The N-terminal sequence directs import of mitochondrial alanine aminotransferase into mitochondria ☆,☆☆

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Abstract Herein, we report cloning and subcellular localization of two alanine aminotransferase (ALT) isozymes, cALT and mALT, from liver of gilthead sea bream (*Sparus aurata*). CHO cells transfected with constructs expressing cALT or mALT as C- or N-terminal fusion with the enhanced green fluorescent protein (EGFP) showed that cALT is cytosolic, whereas mALT localized to mitochondria. Fusion of EGFP to mALT N-terminus or removal of amino acids 1–83 of mALT avoided import into mitochondria, supporting evidence that the mALT N-terminus contains a mitochondrial targeting signal. The amino acid sequence of mALT is the first reported for a mitochondrial ALT in animals.

Keywords: Alanine aminotransferase; Mitochondrial targeting signal; Amino acid metabolism; Liver; *Sparus aurata*

1. Introduction

Alanine aminotransferase (ALT, EC 2.6.1.2) plays an important role in amino acid metabolism and gluconeogenesis by catalyzing reversible transamination between L-alanine and α-keglutarate to form pyruvate and L-glutamate [1]. In mammals, ALT activity is found in liver, muscle, heart, kidney, brain and adipose tissue. The presence of two soluble ALT enzymes, cytosolic and mitochondrial, has been suggested on the basis of biochemical studies [2,3], even though the functional role of ALT isozymes remains unclear. Since mammalian ALT cytosolic activity is predominant in glycolytic tissues and shows lower affinity for L-alanine, it was hypothesized as being mainly involved in the conversion of pyruvate to L-alanine. On the other hand, mitochondrial ALT exhibits higher affinity for L-alanine and was considered to be mainly involved in the reverse reaction [4]. However, recent studies have shown a significant contribution of ALT reaction in glutamate oxidation by rat liver mitochondria and that a part of alanine-derived pyruvate originates in the cytoplasm. Furthermore, contribution of rat liver mitochondrial ALT activity during gluconeogenesis was considered negligible compared with that of the cytoplasmic isozyme [5]. Based on the amino acid sequence of liver cytosolic ALT [6], the human ALT1 gene has been cloned and mapped to human chromosome 8q24.3 [7]. Recently, Yang and co-workers [8] cloned another human ALT isozyme, ALT2, and mapped this gene to chromosome 16q12.1. At present, no data are available regarding subcellular localization of ALT2.

To gain insight of the molecular machinery involved in amino acid metabolism, we cloned and addressed subcellular localization of two ALT isoforms from the liver of gilthead sea bream (*Sparus aurata*).

2. Materials and methods

2.1. Molecular cloning of cALT and mALT from the liver of S. aurata

RT-PCR experiments were carried out with total RNA from liver of *S. aurata*. The synthesized cDNA was used as a template for PCR using primers IMAL03, IMAL04 and IMAL10 (Table 1), which correspond to conserved regions in rat and human liver cytosolic ALT. PCR products were ligated into pGEM T easy (Promega) and sequenced using ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequence analysis revealed cloning of two isozymes, named cALT and mALT, and was thereafter used to design oligonucleotides (Table 1) to obtain full-length cDNAs coding for cALT and mALT by performing RACE experiments with SMART™ RACE cDNA Amplification Kit (Clontech). DNA fragments generated by RACE PCR were ligated into pGEM T easy and sequenced on both strands. RT-PCR allowed amplification of the open reading frame of cALT and mALT using primer pairs IMAL19/IMAL20 and IMAL23/IMAL24, respectively (Table 1). The resulting products were ligated into pGEM T easy to generate pGEM-cALT and pGEM-mALT.

