Characterization of Rat e-N-Trimethyllysine Hydroxylase, the First Enzyme of Carnitine Biosynthesis*

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*e-N-Trimethyllysine hydroxylase (EC 1.14.11.8) is the first enzyme in the biosynthetic pathway of l-carnitine and catalyzes the formation of β-hydroxy-N-e-Trimethyllysine from e-N-trimethyllysine, a reaction dependent on α-ketoglutarate, Fe²⁺, and oxygen. We purified the enzyme from rat kidney and sequenced two internal peptides by quadrupole-time-of-flight mass spectrometry. The peptide sequences were used to search the Expressed Sequence Tag data base, which led to the identification of a rat cDNA of 1218 base pairs encoding a polypeptide of 405 amino acids with a calculated molecular mass of 47.5 kDa. Using the rat sequence we also identified the homologous cDNAs from human and mouse. Heterologous expression of both the rat and human cDNAs in COS cells confirmed that they encode e-N-trimethyllysine hydroxylase. Subcellular fractionation studies revealed that the rat enzyme is localized exclusively in mitochondria. Expression studies in yeast indicated that the rat enzyme is synthesized as a 47.5-kDa precursor and subsequently processed to a mature protein of 43 kDa, presumably upon import into mitochondria. The Michaelis-Menten constants of the purified rat enzyme for trimethyllysine, α-ketoglutarate, and Fe²⁺ were 1.1 mM, 109 mM, and 54 mM, respectively. Both gel filtration and blue native polyacrylamide gel electrophoresis analysis showed that the native enzyme has a mass of approximately 87 kDa, indicating that in rat e-N-trimethyllysine hydroxylase is a homodimer.

Carnitine (3-hydroxy-4-N-trimethylaminobutyrate) is a vital compound, which plays an indispensable role in the transport of activated fatty acids across the inner mitochondrial membrane into the matrix, where β-oxidation takes place (1, 2). Furthermore, carnitine is involved in the transfer of the products of peroxisomal β-oxidation, including acetyl-CoA, to the mitochondria for oxidation to CO₂ and H₂O in the Krebs cycle (3, 4). Apart from the dietary intake of carnitine, most eukaryotes are able to synthesize this compound from trimethyllysine (5–7). The trimethyllysine is generated by the hydrolysis of proteins containing lysines that are trimethylated at their ε-amino group by a protein-dependent methyltransferase using S-adenosylmethionine as a methyl donor. In the carnitine biosynthetic pathway, trimethyllysine is first hydroxylated at the β-position by e-N-trimethyllysine hydroxylase (TMLH¹), after which the resulting β-hydroxytrimethyllysine is cleaved by a specific aldolase into γ-trimethylaminobutyraldehyde and glycine (6, 8). Subsequently, γ-trimethylaminobutyraldehyde is oxidized by γ-trimethylaminobutyraldehyde dehydrogenase to form γ-butyrobetaine (9). In the last step, γ-butyrobetaine is hydroxylated at the β-position by a second hydroxylase, γ-butyrobetaine hydroxylase, yielding l-carnitine (5, 7, 10). In rat and mouse, γ-butyrobetaine hydroxylase is localized exclusively in the liver, whereas in humans, the enzyme is present in kidney, liver, and brain. Although most tissues are capable of converting trimethyllysine into γ-butyrobetaine, liver and kidney are the main sites of carnitine biosynthesis in all mammals, including humans (10–15).

After the recent identification of the cDNAs coding for γ-trimethylaminobutyraldehyde dehydrogenase and γ-butyrobetaine hydroxylase (16–18), we focused our attention on the first enzyme of the carnitine biosynthesis, TMLH. Like γ-butyrobetaine hydroxylase, TMLH is a non-heme ferrous iron dioxygenase that requires α-ketoglutarate, Fe²⁺, and molecular oxygen as cofactors (8, 19–21). In this class of enzymes, the hydroxylation of the substrate is linked to the oxidative decarboxylation of α-ketoglutarate. In both humans and rat, the highest TMLH activity is found in kidney but is also present in liver, skeletal muscle, heart, and brain (13, 20). Subcellular localization experiments using differential centrifugation indicated that the enzyme is predominantly localized in mitochondria (8, 21) in contrast to the other three carnitine biosynthetic enzymes, which are cytosolic. We purified the hydroxylase responsible for the conversion of trimethyllysine to hydroxymethyllysine from rat kidney and determined part of its amino acid sequence by (quadrupole-

