Molecular cloning and tissue distribution of mammalian L-threonine 3-dehydrogenases

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Abstract

**Background:** In mammals, L-threonine is an indispensable amino acid. The conversion of L-threonine to glycine occurs through a two-step biochemical pathway involving the enzymes L-threonine 3-dehydrogenase and 2-amino-3-ketobutyrate coenzyme A ligase. The L-threonine 3-dehydrogenase enzyme has been purified and characterised, but the L-threonine 3-dehydrogenase gene has not previously been identified in mammals.

**Results:** Transcripts for L-threonine 3-dehydrogenase from both the mouse and pig are reported. The ORFs of both L-threonine dehydrogenase cDNAs encode proteins of 373 residues (41.5 kDa) and they share 80% identity. The mouse gene is located on chromosome 14, band C. The amino-terminal regions of these proteins have characteristics of a mitochondrial targeting sequence and are related to the UDP-galactose 4-epimerases, with both enzyme families having an amino-terminal NAD⁺ binding domain. That these cDNAs encode threonine dehydrogenases was shown, previously, by tiling 13 tryptic peptide sequences, obtained from purified L-threonine dehydrogenase isolated from porcine liver mitochondria, on to the pig ORF. These eukaryotic L-threonine dehydrogenases also have significant similarity with the prokaryote L-threonine dehydrogenase amino-terminus peptide sequence of the bacterium, *Clostridium sticklandii*. In murine tissues, the expression of both L-threonine dehydrogenase and 2-amino-3-ketobutyrate coenzyme A ligase mRNAs were highest in the liver and were also present in brain, heart, kidney, liver, lung, skeletal muscle, spleen and testis.

**Conclusions:** The first cloning of transcripts for L-threonine dehydrogenase from eukaryotic organisms are reported. However, they do not have any significant sequence homology to the well-characterised *Escherichia coli* L-threonine dehydrogenase.

**Background**

In husbanded animals, dietary supplements of mixtures of indispensable amino acids regulate their growth and the activity of amino acid metabolising enzymes [1,2]. The liver plays a critical role in regulating amino acid metabolism. Porcine-derived hepatocytes are being used in clinical studies of bioartificial liver organs [3]. The regulation of amino acid supply to bioartificial organs and maintaining the activity of the amino acid-metabolising enzymes will be important in their development. From a metabolic perspective, threonine is one of three indispensable amino acids. In biological tissues, there are two ma-
jor and one minor L-threonine degradation pathways. L-
threonine is either catabolised by L-threonine 3-dehydro-
genase (EC 1.1.1.103; gene abbreviation TDH) to 2-ami-
no-3-ketobutyrate or by L-serine/threonine dehydratase (EC 4.2.1.16; L-threonine deaminase, gene abbreviation SDH) to NH₄⁺ and 2-ketobutyrate in the major pathways
and by threonine aldolase (EC 4.1.2.5; gene abbreviation GLY1) to yield glycine and acetaldehyde in the minor
pathway [4–7]. In both prokaryotic and eukaryotic cells, the
conversion of L-threonine via 2-amino-3-ketobutyrate
to glycine takes place in a two-step process [4,8]. L-threo-
nine dehydrogenase catalyses the reaction: L-threonine +
NAD⁺ = 2-amino-3-ketobutyrate + NADH. The subse-
quent reaction between 2-amino-3-ketobutyrate and
coenzyme A to form glycine and acetyl-CoA is catalysed by
2-amino-3-ketobutyrate coenzyme A ligase (EC 2.3.1.29;
gene name KBL). If not processed by 2-amino-3-keto-
butyrate coenzyme A ligase, the highly reactive intermediate,
2-amino-3-ketobutyrate coenzyme A ligase dimers [5,9,10]. The
three-dimensional structure of Escherichia coli 2-amino-3-
ketobutyrate coenzyme A ligase has been determined re-
cently [11]. Recently, we have cloned the murine and hu-
man 2-amino-3-ketobutyrate coenzyme A ligase cDNAs [12] and wished to clone the first enzyme in this L-threo-
nine catabolic pathway. The E. coli L-threonine dehydro-
genase gene and its activity have been well characterised
[8,13]. I describe the first isolation of eukaryotic cDNA se-
dquences encoding L-threonine dehydrogenases that have
significant identity to 13 peptide sequences from the por-
cine L-threonine dehydrogenase protein, that they had isolated from liver mitochondria and partially peptide, sequenced. This peptide sequence was used to identify mouse ESTs with signif-
ificant homology by a back translation to nucleotides
search. The program ESTblast [16] was used to construct a
tentative mouse contiguous sequence from EST sequenc-
es. PCR primers were designed to match the 5' and 3' ends
of the electronic contiguous sequence and used to amplify
the gene from mouse liver and lung cDNA. After agarose
gel electrophoresis, each primer set produced a single am-
plicon indicating that the gene is not alternatively spliced.
The PCR products were cloned and sequenced. A blast
search with the murine threonine dehydrogenase cDNA
sequence of the pig EST database identified similar 5' and
3' ESTs (accession Nos. BE233801 and BH400146 respec-
tively) and the sequence of these ESTs was utilised to de-
sign primers to amplify the pig L-threonine dehydrogenase from hepatocytes by RT-PCR.