2.2. Fusion protein constructs

To assess subcellular localization of cALT and mALT, the complete coding sequence of both cDNAs was amplified by PCR and fused to the enhanced green fluorescent protein (EGFP). cALT was amplified using primer pairs IMAL18/IMAL20 and IMAL19/IMAL21 (Table 1), whereas mALT was amplified using primer pairs IMAL22/IMAL24 and IMAL23/IMAL25 (Table 1), to clone the generated fragments either into pEGFP-C3 or pEGFP-N1 (Clontech). The pGFP-cALT and pGFP-mALT constructs were generated by ligation of the corresponding amplified product into pEGFP-C3, previously digested with *Eco*RI and *Bam*HI. Digestion of PCR products and pEGFP-N1 with *Nhe*I and *Bam*HI allowed ligation and generation of pcALT-GFP and

☆ Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AY206502 (cALT) and AY206503 (mALT).

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Abbreviations: ALT, alanine aminotransferase; EGFP, enhanced green fluorescent protein; LDH, lactate dehydrogenase; SDH, succinate dehydrogenase

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Table 1
Primers used in the present study

	Primer (5′ to 3′)	Maps to
IMAL03	CATCAACCCAGGAAACCCCAC	cALT, 813–833
IMAL04	ACATGGCTCCCTGCACTGGG	cALT, 1333–1314
IMAL07	CAGCCCTCTGCGTACACATTATCCTG	cALT, 947–922
IMAL08	GAAGGAACGGACAGCAGTGTTAGCAGAG	cALT, 1224–1251
IMAL09	GCAGAGAAGGCCAGGCTAACAGAGCAG	cALT, 1254–1281
IMAL10	GCTGACTGTTGACACCATGAAC	cALT, 156–177
IMAL11	CCGCTCGCTGGACTACGGGACCACGA	cALT, 207–232
IMAL12	TAGCCCGCTGCACGATGGGCCCCCGC	mALT, 408–383
IMAL13	CGAGCTGCAGCGCTCTCTGGACGAG	mALT, 928–953
IMAL14	CGGCAACACTGCAACCCCCGAGC	mALT, 957–979
IMAL15	CCCTCCCTCAGCTCCTTCTCTATCTGC	cALT, 260–234
IMAL17	CAGCAGTTCAGGGTAGGAGCAGAGC	mALT, 557–533
IMAL18	GA**GAATTC**TGTCCCACCAGGCGGCGAACG	cALT, 113–133
IMAL19	GA**GCTAGC**CAACATGTCCCACCAGGCGGCG	cALT, 108–129
IMAL20	GA**GGATCC**TGGGGTTGTTATGAGAACTCTTG	cALT, 1595–1573
IMAL21	GA**GGATCC**CGTGAGAACTCTTGTGTGAAGCG	cALT, 1584–1563
IMAL22	GA**GAATTC**TGTCGGCTACAAGGATGCAG	mALT, 100–119
IMAL23	GA**GCTAGC**CAACATGTCGGCTACAAGGATG	mALT, 96–116
IMAL24	GA**GGATCC**ATTTAAGAATACTGCTGAGTGAAC	mALT, 1766–1742
IMAL25	GA**GGATCC**CGAGAATACTGCTGAGTGAACTTC	mALT, 1760–1739
IMAL28	GAGTTCCACTCCTGTCGAGTG	mALT, 1–21
IMAL29	CTTCTCTTTGGGGAGCCCGC	mALT, 296–277

The following primers contain restriction sites (bold and underlined): IMAL18 and IMAL22 *Eco*RI; IMAL19 and IMAL23 *Nhe*I; IMAL20, IMAL21, IMAL24 and IMAL25 *Bam*HI.
Mapping positions are calculated from *S. aurata* cALT and mALT cDNAs.

pmALT-GFP. These constructs were used for expression of cALT and mALT as a C- or N-terminal fusion with EGFP. pmALTΔ83-GFP was obtained by deletion of 83 amino acids at the N-terminal region of mALT. For this, pmALT-GFP was digested with *Nhe*I and *Sma*I; following purification and end filling-in, the resulting major band was religated. All constructs were checked by cycle sequencing.