¹ The abbreviations used are: TMLH, γ-trimethyllysine hydroxylase; MS, mass spectrometry; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; ORF, open reading frame; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption time of flight; Q-TOF, quadrupole-time-of-flight; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography; ESI, electrospray ionization; CMV, cytomegalovirus; EST, expressed sequence tag; kb, kilobase(s).
time-of-flight) mass spectrometry. Using this sequence information, we identified the cDNAs encoding trimethyllysine hydroxylase from rat, human, and mouse. Finally, we expressed the rat and human cDNAs in COS cells to confirm that the identified cDNAs encode TMLH.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trimethyllysine was purchased from Sigma Chemical Co. Q-Sepharose HP and Butyl-Sepharose 4 Fast Flow were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden), and CHT-II hydroxylapatite was from Bio-Rad (Hercules, CA). All other reagents were of analytical grade. The pMAL-C2X vector was purchased from New England BioLabs (Herts, United Kingdom), the pCDA3 vector was from Invitrogen (San Diego, CA).

**TMLH Assay**—Two methods were used to determine TMLH activity. In the first method, TMLH activity was determined radiochemically by measuring trimethyllysine-dependent release of $^{14}$C CO$_2$ that is produced from the decarboxylation of a-[1-$^{14}$C]ketoglutarate to succinate. The assay mixture (total volume, 250 µl) contained 75 mM Tris/MES (pH 7.25, containing 100 µM a-ketoglutarate, 10 µM a-[1-$^{14}$C]ketoglutarate, 2.5 mM sodium ascorbate, 5.0 mM calcium chloride, 0.5 mM dithiotheitol, 0.5 mM ammonium ion/II/sulfite, 2 mM Mg bovine serum albumin, and 2 mM trimethyllysine. The reaction was started by addition of the enzyme sample to the reaction mixture, which was incubated for 30 min at 37 °C, after which it was terminated by the addition of 100 µl of peroxidol chloride. The released $^{14}$C CO$_2$ was trapped in 0.5 ml of 2 N NaOH, essentially as described by Wanders et al. (22), and the NaOH was counted for radioactivity in a liquid scintillation counter.

In the second method, the amount of hydroxyltrimethyllysine that was enzymatically produced from trimethyllysine was determined by HPLC tandem MS. The reaction mixture and incubation time were the same as in the radiochemical method, except for the a-ketoglutarate concentration, which was 2.5 mM instead of 0.1 mM. This method will be described in detail elsewhere. Briefly, the reaction mixture was applied to a Microcon centrifugal filter unit with a 30-kDa cut-off (Millipore, Bedford, MA) to remove most of the proteins. 100 µl of the filtrate was derivatized with methyl chloroformate at alkaline pH, followed by ethyl acetate extraction. Part of the aqueous phase was injected into an ion trap mass spectrometer (Micromass) equipped with a Z-spray source.

**Cloning and Expression of TMLH in COS Cells**—The complete open reading frame (ORF) of rat TMLH was amplified by the polymerase chain reaction (PCR) from rat kidney cDNA using Pwo DNA polymerase (Roche Molecular Biochemicals) and the following primers: a BamHI-tagged forward primer 5′-aaagattcATGAAGAGAGACATACGTACACAGATTTGTC-3′ and a NotI-tagged reverse primer 5′-tttgatggccCTAGGCGTACCGAGGATGATGCT-3′. The human ORF of TMLH was amplified from human kidney cDNA using the following primers: a BamHI-tagged forward primer 5′-taatggcagtGTGTTAGACCGAGAGATTTGTC-3′ and an NotI-tagged reverse primer 5′-tttgatggccCTAGGCGTACCGAGGATGATGCT-3′. The PCR products were cloned downstream of the PcMV promoter into the BamHI and NotI sites of the mammalian expression vector pCDNA3. Both ORFs were sequenced to exclude sequence errors introduced during the PCR, after the ORF was cloned in the correct orientation.

