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Glutamine synthetase expression in liver, muscle, stomach and intestine of *Bostrichthys sinensis* in response to exposure to a high exogenous ammonia concentration

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Summary

A previous study provided evidence that the adaptive strategy used by the teleost fish *Bostrichthys sinensis* (sleeper) for detoxifying ammonia during extended periods of air exposure was to synthesize and store glutamine, primarily in the muscle, accompanied by an increase in glutamine synthetase (GSase) activity in liver. The aim of the present study was to assess the effect on GSase expression in various tissues of exposure of *B. sinensis* to exogenous ammonia. Exogenous ammonia increases internal ammonia concentrations in fish, mimicking environmental situations such as air exposure that preclude loss of ammonia across the gills, and thus triggering alternative mechanisms for ammonia detoxification. The results reveal relatively high levels of GSase activity, not only in liver but also, unexpectedly, in muscle, and even higher levels in intestine and, in particular, stomach. Exposure to ammonia results in significant increases in GSase activity, GSase protein and GSase mRNA levels in all of these tissues except stomach. The amino acid sequences of GSases from liver and stomach deduced from the cDNA sequences are essentially identical and are >97 % identical to the amino acid sequences of GSases from Gulf toadfish (*Opsanus beta*) and marble goby (*Oxyeleotris marmoratus)*.

Key words: *Bostrichthys sinensis*, teleost, sleeper, nitrogen excretion, enzyme induction, glutamine synthetase, exogenous ammonia exposure.

Introduction

The major end-product of amino acid catabolism in fish is ammonia, the majority of the ammonia being produced by transdeamination in the liver (Wood, 1993; Wilkie, 1997; Ballantyne, 2001; Ip et al., 2001a). Most teleost fishes are primarily ammonotelic, detoxifying ammonia by simply excreting it across their gills into the aqueous environment. Several species of fish have adapted to unique environmental circumstances that preclude this route of ammonia detoxification, however, by expressing high levels of the urea cycle enzymes with an accompanying active urea cycle, and are thus ureotelic. Examples include an alkaline lake tilapia (*Alcolapia grahami*) that thrives in a natural environment highly buffered at pH 10 (Randall et al., 1989; Lindley et al., 1999) (note that ammonia cannot be excreted across the gills to an alkaline external medium; Wright and Wood, 1985), several air-breathing catfish unique to the Indian subcontinent that can live in air for extended periods of time (Saha and Ratha, 1987, 1989; Saha and Das, 1999; Ratha et al., 1995; Saha et al., 1995, 1999, 2000), and the Gulf toadfish (*Opsanus beta*), which becomes primarily ureotelic when confined for extended periods of time (Walsh et al., 1990; Barber and Walsh, 1993; Walsh and Milligan, 1995) (for reviews, see Anderson, 1995a, 2001; Graham, 1997; Walsh, 1997; Saha and Ratha, 1998; Ip et al., 2001a). All of these fish have relatively high levels of urea cycle enzymes, including glutamine synthetase (GSase) and the glutamine-dependent carbamoyl-phosphate synthetase III (CPSase III) characteristic of glutamine-dependent urea cycle activity in fish (Anderson, 1995a,b, 2001).

Several recent studies have suggested, however, that ureotelism (i.e. increased urea cycle pathway activity) is not the universal response of fish to these kinds of environmental circumstances (Ip et al., 2001a). Marble goby (*Oxyeleotris marmoratus)* is a facultative fresh water air-breather from Southeast Asia that can tolerate continuous air exposure for up to a week and has low levels of a full complement of the urea cycle enzymes, but does not increase urea excretion or the level of urea cycle enzyme activity during long-term air exposure. Instead, GSase activity increases and ammonia is converted to glutamine, which accumulates in the tissues (Jow et al., 1999).

Bostrichthys sinensis (sleeper) inhabits brackish water near the mouths of rivers in southeast Asia and is periodically

subjected to air exposure for variable periods of time. This species also synthesizes and stores glutamine, primarily in the muscle, in the first few days of air exposure, which is accompanied by an increase in GSase activity in liver (Ip et al., 2001b). The level of glutamine that accumulates during the first few days is equivalent to the level of ammonia that would normally be excreted during this time. After 3 days of air exposure the fish adopt a reduced rate of ammonia production, possibly *via* reduced rates of proteolysis or amino acid catabolism. Although all urea cycle enzyme activities are present in liver, the levels of activity are very low, considerably lower even than found in marble goby, and are far too low to account for meaningful ammonia detoxification (Ip et al., 2001b). Preliminary studies have indicated that the physiological and biochemical responses of *B. sinensis* to air exposure and related environmental circumstances that preclude ammonotelism are reproducible and readily measurable, indicating that this fish is an excellent model for studying the biochemical mechanisms of these adaptive responses.

The purpose of this study was to extend studies on the effects of air exposure on GSase expression in *B. sinensis* by assessing the response of *B. sinensis* to exogenous ammonia exposure, a strategy that has been used to increase internal ammonia concentrations in fish, mimicking the various environmental situations, such as air exposure, that preclude loss of ammonia across the gills, thus triggering alternative mechanisms for ammonia detoxification (see Kong et al., 1998 and references therein). The sequence of GSase cDNA was determined and changes in GSase activity, GSase protein and GSase mRNA levels, as well as activities of some urea cycle enzymes, urea and ammonia concentrations and excretion rates and amino acid levels, were measured in response to exposure to NH_4Cl. The results show that there are relatively high levels of GSase activity in liver, and also, unexpectedly, even higher levels in muscle, stomach and intestine, and that exposure to ammonia results in significant increases in GSase activity, GSase protein and GSase mRNA levels in all of these tissues except stomach.

Materials and methods

Experimental protocol for exposure of fish to ammonium chloride and collection of samples

Bostrichthys sinensis Lacapède 1801 [also identified as *Bostrychus*, *Philypnus*, *Eleotris* and *Eleotris* (*Bostrychus*) *sinensis* (http://www.fishbase.org/Summary/SpeciesSummary.cfm?ID=14480) and is a member of the same family as marble goby, Eleotridae] (120–190 g), obtained from mouths of rivers in southeast China, were purchased from Yuen Long wet market in Hong Kong and transferred by air to the National University of Singapore for study. Fish were acclimated for at least 48 h before experimentation in aerated water adjusted to 30 % sea water (10 ‰ salinity) at 25–28 °C; water was changed daily and the fish were fed live guppies. Two or three fish were placed in individual containers with 10 l of aerated 30 % sea water (10 ‰ salinity) buffered with 20 mmol l^{-1} Tris-HCl, pH 7.2, at 25 °C and fasted for 48 h prior to experiments; they were not fed during the experimental period. Exposure to ammonia was accomplished by adding NH_4Cl (15 mmol l^{-1}, adjusted to pH 7.2) to the buffered 30 % sea water (10 ‰ salinity) (control); half of the water for both the control and ammonia-exposed fish was changed every 24 h from the start of the experiments. The fish were kept in constant darkness during the experimental period because in their natural habitat they hide in crevices to prey on other organisms.

Water samples were collected at time 0 and at 24 h and 48 h (days 1 and 2, respectively); the samples were acidified with 0.1 mol l^{-1} HCl to prevent loss of NH_3 and stored at 4 °C for analysis of ammonia and urea concentration within 48 h. After 48 h the ammonia-exposed fish were returned to control conditions and the water was analyzed for ammonia and urea concentration 24 h later (day 3).

