GLUTAMINE METABOLISM IN REGULATING AMMONIA LEVEL *IN VIVO* IN AN AIR-BREATHING FRESHWATER TELEOST, *Heteropneustes fossilis*

ABSTRACT

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ABSTRACT

Glutamine occupies a central position in nitrogen metabolism. Besides being used in protein synthesis, it serves as a substrate for the synthesis of many biologically important molecules in the organisms such as purines and pyrimidines. It helps in storage and transport of both glutamate and ammonia. Glutamine synthesis plays a major role in detoxifying ammonia at cellular level maintaining it at a lower non-toxic level besides controlling the level of glutamate a neurotransmitter in brain. Ammonia is produced continuously during catabolism of nitrogen containing biomolecules *in vivo*. It is highly toxic for the normal physiological function of living organism even at low concentrations. Therefore, the cells try to eliminate the toxic ammonia immediately or detoxify it by conversion to some less toxic/non-toxic substances such as amino acid, urea or uric acid for temporary storage *in vivo*.

The nature of nitrogen excretory products in animals has evolved depending upon the availability of water in the surrounding medium. Animals are thus classified as ammoniotelic (aquatic), ureotelic (amphibians and terrestrial) and urecotelic (desert and aerial). The fishes are primarily ammoniotelic and excrete ammonia to the aquatic medium. However, african lungfishes, marine teleosts and elasmobranchs

synthesize and retain urea in their body with a functional ornithine-urea (o-u) cycle. African lungfishes, mudskippers and aquatic amphibians showed transition from ammoniotelism to ureotelism mainly to avoid ammonia toxicity during water restricted conditions.

Ornithine-urea cycle could not be detected in the freshwater teleosts and hence it was suggested to have been deleted in freshwater teleosts during its evolution from marine ancestors. However, presence of a complete o-u cycle with appreciable level of activity of all the enzymes were reported from this laboratory in four species of fresh-water air-breathing teleosts including *Heteropneustes fossilis*. Further studies on ammonia metabolism with *H. fossilis* indicated its high tolerance for ambient ammonia (upto 75mM NH_4Cl), longer periods (60-70hrs) of water deprivation and hyper-osmolar (300mOsm) ambient medium without any apparent deleterious effect. During such exposures, the fish suppressed excretion of ammonia and accumulated excess ammonia *in vivo*. The induction of o-u cycle was followed by enhanced rate of conversion of accumulated ammonia to urea in liver and kidney. The high ammonia tolerance limit in this fish was suggested to be due to the existence of better ammonia management mechanisms including induction of ureogenesis in *H. fossilis*.

A direct correlation between the urea production and glutamine synthesis has been reported in marine fishes

where CPS III utilized glutamine as the amino donating substrate for urea cycle. The synthesis of glutamine and its degradation has been suggested to play a major role in the regulation of ammonia besides serving as a amino donating substrate for different metabolic pathways. Ammonia produced in different tissues is fixed in the form of glutamine by the enzyme glutamine synthetase (GS) and transported to the target organs for its direct entry to o-u cycle through CPS III or deaminated to release ammonia by glutaminase (Glnase) either for its excretion or utilization by o-u cycle through CPS I. Therefore, the role of glutamine metabolism was evaluated in ammonia management in the ammoniotelic but ureogenic fresh water teleost, *Heteropneustes fossilis*.

The present study conducted in *H. fossilis* included the following:

1. Normal distribution of glutamine synthetase (GS) and glutaminase (Glnase) activity in various tissues such as brain, liver, kidney, muscle and gill.
2. The sub-cellular localization of GS and Glnase in brain, liver and kidney tissues.
3. The diurnal variation of GS and Glnase activity in brain, liver and kidney tissues.
4. The alteration in the levels of ammonia and glutamate and the activity of GS and Glnase in different

tissues during starvation, exposure to hyper ambient ammonia and various periods of dehydration.

5. GS was purified from the brain tissue and its molecular characters, kinetics and regulation were studied *in vitro*.

GS and Glnase activity were assayed in five tissues of *H. fossilis* with brain showing the highest level of enzyme activity followed by liver kidney and gill. Only GS but not Glnase activity could be detected in muscle. High GS and Glnase activity in brain might be helping in prompt detoxification of ammonia and maintenance of glutamate-glutamine pool. The GS activity in liver and kidney was 2-3 times higher than those reported in ammoniotelic freshwater fishes but lower compared to ureo-osmotic marine fishes. Thus, *H. fossilis* occupies an intermediate position between the ammoniotelic freshwater fishes and ureo-osmotic marine fishes with respect to level of GS activity. The ratio of the activity of GS/Glnase in various tissues of *H. fossilis*, in general, favoured detoxification of ammonia for the production of metabolically important glutamine.