Analysis of murine and porcine L-threonine dehydrogenase
cDNAs

The 1508 bp mouse sequence has an ORF which encodes a
373 residue protein and has a ATTAAA polyadenylation
signal at 1460–1465 (GenBank accession No. AY116662)
(Fig. 1). A second clone (accession No. AF134346) in-
cludes 63 bp of 5'UTR and utilises a more 5' ATTAAA poly-
adenylation signal at 1350–1355. The predicted protein has
a 41,611 Da molecular mass and an isoelectric point 8.45.
The mouse genomic sequence for this cDNA is locat-
ed on chromosome 14, band C (accession No. NW_000100, The Sanger Institute, UK). The gene spans
16.4 kb and consists of 9 exons. There is a 329 bp CpG is-
land (64% CG) spanning the 5' untranslated exon (Fig.
2).

The pig sequence (GenBank accession No. AY095535)
also has an ORF that encodes a 373 residue protein with a
41,432 Da molecular mass and an isolectric point 7.67
(Fig. 3). At the nucleotide level, the porcine and mouse
ORFs have 78% identity and at the protein level have 81%
identity and 94% similarity. The potential polyadenyla-
tion signal on the pig sequence is homologous to the most
5' signal on the mouse sequence.

Comparison of the porcine L-threonine dehydrogenase
ORF with sequenced peptides from the porcine L-threo-
nine dehydrogenase enzyme

Evidence that the porcine cDNA encodes for L-threonine
dehydrogenase comes from the high degree of similarity
to sequenced peptides from the purified and structurally
classified porcine L-threonine dehydrogenase protein
isolated from liver mitochondria [14]. The sequences of
13 porcine peptides have been aligned with the porcine

Results

Previously we identified, by homology to the E. coli gene,
both the mouse and human cDNAs for 2-amino-3-keto-
butyrate coenzyme A ligase, the second enzyme in the bio-
chemical pathway that converts L-threonine to glycine
[12]. In a search for the mouse cDNA of L-threonine dehy-
rogenase, which is the first enzyme in this pathway, I ini-
tially used the same approach. However, only expressed
sequence tags belonging to sorbial dehydrogenase (and,
with a much lower degree of homology, numerous isoforms
of alcohol dehydrogenase) were identified. Nor were
other candidate genes found in the human genomic se-
quence. Fortunately, Kao and Davis (1994) [14] had pre-
viously purified and characterised the porcine L-threonine
dehydrogenase protein, that they had isolated from liver
mitochondria and partially peptide, sequenced. This pep-
tide sequence was used to identify mouse ESTs with sig-
ificant homology by a back translation to nucleotides
search. The program ESTblast [16] was used to construct a
tentative mouse contiguous sequence from EST sequenc-
es. PCR primers were designed to match the 5' and 3' ends
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the gene from mouse liver and lung cDNA. After agarose
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plicon indicating that the gene is not alternatively spliced.
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isolated from liver mitochondria [14]. The sequences of
13 porcine peptides have been aligned with the porcine
Figure 1
The cDNA sequence and translation of murine L-threonine dehydrogenase. There are two potential polyadenylation signals (attaaa at 1350–1355 and 1460–1465) shown in bold and underlined with the polyadenylation sites indicated by a . An * indicates the taa stop codon. The bold type and underlined nucleotide pairs indicate the positions of the exon/exon boundaries.
ORF protein and have 98% identity over 212 residues (Fig. 4). The 5 mismatched residues are probably due to errors in peptide sequencing since they are located towards the end of the sequences.

Import into mitochondria
Mammalian L-threonine dehydrogenase is a nuclear encoded gene; the protein is synthesised in the cytoplasm and imported into mitochondria. The amino-terminal of the mature porcine L-threonine dehydrogenase protein isolated from mitochondria [14] corresponds to amino acid residue 51 on the porcine L-threonine dehydrogenase ORF (Fig. 4), which suggests that the pro-protein is cleaved to produce a 36 kDa mature enzyme. This value is close to that which would be expected since the mature porcine enzyme has a subunit molecular mass of 37 kDa on SDS-PAGE [14]. The amino-terminal region of the mouse, fly and nematode L-threonine dehydrogenase proteins all have characteristics of mitochondrial targeting sequences (Fig. 5), despite being the region of lowest similarity within the protein, having a high content of basic amino acids and few acidic amino acids [17].