**Protein and Peptide Analysis**—For MALDI-TOF MS analysis, procollagen type I was digested with Factor B from porcine pancreatic trypsin using a 1:50 enzyme to substrate ratio. The digested peptide solution (2 µg) was injected onto a micro-HPLC column. The resulting peptide spectra were used to sequence the peptide using a Micromass TofSpec 2EC (Micromass, Wythenshawe, UK) equipped with a Z-spray source. For ES1-Q-TOF MS analysis, the peptide solution (2 µl) was injected into a nanospray capillary, and the peptide mass spectra were recorded using a Q-TOF mass spectrometer (Micromass) equipped with a Z-spray source.

**Characterization of the Purified TMLH**—The Michaelis-Menten constants ($K_{m}$) of purified TMLH for trimethyllysine, a-ketoglutarate, and Fe$^{2+}$ were determined using the radiometric assay described above. For the determination of the $K_{m}$ of a-ketoglutarate and Fe$^{2+}$, a fixed concentration of 2 mM trimethyllysine was used. The pH optimum was determined by changing the pH of the reaction mixture using 75 mM bis-tris-propane buffer instead of the Tris/MES buffer, at pH values ranging from 5.5 to 9.5 in steps of 1 pH unit.

**Protein and Peptide Analysis**—For MALDI-TOF MS analysis, protein-containing gel slices were S-alkylated, digested with trypsin (Roche Molecular Biochemicals, sequencing grade), and extracted according to Shevchenko et al. (26). Extracted peptides were purified and concentrated using Zip-Tips (Millipore). Peptides were eluted from the Zip-Tips with 10 µl of 1% (w/v) formic acid, 80% acetonitrile. The peptide solution was mixed with an equal volume of 10 mg/ml a-cyanohydrinylaminic acid (Sigma Chemical Co.) solution in acetonitrile/ethanol (1:1, v/v). Aliquots of 0.5 µl were spotted on the target and allowed to dry at room temperature. MALDI-TOF MS spectra were acquired on a MicroMass ToFSpec 2EC (Micromass, Wythenshawe, UK) equipped with a 2-GHz digitizer. The resulting peptide spectra were searched against a non-redundant sequence database (Swiss-Prot/TREMBL) using the Proteinprobe program. For ES1-Q-TOF MS analysis, the peptide solution (2 µl) was introduced into a nanospray capillary, and positive mode spectra were recorded with a Q-TOF mass spectrometer (Micromass) equipped with a Z-spray source.
TMLH in Carnitine Biosynthesis

Overview of the various steps involved in the purification of TMLH from rat kidney

| Purification step | Protein Specific activity | Activity | Yield | Purification fold |
|-------------------|-------------------------|----------|-------|------------------|
| Homogenate        | mg                      | nmol/min/mg | nmol/min | %     |                  |
| Low salt precipitate | 6289                    | 0.022    | 139   | 100              |                  |
| Q-Sepharose       | 613                     | 0.17     | 105   | 76               | 7.8              |
| Butyl-Sepharose   | 21                      | 1.34     | 28    | 20               | 61               |
| CHT-II hydroxylapatite | 0.8                    | 10.3     | 8     | 6                | 467              |

The bacterial expression vector pMAL-C2X, to express the TMLH as a fusion protein with maltose-binding protein. The ORF was sequenced to determine its amino acid sequence. The presence of ascorbate was essential for the expression of the TMLH protein. After 24 h, the cells were pelleted and lysed in 1/10 of the culture volume in a 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl by sonication for 1 min at 14,000 × g, and the pellet was discarded. Fusion proteins were purified from the supernatant following the specifications of the manufacturer (New England BioLabs) and stored at −20 °C. This fusion protein was used to raise an antiserum in a rabbit as described earlier (32). Density Gradient Analysis—Kidneys were obtained from male Wistar rats and homogenized in 5 mM MOPS buffer, pH 7.4, containing 250 mM sucrose and 2 mM EDTA. A postnuclear supernatant was produced by centrifugation of the homogenate at 600 × g for 10 min at 4 °C and subfractionated by equilibrium density gradient centrifugation in a linear Nycodenz gradient as described previously (31). Glutamate dehydrogenase, catalase, β-glucuronidase, phosphogluco isomerase, and cytosol, respectively. The activity of the marker enzymes was determined as described previously (31, 32).