The sub-cellular distribution of GS and Glnase were studied in brain, liver and kidney by separating the sub-cellular fractions by differential centrifugation and identifying them using marker enzymes such as GDH, LDH, G-6-Pase for mitochondrial, cytosolic and microsomal fractions

respectively. The cytosolic GS in brain and mitochondrial Glnase in all the tissues were similar to the earlier reports in other animals. However, the mitochondrial GS in the liver and kidney of H. fossilis was a unique observation for any ammoniotelic animal showing close similarity with ureo-osmotic marine elasmobranchs. It suggested that active glutamine utilization might exist in the mitochondria of liver and kidney of this fish.

Diurnal variation of GS and Glnase activity in brain, liver and kidney of H. fossilis was studied at 3 hrs interval during 24 hrs cycle. There was no significant variation in any of the tissues studied indicating that glutamine metabolism operated at the same rate throughout the 24 hr cycle in this freshwater teleost.

The high GS activity besides its unique pattern of sub-cellular localization in the liver and kidney suggested the effective involvement of glutamine metabolism in ammonia management in H. fossilis. A direct experiment was conducted to study the effect of higher ambient ammonia on GS and Glnase activity and levels of ammonia and glutamate in various tissues. The fish was exposed to 50, 75 and 100 mM concentration of NH_4Cl for 14 days. The effect of treatment with 100 mM NH_4Cl was restricted to 4 days due to complete mortality of fishes after 4 days. Ths fish did not take any food and continued to starve during their exposure to NH_4Cl. As starvation does influence amino acid metabolism,

the effect of starvation was also monitored simultaneously with the treatment with NH_4Cl.

The metabolic ammonia and the activity of GS did not change significantly in any of the tissues during starvation. However, tissue specific accumulation of glutamate and the induction of Glnase activity was observed in different tissues. Non-accumulation of ammonia might have been the cause of unchanged GS activity during starvation. The induction of Glnase activity in brain and kidney during starvation might have produced sufficient glutamate in liver and kidney. The induction of GDH activity for ammonia detoxification by glutamate formation might have contributed to the accumulation of glutamate. Brain glutamate level remained unchanged during starvation period. GS/Glnase activity decreased significantly favouring also glutamate formation which might have been needed for energy production.

Exposure of *H. fossilis* to various concentrations of NH_4Cl resulted in significant accumulation of ammonia and glutamate in different tissues accompanied by high induction of GS activity. Brain showed early induction of GS activity followed by liver and kidney. Brain being most sensitive to ammonia toxicity and also the o-u cycle being absent, the early induction of GS activity might have controlled the ammonia level in the brain by converting it to glutamine. In liver and kidney, inspite of the presence

of functional o-u cycle, induction of GS activity in both the tissues might have helped in accelerating the ammonia detoxification process under hyper-ammonia stress. The glutamate accumulation in these tissues might have been caused due to induction of GDH activity during hyper-ammonia stress. Glutaminase activity was significantly inhibited in brain and kidney exposed to 75 and 100 mM. NH_4Cl There was no change at 50 mM ambient NH_4Cl Glnase activity in liver significantly increased only at early stages of exposure to NH_4Cl. The inhibition of Glnase activity might have been an adaptation to decrease addition of metabolic ammonia and hence, to tolerate the stress of higher ambient ammonia. The increase in the ratio of GS/Glnase activity in all the tissues at various concentrations of NH_4Cl indicated more synthesis of glutamine rather than its hydrolysis to glutamate and ammonia.

The effect of dehydration was studied in *H. fossilis* kept in glass jars covered with bilayer cheese cloth for 36 hrs. There was significant accumulation of glutamate accompanied by induction of GS activity and inhibition of Glnase activity in the three tissues studied. The results were similar to those obtained for NH_4Cl experiment. The increase in accumulation of ammonia *in vivo* during emersion has been shown earlier in this fish. This increase might have induced GS activity to detoxify the excess ammonia.

Brain being highly sensitive to ammonia, and in absence of o-u cycle there was early induction of GS activity within 3 hr of dehydration. Induction of GS was comparatively late in liver and kidney (with 9-12 hrs). These two organs are the primary ammonia metabolizing organs and hence, might have high ammonia tolerance limit. Significant accumulation of glutamate along with the synthesis of glutamine by GS might be due to higher induction of GDH activity for ammonia detoxification. The inhibition of Glnase in all the tissues might be an adaptation to avoid undue ammonia formation by hydrolysis of glutamine. The ratio of GS/Glnase increased with the increase in duration of dehydration indicating increase in ammonia detoxification through glutamine synthesis.