For the collection of plasma and tissues, the fish were killed by a sharp blow to the head. The caudal peduncle was severed and blood from the caudal artery was collected in heparinized micro-hematocrit capillary tubes. The plasma obtained after centrifugation at 5000 ***g*** for 5 min was deproteinized by addition of an equal volume of 6 % trichloroacetic acid (TCA) and centrifugation at 5000 ***g*** for 10 min; the supernatant was stored at –80 °C for subsequent analysis of ammonia, urea and amino acids. The liver, a large section of lateral muscle, stomach and intestine were excised, immediately freeze-clamped in liquid nitrogen, wrapped in aluminum foil and stored at –80 °C until analysis (stomach and intestine were removed together, flushed well with deionized water, and cut into two sections, corresponding to stomach and intestine; where indicated the latter was cut into two halves, foregut and hindgut). For ammonia, urea and amino acid analysis, weighed frozen liver and muscle tissue samples were powdered at –80 °C and added to 5 volumes of 6 % TCA. After homogenizing for three 20 s intervals (cooling between intervals) with an Ika-werk Staufen Ultra-Turrax homogenizer at 24 000 revs min^{-1} the sample was centrifuged for 10 min at 10000 ***g*** at 4 °C; the supernatant was stored at –80 °C for subsequent analysis.

Ammonia and urea analysis

Acidified water samples were thawed and neutralized with 2 mol l^{-1} $KHCO_3$ to pH 5.5–6.0; acidified liver, muscle and plasma samples were similarly neutralized with 5 mol l^{-1} K_2CO_3. Ammonia concentration was measured as described by Kun and Kearney (1974), by conversion to glutamate in the presence of α-ketoglutarate, NADH and glutamate dehydrogenase and measuring the decrease in absorbance at 340 nm accompanying the stoichiometric conversion of NADH to NAD^+. The reaction mixtures contained 115 mmol l^{-1} ethanolamine, pH 8, 11 mmol l^{-1} α-ketoglutarate, 0.12 mmol l^{-1} NADH, 0.6 mmol l^{-1} ADP, excess units of glutamate dehydrogenase, and 0.45 ml of sample in a final volume of 1.55 ml. For urea analysis, 0.5 ml of the neutralized samples was mixed with 0.5 ml of 20 mmol l^{-1} imidazole

buffer, pH 7.2; a second 0.5 ml of sample was mixed with 0.5 ml of this same buffer containing 2 units of urease to break down all urea and serve as a blank to correct for any other colour-contributing components. After 15 min incubation at 30 °C, the urea concentration in each of the 1 ml solutions was determined as described by Kong et al. (1998).

Amino acid analysis

Acidified liver, muscle and plasma samples were thawed and diluted with an equal volume of 0.2 mol l^{-1} lithium citrate buffer and the pH adjusted to 2.2 with 4 mol l^{-1} LiOH. These samples were then analyzed for free amino acids using a Shimadzu LC-6A Amino Acid Analysis System with a Shim-pack ISC-07/SI504 Li type column. The concentrations of free amino acids are expressed as μmol g^{-1} wet mass for muscle and liver samples and as μmol ml^{-1} for plasma samples; the total free amino acid concentrations are expressed as the sum of the free amino acids The detection limit of the assay was 0.002 μmol g^{-1}.

Enzyme assays

CPSase III activity was measured as previously described, with appropriate precautions taken to assure that CPSase II, which is related to pyrimidine nucleotide biosynthesis and normally also present in tissues (Anderson, 1995b), was not the primary CPSase activity being measured (Korte et al., 1997; Anderson, 1995a, 2001). GSase (Shankar and Anderson, 1985), ornithine carbamoyltransferase (Xiong and Anderson, 1989), and argininosuccinate synthetase and lyase (Cao et al., 1991) were measured as previously described (Korte et al., 1997; Anderson, 1995a, 2001) generally following the protocols described by Anderson and Walsh (1995). Glyceraldehyde-3-phosphate dehydrogenase (Low et al., 1993), citrate dehydrogenase (Moon and Ouellet, 1979) and glutamate dehydrogenase (amination direction) (Ip et al., 1993) activities were assayed essentially as described (in the indicated references for each enzyme). Extracts for measuring the levels of GSase and CPSase III in frozen tissues stored at –80 °C were prepared by homogenizing tissue (powdered at –80 °C) with 5 volumes of extract buffer (3 mmol l^{-1} EGTA, 3 mmol l^{-1} EDTA, 50 mmol l^{-1} NaF, 50 mmol l^{-1} KCl, 50 mmol l^{-1} Hepes, pH 7.6, 50 mmol l^{-1} glutamine, 2 mmol l^{-1} dithiothreitol, plus 0.2 mmol l^{-1} phenylmethanesulfonyl fluoride (PMSF) added just before homogenization in a small volume of ethanol) for 60 s using the homogenizer described above at 24 000 revs min^{-1}. The homogenate was sonicated for 20 s and then centrifuged at 10 000 ***g*** for 10 min. A 3 ml sample of supernatant was then added to a 10 ml column of Sephadex G-25 equilibrated with 50 mmol l^{-1} Hepes, pH 7.6, 50 mmol l^{-1} KCl, 1 mmol l^{-1} EDTA, 50 mmol l^{-1} glutamine and 1 mmol l^{-1} dithiothreitol, and eluted. The first 1 ml of eluate was discarded and the next 2.8 ml, which contained the majority of the protein well separated from lower molecular weight components in the homogenate, was collected; correction for dilution was made by measuring the protein concentration in the homogenate and the eluate. All steps were carried out at 4 °C and activities were measured immediately. The unusual buffer (i.e. presence of NaF, high concentrations of EDTA and EGTA) used for homogenization was based on the efforts of another study to prevent possible dephosphorylation of a putative covalently modified GSase; control studies using the buffer employed in the gel-filtration column indicated that these components had no effect on the results.

For measurement of all urea cycle enzyme activity in liver samples, extracts were made from freshly excised liver and the extract fractionated into cytosolic and mitochondrial fractions. The sample was homogenized with 10 volumes of mitochondrial extraction buffer (285 mmol l^{-1} sucrose, 3 mmol l^{-1} Tris-HCl, pH 7.2, 3 mmol l^{-1} EDTA) using a Potter-Elvejhem-type glass homogenizer with a Teflon pestle and a variable speed motor using 1–3 strokes at moderate revs min. The homogenate was centrifuged at 600 ***g*** for 15 min and the resulting supernatant at 10 000 ***g*** for 15 min, three times. After each of the first two centrifugations the supernatant was discarded and the pellet gently resuspended in the same volume of mitochondrial extraction buffer. After the third centrifugation the pellet (mitochondrial fraction) was suspended in 1 ml of buffer containing 50 mmol l^{-1} Hepes, pH 7.6, 50 mmol l^{-1} KCl, 0.5 mmol l^{-1} EDTA, 1 mmol l^{-1} dithiothreitol, sonicated for 1 min, centrifuged at 10 000 ***g*** for 10 min, and the supernatant passed through a gel-filtration column equilibrated with the same buffer. A cytosolic fraction was prepared by homogenizing with 5 volumes of mitochondrial extraction buffer and centrifugation at 10 000 ***g*** for 15 min; the supernatant (cytosolic fraction) was also passed through a gel-filtration column equilibrated with the same buffer. Enzyme assays were performed immediately.