Sequence homology in other species
A database search revealed the presence of L-threonine dehydrogenase genes in the genome of other organisms. The fly, *Drosophila melanogaster* has a 6 exon gene located on chromosome 3L which translates into a cDNA of 1288 bp, encoding the 367 residue CG5955 protein (accession No. AAF51607) (Fig. 5). The nematode, *Caenorhabditis elegans* has a 5-exon gene located on chromosome V (encompassing only the first 5 exons of the predicted 10 exons of the hypothetical gene product F08F3.4, accession No. AAB04871). By extending the fifth exon to the next polyadenylation site a 1217 bp cDNA is formed, encoding a 359-residue protein (Fig. 5). The cDNA sequences of both these genes are supported by EST data. The fly and nematode proteins have over 52% identity and 88% similarity to the mammalian proteins in the 306-residue, central core of the enzyme. Four exon/exon boundaries are conserved in two or more of the genes (Fig. 5). The search also revealed similar L-threonine dehydrogenase ESTs in amphibians, bony fishes, tunicates, flies, moths, mites, nematodes and trypanosomes, but not in higher plants and yeasts. Similarly, the gene for the second enzyme in this pathway, KBL, is also absent from the yeast, *Saccharomyces cerevisiae* [12], and no L-threonine dehydrogenase activity has been found in *S. cerevisiae* [18].
Figure 3

The porcine L-threonine dehydrogenase cDNA sequence and translation. The ORF (accession No. AY095535) is shown in bold and the 5' EST (accession No. BE233801) and the 3' EST (accession No. BI400146) are shown in lower case. The potential polyadenylation signal (attaaa) is shown in bold and underlined.
That L-threonine dehydrogenase sequences have been evolutionarily conserved between the Gram+ bacteria and mammals is shown by the homology between mouse and the amino-terminus peptide sequence from the threonine dehydrogenase of the Gram+ Firmicutes bacteria, *C. sticklandii*, which has 54% identity and 93% similarity over 28 residues [15] (Fig. 6). *C. sticklandii* is an amino acid fermenting anaerobic bacterium that can grow on threonine as a sole substrate. Together, the mouse and *C. sticklandii* sequences enabled the identification of putative L-threonine dehydrogenase genes in a number of bacterial species such as *Thermoplasma acidophilum*, *T. volcanium* and *Staphylococcus epidermidis*. An alignment with the putative L-threonine dehydrogenase sequence, the SAV0542 gene, from *S. aureus* [19] that has 41% identity and 75% similarity to the mouse protein is shown in Fig. 6.

**Figure 4**
Comparison of the translation of the porcine L-threonine dehydrogenase cDNA and sequenced peptides from porcine L-threonine dehydrogenase. Porcine cDNA sequence, Ss cDNA; porcine sequenced peptides (Pig PEP)[14]. Individual pig peptides are alternatively, not underlined, then underlined and shown in bold. Conserved residues are shown by an (*) and strongly conserved residues by (:).

| Ss cDNA  | MPVKMLKQAVKRTLSAPACGQPPTLPRFGLTSPQRIPADANFHSTSFSEQANQPRVL | 60 |
|----------|-------------------------------------------------------------|----|
| Pig PEP  | SEANQPRVL                                                  |----|

| Ss cDNA  | TGLGQLGVLASLLRKRFGKDNVILSDIRKFPEHYFVLSGIPFIESDILDYK | 120 |
|----------|------------------------------------------------------|----|
| Pig PEP  | TGLGQLGVLASLxKRFFGKDVNLSDIRKFPEHYFVLSGIPFIESDILDYK |    |

| Ss cDNA  | RVTWLFHYSALLSAGVEANVSLARAVNITGLHNVLDVAAEHGLRFVPSTIGAFGTPSR | 180 |
|----------|-------------------------------------------------------------|----|
| Pig PEP  | AVNITGLHNVLDVAAEHGLRFVPSTIGAFGTPSR                         |----|

| Ss cDNA  | NPTFDLCIQRPRTIYGVSKVHAELMGYRYYRGDLFRCRLYPGIIASADSEQGGTTDYA | 240 |
|----------|-------------------------------------------------------------|----|
| Pig PEP  | VHAELMGYRYRGLDFR YPGIADSEQGGTTDYA                          |----|

| Ss cDNA  | VQIFQDAVKNGRFECNLNPARTLAPMEAPAEALSRTYNVNAF5FTP            | 300 |
|----------|-------------------------------------------------------------|----|
| Pig PEP  | VQIFQDAVK ATLEVMEAPAEALSRTYNVNAF5FTP                       |----|

| Ss cDNA  | AELAQEVLRHIFPEQITYNDSVRQAIADSWFMNNFFDOTARRDGWKGDFLDLFELVTML | 360 |
|----------|---------------------------------------------------------------|----|
| Pig PEP  | AELAQEVLRHIFPEQITYNDSVRQAIADSWFMNNFFDOTARRDGWKGDFLDLFELVTML |    |

