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9. Alanine aminotransferase: A target to improve utilisation of dietary nutrients in aquaculture

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Abstract. Alanine aminotransferase (ALT) is a sensitive marker of dietary protein utilisation in fish. Three ALT isoforms (cALT1, cALT2 and mALT) encoded by two genes have been isolated from gilthead sea bream (*Sparus aurata*). Molecular characterization of ALT isozymes and gene promoters suggest involvement of cALT1 and mALT in postprandial use of dietary amino acids, while cALT2 seems associated to hepatic gluconeogenesis. Inhibition of hepatic cytosolic ALT activity stimulates pyruvate kinase and decreases the renewal rate of alanine in *S. aurata*. These findings point to ALT as a target to spare protein and improve catabolism of dietary carbohydrates in cultured fish.

Introduction

Feeds supplied to fish in culture typically represent the largest variable cost in aquaculture producers' budget. Nowadays an important concern in

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aquaculture is to reduce the amount of dietary protein and to increase the content of cheaper nutrients to replace fishmeal [1]. In addition, since a large quantity of wild-caught fish is used as a source of fishmeal for aquafeeds, a reduction in the amount of protein supplied with the diet to fish in culture will alleviate pressure and dependence on marine fisheries, which are often overexploited [2–4]. Moreover, an excess of protein in the diet of farmed fish enhances amino acid degradation and eutrophication of local waters as a result of increased excretion of ammonia and organic compounds [5,6].

Most fish species produced by aquaculture, such as gilthead sea bream (*Sparus aurata*), turbot and sea bass, are carnivorous ectothermic species and efforts to partially substitute dietary protein by other nutrients are limited by the metabolic features of these animals. Carnivorous fish present low capacity to metabolise dietary carbohydrates [7–9]. The metabolism of carnivorous fish is adapted to a preferential use of dietary amino acids as the main source for metabolic energy production.

Alanine aminotransferase (ALT; EC 2.6.1.2), also known as glutamate pyruvate transaminase, plays a major role in amino acid metabolism and gluconeogenesis by catalysing the reversible transamination between L-alanine and α-ketoglutarate to form pyruvate and L-glutamate. Therefore, ALT constitutes an important link between amino acids, carbohydrates and energy metabolism (Fig. 1).

ALT is a homodimer formed by two subunits of 50 kDa. Each subunit binds a molecule of the coenzyme pyridoxal-5-phosphate (PLP), which is necessary for enzyme activity. In mammals, the hepatic activity of ALT is regulated by hormonal and nutritional status, and its expression levels increase in rats fed with high protein diets, during starvation, after treatment with cortisol or β-adrenergic agonists, and in diabetic animals [10,11]. ALT and aspartate aminotransferase (AST) are quantitatively the most important aminotransferases in the fish liver [12]. Nevertheless, ALT activity is more sensitive to changes in the nutritional status than AST in *S. aurata* and other fish species.

In the present review we will address current knowledge of the nutritional regulation of ALT expression in fish and its potential use as a biotechnological target to enhance carbohydrate catabolism for energy purposes, and preserve dietary amino acids for growth in order to spare protein and improve the sustainability of aquaculture.

