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INDUCTION OF CARBAMOYL PHOSPHATE SYNTHETASE III AND GLUTAMINE SYNTHETASE mRNA DURING CONFINEMENT STRESS IN GULF TOADFISH (*OPSANUS BETA*)

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Summary

Gulf toadfish (*Opsanus beta*) rapidly switch to excretion of urea as their main nitrogenous waste product under several laboratory conditions, including confinement to small volumes of water. Prior evidence suggested that the activities of two key enzymes of urea synthesis exhibited potentially different modes of upregulation during this switch, with carbamoyl phosphate synthethase III (CPSase III) activated allosterically by *N*-acetylglutamate, and glutamine synthetase (GSase) activated by increases in the concentration of protein. The present study was undertaken to examine additional aspects of the regulation of these enzymes. The sequence for *O. beta* CPSase III cDNA was obtained, and it was found to be similar to that of other piscine CPSases. The sequence also allowed us to develop riboprobes for CPSase III mRNA analysis using ribonuclease protection assays (RPAs). CPSase III mRNA was expressed in liver, muscle, kidney and intestine, in agreement with prior enzymatic measurements. Levels of CPSase III mRNA increased five- to tenfold (relative to β-actin mRNA) in liver (but not muscle) following 48 h of confinement stress. Measured by western analysis using an antibody to chicken GSase, confined *O. beta* GSase protein concentrations increased eightfold over control levels, in agreement with prior and present measurements of increases in GSase activity. Furthermore, RPAs of GSase mRNA levels demonstrated an increase of fivefold during confinement.

Key words: gulf toadfish, *Opsanus beta*, nitrogen excretion, enzyme induction, urea synthesis, carbamoyl phosphate synthethase, glutamine synthetase, stress.

Introduction

The mode of nitrogen excretion for most non-elasmobranch species of fish is ammoniotelic, i.e. nitrogen wastes are excreted mostly as ammonia across the gills into the surrounding aqueous environment. However, recent studies have demonstrated in certain teleostean species of fish a primarily ureotelic rather than ammoniotelic mode of nitrogen excretion, either normally or under specific physiological circumstances, accompanied by the presence of physiologically significant levels of the full complement of urea cycle enzymes. This group includes the alkaline-lake-adapted tilapia (*Oreochromis alcalicus grahami*) (which has adapted to an aqueous environment at pH>10, where it is not possible to excrete ammonia across the gills) (Randall et al., 1989; Lindley et al., 1999), an Indian air-breathing catfish (*Heteropneustes fossilis*) when exposed to a non-aqueous environment for an extended period (Saha and Ratha, 1987, 1989) and the gulf toadfish (*Opsanus beta*) when subjected to moderate stress such as confinement (Mommsen and Walsh, 1989; Walsh et al., 1990, 1994; Anderson and Walsh, 1995). A few species of teleost fishes have the full complement of urea cycle enzyme activities in liver extracts (although at lower levels than in the three species noted above shown to be capable of ureotelic nitrogen excretion) but have been shown to be primarily ammoniotelic (Anderson, 1995a). In largemouth bass (*Micropterus salmoides*), the level of the enzyme catalyzing the first step of the urea cycle (the urea-cycle-related mitochondrial glutamine- and acetylglutamate-dependent carbamoyl phosphate synthetase III; CPSase III) in liver is too low to account for the urea that is excreted (approximately 30 % of the total ammonia plus urea nitrogen is excreted as urea). Surprisingly, although CPSase III activity is present at low levels in muscle extracts, the levels are sufficient to account for the urea excreted (Kong et al., 1998). Low levels of CPSase III activity are also present in muscle from gulf toadfish (Julsrud et al., 1998) and in muscle of rainbow trout (*Oncorhynchus mykiss*) (Korte et al., 1997).

Gulf toadfish are useful as a model for studying ureotelism in a teleost fish because the levels of urea cycle enzyme activities in liver are quite high, comparable with those found in the ureo-osmotic elasmobranchs (Mommsen and Walsh, 1989; Anderson and Walsh, 1995). Isolated hepatocytes have a high capacity for [^{14}C]urea synthesis when incubated with [^{14}C]bicarbonate and other appropriate substrates; when fully induced, urea synthesis by hepatocytes occurs at rates comparable with those observed in elasmobranchs (Walsh et al., 1989). Ureotelism in gulf toadfish appears to function primarily at times when normal pathways of ammonia excretion are blocked (Walsh et al., 1990; Barber and Walsh, 1993). Previous studies have shown that ureotelism in gulf toadfish is not linked to osmoregulation (Walsh et al., 1990), acid–base balance (Walsh et al., 1990; Barber and Walsh, 1993) or air-exposure during low tides (Hopkins et al., 1999). In the laboratory, ureotelism is inducible by exposure to ammonia in the water, by exposure to air or by confinement/crowding (Walsh et al., 1990, 1994; Walsh and Milligan, 1995). Confinement/crowding leads to an acute plasma cortisol surge at 2 h, followed in less than 24 h by a cortisol-mediated several-fold increase in hepatic glutamine synthetase (GSase) activity (Walsh et al., 1994; Hopkins et al., 1995), an apparent rate-limiting enzyme of glutamine-dependent ureotelism in toadfish (Anderson and Walsh, 1995; Walsh and Milligan, 1995). Elevated GSase activity is thought to trap ammonia, thereby reducing ammonia excretion, and may subsequently serve to feed this nitrogen into the urea cycle as the primary substrate for CPSase III. However, similar conditions do not result in an increase in the level of CPSase III activity in liver or in muscle; in liver, there is an increase in the concentration of acetylglutamate, a positive allosteric effector for CPSase III (Julsrud et al., 1998).