Subcellular localization

The following fractionation method was used to determine if GSase was localized in the mitochondrial or cytosolic fractions and if the increase in GSase activity observed in liver occurred primarily in the cytosolic or the mitochondrial fraction. Liver of freshly killed control fish was cut into small pieces and homogenized as described above in 10 volumes of homogenization buffer (50 mmol l^{-1} Tris-HCl, pH 7.6, 100 mmol l^{-1} sucrose, 3 mmol l^{-1} EDTA, 3 mmol l^{-1} EGTA, 50 mmol l^{-1} NaF, 20 mmol l^{-1} KCl, plus 0.5 mmol l^{-1} PMSF, added as described above). The homogenized sample was passed through four layers of coarse cheesecloth and centrifuged at 42 ***g*** for 15 min at 4 °C to remove unbroken cells and debris. The supernatant, regarded as the homogenate, was centrifuged at 1000 ***g*** for 15 min at 4 °C, and the pellet, regarded as the nuclear fraction, was resuspended in homogenization buffer. The supernatant was centrifuged at 8000 ***g*** for 15 min to give a further pellet and supernatant (cytosolic fraction). This pellet was gently resuspended in homogenization buffer and centrifuged at 8000 ***g*** for 15 min; the resulting pellet was resuspended in homogenization buffer (mitochondrial fraction). The three fractions (nuclear, mitochondrial and cytosolic) were each sonicated for 60 s and then passed through 10-ml columns of

Sephadex G-25 (as described above) equilibrated with 50 mmol l^{-1} Tris-HCl, pH 7.2, containing 50 mmol l^{-1} KCl, and the following enzyme activities were assayed immediately: glyceraldehyde-3-phosphate dehydrogenase as a cytosolic marker, glutamate dehydrogenase as a mitochondrial marker, and GSase. The results of trial experiments showed that (1) only 1 % of glyceraldehyde-3-phosphate dehydrogenase activity was present in the mitochondrial fraction, indicating that mitochondria were quite free of cytosolic components, and (2) more than 70 % of the glutamate dehydrogenase activity was present in the mitochondrial fraction, indicating that the mitochondrial fraction represented a high percentage of the mitochondrial enzymes (the majority of the remainder was in the nuclear fraction, suggesting that there had been some loss of mitochondria to this fraction, but some was also found in the soluble fraction, suggesting some breakage of mitochondria had occurred).

GSase cDNA sequence

mRNA was isolated from stomach and liver tissue (stored at –80 °C) using Poly (A) Pure mRNA kit 1915 from Ambion, Inc. (Austin, TX, USA). First- and second-strand cDNA was prepared from this mRNA and ligated to the kit-supplied adapter oligonucleotide using Marathon cDNA Amplification kit K1802-1 from Clontech (Palo Alto, CA, USA). Two overlapping fragments of GSase cDNA were amplified by the polymerase chain reaction (PCR) using AdvanTAge cDNA Polymerase Mix (Clontech 8417-1). The primers used for the amplification were adapter-specific AP1 from the kit and one (right primer) or the other (left primer) of two different consensus primers (see Fig. 1), designed on the basis of identifying highly conserved regions of several aligned mammalian and fish Gsases. The two primers were TGRCASCCDGCHCCRTTCCAGTT (RcGS1, right primer, giving a 5′ fragment of the cDNA that overlaps the 3′ fragment) and CCDTGGTTTGGVATGGARCARGA (LcGS1, left primer, giving a 3′ fragment of the cDNA that overlaps the 5′ fragment). PCR conditions were varied to maximize the yield and purity of the band of the correct size as estimated by agarose gel electrophoresis (to obtain the product of interest, touchdown PCR cycling parameters were used initially, followed by a two-step PCR procedure using a higher annealing temperature of 64–68 °C to increase specificity and stringency). After conditions were maximized, the product from the first amplification was re-amplified using the nested internal primer adapter-specific AP2 from the kit in place of AP1. Clontech AdvanTAge PCR Cloning kit K1901-1 was used to ligate the amplified products into the kit-supplied pT-Adv Vector and to clone this fragment-containing Vector by transformation into kit-supplied TOP10F′ competent *Escherichi coli* cells and growing on LB/ampicillin/X-gal/IPTG agar plates. White colonies were selected and grown on LB/ampicillin cultures and plasmid DNA was isolated using QIAprep Spin Miniprep kit 27104 (QIAGEN, Valencia, CA, USA). The presence of the inserted fragment of correct size was confirmed by cutting with *Eco*RI to release the insert, followed by electrophoresis on 1 % agarose and comparison of the migration distance with that of fragments from a DNA ladder of known sizes and the uncut plasmid as control.

The insert in the plasmid was sequenced by primer walking, beginning with AP2 and/or the consensus primer as the sequencing primers, respectively; the sequence for the entire 5′ UTR region through the coding region and into the 3′ UTR was obtained for both strands. Automatic sequencing was carried out by the University of Minnesota Advanced Genetic Analysis Center. Primers were purchased from Integrated DNA Technologies (Coraville, IA, USA). PCRs were carried out with a Perkin Elmer Gene Amp 2400 PCR instrument. The Advantage cDNA polymerase mix from Clontech was used for all PCR amplifications. The nucleotide and deduced amino acid GSase sequences are available from GenBank under Accession numbers AY071837 (liver) and AY071838 (stomach). Multiple sequence alignments were performed using the program ClustalW.

Partial sequence of β-actin cDNA

In another project, the partial sequence of β-actin cDNA from liver of marble goby had been determined, and a probe prepared from this cDNA was used for measuring β-actin mRNA by ribonuclease protection assays as described below. This was possible because this portion of the β-actin sequence is very highly conserved (Kong et al., 2000). Liver cDNA from marble goby was used as template in the PCRs carried out for generating the β-actin DNA fragment; primers used for PCR and for sequencing were the same as previously described (Kong et al., 2000). The base sequence of the portion sequenced is:

ATCCGTAAGGACCTGTATGCCAACACTGTGCTGTCT-
GGAGGTACCACCATGTACCCTGGCATTGCTGACAGG-
ATGCAGAAGGAGATCACAGCCCTGGCTCCATCCACC-
ATGAAGATCAAGATCATTGCCCCTCCAGAGCGTAAA-
TACTCTGTCTGGATCGGAGGCTCCATCCTGGCCTCTC-
TGTCCACCTTCCAACAAATGTGGATC.

The deduced amino acid sequence based on the reading frame beginning at the first codon (ATC) is:

IRKDLYANTVLSGGTTMYPGIADRMQKEITALAPSTM-
KIKIIAPPERKYSVWIGGSILASLSTFQQMWI.