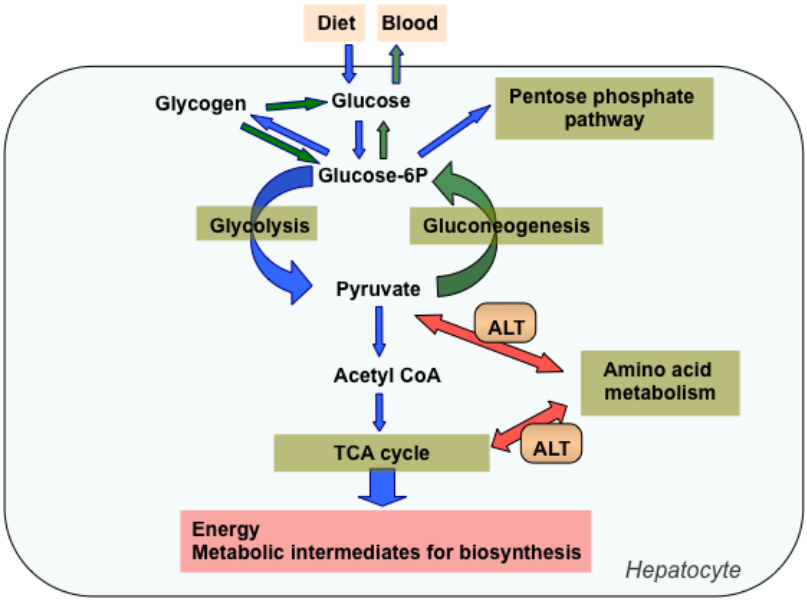

Figure 1. Role of ALT in the hepatic metabolism.

1. Hepatic ALT activity as a marker of dietary protein utilisation in fish

Partial substitution of dietary protein by carbohydrates promotes a metabolic adaptation that involves stimulation of key enzymes in glycolysis and pentose phosphate pathway in the liver of *S. aurata* [13–17]. Furthermore, supply of high protein/low carbohydrate diets increased ALT activity, while AST remained unaffected (Fig. 2A) [13,17]. Similar results were reported for Atlantic salmon [18] and rainbow trout [19,20]. This finding argues for a central role of ALT in the metabolic adaptation to changes in dietary nutrient composition. The increase in liver ALT activity in fish fed high protein diets may contribute to an efficient use of dietary amino acids either for growth or as a substrate for gluconeogenesis.

Hepatic ALT activity decreases in *S. aurata* following long-term starvation, whereas AST activity shows a slight tendency to increase [13]. The effect of starvation on ALT and AST activities in other fishes is variable and does not follow a clear pattern among different species.

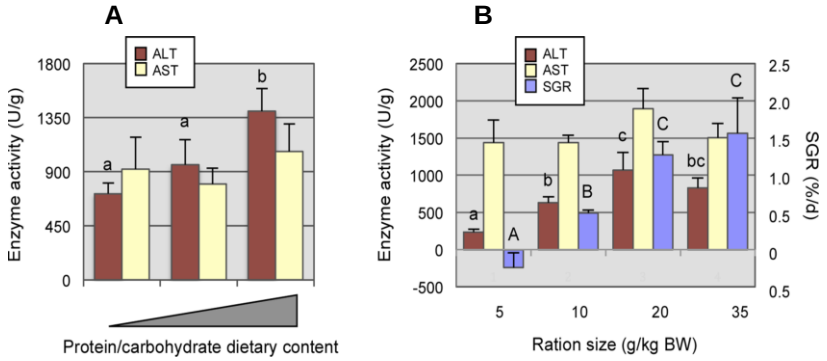

Figure 2. Nutritional regulation of ALT activity in the liver of *S. aurata*. (A) Effect of protein/carbohydrate dietary content on ALT and AST activity. Different letters indicate significant differences for ALT activity (lower-case letters) among diets. No significant differences were found for AST [17]. (B) Effect of ration size on somatic growth rate (SGR, expressed as weight gain percentage per day) and the hepatic activity of ALT and AST in *S. aurata*. Different letters indicate significant differences for ALT activity (lower-case letters) and SGR (upper-case letters) among ration sizes. No significant differences were found for AST [13].

Feeding *Sparus aurata* with different energy levels showed that liver ALT enzyme activity correlates with ration size and somatic growth rate. In contrast, regardless of the ration size no significant differences in AST activity were observed (Fig. 2B) [13].