2.3. Expression in CHO cells and confocal fluorescence microscopy

CHO cells grown on glass coverslips in Ham's F-12 Nutrient Mixture (Invitrogen), supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2, were transiently transfected with the various GFP-fusion protein constructs using FuGene 6 (Roche), according to the manufacturer's instructions. For mitochondrial staining, 1 μM MitoTracker® Red CM-H_2XRos (Molecular Probes, Eugene, OR) was added to medium for 45 min at 37 °C at 48 h post-transfection. The cells were fixed with 4% paraformaldehyde and mounted in Mowiol (Aldrich). The cells were examined by sequential excitation at 488 nm (EGFP) and 543 nm (MitoTracker) by laser scanning confocal fluorescence microscopy (Olympus Fluoview FV600, Olympus, Middlesex, UK) with a PLAN APO 100×1.40 oil objective (Olympus). The images were acquired and merged by using the Fluoview application software (Olympus).

2.4. Subcellular fractionation of CHO cells

Subcellular fractionation of CHO cells transfected with constructs pGFP-cALT, pcALT-GFP, pGFP-mALT, pmALT-GFP or pmALTΔ83-GFP, was performed using the Subcellular Proteome Extraction Kit (Calbiochem). Proteins in cytosolic, mitochondrial, nucleic and cytoskeleton fractions were solubilized in SDS buffer and separated on 10% SDS–PAGE gels. Proteins were transferred to nylon membranes and immunoblotted with a rabbit polyclonal anti-GFP (Clontech, diluted 1:100). An anti-rabbit peroxidase-conjugated secondary antibody was used for chemiluminescent detection with SuperSignal West Dura Kit (Pierce). To confirm the quality of subcellular fractionation, we performed assays for cytosolic (lactate dehydrogenase, LDH) and mitochondrial (succinate dehydrogenase, SDH) marker activities. LDH was assayed using a kit from Linear Chemicals. The activity of SDH was measured according to Dumas et al. [9].

2.5. Subcellular fractionation of S. aurata *liver extracts*

Mitochondrial fractions were prepared, at 4 °C, from liver homogenized (1/5, w/v) in buffer A (50 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 50 mM NaF, 0.5 mM PMSF, 1 mM DTT, 200 mM mannitol and 70 mM sucrose). To remove nuclear and cell debris, the homogenate was centrifuged at $500 \times g$ for 10 min. The mitochondrial fraction was pelleted by centrifugation at $9000 \times g$ for 20 min. The resulting supernatant contained the cytosolic fraction. The pellet was washed twice with buffer A, resuspended in the same buffer and mitochondria were disrupted using a Dounce homogenizer. ALT activity in cytosolic and mitochondrial fractions was determined at 30 °C [10]. LDH and SDH were assayed to confirm the quality of the isolated fractions.

2.6. Northern blotting analysis

Total RNA was isolated from *S. aurata* liver using Total Quick RNA Cells & Tissues Kit (Talent), electrophoresed on a 1% agarose gel containing 4.75% formaldehyde, transferred to a nylon membrane and hybridized with homologous probes for cytosolic and mitochondrial ALT. The probes were labelled by incorporation of digoxigenin-11-dUTP during PCR with primer pairs IMAL10/IMAL07 or IMAL28/IMAL29 (Table 1) and pGEM-cALT or pGEM-mALT, respectively, as template. Labelled probes were immunodetected with anti-digoxigenin conjugated to alkaline phosphatase and CDP-Star™ (Roche) as chemiluminiscent substrate.

3. Results and discussion

3.1. Molecular cloning of S. aurata *liver cDNAs encoding cALT and mALT*

RT-PCR and RACE PCR approaches allowed us to clone two ALT isozymes from the liver of *S. aurata*, named cALT and mALT. The 2018-bp cALT cDNA contains a 1476-bp ORF, whereas the 2349-bp mALT cDNA includes a 1665-bp ORF. A consensus polyadenylation-like signal (ATTAAA) is found 19 bp and 15 bp upstream the cALT and mALT poly(A^+) tails, respectively. The deduced amino acid sequences of cALT and mALT ORFs predict polypeptides of 491 and 554 amino acids with calculated molecular masses of 54.865 and 61.161 kDa, respectively. The fish liver cALT and mALT proteins share an identity of 78%. Alignment of the inferred peptide sequences of *S. aurata* liver cALT and mALT with human and rat ALT1 and human ALT2 gave an identity of