| Ss cDNA  | NFHGAHSRVAQAN | 373 |
|----------|---------------|----|
| Pig PEP  |               |----|

**Mammalian threonine dehydrogenases have an NAD+ binding domain**
A search of the protein structural database revealed that the closest matches with 19% identity were UDP-galactose 4-epimerases (GALE) from *E. coli* and *Homo sapiens* [20,21]. GALE is a mixed alpha-helices/beta-sheet protein with a N-terminal NAD+ binding Rossmann-fold and belongs to the tyrosine-dependent oxidoreductase protein family (also known as short-chain dehydrogenases). The characteristic Tyr-X-X-Lys couple (residues 195 and 199) found in all family members are important for catalysis with the conserved tyrosine serving as the active-site base [21]. By comparison with the crystal structures of the GALE proteins, two domains were identified and it is likely that the substrate, L-threonine, is located in the cleft between the two domains. The larger amino-terminus domain (residues 58–231 on the mouse sequence) has a...
Comparison of eukaryotic L-threonine dehydrogenase protein sequences. The L-threonine dehydrogenase sequences are:

| Protein      | Sequence                                                                 |
|--------------|---------------------------------------------------------------------------|
| MmTDH        | MLFLGMLKQVNVQTAQSASCRKLVLPLKPLGTSQHRPADANFSTIASEPPVRVLI 60               |
| SsTDH        | MFPVKMLKQVASRTLGSPACGCQPFPLPRRFGLSTSPQIPADANFHSTFSEANQPRVLI 60          |
| DmCG5955     | MFRKCLASALAPVA--PILPLR--TPIASQLVQRRAFHQVFR--ESFRPKILI 50              |
| CeF3.4       | MGNLNVFGR1HNARHNYSLPVLVDP----------LLAFKTIQSQ--TQAQPRVLI 42            |

NAD

| Protein      | Sequence                                                                 |
|--------------|---------------------------------------------------------------------------|
| MmTDH        | TGGLQGLDQGLALNLRKFRGKDVNLSDARKPADANFSTIASEPVRVLI 120                   |
| SsTDH        | TGGLQGLDVGLASLLRFRKDGKFDNLSDARKPADANFSTIASEPVRVLI 120                 |
| DmCG5955     | CGGLQGIIEAKLLRTQYGASNWLSDIKPSQSLNGPYPFADILDQFQKLQKVDH                |
| CeF3.4       | TGQGLQGRGLNSVYKMGSCVMSDIVLPANATDSDYNYLIDILNQGBIEIYVN 120             |

NAD

| Protein      | Sequence                                                                 |
|--------------|---------------------------------------------------------------------------|
| MmTDH        | RISWLFHYSALLSVPALFVNLDTIGAFGSFQSPR                                      |
| SsTDH        | RVTLWFLHYSALLSVPALFVNLDTIGAFGSFQSPR                                      |
| DmCG5955     | RIDWLIHFSSALLSVPALFVNLDTIGAFGSFQSPR                                      |
| CeF3.4       | NIDTIVHFSALLSVPALFVNLDTIGAFGSFQSPR                                      |

NAD

| Protein      | Sequence                                                                 |
|--------------|---------------------------------------------------------------------------|
| MmTDH        | NAPDQLCQRFTPRTTGVSvkVH1ELMGEYYYYYYGLDFCRLYPGIIADSQPGGTTDYA 240          |
| SsTDH        | NTPDQLCQRFTPRTTGVSvkVH1ELMGEYYYYYYGLDFCRLYPGIIADSQPGGTTDYA 240          |
| DmCG5955     | NPTNQVTQRPRTTYGGVSvkVH1ELMGEYYYYYYGLDFCRLYPGIIADSQPGGTTDYA 229          |
| CeF3.4       | ENTDPDLTVCPTTYYGVSvkV1AEFLKMDYrPGFVDRSAGMPGGIIATS--PGGTTDYA 221         |

NAD

| Protein      | Sequence                                                                 |
|--------------|---------------------------------------------------------------------------|
| MmTDH        | VQQHAAAKNTFECNLGAQTRLMYISDCLATLVEVMAEAPRISLRKTM1AMSFTP 300              |
| SsTDH        | VQIFQDAVNRFECLNAPGKLMYIDDCCLATLVEVMAEAPRISLRKTM1AMSFTP 300              |
| DmCG5955     | VAVFHELKRNKTVCTYLRDQTRLMYIECDLRALEIRFCSRQNLARVYRVTMSTP 289             |
| CeF3.4       | IQIFDALKQKHTCHYLDQTRLMYIDDOMASVIQLLAADSQSLKRTYNVGFSTP 281              |