2. Molecular cloning, subcellular localization, and kinetic properties of *Sparus aurata* ALT isozymes

Biochemical studies on rat and human ALT suggested the presence of soluble isoforms of ALT in cytosol and mitochondria [21–24]. The fact that K_m for alanine of rat mitochondrial ALT is one order of magnitude lower than the value of cytoplasmic ALT, led to the assumption that pyruvate formation from alanine is a major function of mitochondrial ALT [25]. However, later reexamination of the subject indicated that a considerable part of alanine-derived pyruvate originates in cytoplasm and that contribution of rat mitochondrial ALT activity during gluconeogenesis is negligible compared with that of the cytoplasmic enzyme [26]. In addition, presence of several cytosolic ALT and mitochondrial ALT isozymes have been demonstrated in mammalian tissues [27–30]. The metabolic function of

each ALT isoform in the cell and the molecular mechanism that generates ALT isoforms in mammals remain unknown.

Similarly as in mammals, distribution of ALT activity in liver extracts from *S. aurata* showed that more than 85 % of total ALT activity localizes to the cytosol, whereas mitochondrial ALT corresponds to about 14% of total ALT activity [31]. Three ALT isozymes were isolated from *S. aurata* tissues, named cALT1, cALT2 and mALT [31,32]. Alternative splicing of cALT gene generates cALT1 and cALT2, while a separate gene encodes mALT. Compared to cALT1 messenger, cALT2 mRNA contains an extra exon with an upstream translational start site that results in the inclusion of 23 amino acid residues at the N-terminus of the protein [32]. Considering that polyasparagine and polyglutamine regions are involved in protein-protein interaction through the formation of a polar zipper of hydrogen bonds between the side chains [33], presence of an asparagine-rich region in cALT2 suggests regulation of the enzyme activity by aggregation or interaction with effector proteins. In this regard, aggregation of ALT molecules gives rise to active oligomers during the purification of the rat liver enzyme [34].

The primary structure of *S. aurata* mALT protein shares an identity of 77 and 74 % with cALT1 and cALT2, respectively. Alignment of peptide sequences of piscine cALTs and mALT with mammalian ALT1 and ALT2 shows an identity of 66-71 %. The overall similarity with mammalian ALTs suggests a high degree of conservation of structure and reaction mechanism in vertebrate evolution. In the rat liver cytosolic ALT, Lys^{313} resides in the active site and participates in binding to the coenzyme PLP [35,36]. This residue is conserved in *S. aurata* ALTs and corresponds to Lys^{309}, Lys^{332} and Lys^{372}, in cALT1, cALT2 and mALT, respectively.

Subcellular localization of *S. aurata* ALT isoforms was examined by means of confocal fluorescence microscopy and immunodetection after expression of enhanced green fluorescent protein (GFP)-fusion proteins in transiently transfected eukaryotic cells. Both cALT1 and cALT2 show a diffuse distribution in the cell, indicating that both isoforms mostly exhibit cytosolic localization. A minor part of the two proteins is also present in vesicle-like structures [31,32].

Consistent with the physicochemical properties of a mitochondrial targeting signal presequence [37–40], the N-terminus of mALT comprises twelve positively charged, seventeen hydroxylated, and many hydrophobic residues within the first 70 amino acids. Indeed, fusion of GFP to the C-terminus of mALT (mALT-GFP) leads to colocalization of the fusion protein with mitochondrial markers. On the contrary, fusion of GFP to

mALT N-terminus or to the C-terminus of a mALT mutant lacking the N-terminal end blocks entry of the protein into mitochondria, and confirms presence of a mitochondrial targeting signal at the N-terminus of mALT. Immunodetection of CHO cells transfected with mALT-GFP revealed that although the protein is mostly found in the mitochondrial fraction, it is also detected in cytosol, although to a lesser extend and with higher molecular weight [31]. This finding argues for mALT import and processing into mitochondria according to the presequence import pathway, where basic N-terminal presequences are directed into mitochondria through interaction with translocases of the outer and inner membranes [41–43].

Expression of cALT1 in *S. aurata* is mainly found in liver, brain, skeletal muscle, intestine and kidney. No expression of cALT1 is detected in heart, gill or spleen. In contrast, maximal expression of cALT2 occurs in these three organs. Moderate cALT2 expression is observed in intestine, kidney and liver, whereas low cALT2 mRNA levels are detected in brain and skeletal muscle. mALT is ubiquitously expressed, with the higher levels in kidney, followed by liver, and intestine [32,44].