Immunoblot Analysis—A Multiphor II Nova Blot electrophoretic transfer unit (Amersham Pharmacia Biotech) was used to transfer proteins onto a Nitrocellulose sheet (Schleicher & Schuell, Dassel, Germany) as described by the manufacturer. After blocking of nonspecific binding sites with 5% bovine serum albumin (BSA) in 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 2 mM EDTA. Post-nuclear supernatant was used for detection, according to the manufacturer's instructions (Bio-Rad).

Gel Filtration and Blue Native PAGE—For gel filtration analysis a Superdex 200 column (Amersham Pharmacia Biotech) was used. A 20 ml elution buffer was pH 9.5, containing 100 mM glycerol, 2 mM sodium ascorbate, and 5 mM DTT was used as eluant at a flow rate of 0.4 ml/min. All analyses were performed at 4 °C. The column was calibrated under identical conditions with the following protein standards: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), chymotrypsigen A (25 kDa), and ribonuclease A (14 kDa), all from Amersham Pharmacia Biotech. The 10log of the molecular mass of the protein standards was plotted against the corresponding elution fraction, and the molecular mass of TMLH was calculated by interpolation.

Blue native PAGE was performed as described previously using a 6–14% polyacrylamide gradient gel (33, 34). Citrate synthase (87 kDa) from pig heart (Sigma) and bovine serum albumin (66 kDa) were used as protein standards.

RESULTS

Purification of TMLH from Rat Kidney—Because kidney contains the highest TMLH activity in the rat (35), this tissue was used as source of enzyme for the purification of TMLH using liquid chromatography. An overview of the purification scheme is given in Table I. TMLH activity was retained completely by all columns used and eluted as a single peak during all purification steps. The presence of ascorbate was essential for preserving the enzymatic activity during the later purification steps and subsequent storage at −80 °C. Samples obtained after each purification step were analyzed by SDS-PAGE followed by silver staining (Fig. 1). A single protein band with an apparent molecular mass of 43 kDa was observed after the last purification step.

Identification of the cDNA Encoding TMLH—Attempts to directly sequence the protein by Edman degradation failed, suggesting that the N terminus of TMLH is blocked. Therefore, the purified protein was digested with trypsin and analyzed by MALDI-TOF MS. Because no match was found in the nonredundant database (Swiss-Prot/TREMBL), two peptides were selected for sequencing by Q-TOF MS, which resulted in the following sequences: TLLVDGYAAQQVLQR (1821.99 Da) and MWYFTSFDRS (1339.58 Da). When the non-redundant database was searched with these peptide sequences, both showed high homology with the human hypothetical protein FLJ10727 (GenBank® accession number: NP_060666). Subsequent searches in the EST (Expressed Sequence Tag) data base identified several rat, mouse, and human EST clones with high homology to the peptide sequences. The homologous human ESTs all corresponded to the FLJ10727 cDNA (GenBank® accession number: AK001589). Interestingly, the translated FLJ10727 cDNA showed high homology with human, rat, and Pseudomonas sp. AR-1 γ-butyrobetaine hydroxylase. Based on the EST data, primers were selected to amplify the ORFs from rat, human, and mouse kidney cDNA. The rat and mouse amplicons both contained an ORF of 1218 base pairs, coding for a polypeptide of 405 amino acids with a predicted molecular mass of 47.5 kDa (GenBank® accession numbers: AF374406 and AF033513, respectively). When the theoretical tryptic digest of the translated rat ORF was compared with the MALDI-TOF MS spectrum of the purified TMLH, 12 of the 27 theoretical peptides could be matched, which corresponds with a protein coverage of 34%.

The human amplicon contained an ORF of 1266 base pairs, coding for a polypeptide of 421 amino acids with a predicted molecular mass of 49.5 kDa (GenBank® accession number: AF373407). The human TMLH cDNA sequence was identical to the FLJ10727 cDNA. This sequence is derived from genomic clone NT025307, which has been mapped to Xq28. BLASTn analysis of the human genome data base using the human TMLH cDNA as query showed that the TMLH gene spans about 130 kb and consists of at least eight exons.

The translated ORFs of rat and mouse both have 88% positional identity with the human TMLH protein. The rat and mouse proteins are also highly homologous and share 92% positional identity.