NAD

| Protein      | Sequence                                                                 |
|--------------|---------------------------------------------------------------------------|
| MmTDH        | EEIALQARHGDPRFQQTYCVDPLQRAITAQESPWMILHDSNARCDWQKHPDLPVETATL 360         |
| SsTDH        | EEIALQAVKHLKIPFOITYVDSVRQATADSWPNFDSSTARCDWQKHPDLPVETATL 360           |
| DmCG5955     | EEALFQGKCVPLNTHYVPS--QQLIADAWQVTPDSSTARCDWQKHPDLPVETATL 360           |
| CeF3.4       | EEALADAIRVMPSGELSEYDQICPRTQSIADWPSMSLDSERCDWQKHPDLPVETATL 360         |

NAD

| Protein      | Sequence                                                                 |
|--------------|---------------------------------------------------------------------------|
| MmTDH        | N-------PHGVSTRTAQN 373                                                   |
| SsTDH        | N-------PHGAVSRAQAN 373                                                   |
| DmCG5955     | KDVQNYNIVQPEEQQLIQ 367                                                   |
| CeF3.4       | ALLR-REEDKPTKISTA 358                                                   |

Figure 5
Comparison of eukaryotic L-threonine dehydrogenase protein sequences. The L-threonine dehydrogenase sequences are: mouse, MmTDH; pig, SsTDH; Drosophila melanogaster, DmCG5955; Caenorhabditis elegans, CeF3.4 (corrected as described in results). NAD indicates those conserved residues likely to contact the nicotinamide cofactor by homology with the crystal structure of the UDP-galactose 4-epimerases. The locations of the exon/exon boundaries are shown on the translated protein as underlined residues.
NAD⁺ binding motif. There are 12 conserved residues in the murine L-threonine dehydrogenase protein that are likely to contact the nicotinamide cofactor (Gly-62, Gly-65, Gly-68, Asp-88, Ile-107, His-127, Leu-131, Asn-147, Ser-169, Tyr-195, Lys-199 and Tyr-222) (Fig. 5). The smaller carboxy-terminus domain (residues 232–335) has little similarity to GALE and is likely to be involved in substrate binding.

Expression of L-threonine dehydrogenase mRNA in mouse tissues

To identify which tissues are likely to contribute to L-threonine dehydrogenase activity in the mouse, reverse-transcriptase real time PCR was used to examine the tissue distribution of L-threonine dehydrogenase mRNA. By reverse-transcriptase real time PCR L-threonine dehydrogenase expression was found in all tissues examined, being highest in liver, high in testis and spleen and lowest in skeletal muscle, relative to the expression of β-actin (Fig. 7A). Similar results were also obtained with another set of L-threonine dehydrogenase primers (located on exons 6 and 7) (data not shown). The expression of 2-amino-3-ketobutyrate coenzyme A ligase was also found in all tissues examined, being highest in liver and high in kidney. The expression level of the housekeeping gene β-actin was similar in all tissues examined. Another housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was also used to standardise expression levels, having higher expression in heart and skeletal muscle and lower expression in testis relative to β-actin (data not shown). After 40 cycles of PCR amplification, the amplicons were specific as verified by melting curve analysis (data not shown) and agarose gel electrophoresis (Fig. 7B).

Discussion

L-threonine dehydrogenase is the first enzyme in the biochemical pathway converting L-threonine, via 2-amino-3-ketobutyrate, to glycine. In a search for mammalian L-threonine dehydrogenase genes, clones were isolated from mouse and pig with ORFs very similar to sequenced
peptides from the pig L-threonine dehydrogenase protein [14]. The mouse and pig L-threonine dehydrogenase proteins also have significant homology to amino-terminus peptide sequences from chicken (Gallus gallus) and the bacteria, C. sticklandii [15,22]. I conclude that the cloned cDNAs encode L-threonine dehydrogenase genes. The mammalian proteins belong to the tyrosine-dependent oxidoreductase protein family (also known as short-chain dehydrogenases). They do not have any significant homology to the well-characterised bacterial L-threonine dehydrogenase enzymes from E. coli and the Gram-negative plant pathogenic bacteria Xanthomonas campestris [13,23,24], which belong to the medium-chain, zinc-binding alcohol/polyol dehydrogenase family. However, they are distantly related with both short and long-chain dehydrogenases having characteristics of the NAD(P)-binding Rossmann-fold. Both short- and long-chain L-threonine dehydrogenases have no significant homology to the D-threonine dehydrogenase gene from Pseudomonas.

Figure 7
Expression of L-threonine dehydrogenase mRNA in tissues by semiquantitative PCR. (A) The expression level in each tissue cDNA was normalised to the expression level of the housekeeping gene β-actin. The ratio of L-threonine dehydrogenase mRNA to β-actin mRNA (Y axis, arbitrary units) from each tissue was standardised to that of liver, which was taken as 100. (B) Ethidium bromide stained agarose gel of PCR products after 40 cycles of amplification. Threonine dehydrogenase, TDH; 2-amino-3-ketobutyrate coenzyme A ligase, KBL. The tissues examined were: brain, Br; Heart, He; kidney, Ki; liver, Li; lung, Lu; skeletal muscle, SM; spleen, Sp; testis, Te; and no cDNA control, -c; 100 bp ladder, m.
cruccitiae [25] that is a member of the 6-phosphogluconate dehydrogenase family.