The occurrence of various ALT isozymes in tissues such as the liver supports the notion that ALT isoforms may play different roles in the cell. To unravel the molecular functions of *S. aurata* ALT proteins, kinetic characterisation of ALT isozymes was performed after expression of fish enzymes in *Saccharomyces cerevisiae* (Table 1) [32,44]. Kinetic properties of ALT isoforms were determined considering catalysis of the reaction in the direction of L-glutamate formation (forward reaction) and the direction of L-alanine formation (reverse reaction). Analysis of the forward/reverse ALT activity ratio and the fact that the reverse reaction mechanism of cALT2 followed a ping-pong bireactant system with, in contrast to cALT1, strong double substrate inhibition suggest that cALT2 preferentially converts L-alanine to pyruvate. Mutagenesis analysis demonstrated that residues 3-13 of cALT2 are essential for the reaction direction preference exhibited by this enzyme [32].

Taking into consideration that cALT1 and cALT2 expression in *S. aurata* is found in tissues that can undergo both glycolysis and gluconeogenesis, such as liver, kidney and intestine, it is conceivable that in these particular tissues cALT2 expression can be more restricted to metabolic conditions that favour gluconeogenesis. In contrast, cALT1 prevails during the fed state, which is characterised by elevated glycolysis and deviation of excess pyruvate to form L-alanine and provide 2-oxoglutarate to replenish the citric acid cycle in a metabolic situation where intermediates of this pathway can be used for biosynthetic purposes.

Table 1. Kinetic parameters of *S. aurata* cALT1, cALT2 and mALT [32,44].

	cALT1	cALT2	mALT
Forward reaction			
K_m^{Ala} (mM)	1.8	2.2	2.2
$K_m^{2\text{-Oxo}}$ (mM)	0.05	0.05	0.21
V_{max} (mmol/min/g)	57	14697	403
V_{max} / K_m^{Ala}	0.03	6.65	0.18
V_{max} / $K_m^{2\text{-Oxo}}$	1.18	288	1.89
Reverse reaction			
K_m^{Glu} (mM)	15.9	4.5	11.3
K_m^{Pyr} (mM)	0.69	0.15	0.32
V_{max} (mmol/min/g)	11.6	20.9	534
V_{max} / K_m^{Glu}	0.001	0.005	0.047
V_{max} / K_m^{Pyr}	0.02	0.14	1.68
K_i^{Glu} (mM)		34.9	
K_i^{Pyr} (mM)		36.5	

cALT2 was the only cALT isoform detected in non-gluconeogenic tissues such as heart, gill and spleen. This may be associated with the higher regulatory and kinetic versatility exhibited by this enzyme [32]. The metabolic function of cALT2 in these tissues seems to differ from the role exerted in liver, kidney or intestine, and might be related with processes such as amino acid interconversion and deamination.

Kinetic characterisation of *S. aurata* mALT indicates that this enzyme preferentially catalyzes the reaction in the pyruvate-forming direction [44].

3. Nutritional and hormonal regulation of cALT1, cALT2 and mALT expression

To analyse the functional role of ALT isozymes in intermediary metabolism, the effect of nutritional status and hormonal regulation was addressed on the expression of cALT1, cALT2 and mALT in *S. aurata*. Long-term starvation increases cALT2 mRNA levels and reduces cALT1 expression in liver of *S. aurata* [32]. Up-regulation of cALT2 expression in starved fish is consistent with preference of cALT2 in catalysing the production of pyruvate from L-alanine in a metabolic condition with decreased levels of hepatic pyruvate [45]. Indeed, alanine is the main amino acid released by skeletal muscle and taken up by the liver in starvation [46].