Measurement of GSase mRNA by ribonuclease protection assays

Templates for preparing RNA probes for measuring GSase and β-actin mRNA levels using specific GSase primers (see Fig. 1; left or sense primer = GGCATGGAACAAGAGTACAC-GATT; right or antisense primer = GCTTGGGGTC-AAATGAGGCAAC) and specific β-actin primers (left or sense primer = ATCCGTAAGGACCTGTATGCCAAC; right or antisense primer = GATCCACATTTGTTGGAAGG-TGGA), respectively, with a T7 promoter appended to the 5′ end of the antisense primers, were made from liver cDNA from *B. sinensis* and marble goby, respectively, using MAXIscript

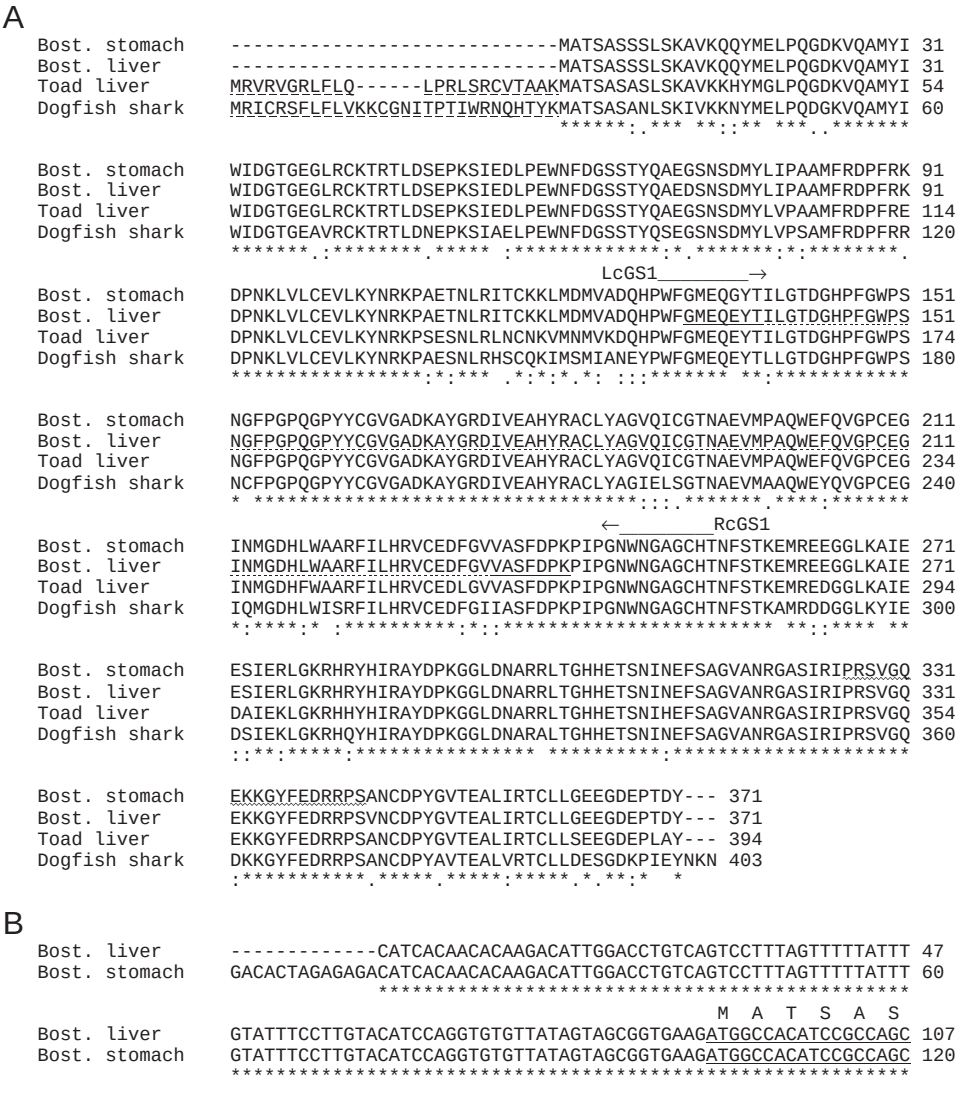

Fig. 1. Alignment of the deduced amino acid sequences of glutamine synthetase (Gsase) from liver and stomach of *B. sinensis* and two other GSases. (A) The amino acid sequence that corresponds to the oligopeptide used for preparing antibody to the GSase is underlined with a wavy line (*B. sinensis* stomach GSase). The amino acid sequences with codons that correspond to the two consensus primers, LcGS1 and RcGS1, used for obtaining the two complete fragments of the cDNA from stomach and liver by PCR with each of the consensus sequences and AP1, respectively, are indicated by lines; arrows indicate the direction copied above the corresponding sequences for *B. sinensis* stomach GSase. Amino acid sequences with codons that correspond to the two specific primers used for preparing the probe for RPAs are indicated by solid lines below the corresponding sequences for *B. sinensis* liver GSase; they are connected by a dotted line corresponding to the entire probe sequence (110 amino acids, 330 base pairs). Mitochondrial targeting sequences are identified by a dashed underline. (B) Base sequences of the 5′ end of the *B. sinensis* liver and stomach mRNA GSase transcripts. The beginning of the open reading frame is underlined with the corresponding N-terminal amino acid sequence indicated above the sequence. Bost., *B. sinensis*; Toad, *Opsanus beta*; Dogfish shark, *Squalus acanthias*. *Identical residues in the alignment; colons, conservative amino acid replacements in the alignment (i.e. similar amino acids); stops, dissimilar amino acids in the alignment.

T7 kit 1312 from Ambion, Inc., as previously described (Kong et al., 2000). The probes were isolated and labeled with psoralen-biotin using BrightStar Psoralen-Biotin labeling kit 1480 from Ambion, Inc., as previously described (Kong et al., 2000). GSase and β-actin mRNA levels in total RNA isolated from *B. sinensis* were measured by ribonuclease protection assays using these probes with RPA III Ribonuclease Protection Assay kit 1414 and BrightStar BioDetect detection kit 1930 from Ambion, Inc., following the instructions provided with the kits and as previously described (Kong et al., 2000). Total RNA was isolated from approximately 0.5 g tissue using RNAwiz^{TM} from Ambion, Inc. (9736) according to the instructions provided. The relative intensities of the GSase and β-actin bands were determined using a FluorChem^{TM} 8000 Advanced Imaging system (Alpha Innotech Corp., San Leandro, CA, USA) for quantification of the chemiluminescence signal.

GSase antibody and western blotting

Affinity-purified rabbit antibodies against the KLH-conjugated highly conserved oligopepetide GSase sequence (acetylcysteinyl-CPRSVGQEKKGYFEDRRPS-amide) identified in Fig. 1 were purchased from Quality Controlled Biochemicals (Hopkinton, MA, USA) and stored at –20 °C. Immunoblotting was carried out by standard protocols using SDS-PAGE [7.5 % acrylamide/bisacrylamide (19:1)] and electroblotting apparatus and reagents from Bio-Rad Laboratories (Hercules, CA, USA). Immunodetection of the GSase on the blotted nitrocellulose membrane was carried out using western blotting detection reagents (RPN21OT) from Amersham Pharmacia Biotech (Piscataway, NJ, USA) according to the manufacturers directions, except 5 % dry milk powder in TBST (50 mmol l^{-1} Tris, pH 8.0, 0.9 % NaCl, 0.1 % Tween 20) was used as the blocking agent instead of the kit reagent. After exposure to X-ray film for an optimal period of

Table 1. *Change in glutamine synthetase activities in various tissues of* B. sinensis *after exposure to NH_4Cl for 48 h*

	GSase activity					
	μmol min^{-1} g^{-1} tissue			μmol min^{-1} mg^{-1} protein $\times10^{-3}$		
Tissue	Control	+NH_4Cl	Increase (-fold)	Control	+NH_4Cl	Increase (-fold)
Liver	0.28±0.03	1.51±0.32[1,*]	5.4	3.7±0.5	16.4±2.9[1,*]	4.4
Intestine (anterior)	1.75±0.54	4.70±0.44*	2.7	21.2±5.8	55.3±8.2*	2.6
Intestine (posterior)	1.42±0.22	3.24±0.42*	2.3	21.0±5.9	39.8±4.3*	1.9
Stomach	12.15±1.47	11.39±1.07	0.9	185.5±21.9	163.8±25.5	0.9
Muscle	0.30±0.02	0.81±0.20*	2.7	4.1±0.4	10.5±2.7*	2.6

GSase, glutamine synthetase.
*Significantly different from control values (P<0.05).
Values are means ± S.E.M. (N=4); [1]N=5.

time, the intensity of the images was quantified as described above.