However, both types of L-threonine dehydrogenase utilise NAD$^+$ as a co-factor, but the C. sticklandii enzyme only utilises NAD$^+$ as an electron acceptor whereas the E. coli enzyme also catalyses the reduction of 3-acetylpyridine adenine dinucleotide and thionicotinamide adenine dinucleotide [13,15]. In L-threonine dehydrogenases purified from vertebrate livers NAD$^+$ is the lead substrate followed by L-threonine and the products are released in the order CO$_2$, aminoacetone and NADH [5,10]. All these reaction products inhibit threonine dehydrogenase activity. The short-chain and medium-chain threonine dehydrogenase also differ in their requirement for zinc ions with the E. coli enzyme requiring one ion per subunit and the C. sticklandii enzyme being inhibited by zinc ions [13,15]. Their substrate specificity differs since the E. coli enzyme will also oxidizes L-serine in addition to L-threonine [8,15].

In the mouse, expression of both threonine dehydrogenase and 2-amino-3-ketobutyrate coenzyme A ligase mRNAs were found in all tissues examined, both being highest in liver. In vertebrates, threonine dehydrogenase enzymatic activity is thought to be mostly confined to the liver when the mass of the organ is taken into consideration. In the rat, the activity in kidney and heart was 30% and in brain 10% of the hepatic activity; no activity was detected in other tissues [18]. In the pig, activity in the pancreas was similar to that of liver, but no activity was found in other tissues [26]. The mRNA data supports the suggestion [12] that this pathway has a wider tissue distribution than had been thought previously.

In vertebrates, L-threonine is degraded by two major enzymatic pathways. In normally-fed pigs and rats 80 to 87% of L-threonine catabolism occurs via threonine dehydrogenase [6,27]. Probably, the 2 major pathways have different physiological roles with the cytosolic L-serine/threonine dehydratase enzyme [28] being highly inducible by a high protein diet, starvation and cortisone, and thought to aid the homoeostasis of blood glucose by mobilising threonine and serine for hepatic gluconeogenesis. Whereas the mitochondrial threonine dehydrogenase enzyme is thought to act in the maintenance of free somatic threonine concentration derived from dietary threonine [6]. With the identification of threonine dehydrogenase and L-serine/threonine dehydratase [29] genes from rodents, the way is open to study their relative contribution to threonine catabolism under different physiological states.

Conclusions

The mouse and pig L-threonine 3-dehydrogenase cDNAs have been cloned. They encode 373 residue proteins. The mouse gene is located on chromosome 14, band C. Mammalian L-threonine 3-dehydrogenases are mitochondrial enzymes that utilise NAD$^+$. In agreement with this, the predicted proteins have mitochondrial targeting sequences in their amino-termini followed by an NAD$^+$ binding domain. In murine tissues, the expression of L-threonine dehydrogenase mRNA was highest in the liver.

Materials and Methods

Molecular cloning of murine and porcine L-threonine dehydrogenase cDNAs

Clones encoding the mouse L-threonine dehydrogenase cDNA sequence were obtained by touchdown PCR amplification from mouse liver and lung cDNAs using the Advantage cDNA polymerase mix (Clontech, UK) on a Perkin-Elmer 2400 thermocycler. The cycle conditions for the first 10 cycles were 94°C for 5 sec, 72°C less 0.4°C per cycle for 3 min and for the next 20 cycles 94°C for 5 sec, 68°C for 10 sec, 72°C for 3 min per cycle using primers (100 nM) derived from the sequence of the mouse ESTs D21787, forward 5’-CCGGCTCCCGCGTGGCTTCTCAGCATCCA-3’ and AV100443, first reverse 5’-TTTTTTTTTTTTTTTGTATCTAAATTG-3’ and second reverse 5’-TTTTTTTTTTTGCAAAGCGATCGTT-3’ (Amersham-Pharmacia Biotech, UK).

The porcine (Sus scrofa) L-threonine dehydrogenase cDNA was cloned from primary hepatocytes [16] (generously provided by Dr Leonard J. Nelson, Liver Cell Biology Laboratory, University of Edinburgh, UK). Total RNA was extracted using guanidine thiocyanate and treated with DNase-I to remove any contaminating genomic DNA (SV total RNA isolation system, Promega, UK).

A comparison of the murine threonine dehydrogenase cDNA sequence with the pig EST database identified similar 5’ and 3’ ESTs (accession Nos. BE233801 and BI400146 respectively). From these EST sequences a gene-specific reverse transcriptase primer and PCR primers were designed to amplify a cDNA region encompassing the porcine L-threonine dehydrogenase ORF.