Expression of the Rat and Human TMLH cDNA—When the putative rat and human TMLH cDNAs were expressed in either E. coli (as maltose-binding fusion protein) or S. cerevisiae, no TMLH activity could be detected in lysates of these cells. Therefore, both ORFs were cloned into the eukaryotic expression vector pcDNA3 and transiently transfected to COS cells. As a negative control, the pcDNA3 vector without insert was included in the transfection experiment. After 48 h, TMLH activity was measured in the lysates of the transfected cells employing the TMLH assay, which is based on the measure-
ment of hydroxytrimethyllysine by HPLC tandem MS. Incubations were performed both in the presence and absence of substrate to show that the formation of hydroxytrimethyllysine was trimethyllysine-dependent. High TMLH activity could be measured in lysates of COS cells transfected with the putative rat and human TMLH cDNA, whereas only low (endogenous) TMLH activity was measured in lysates of cells transfected with pcDNA3 vector without insert (Fig. 2A).

Subsequent immunoblot analysis using the TMLH antibody showed a band with the same molecular mass as the purified TMLH in cells transfected with the rat and human ORF, which was hardly detectable in lysates of cells transfected with pcDNA3 without insert. Additionally, the amount of immunoreactive material was proportional to the TMLH activity (Fig. 2B).

Subcellular Localization of TMLH—To investigate the subcellular localization of TMLH, a density gradient analysis was performed with rat kidney homogenate. All the TMLH activity was associated with the particulate fraction, which was loaded on a Nycodenz density gradient. The activity profile in the gradient exactly coincided with that of the mitochondrial marker glutamate dehydrogenase, confirming the mitochondrial localization of TMLH (Fig. 3). When we analyzed the gradient-fractions by immunoblot analysis using antibodies raised against recombinant TMLH, the pattern of the immunoreactive material corresponded exactly with the TMLH activity profile (Fig. 3).

Processing of TMLH—With a calculated molecular mass of 47.5 kDa, the size of the translated ORF of rat TMLH is not in agreement with that of the purified protein, which has an apparent molecular mass of 43 kDa. Additionally, the COS cell transfection experiment showed that the produced human and rat TMLH both have the same apparent molecular mass as the purified rat TMLH, although the calculated molecular masses are 47.5 and 49 kDa, respectively. The 5' end of the rat cDNA, as well as the mouse and human cDNAs, contained a second putative start codon. The use of this methionine would result in a protein of 42 kDa, and we therefore expressed the shorter rat protein in S. cerevisiae to investigate whether translation starts at this methionine. Immunoblot analysis of the yeast lysate with the TMLH antibody, however, clearly showed that the size of the expressed protein is smaller than the purified rat TMLH, indicating that this methionine is not used as start codon (Fig. 4). Another possibility is that TMLH is synthesized as a 47.5-kDa precursor, which is processed after import into the mitochondrion. The protein sequences of rat, mouse, and human TMLH indeed contain a putative N-terminal mitocho-
drial targeting sequence as determined by the Predotar version 0.5 prediction program. Immunoblot experiments support this hypothesis, because expression of the full-length rat TMLH in *S. cerevisiae* resulted in a protein of 47.5 kDa (the predicted molecular mass of the translated rat ORF), but also showed a band with the same molecular mass as the purified rat protein (Fig. 4). Together, these results suggest that a 47.5-kDa precursor protein is synthesized and subsequently processed between the first and second methionine, resulting in a mature protein of \(43 \text{kDa}\).

**Characterization of the Purified TMLH**—The enzyme has a broad pH optimum between 6.5 and 7.5 at 37 °C, which is in agreement with previous results (20). \(K_m\) values of trimethyllysine, \(\alpha\)-ketoglutarate, and Fe\(^{2+}\) were determined for the highly purified enzyme from Lineweaver-Burk double-reciprocal plots and were 1.1 mM, 109 \(\mu\)M, and 54 \(\mu\)M, respectively (Fig. 5). The \(K_m\) value of trimethyllysine is in agreement with the results of Sachan *et al.* (21), who determined a \(K_m\) value of 1.6 mM for the partially purified rat liver enzyme. Two other groups have determined \(K_m\) values of 0.1 mM (20) and 0.13 mM (36) for the rat and bovine liver enzymes, respectively, which are considerably lower than the \(K_m\) value determined in this study. The \(K_m\) values found previously for \(\alpha\)-ketoglutarate (480 and 220 \(\mu\)M) and Fe\(^{2+}\) (21 and 60 \(\mu\)M) are in agreement with our results (20, 36).