Statistical analyses

Results are presented as means ± standard errors (S.E.M.). Student's *t*-test or analysis of variance (ANOVA) followed by multiple comparisons of means by Duncan's procedure were used to evaluate differences between means where applicable. Arcsine transformation was performed on the 'percentage' data before statistical analyses. Differences were regarded as statistically significant at P<0.05.

Results

GSase sequences

The amino acid sequences of the liver and stomach GSase deduced from the cDNA sequenced in this study are shown in Fig. 1A, in alignment with two other GSases. The sequences are clearly homologous to other animal GSases, the *B. sinensis* liver GSase giving scores of 97, 98, 84, 86 and 86 % identity when aligned with GSases from marble goby, Gulf toadfish, spiny dogfish shark *Squalus acanthias*, mouse and the toad *Xenopus laevis*, respectively. The GSase sequences from *B. sinensis* liver and stomach are essentially identical, differing in amino acid sequences at only three positions, each arising from a single base change in the open reading frame (ORF). As shown in Fig. 1B, the 89-base 5′ untranslated region of the liver transcript is identical to the corresponding region of the stomach transcript, but the latter appears to have an additional 13 bases; both have a single translation start site. The 3′ sequences of the 341 bases sequenced beyond the end of the ORF of the two transcripts are identical except that three bases are absent in the stomach transcript at position 58–61 beyond the end of the ORF.

Increased expression of GSase

As shown in Table 1, exposure to 15 mmol l^{-1} NH_4Cl for 2 days results in a significant increase in GSase activity (units g^{-1} tissue and units mg^{-1} protein) in liver, and a smaller but significant increase in intestine and muscle, but no increase in stomach levels. The levels of GSase activity were found to be unexpectedly high in muscle and intestine and, especially, in stomach. The increases in GSase activity are accompanied by a correspondingly significant increase in GSase protein levels in liver and a small increase in intestine and muscle (Fig. 2 and Table 2) and a significant increase in GSase mRNA concentration in all tissues (Fig. 3, Table 2); although GSase activity and protein levels did not increase in stomach, it is notable that there was a small increase in GSase

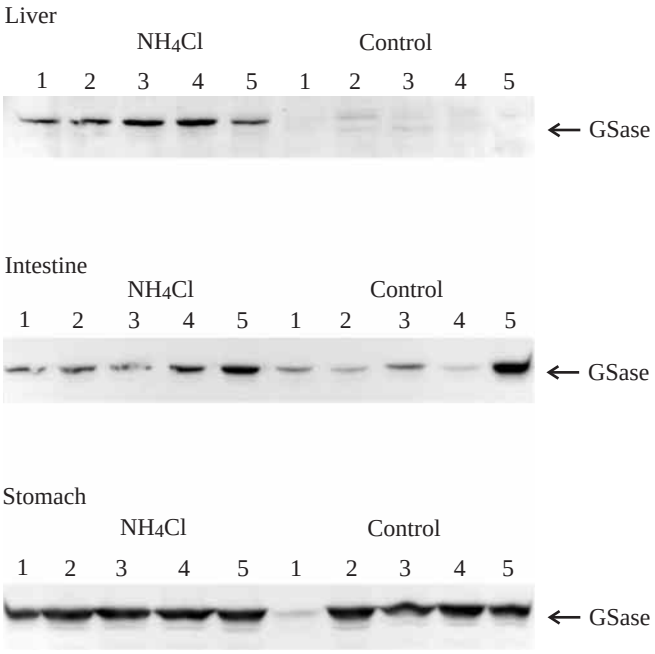

Fig. 2. Western blots showing changes in level of expression of GSase protein following exposure of *B. sinensis* to NH_4Cl. Total protein (0.5 μg) loaded onto the gels is equivalent in all lanes. The bands correspond to the expected molecular size of GSase as determined by the migration of standards and the predicted molecular mass of *B. sinensis* Gsase, based on its amino acid sequence.

Table 2. *Summary of changes in expression of glutamine synthetase protein (western blots) and mRNA (ribonuclease protection assays) in various tissues of* B. sinensis *after exposure to NH_4Cl for 48 h*

	Western blots (arbitrary numbers)			Ribonuclease protection assays (GSase/actin ratio)		
Tissue	Control	+NH_4Cl	Increase (-fold)	Control	+NH_4Cl	Increase (-fold)
Liver	1662±231	9753±1881*	5.9	0.049±0.004	0.988±0.201*	20.2
Intestine	1642±558	2021±375	1.2	0.187±0.048	1.326±0.194*	7.1
Stomach	4532±245[1]	4617±188	1.0	0.300±0.051	0.676±0.062*	2.3
Muscle	10 539±2479	12 500±1855	1.2	0.077±0.036	0.678±0.084*	8.8

GSase, glutamine synthetase.
*Significantly different from the control values (P<0.05).
Values are means ± 1 S.E.M. (N=5); [1]N=4.

mRNA. As shown in Table 3, virtually all GSase activity in liver both before and after exposure to NH_4Cl is localized in the cytosol.

Effect on CPSase III activity and other urea cycle enzymes

The levels of CPSase III activity were found to be very low in freshly excised liver tissue, i.e. <1 nmol min^{-1} g^{-1} liver, as compared, for example, to 500, 60 and 10 nmol min^{-1} g^{-1} liver in Gulf toadfish (Anderson and Walsh, 1995), the alkaline lake tilapia (Lindley et al., 1999) and largemouth bass *Micropterus salmoides* (Cao et al., 1991), and even lower or not detectable in muscle, stomach and intestine; an increase in CPSase III activity upon ammonia exposure was not observed for any of these tissues (data not shown). Ornithine carbamyltransferase (mitochondrial) and argininosuccinate synthetase and lyase activities (cytosolic) in liver were also very low (300 and 0.4 nmol min^{-1} g^{-1} liver, respectively) and did not increase in fish exposed to ammonia (data not shown).

Effect on various metabolite levels

As expected, exposure to ammonia resulted in a net influx of ammonia, which was reversed by removal of ammonia from the external medium, and a dramatic increase in ammonia concentration in liver, plasma and muscle (Table 4). There was no significant change in the rate of urea excretion and little change in the urea concentration in these three tissues (Table 5). However, there was a significant increase in the concentration of glutamine in these three tissues after 48 h of exposure to ammonia, which was accompanied by a decrease and increase in glutamate concentration in muscle and liver, respectively (Table 6). Total free amino acids increased only in muscle, which may be significant given the large muscle mass of fish relative to total body mass (>50 %). The concentrations of several other amino acids changed, as noted in Table 6, including an increase in alanine in muscle, but none were as dramatic and represented much lower levels of total nitrogen than glutamine. This result is consistent with the noted increase in GSase activity.