Food intake participates in short-term modulation of hepatic cALT1 and cALT2 expression. Consistent with the expression pattern in starved fish, cALT2 mRNA abundance decreased to minimum levels after a postprandial period of 4-8 hours. In contrast, the hepatic expression of cALT1 reached maximal values 2-8 hours following food intake. Likewise, administration of glucose and insulin to *S. aurata* significantly decreased cALT2 mRNA 6 hours after treatment, while cALT1 mRNA levels remained unaffected. Considering together kinetic data and nutritional regulation of cALTs expression, these observations point to up-regulation of cALT2 in conditions associated with increased gluconeogenesis, whereas cALT1 seems more involved in postprandial utilisation of dietary nutrients [32].

Similarly as for cALT1, starvation decreased mALT mRNA levels in liver and kidney of *S. aurata* [44,47]. Analysis of mALT expression in liver of fed *S. aurata* indicated a tendency to increase at postprandial time 6 hours [47]. Possibly, in the fed state cooperation of cALT1 and mALT allows addressing of dietary amino acids into the mitochondria to provide substrates for energy purposes. The fact that feeding *S. aurata* with high protein diets increases hepatic cALT1 expression while does not affect mALT [47,48], suggests that mALT is more involved in energy production from amino acids to maintain basal metabolism, whereas cALT1 would metabolise excess of dietary amino acids in the cytosol.

In mammals, type 2 diabetes leads to increased gluconeogenesis in the liver [49–51], and among the amino acids, alanine is the most effective gluconeogenic precursor [52]. Consistent with up-regulation of cALT2 expression in gluconeogenic conditions in liver, the diabetogenic action of streptozotocin (STZ), a glucosamine-nitrosourea derivative that causes β cell necrosis and that is widely used to generate diabetic animal models [53–55], leads to a marked increase in the hepatic expression of cALT2 in *S. aurata*, whereas cALT1 and mALT mRNA levels show a tendency to decrease [32,47]. The requirement for gluconeogenic substrates may be critical to enhance hepatic cALT2 expression in STZ-induced diabetic *S. aurata*.

4. Transcriptional control of *Sparus aurata* cALT and mALT gene promoters

To gain insight into the transcriptional regulation of ALT genes in fish, the promoter regions of cALT and mALT from *S. aurata* were isolated and characterised. The cALT and mALT promoters were the first ALT gene promoters reported for animals others than humans [44,56].

The *S. aurata* cALT promoter contains an initiator (Inr)-like element that overlaps the transcription start site. Sequential 5' deletions of promoter reporter constructs and analysis of transcriptional activity in sea bass larvae-derived SBL cells, allowed to conclude that the promoter region within 89 bp upstream from the transcriptional start constitutes the core functional promoter of this gene. Transient transfection experiments and EMSA analysis demonstrated that p300, a transcriptional coactivator that modulate the activity of a wide array of transcription factors [57,58], confers an activating signal on the cALT promoter by forming part of a complex that binds to a response element located at position -73 to -60 bp upstream from the transcription start site [56]. Recruitment of proteins with histone acetyltransferase activity, such as p300, to the Inr element of cALT promoter may provide an open DNA configuration that makes the site of transcriptional initiation more accessible to other transcription factors and the basal transcriptional machinery. In this regard, targeting of p300 to the Inr element might enable the recruitment of TFIID to the cALT promoter, as suggested for other TATA-less Inr-containing promoters [59,60].

Apart from acetylating histones, p300 acetylates several transcription factors in a regulated manner [61]. Transfection experiments in SBL cells showed that an acetyltransferase-deficient mutant of p300 failed to induce *S. aurata* cALT promoter activity, indicating that acetyltransferase activity of p300 is essential for p300-mediated transcriptional activation of cALT [56]. Thus, p300 may induce cALT promoter activity by acetylating histones and transcription factors. Indeed, EMSA assays and site-directed mutagenesis revealed that c-Myb transactivates *S. aurata* cALT promoter by binding to a c-Myb box at position -52 to -35 bp upstream from the transcriptional start. c-Myb-dependent transactivation of cALT occurred irrespective of the p300 response element [56]. However, since p300-mediated acetylation increases DNA binding activity of c-Myb and replacement of acetylated residues (lysines) in c-Myb by arginine dramatically decreases the transactivating capacity of c-Myb [62,63], p300-mediated acetylation of c-Myb possibly enhances binding of c-Myb to the *S. aurata* cALT promoter (Fig. 3).