68–71% (see Supplementary materials) [7,8,11]. The overall similarity with mammalian ALTs suggests a high degree of conservation of the structure and conceivably the reaction mechanism during vertebrate evolution. Highest sequence divergence was observed within the N-terminal region of mALT, being 31, 58 and 63 amino acids longer than human ALT2, human and rat ALT1 and cALT, respectively. Lys313 in the rat liver cytosolic ALT has been suggested to reside in the active site and to participate in binding to the coenzyme pyridoxal-5′-phosphate [6,11]. This residue is conserved in both cytosolic and mitochondrial ALT proteins in *S. aurata* and corresponds to Lys309 and Lys372, respectively, in the fish isozymes. Recently, glycation of Lys313 in the mammalian cytosolic ALT has been suggested that may contribute to an inhibitory effect on ALT activity [12]. However, it remains unclear whether post-translational modifications have any regulatory implication in ALT function. Computer analysis with MitoProt II 1.0a4 [13] pointed to a probability of 98.36% that mALT cDNA encodes a protein that localizes to mitochondria, containing a putative cleavage site at position 70. Using PSORT II algorithm [14], cALT was predicted to be cytoplasmic. Consistent with the size of cALT and mALT cDNA sequences, Northern blots carried out using homologous probes for *S. aurata* cALT and mALT showed hybridization to single bands of about 2.1 and 2.4 kb, respectively (Fig. 1a).

3.2. Subcellular localization of cALT and mALT isozymes

The activity of ALT isoforms in vivo was analyzed in subcellular fractions of *S. aurata* liver extracts. Distribution of ALT activity indicated that more than 85% of total ALT activity localized to cytosolic fraction, whereas mitochondrial ALT corresponded to about 14% of total ALT activity (Fig. 1b). Subcellular localization of the two proteins cloned from the liver of *S. aurata*, cALT and mALT, was examined with EGFP-fusion proteins in transiently transfected CHO cells by means of confocal fluorescence microscopy. To determine whether cALT and mALT behave as cytosolic and mitochondrial proteins, respectively, EGFP was fused to the N- and C-terminus of both proteins. Irrespective of the position of EGFP, cALT showed a diffuse distribution in the cells, indicating cytosolic localization (Fig. 2). The mALT-GFP fusion protein exhibited colocalization with the mitochondrial marker MitoTracker. This result denoted mitochondrial localization of mALT. In contrast, GFP-mALT showed a diffused distribution and did not colocalize with MitoTracker, suggesting that fusion of EGFP to the N-terminus of mALT blocked entry into mitochondria (Fig. 3). The subcellular localization of the EGFP-fusion proteins was confirmed by immunodetection of EGFP in cytosolic, mitochondrial, nucleic and cytoskeleton fractions obtained from transiently transfected CHO cells (Fig. 4). Remarkably, immunodetection of CHO cells transfected with mALT-GFP revealed that the protein was mostly found in the mitochondrial fraction and to a lesser extent in cytosol. The fact that mALT-GFP associated to the mitochondrial fraction exhibited a lower molecular weight than the protein immunodetected in cytosol argues for protein import and processing into mitochondria. Our findings are consistent with kinetic and chromatographic studies that indicate the presence of ALT isozymes in cytosol and mitochondria [2,3]. Indeed, mALT is the first amino acid sequence reported for a mitochondrial ALT in animals.