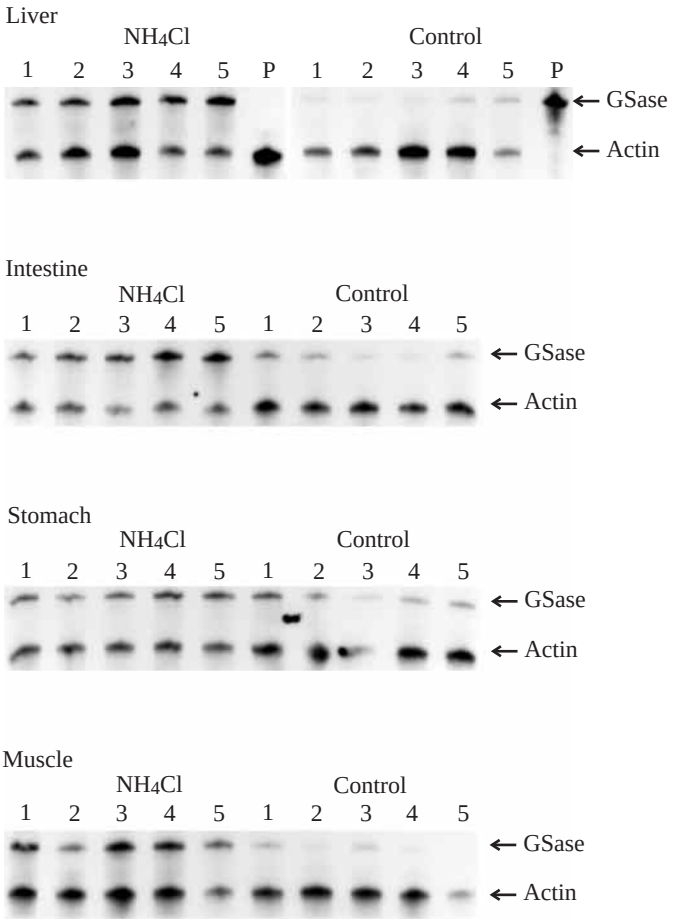

Fig. 3. Expression of GSase mRNA in control- and NH_4Cl-exposed *B. sinensis* in different tissues. mRNA was detected using ribonuclease protection assays as described in the text. Each lane was loaded with sample that originally contained 10 µg of total RNA; when yeast RNA was substituted for mRNA from *B. sinensis* as a control, no visible band was obtained (data not shown). Lanes P correspond to either the GSase or β-actin probe loaded with yeast RNA without the RNase step.

Table 3. *Changes in glutamine synthetase activities in the cytosolic and mitochondrial fractions of liver in* B. sinensis *after exposure to NH_4Cl for 48 h*

	GSase activity (μmol min^{-1})			
	g^{-1} tissue		mg^{-1} protein	
Compartment	Control	+NH_4Cl	Control	+NH_4Cl
Cytosol	0.66±0.17	2.15±0.62*	5.68±0.52	29.9±7.3*
Mitochondria	0.007±0.001	0.023±0.007*	0.53±0.03	2.25±0.45*

GSase, glutamine synthetase.
*Significantly different from control value (P<0.05).
Values are means ± S.E.M. (N=3).

Table 4. *Effects of exposure to NH_4Cl in various tissues of* B. sinensis

Variable	Control	+NH_4Cl
Ammonia excretion rate (μmol h^{-1} g^{-1} fish)		
Day 0	0.20±0.04 (3)	0.18±0.04 (7)[1]
Day 1	0.22±0.03 (4)	0* (6)
Day 2	0.22±0.06 (4)	0* (7)
Day 3[2]	0.23±0.02 (4)	0.45±0.05* (3)
Ammonia concentration[3] (μmol g^{-1} tissue)		
Muscle	1.85±0.21 (3)	13.2±0.51* (4)
Liver	1.15±0.10 (4)	8.87±0.46* (4)
Plasma	0.26±0.08 (3)	2.62±0.43* (3)

N values are given in parentheses.
*Significantly different from control values (P<0.05).
[1]Same as control set, before exposure to NH_4Cl.
[2]Transferred to 30 % sea water, pH 7.2, for recovery after day 2.
[3]After exposure for two days.

Discussion

In elasmobranchs, the GSase in liver is localized in the mitochondria along with the urea cycle-related glutamine-dependent CPSase III (Casey and Anderson, 1982); ammonia, therefore, enters the urea cycle pathway as glutamine *via* carbamoyl phosphate formation (Anderson and Casey, 1984). In brain and extrahepatic tissues, however, the GSase activity is localized in the cytosol (Smith et al., 1987). There is a single gene for GSase in the elasmobranch *S. acanthias* and the GSase transcript has two potential start codons: the first gives rise to a protein with an N-terminal mitochondrial targeting sequence and two putative proteolytic cleavage sites which, when cleaved after being transported into mitochondria, would yield a mature protein of the same size as the mitochondrial GSase; the second potential start codon yields a protein that lacks the mitochondrial targeting signal and is the same size as the cytosolic GSase (Campbell and Anderson, 1991; Laud and Campbell, 1994). Thus, the two isozymes arise either from two mRNAs generated by differential splicing or from the same mRNA by differential translation. GSase expressed in Gulf toadfish liver occurs in both the cytosol and mitochondria, and only the cytosolic enzyme appears to be subject to regulation related to ammonia metabolism (Walsh and Milligan, 1995; Walsh, 1996, 1997). As in *S. acanthias*, there appears to be a single gene and the mechanism for expression of isozymes targeted for different subcellular compartments appears to be analogous to that in elasmobranchs (Walsh et al., 1999). These are the only two fish GSases that have been highly purified and characterized and the complete coding sequence determined (Shankar and Anderson, 1985; Campbell and Anderson, 1991; Laud and Campbell, 1994; Walsh, 1996; Walsh et al., 1999). GSases in mammalian species are localized in the cytosol in all tissues and this appears to be the case for most teleost non-ureotelic fish species as well (Campbell and Anderson, 1991; Felskie, 1998; Anderson, 2001). Mitochondrial localization of GSase in fish may be related to ureotelic capability associated with expression of mitochondrial CPSase III.

In contrast to these two fish species (*S. acanthias* and Gulf toadfish, as noted above), the sequences of the GSase cDNAs derived from mRNA from liver and stomach, respectively, of *B. sinensis* reported here are characterized by a single start codon for translation. This would be consistent with the observation that the liver GSase appears to be localized exclusively in the cytosol and that *B. sinensis* is not ureotelic. GSase cDNA from stomach was sequenced as well as GSase cDNA from liver because of the unexpectedly high levels of GSase activity found to be present in stomach. The results suggest that the mRNAs for the two tissues are coded for by the same gene; the significance of the extra 13 bases on the 5′ end of the stomach cDNA is not known, perhaps reflecting differential splicing during transcription related to specific tissue expression or an artifact of experimental reverse transcription of the mRNA and subsequent adapter ligation. The sequence of the 5′ untranslated region of the GSase cDNA from stomach bears little relationship to the 5′ untranslated region of Gulf toadfish cDNA upstream from the second start

Table 5. *Changes in urea excretion and in urea concentration in various tissues of* B. sinensis *during exposure to NH_4Cl*

Variable	Control	+NH_4Cl
Urea excretion rate (μmol h^{-1} g^{-1} fish)		
Day 0	0.015±0.002 (4)	0.018±0.004 (7)[1]
Day 1	0.015±0.002 (4)	0.023±0.006 (7)
Day 2	0.014±0.002 (4)	0.011±0.004 (7)
Day 3[2]	0.012±0.001 (4)	0.010±0.001 (7)
Urea concentration[3] (μmol g^{-1} tissue)		
Muscle	0.22±0.04 (4)	0.24±0.04 (4)
Liver	0.16±0.05 (4)	0.31±0.06* (4)
Plasma	0.36±0.09 (4)	0.25±0.05 (3)

N values are given in parentheses.
*Significantly different from control values.
[1]Same as control set, before exposure to NH_4Cl.
[2]Transferred to 30 % sea water, pH 7.2, for recovery after day 2.
[3]After exposure for two days.