Total RNA was reversed transcribed with AMV RNase H-reverse transcriptase (ThermoScript, Life Technologies, UK) at 50°C using primer 5’-GTGAATACAAATGTCAAGCGATCGTT-3’. The cDNA was amplified by touchdown PCR, as described above, with a final annealing temperature of 58°C using the PCR primers 5’-GGAGGCTGTGTCGAGAGACC-3’ and 5’-CGCCCTTCCCGGCATCTCA-3’. PCR products were examined by agarose gel electrophoresis and stained with ethidium bromide. For cloning, PCR products were excised from low-melting gel electrophoresis and stained with ethidium bromide.
point agarose gels and the agarose digested with agarase (Promega, U.K.). The PCR products were cloned into the T-A vector pCR-II-TOPO (Invitrogen, The Netherlands) and sequenced in both directions using the big dye terminator cycle sequencing ready reaction kit and AmpliTaq DNA polymerase FS and run on an ABI 373XL Stretch Sequencer (both from PE Applied Biosystems, UK).

**Tissue distribution of L-threonine dehydrogenase mRNA by semiquantitative PCR**

Murine cDNA from 8 tissues (BD Clontech, UK) was analysed for the relative expression of L-threonine dehydrogenase and the housekeeping gene, β-actin, by real time PCR. This was carried out on a GeneAmp 5700 Sequence Detection System using a SYBR Green I double-stranded DNA binding dye assay (both from AB Applied Biosystems). Approximately 0.4 µg of cDNA from each tissue was amplified by PCR using Taq Gold polymerase. Tissue master mixes were divided into gene specific mixes with the addition of PCR primers to a final concentration of 300 nM. The L-threonine dehydrogenase primers were: sense 5’-AACACCGGCGCTGCTCTC-3’ and antisense 5’-CCGACATTGCGTATCGAGA-3’ and produced a 99 bp ampiclon. The 2-amino-3-ketobutyrate coenzyme A ligase primers were: sense 5’-TGACGGTTGCTCTGCTGCTGCTCAATGCTGCTGCT-3’ and antisense 5’-ACACCGGCGCTGCTCTC-3’ and produced a 150 bp ampiclon. The β-actin primers were: sense 5’-CAGAGAATTCTGCTGGGTGCT-3’ and antisense 5’-GGAGGCCCCATCGGAC-3’ and produced a 93 bp ampiclon. The PCR primers were designed (with the aid of the Primer Express program, AB Applied Biosystems) to amplify cDNA with both sense primers located on the penultimate exon and the antisense primers located on the last exon. The β-actin primers were designed to amplify β-actin specifically and not other actin isoforms or pseudogenes. The amplification conditions were: a 10 min hot start to activate the polymerase followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The number of cycles required for the SYBR Green I dye fluorescence to become significantly higher than background fluorescence (termed cycle threshold [Ct]) was used as a measure of abundance. A comparative Ct method was used to determine L-threonine dehydrogenase gene expression. Expression levels of L-threonine dehydrogenase in each tissue cDNA sample was normalised to the expression levels of the housekeeping gene β-actin (ΔCt). The ratios of L-threonine dehydrogenase mRNA/β-actin mRNA from each tissue were standardised to that of liver which was taken as 100% (ΔΔCt). The formula 2−ΔΔCt was used to calculate relative expression levels assuming a doubling of the DNA template per PCR cycle. To confirm amplification specificity, the PCR products from each sample were examined by melting curve analysis and subsequent agarose gel electrophoresis.