3.3. The amino terminus of mALT contains a mitochondrial targeting signal

The N-terminus of mALT comprises 12 positively charged, 17 hydroxylated and many hydrophobic residues within the first 70 amino acids. It fulfills the physicochemical properties expected for a mitochondrial targeting signal presequence: enrichment in positively charged, hydroxylated and hydrophobic residues to usually form an amphiphilic secondary structure [15–20]. According to the ''acid chain hypothesis'', the basic N-terminal presequences are directed into mitochondria through interaction with translocases of the outer

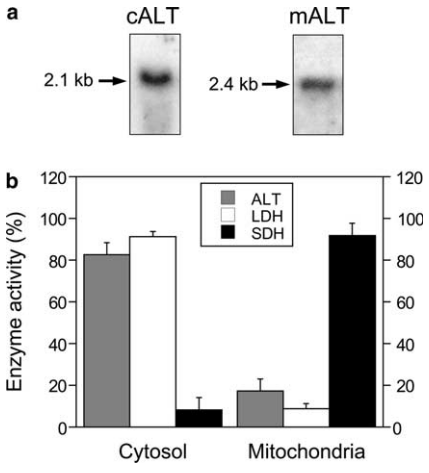

Fig. 1. Northern blots of cALT and mALT, and in vivo ALT activity in subcellular fractions of *S. aurata* liver extracts. (a) Representative Northern blots of cALT and mALT in *S. aurata* liver. 20 μg of total hepatic RNA was loaded. (b) ALT, LDH and SDH activities in cytosolic and mitochondrial fractions from the liver of *S. aurata*. Percentage of activity in each fraction is presented as means ± S.D. ($n = 5$–11).

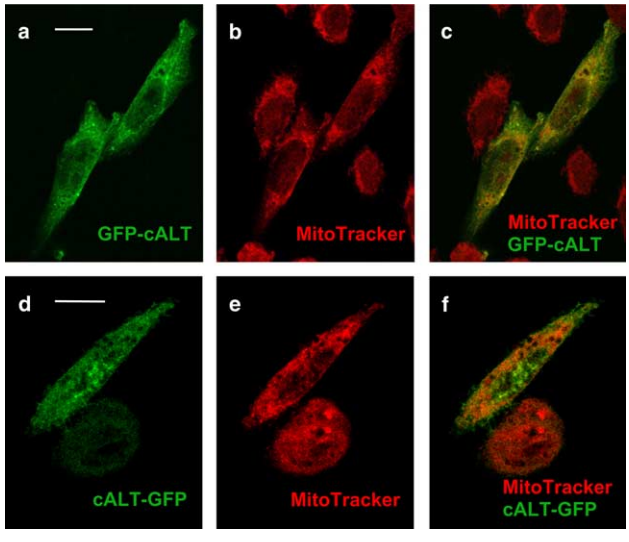

Fig. 2. Cytosolic localization of cALT. CHO cells grown on coverslips were transiently transfected with either pGFP-cALT (a–c) or pcALT-GFP (d–f), cultured for 48 h and stained with the mitochondrial marker MitoTracker® Red CM-H_2XRos. (a,d) Green fluorescence from EGFP; (b,e) red fluorescence from CM-H_2XRos staining; (c,f) merged images. *Bar*, 10 μm.