**Native Molecular Mass Determination of Purified TMLH**—Gel filtration analysis showed that the native enzyme has a molecular mass of \(87 \text{kDa}\), suggesting that TMLH has a dimeric configuration (Fig. 6A). This result was supported by blue native PAGE analysis (Fig. 6B), which showed that TMLH has a similar size as citrate synthase (87 kDa; lane 1, citrate synthase (87 kDa); lane 2, TMLH; and lane 3, bovine serum albumin (66 kDa)).

**DISCUSSION**

To identify the genes encoding the enzymes of the carnitine biosynthetic pathway we previously purified rat liver \(\gamma\)-trimethylaminobutyraldehyde dehydrogenase and \(\gamma\)-butyrobetaine hydroxylase, the penultimate and ultimate enzymes in carnitine biosynthesis, respectively. We used protein sequence data in combination with EST data base searching to identify the corresponding rat and human cDNAs (16, 17). In this study the same approach was used to identify TMLH, which mediates
the first step in carnitine biosynthesis. The enzyme was purified from rat kidney to near homogeneity and used for peptide sequencing. Subsequently, the resulting peptide sequences were used to search the EST data base, and ORFs were identified from rat, mouse, and human origin with high homology to the peptide sequences. The following observations demonstrated that the identified rat cDNA truly encodes TMLH. First, the peptide sequences obtained from the purified kidney TMLH exactly matched two stretches of sequence from the translated coding region of the rat cDNA. Second, the heterologously expressed rat cDNA exhibited high TMLH activity. Third, the peptide pattern of the purified rat TMLH determined by MALDI-TOF analysis matched the theoretical trypsin digest of the translated rat ORF. Finally, the antibody raised against recombinant TMLH recognized the purified enzyme.

Because the human homologue of the rat TMLH has 88% positional identity with the rat protein and exhibited high TMLH activity in the heterologous expression system, the corresponding cDNA encodes human TMLH. Data base searching showed that the TMLH cDNA is identical to the FLJ10727 cDNA and that the TMLH gene is localized at Xq28. Although we did not express the mouse ORF, it has 92% positional identity with the rat TMLH, and therefore most likely represents the mouse homologue of TMLH.

Purified TMLH behaves as an 87-kDa enzyme in both gel filtration and blue native PAGE analysis. Because a single protein of 43 kDa was present in the final purification sample and the MALDI-TOF analysis of the blue native PAGE sample demonstrated that the dimer consisted of a single protein, TMLH appears to be homodimer. The last enzyme of carnitine biosynthesis, γ-butyrobetaine hydroxylase, has considerable homology with TMLH and has also been reported to function as a homodimer (37–39). Analysis of the non-redundant data base with the BLASTp algorithm using rat TMLH as query, only retrieved γ-butyrobetaine hydroxylase sequences from several organisms. No homology was found with other α-ketoglutarate-dependent dioxygenases, suggesting that TMLH and γ-butyrobetaine hydroxylase belong to a separate subclass of dioxygenases.

TMLH has been reported to be localized in mitochondria (8, 21), although this conclusion was drawn from relatively crude experiments involving differential centrifugation. Therefore, the subcellular localization of TMLH in rat kidney was re-investigated by subcellular fractionation using density gradient centrifugation. The TMLH activity profile and the distribution of immunoreactive material clearly showed that TMLH is localized exclusively in mitochondria. The expression studies of TMLH in S. cerevisiae suggest that translation of TMLH starts at the first available start codon, which results in the formation of a 47.5-kDa precursor protein. This precursor is subsequently processed to the mature 43-kDa protein, presumably upon import into mitochondria where the mitochondrial import machinery removes the N-terminal prosequence.

The mitochondrial localization of TMLH is remarkable, because the other three enzymes of the carnitine biosynthesis are localized in the cytosol. The submitochondrial localization of TMLH will have implications for the substrate flow and regulation of the carnitine biosynthesis. If TMLH is localized in the mitochondrial matrix, the existence of transport system to shuttle substrate and product over the inner mitochondrial membrane would be required. In contrast, if TMLH is present in either the inner membrane space or the outer mitochondrial membrane, no transport system would be needed because the outer mitochondrial membrane is permeable for small molecules.

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