Table 6. *Concentrations of free amino acids (FAA) and total free amino acids (TFAA) in liver of various tissues of* B. sinensis *after exposure to NH_4Cl for 2 days*

	Muscle (μmol g^{-1})		Liver (μmol g^{-1})		Blood plasma (μmol ml^{-1})	
FAA	Control	+NH_4Cl	Control	+NH_4Cl	Control	+NH_4Cl
Ala	1.604±0.146	2.865±0.310(3)*	0.327±0.104	0.190±0.017	0.117±0.012	0.111±0.019
Arg	0.726±0.065	0.572±0.032	0.046±0.004	0.045±0.007	0.016±0.006	0.013±0.002
Asg	1.072±0.114	1.005±0.152	n.d	n.d.	0.028±0.005	0.010±0.001*
Asp	1.161±0.119	0.259±0.050*	0.539±0.139	0.188±0.037*	0.041±0.007(3)	0.021±0.004
Cys	n.d.	n.d.	n.d.	n.d.	0.010±0.003	0.011±0.006
Gln	2.639±0.247	8.118±0.067*	n.d.	3.307±0.327(3)	0.079±0.023	0.280±0.045(3)*
Glu	0.667±0.069	0.306±0.045*	3.803±0.013	6.984±0.233*	0.039±0.003(3)	0.040±0.002(3)
Gly	14.36±1.85	16.61±2.54	2.503±0.474	1.669±0.231	0.497±0.086	0.401±0.033
His	6.580±0.625	6.755±0.992	0.328±0.042	0.190±0.022*	0.130±0.016(3)	0.123±0.023(3)
Ile	0.081±0.012	0.049±0.004	0.022±0.002(3)	0.021±0.005	0.074±0.012	0.020±0.010*
Leu	0.144±0.018	0.097±0.008	0.042±0.003(3)	0.048±0.006(3)	0.131±0.020	0.061±0.009(3)*
Lys	6.580±0.455	5.521±0.091	0.397±0.058	0.238±0.041	0.351±0.073	0.287±0.039
Met	n.d.	n.d.	n.d.	n.d.	0.035±0.008	0.022±0.007(3)
Phe	0.130±0.005	0.096±0.014*	0.055±0.006	0.035±0.003	0.069±0.004	0.024±0.005(3)*
Pro	0.227±0.003	0.351±0.098(3)	0.129±0.024	0.121±0.019	0.090±0.056	0.019±0.004
Ser	0.906±0.042	1.156±0.154	0.084±0.013	0.050±0.006	0.038±0.005	0.026±0.004
Tau	16.70±1.46	18.30±2.44	17.00±1.12	14.17±0.42	0.387±0.074	0.321±0.041
Thr	1.290±0.129	1.337±0.302	0.352±0.078(3)	0.485±0.082	0.451±0.078(3)	0.184±0.037(3)*
Try	0.058±0.005	0.031±0.005*	0.042±0.008	0.021±0.004	0.060±0.003	0.027±0.003(3)*
Tyr	0.064±0.004	0.053±0.005	0.055±0.006	0.058±0.005	0.036±0.002	0.019±0.005(3)*
Val	0.145±0.016	0.100±0.013	0.054±0.017	0.032±0.007	0.118±0.016	0.099±0.025
TFAA	55.12±4.00	69.69±4.29*	26.62±1.76	25.27±3.06(3)	2.786±0.310	2.267±0.241

Values are means ± S.E.M. (N=4, unless indicated otherwise in parentheses).
*Significantly different from control values (P<0.05).
n.d., not detectable.

codon. As noted in the Results and in Fig. 1, the derived amino acid sequences of the open reading frame of both cDNAs are very similar to the sequences of GSases from Gulf toadfish and *S. acanthias*, as well as to GSases from many mammalian species. These results do not exclude the possibility that additional GSase genes are expressed in each of these tissues in *B. sinensis*, since only one cDNA may have been amplified in each tissue; multiple GSase genes have recently been identified in rainbow trout (*Oncorhynchus mykiss*) (B. Murray, E. Busby, T. Mommsen, and P. Wright, personal communication) and Gulf toadfish (P. Walsh, personal communication).

A major effect seen when *B. sinensis* is exposed to air for 2 days is increased GSase activity in liver and increased levels of glutamine in several tissues, muscle in particular (Ip et al., 2001b). The increase in glutamine levels in muscle and other tissues is equivalent to the amount of ammonia that would have been excreted, but could not be excreted due to air exposure; it has been proposed that this increased glutamine synthesis capability and glutamine accumulation represents the normal short-term adaptive response as an alternative pathway for ammonia detoxification during air exposure in this fish (Ip et al., 2001b). The results reported here when the fish is exposed to exogenous ammonia are similar, but more dramatic. This may not be unexpected if increased ammonia concentration is the primary regulatory signal in both situations, since ammonia exposure leads to considerably higher tissue ammonia levels than occur as a result of air exposure. GSase activity increased 5.4-fold in liver and glutamine concentration in muscle increased 3.1-fold compared to 1.8-fold and 1.7-fold, respectively, when exposed to air. The increase in GSase activity in liver was accompanied by a 5.9-fold increase in GSase protein and a 20-fold increase in GSase mRNA, suggesting that the increased activity is due primarily to transcriptional regulation. These effects are similar to results obtained when GSase protein and mRNA are measured in Gulf toadfish subjected to conditions that promote induced GSase activity (Kong et al., 2000).

Brain tissue of virtually all fish has high GSase activity, which functions to detoxify ammonia (Webb and Brown, 1976; Webb, 1980). In contrast, the level of GSase activity in other fish tissues is variable (Webb, 1980; Campbell and Anderson, 1991); it is high in liver of ureo-osmotic elasmobranchs and a few teleosts reported to be ureotelic, low or non-detectable in liver, somewhat higher in intestine, and very low or non-detectable in muscle of non-ureotelic/non-ureogenic fish (Table 7). The level of GSase activity in *B. sinensis* liver is comparable to measurable levels of GSase activity reported in liver of several other non-ureotelic/non-ureogenic fishes (Table 7). However, the level of GSase activity in muscle is about

Table 7. *Reported glutamine synthetase activities in various tissues of different species of fish*