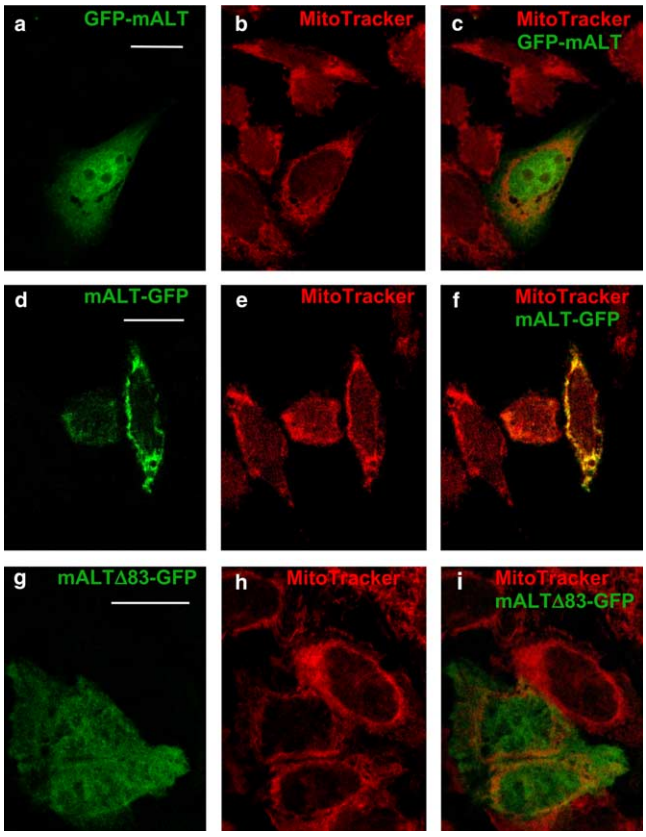

Fig. 3. The N-terminus of mALT contains a mitochondrial target signal. CHO cells were grown on coverslips, transiently transfected with pGFP-mALT (a–c), pmALT-GFP (d–f) or pmALTΔ83-GFP (g–i), cultured for 48 h and stained with the mitochondrial marker MitoTracker® Red CM-H_2XRos. (a,d,g) Green fluorescence from EGFP; (b,e,h), red fluorescence from CM-H_2XRos staining; (c,f,i), merged images. *Bar*, 10 μm.

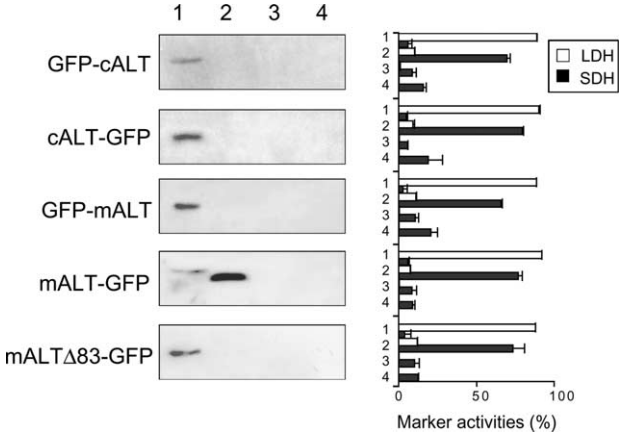

Fig. 4. Immunodetection of EGFP in subcellular fractions of CHO cells transfected with pGFP-cALT, pcALT-GFP, pGFP-mALT, pmALT-GFP or pmALTΔ83-GFP. Following sequential extraction of cytosolic (1), mitochondrial (2), nucleic (3) and cytoskeleton fractions (4), proteins were analyzed by SDS–PAGE and immunodetection was performed using anti-GFP antibody (left part of the figure). Percentage of cytosolic and mitochondrial marker activities LDH and SDH, respectively, represented in the right part of the figure, was assayed in each fraction.

and inner membranes, termed TOM and TIM complexes, respectively [21,22]. To further investigate the involvement of N-terminus of mALT in the subcellular localization of the protein, CHO cells were transiently transfected with a construct expressing a fusion of EGFP to the C-terminus of mALT lacking 83 amino acids of the N-terminal end (pmALTΔ83-GFP). The resulting polypeptide exhibited a diffuse distribution in the cells and did not colocalize with MitoTracker (Fig. 3). Immunodetection of EGFP in cytosolic, mitochondrial, nucleic and cytoskeleton fractions confirmed that mALTΔ83-GFP localized to cytosol (Fig. 4). Thus, removal of the N-terminus of mALT avoided entry of the protein into mitochondria and confirmed that amino acids 1–83 of mALT contain a mitochondrial targeting signal that is necessary for import into mitochondria.

Availability of nucleotide sequences of ALT isoforms will be useful to analyze modulation of cytosolic and mitochondrial ALT expression in different nutritional conditions and clinical disorders associated to high ALT activity levels, such as obesity, muscle diseases and diabetes. Future studies are needed to examine kinetic properties of cytosolic and mitochondrial ALT and clarify the link between structure, localization and function of ALT isozymes.

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