Given that conditions associated with increased gluconeogenesis, such as starvation and treatment with STZ, result in a marked increase of cALT2 mRNA levels in the liver of *S. aurata*, cALT2 up-regulation can be responsible for serum increase of ALT activity under conditions associated with insulin resistance and development of type-2 diabetes [32]. In agreement with cALT2 expression, mRNA abundance of p300 and c-Myb increased in starved *S. aurata* and decreased in fish liver after the administration of insulin [56].

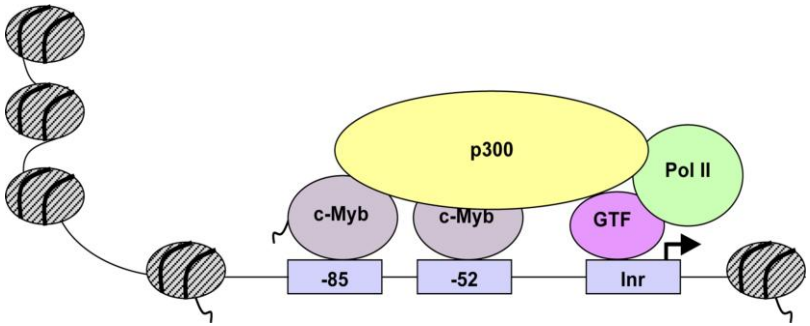

Figure 3. Coactivator p300 and c-Myb transactivate the *S. aurata* cALT promoter. Schematic representation of the cALT promoter region, with the DNA wrapped around nucleosomes forming histones (depicted in a grey circle). By acetylating the histone tails and transcription factors, i.e. c-Myb, p300 can make the DNA more accessible to other regulatory proteins. In addition, p300 can form a physical bridge between transcription factors and the general transcription factors and RNA polymerase II (Pol II). Inr, initiator element. GTF, General Transcription Factors. Acetylation is represented by the symbol`ㄣ . Figure adapted from [64].

Functionality of the promoter region of *S. aurata* mALT was assayed in human kidney-derived HEK293 cells transfected with fusion constructs containing sequencial 5'-deletions of the isolated fragment. The promoter region within 49 bp upstream from the transcription start site constitutes the core functional promoter for mALT gene. HNF4α transactivates the *S. aurata* mALT gene promoter by binding to a response element located at position -63 to -39 bp upstream from the transcriptional start [44]. Tissue distribution of *S. aurata* HNF4α expression, mainly in kidney, liver and intestine, correlates well with that observed for mALT [44].

There is increasing evidence suggesting a major role for HNF4α in the kidney [65–67], and involvement of this transcription factor in transactivation of genes involved in amino acid metabolism such as tyrosine aminotransferase and ornithine aminotransferase [68,69]. In the fish kidney, dietary amino acids are used as substrates for oxidation and gluconeogenesis [70]. Under gluconeogenic conditions, such as starvation and STZ-treatment, down-regulation of HNF4α expression correlates with decreased mALT mRNA levels in kidney of *S. aurata*, which suggest a central role of HNF4α in the transcriptional regulation of mALT. Expression of mALT in this tissue might be mainly involved in the oxidation of amino acids for energetic purposes rather than for providing gluconeogenic substrates [44].