Species	GSase activity (μmol min^{-1} g^{-1} tissue) Brain	Liver	Muscle	Intestine	Stomach	Reference
Ureoosmotic						
Squalus acanthias	18.0	18.4	1.0	–	–	Webb, 1980
Taeniura lymma	7.4	25.0	1.4	–	–	Webb, 1980
Ureotelic						
Oreochromis alcalicus grahami	–	6.2	0.3	–	–	Lindley et al., 1999
Opsanus beta	109.5	7.0	–	–	–	Wang and Walsh, 2000; Anderson and Walsh, 1995
Opsanus beta	–	–	0.12	3.10	6.6	P. J. Walsh, personal communication
Ureogenic						
Porichthys notatus	33.2	1.0	0.4	–	–	Webb, 1980
Porichthys notatus	59.4	1.3	–	–	–	Wang and Walsh, 2000; Anderson and Walsh, 1995
Opsanus tau	158.3	1.3	–	–	–	Wang and Walsh, 2000; Anderson and Walsh, 1995
Mircopterus salmoides	–	0.44	0.12	0.36	–	Kong et al., 1998
Heteropneustes fossilis	8.3	1.0	–	–	–	Chakravorty et al., 1989
Nonureotelic/nonureogenic						
Potamotrygon circularis	10.9	0.2	0.0	–	–	Webb, 1980
Oncorhynchus mykiss	–	0.29	0.03	0.06	–	Korte et al., 1997
Oncorhynchus mykiss	–	0.86 (1.09)[1]	0.04 (0.10)[1]	–	–	Todgham et al., 2001
Platichthys stellatus	49.7	0.50	0.0	–	–	Webb, 1980
Ictalurus punctatus	–	0.22	0.03	0.33	–	Felskie et al., 1998
Cyprinus carpio	–	0.41	0.07	0.99	–	Felskie et al., 1998
Carassius auratus	–	0.14	0.04	1.02	–	Felskie et al., 1998
Amia calva	–	0.28	0.21	0.50	–	Felskie et al., 1998
Amia calva	7.2	0.08	0.09-0.14	0.11	–	Chamberlin et al., 1991
B. sinensis	–	0.28 (1.51)[2]	0.30 (0.81)[2]	1.6 (4.0)[2]	12.2	This study

GSase, glutamine synthetase.
[1]Number in parentheses is after exercise.
[2]Number in parentheses is after exposure to exogenous ammonia (from Table 1).

tenfold higher than in muscle of most of these other species and is as high as the levels in *B. sinensis* liver; the level of GSase in intestine (posterior and anterior) is also higher than in the intestine of these other species (Table 7). Exposure to ammonia resulted in increased GSase activity, GSase protein and GSase mRNA levels in these tissues (intestine and muscle), as well as in liver. The total amino acid concentration increased significantly only in muscle, due primarily to the large increase in glutamine concentration. Given that muscle represents >50 % of the body mass (Ip et al., 2001b), muscle GSase in *B. sinensis* is likely to play the major role in ammonia detoxification in *B. sinensis* under conditions that limit ammonotelism. This function may, in fact, explain the uniquely high levels of GSase in muscle of this species and might partially explain the capability of *B. sinensis* to survive exposure to high concentrations of environmental ammonia (up to 50 mmol l^{-1} NH_4Cl at pH 7; S. F. Chew and Y. K. Ip, unpublished results) and extended periods of air exposure (Ip et al., 2001b). The observed relatively large increase in GSase mRNA with little increase in GSase protein in muscle (as well as intestine) is worth noting, perhaps reflecting additional regulatory control mechanisms, such as post-translational covalent modification.

Unexpectedly high levels of GSase activity were found in stomach tissue and, unlike liver, intestine and muscle, the levels of GSase activity and protein were not affected by exposure to ammonia, although a small increase in GSase mRNA was observed. As noted in Table 7, P. J. Walsh (personal communication) has observed high levels of GSase in stomach of Gulf toadfish and this may be characteristic of fish in general, reflecting a role unrelated to that in liver and muscle. As noted above, the GSase sequence is essentially identical to that from liver, but the observations that the mRNA appears to be 13 bases longer on the 5′ end and that GSase

activity is not induced during ammonia exposure could reflect unique stomach transcriptional regulatory factors. Further study aimed at elucidating the physiological role of this high GSase activity in stomach of this fish, and perhaps other fish, is clearly needed. Measurement and characterization of the properties of the biosynthetic activity, as opposed to the transferase activity assay used here and by most investigators, may be especially important; the ratio of transferase to biosynthetic activity has been reported to vary with different GSase isozymes (Walsh, 1996), and it is not impossible that stomach may have unique glutamine-related transferase activities that are not actually due to GSase.

The brain is often the organ undergoing the largest increases in glutamine concentration in fish exposed to ammonia, hence the high levels of GSase in the brain. Increases in glutamine concentrations in brain of more than tenfold resulting from ammonia exposure have been reported in rainbow trout *Salmo gairdneri* (Arillo et al., 1981), goldfish *Carassius auratus* (Levi et al., 1974) and common carp *Cyprinus carpio* (Dabrowska and Wlasow, 1986). For mudskippers exposed to sublethal concentrations of ammonia, the glutamine levels in the brains increase from 2.46 to 28 $\mu mol\,g^{-1}$ (*Periophthalmodon schlosseri*) and 2.77 to 15 $\mu mol\,g^{-1}$ (*Boleophthalmus boddaerti*); although accumulation of glutamine in the liver also occurs, the levels attained were much lower than in the brains (Peng et al., 1998). Thus, Korsgaard et al. (1995) proposed glutamine formation in the brain as one of the strategies available to fishes to deal with increasing concentrations of internal ammonia. Ammonia presumably exerts its toxic effects in the brain, and it is essential to have mechanisms to protect the brain against ammonia toxicity. For fishes that adopt this strategy, there is usually high ammonia tolerance in non-cerebral tissues (Korsgaard et al., 1995). Not unexpectedly, ammonia is also detoxified to glutamine in the brain of *B. sinensis* (Y. K. Ip and S. F. Chew, unpublished data). The results reported here with *B. sinensis*, however, represent the first report of a non-ureotelic teleost fish responding to environmental ammonia by increasing the expression of GSase in non-cerebral tissues. Previously, it was believed that only cerebral GSase was inducible by sublethal concentrations of environmental ammonia (Korsgaard et al., 1995; Peng et al., 1998).

The changes in levels of amino acids other than glutamine in muscle, liver and plasma, and the observed lack of urea cycle activity after exposure to ammonia reported here, are also similar to the changes observed when *B. sinensis* is exposed to air (Ip et al., 2001b). In addition to the large increase in glutamine concentration in muscle, there was also a significant increase in the total free amino acid content in this tissue; 72 % of this increase is attributable to the increase in glutamine content, 18 % to glycine and 10 % to alanine. As with exposure to air, this indicates a possible decrease in the rate of amino acid catabolism (Lim et al., 2001), reducing the rate of endogenous ammonia accumulation, or an increase in the synthesis of certain amino acids (e.g. glutamine), or both. There might also be a slight increase in partial amino acid catabolism, leading to the formation of alanine (Ip et al., 2001c). The glutamine concentration in liver of *B. sinensis* exposed to ammonia increased from 0 to 3.3 $\mu mol\,g^{-1}$. In comparison, less than a 20 % increase in hepatic glutamine concentration, with a corresponding decrease in glutamate concentration, was observed in rainbow trout exposed to ammonia (Arillo et al., 1981). For goldfish exposed to 0.75 $mmol\,l^{-1}$ NH_4Cl, the liver glutamine concentration increased only slightly (Levi et al., 1974).

The high levels of GSase in liver, intestine and, particularly, muscle, and the resulting significant increases in the level of GSase activity in these tissues accompanied by significant increases in tissue glutamine concentrations in *B. sinensis* upon exposure to exogenous ammonia appear to reflect a unique strategy to handle ammonia detoxification when tissue levels of ammonia increase. This appears to be the same strategy as that used by this fish to detoxify the increased endogenous ammonia concentrations that occur during air exposure (Ip et al., 2001b). Presumably the underlying cause of the effects induced by both exposure to exogenous ammonia and extended periods of air exposure is increased ammonia concentrations in the tissues. Since ammonia exposure results in higher tissue levels of ammonia than air exposure and the effects are greater and more reproducible than air exposure, ammonia exposure of *B. sinensis* represents an excellent model for investigating the underlying molecular mechanisms that regulate detoxification of ammonia to glutamine in non-cerebral tissues.

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