Glutamine synthetase was purified from the brain of *H. fossilis* acclimatised to laboratory conditions. The protocol followed for purification such as $(NH_4)_2SO_4$ precipitation, DEAE Sephacel and Sephadex G-200 column chromatography resulted in 62% recovery of activity with 58 fold of purification. It showed a single band on protein staining and specific staining on PAGE. The enzyme was probably a single species of protein like the GS reported from other sources. The molecular weight was found out to be 3.91×10^5 by gel filtration. It was similar to the molecular weight of GS from animal origin. The purified GS showed instability

in the purification buffer (Tris-HCl pH 7.4). Presence of 10% glycerol and 0.25 N NaCl separately or in combination could give better stability to the pure enzyme for more than a month. A broader pH maxima of 7.2-11 was observed for H. fossilis brain GS compared to the pH optima of GS known from other sources. The broader pH range indirectly indicate better adaptation of the brain enzyme of H. fossilis to fluctuations in pH in derelict water bodies. The temperature maxima was found to be 45°C. However, the native enzyme showed decline in activity after 15 minutes at 45°C. The prevention of thermal denaturation of the pure enzyme was observed in presence of the reaction mixture. Among the substrates/cofactors studied Mg^{2+} alone and in combination with ATP were found to stabilize the pure enzyme. Mg^{2+} in presence or in absence of ATP could bind to the enzyme to protect the enzyme from thermal denaturation. The substrate and cofactors of GS reaction could also protect the enzyme from 2-mercaptoethanol denaturation. The Km values of the enzyme were determined for ATP (2.3 mM), Mg^{2+} (6.25 mM), Glutamate (50 mM) and hydroxylamine (0.5 mM). Higher Km values for glutamate and hydroxylamine showed lower affinity of the enzyme towards these substrates. This might have been compensated by the presence of higher (2-60 times higher) GS specific activity compared to specific activity reported for ureotelic animals. The purified brain GS

activity has been shown to be modulated by variety of metabolites. The purified GS showed higher affinity towards Mg^{2+} for its optimum activity. In addition to Mg^{2+}, Co^{2+} and Mn^{2+} could activate the enzyme to a lesser extent in absence of Mg^{2+}. However, Co^{2+} and Mn^{2+} inhibited the enzyme at higher concentrations. Cu^{2+}, Zn^{2+}, Po_4^{-2}, Co^{2+}, Ca^{2+} showed inhibitory effect on GS. Thus, divalent cations showed both activatory or inhibitory effects. The purified enzyme was inhibited by several amino acids, such as Ala, Gly, CP, Asp, Asn, Orn, Arg and Cit. The inhibition by amino acids might be due to the feedback or product inhibition mechanisms suggested in other animals and micro-organisms. In addition to metal ions and amino acids some nucleotides such as ADP, AMP and IMP inhibited GS activity. The effect of inhibition was ADP > AMP > IMP. ADP inhibited the enzyme significantly with non-competitive type of inhibition with Ki value of 3.15 mM. The inhibition of GS by ADP is of physiological significance as it could aggravate taxaemic condition at lower energy level.

Higher tissue level of GS compared to Glnase suggested capability of H. fossilis to effectively detoxify ammonia by converting it to glutamine. The high activity of GS and Glnase in the brain where o-u cycle was absent, indicated the major role of this reaction in ammonia management besides maintaining the level of glutamate a neurotransmitter.

The mitochondrial localization of GS in liver and kidney tissues unlike the ammoniotelic species was a unique finding resembling with uricotelic birds and reptiles and ureo-osmotic marine elasmobranchs. The induction of GS activity and general inhibition of Glnase activity and the accumulation of glutamate during dehydration and hyper-ambient ammonia treatment strongly suggested the synthesis of glutamate and glutamine as an important pathway for ammonia detoxification besides o-u cycle in the ureogenic teleost. Localization and induction of mitochondrial glutamine synthetase in liver and kidney suggested the possibility of utilization of glutamine for urea synthesis through the CPS III isoenzyme like ureo-osmotic marine fishes. The inhibition of glutaminase during ammonia accumulation might be a physiological adaptation to avoid the undue formation of ammonia in addition to already high accumulated ammonia level _in vivo_. Thus metabolism of glutamine plays an important role in ammonia management in _H. fossilis_ besides o-u cycle and thereby providing higher tolerance limit for ammonia. Some of the characters of GS also indicate evolutionary closeness of this species with marine ancestors.

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