5. Effect of ALT inhibition on the intermediary metabolism of *Sparus aurata*

Bearing in mind the central role of ALT linking amino acids, carbohydrates and energy metabolism, ALT was considered a candidate gene target to spare protein and improve the metabolic utilisation of dietary carbohydrates. To test this hypothesis, the metabolic effect of inhibiting cytosolic ALT activity on intermediary metabolism was addressed in *S. aurata* using amino-oxyacetate (AOA), an inhibitor of PLP-dependent transaminases [71]. Although *in vitro* AOA inhibits ALT activity in cytosolic and mitochondrial fractions isolated from liver of *S. aurata*, AOA only affects cytosolic ALT activity *in vivo*, which suggests that AOA does not enter mitochondria and thus cannot inhibit mALT [72]. Consequently, the effect of AOA *in vivo* may be restricted to cytosolic ALT activity and, during the fed state, essentially to cALT1, the cALT isoform mainly involved in postprandial utilisation of dietary nutrients [32]. Considering that mALT activity accounts for as less as about 14 % of total ALT activity in the liver of fed *S. aurata* [31], the use of AOA *in vivo* appeared to be suitable to specifically inhibit ALT activity of cytosolic fractions.

Dietary AOA supplementation to *S. aurata* for 30 days causes a significant inhibition on ALT activity, without affecting ALT protein levels and irrespective of nutrient composition of the diet. The most remarkable effects of AOA-dependent ALT inhibition on the intermediary metabolism is a significant increase in pyruvate kinase (PK) activity, a key enzyme in glycolysis, without affecting the gluconeogenic fructose-1,6-bisphosphatase activity nor glycemia [44].

It was reported that glutamate behaves as an inhibitor of PK activity, while alanine is a strong allosteric inhibitor of hepatic PK [73]. Taking into account that dietary AOA supplementation decreased glutamate and alanine levels in the liver of *S. aurata*, increased PK activity in the liver after long-term exposure to AOA may result from: i) low glutamate levels and the consequent loss of the glutamate-dependent PK inhibition; and ii) reduction of alanine concentration as a consequence of ALT inhibition. ^{1}H NMR studies showed that inclusion of AOA to the diet also decreased hepatic glycogen. Moreover, ^{2}H NMR analysis indicated decreased ^{2}H-enrichment level of alanine methyl hydrogens in the liver of fish exposed to AOA irrespective of the composition of the diet [72].

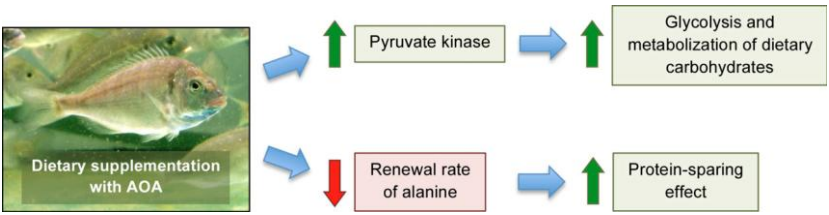

Figure 4. Effect of dietary amino-oxyacetate supplementation on the intermediary metabolism of *S. aurata*.

Altogether, these results suggest that AOA-dependent inhibition of cytosolic ALT activity in the liver stimulates glycolysis and decreases the renewal rate of alanine, which in turn may improve metabolism of dietary carbohydrates and spare protein (Fig. 4).

6. Conclusion

Molecular characterisation of *S. aurata* ALT isozymes including cloning and subcellular localisation of ALT isoforms, nutritional and hormonal regulation of ALT expression, enzyme kinetics, and transcriptional regulation of cALT and mALT gene promoters led to hypothesize that inhibition of cytosolic ALT activity could be useful to improve the use of dietary carbohydrates and spare protein in aquaculture. Preliminary studies showed that AOA-dependent inhibition of cALT activity gave rise to promising results including stimulation of PK activity and decreased renewal rate of alanine in the liver. To perform a more robust protein-sparing effect in order to promote an important increase in the use of dietary carbohydrates for energy production and preserve dietary amino acids for growth in cultured fish, strategies to apply in future studies should consider: i) use of interference RNA (iRNA) to specifically down-regulate the expression of cALT messengers; ii) explore the impact of post-translational mechanisms that may control ALT activity, such as protein-protein interactions; and iii) increase genomic and transcriptomic information available for *S. aurata* and other fish species with commercial interest to identify other candidate genes to perform a multifactorial action to improve the utilisation of dietary nutrients in fish farming.

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