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**References**

1. Keene JC, Austin RE: Dietary supplements of mixtures of indispensible amino acids lacking threonine, phenylalanine or histidine increase the activity of hepatic threonine dehydrogenase, phenylalanine hydroxylase or histidase, respectively, and prevent growth depressions in chicks caused by dietary excesses of threonine, phenylalanine, or histidine. J Nutr Biochem 2001, 12:274-284
2. Yuan JH, Austin RE: The effect of dietary protein level on threonine dehydrogenase activity in chickens. Poult Sci 2001, 80:1333-1336
3. Strain AJ, Neuberger JM: A bioartificial liver – state of the art. Science 2002, 295:1005-1009
4. Dale RA: Catabolism of threonine in mammals by coupling of L-threonine 3-dehydrogenase with 2-amino-3-oxobutyrate-CoA ligase. Biochim Biophys Acta 1978, 544:496-503
5. Aoyama Y, Motokawa Y: L-threonine dehydrogenase of chick liver. Purification, characterization, and physiological significance. J Biol Chem 1981, 256:12367-12373
6. Bird MI, Nunn PB: Metabolic homoeostasis of L-threonine in the normally-fed rat. Importance of liver threonine dehydrogenase activity. Biochem J 1983, 214:687-694
7. Ravnikar PD, Somerville RL: Genetic characterization of a highly efficient alternate pathway of serine biosynthesis in Escherichia coli. J Bacteriol 1987, 169:2611-2617
8. Boylton SA, Dekker EE: L-threonine dehydrogenase. Purification and properties of the homogenous enzyme from Escherichia coli K-12. J Biol Chem 1981, 256:809-818
9. Pagani R, Leoncini R, Righi S, Guerranti R, Lazzeretti L, Marinello E: Mitochondrial L-threonine dehydrogenase. Biochem Soc Trans 1992, 20:3795
10. Tressel T, Thompson R, Zieske LR, Menendez MI, Davis L: Interaction between L-threonine dehydrogenase and aminoaacete synthetase and mechanism of aminoaacete production. J Biol Chem 1986, 261:16428-16437
11. Schmidt A, Sivarajan J, Li Y, Laroque R, Barbosa JA, Smith C, Matte A, Schrag JD, Cogler M: Three-dimensional structure of 2-amino-3-ketobutyrate CoA ligase from Escherichia coli complexed with a PLP-substrate intermediate: inferred reaction mechanism. Biochemistry 2001, 40:5151-5160
12. Edgar AJ, Polak JM: Molecular cloning of the human and murine 2-amino-3-ketobutyrate coenzyme A ligase cDNAs. Eur J Biochem 2000, 267:1805-1812
13. Johnson AR, Chen YW, Dekker EE: Investigation of a catalytic zinc binding site in Escherichia coli L-threonine dehydrogenase by site-directed mutagenesis of cysteine-38. Arch Biochem Biophys 1998, 358:211-221
14. Kao YC, Davis L: Purification and structural characterization of porcine L-threonine dehydrogenase. Protein Expr Purif 1994, 5:423-431
15. Weinreb M, Andreeen JR: Purification and characterization of threonine dehydrogenase from Clostridium sticklandii. Arch Microbiol 1995, 163:286-290
16. Gill RW, Hodgman TC, Littler CB, Oxer MD, Montgomery DS, Taylor S, Saraupe P: A new dynamic tool to perform assembly of expressed sequence tags (ESTs). Comput Appl Bios 1997, 13:453-457
17. Claros MG, Vincens P: Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem 1996, 241:779-786
18. Green ML, Elliott WT: The enzymic formation of aminoaacete from threonine and its further metabolism. Biochem J 1964, 92:537-549
19. Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, Kobayashi I, Cui L, Oguchi A, Aoki K, Nagai Y, et al: Whole genome sequencing of methicillin-resistant Staphylococcus aureus, the major hospital pathogen. Lancet 2001, 357:1225-1240
20. Bauer AJ, Raymont I, Frey PA, Holden HM: The molecular structure of UDP-galactose 4-epimerase from Escherichia coli determined at 2.5 Å resolution. Proteins 1992, 12:372-381

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21. Thoden JB, Wohlers TM, Fridovich-Keil JL, HM Holden: Crystallographic evidence for Tyr 157 functioning as the active site base in human UDP-galactose 4-epimerase. Biochemistry 2000, 39:5691-5701

22. Yuan JH, Austic RE: Characterization of hepatic L-threonine dehydrogenase of chicken. Comp Biochem Physiol B Biochem Mol Biol 2001, 130:65-73

23. Aronson BD, Somerville RL, Epperly BR, Dekker EE: The primary structure of Escherichia coli L-threonine dehydrogenase. J Biol Chem 1989, 264:5226-5232

24. Liu YS, Tseng YH, Lin JW, Weng SF: Molecular characterization of the gene coding for threonine dehydrogenase in Xanthomonas campestris. Biochem Biophys Res Commun 1997, 235:300-305

25. Ashiuchi M, Pachdibamrung K, Miyaji T, Nagata S, Misono H: Nucleotide sequence, cloning, and overexpression of the D-threonine dehydrogenase gene from Pseudomonas crucivaea. FEMS Microbial Lett. 1998, 167:75-80

26. Le Floch N, Thibault JN, Seve B: Tissue localization of threonine oxidation in pigs. Br J Nutr 1997, 77:593-603

27. Ballevre O, Cadenhead A, Calder AG, Rees WD, Lobley GE, Fuller MF, Garlick Pj: Quantitative partition of threonine oxidation in pigs: effect of dietary threonine. Am J Physiol 1990, 259:E483-491

28. Goldstein L, Knox WE, Behrman EL: Studies on the nature, inducibility, and assay of the threonine and serine dehydrase activities of rat liver. J Biol Chem 1962, 237:2855-2860

29. Ogawa H, Fujitaka M, Date T, Mueckler M, Su Y, Pitot HC: Rat serine dehydratase gene codes for two species of mRNA of which only one is translated into serine dehydratase. J Biol Chem 1990, 265:14407-14413

30. Dabos KJ, Nelson LJ, Bradnock TJ, Parkinson JA, Sadler IH, Hayes PC, Plevis JN: The simulated microgravity environment maintains key metabolic functions and promotes aggregation of primary porcine hepatocytes. Biochim Biophys Acta 2001, 1526:119-130