The present study was undertaken to begin to examine the molecular mechanisms underlying these responses of *O. beta* GSase and CPSase III to confinement/crowding. The expression of CPSase III in largemouth bass (Kong et al., 1998) and rainbow trout (Korte et al., 1997) has been confirmed by sequencing the cDNA for CPSase III and measuring CPSase III mRNA levels in extracts. Here, we report similar studies with gulf toadfish, extending the studies to measurement of the levels of CPSase III and GSase mRNA before and after confinement. The sequence for CPSase III cDNA and the partial sequence for the cytosolic pyrimidine-pathway-related glutamine-dependent acetylglutamate-independent CPSase II (aspartate transcarbamoylase and dihydro-orotase) and β-actin as a housekeeping gene are reported.

Materials and methods

Animals and acclimation conditions

Sexually mature gulf toadfish (*Opsanus beta* Goode and Bean) were captured by roller trawl by local fishermen in Biscayne Bay, Florida, USA, in the summer of 1997 and 1998. At the University of Miami, fish were maintained initially in glass aquaria supplied with flowing Biscayne Bay sea water (29–34 ‰, temperature 25±1 °C). Each aquarium contained a sand/gravel substratum (2–5 cm depth) and several polyvinyl chloride tubes that acted as individual shelters. Typically, 3–4 fish inhabited a single 45 l aquarium corresponding to a density of approximately 7–10 g fish l^{-1}. On days 1 and 3 after arrival, the fish were bathed in a mixture of Malachite Green and formalin (Wood et al., 1995) as a prophylactic treatment against the ciliate *Cryptocaryon irritans*. Fish were maintained under these conditions for at least 1 week and were fed *ad libitum* with frozen squid on alternate days. Food was withheld for 48 h prior to experimentation.

Control (unconfined) toadfish remained in 45 l aquaria as above and were sampled at the same time as confined fish. To induce ureotelism, fish weighing between 42 and 88 g (mass 63±2.0 g; mean ± S.E.M., N=33) were subjected to a standardized ‘confinement’ protocol (Walsh et al., 1994; Wood et al., 1995; Hopkins et al., 1995) 48 h prior to tissue harvesting. We chose 48 h as a time point because it is intermediate between prior studies examining changes in GSase and CPSase III activity at 24 h (Walsh et al., 1994), 72 h (Julsrud et al., 1998) and 96 h (Walsh and Milligan, 1995). Briefly, the confinement procedure involved placing fish and their tube shelters in small plastic tubs (volume approximately 6 l) to achieve densities exceeding 80 g fish l^{-1}. The tubs were aerated continually and supplied with flowing sea water. The fish were not fed during the period of crowding. To harvest tissues, fish were rapidly anesthetized with 1.5 g l^{-1} MS-222, and tissues were rapidly dissected, frozen in liquid nitrogen and stored in aluminum foil at −80 °C for up to several months before analysis.

Molecular studies

Primers for polymerase chain reactions (PCRs) were either synthesized using a PCR-Mate 391 DNA synthesizer (Applied Systems, Foster City, CA, USA) or purchased from Integrated DNA Technologies (Coralville, IA, USA). PCRs were carried out in DNA thermal cyclers from Perkin-Elmer, Norwalk, CT, USA.

Isolation of mRNA (from toadfish liver for CPSase III and GSase and from toadfish intestine for CPSase II and β-actin), subsequent cDNA synthesis and cDNA sequencing, and sequence analysis were accomplished using previously published strategies and procedures (Hong et al., 1994; Korte et al., 1997; Kong et al., 1998). The nucleotide and deduced amino acid sequences are available from GenBank under accession numbers AF169248 (CPSase III) and AF169249 (CPSase II). Numbering of amino acid residues for CPSase III began with the initial methionine residue of the entire translated product, i.e. including the mitochondrial targeting sequence. The multiple sequence alignments were performed using the programs ClustalW and MacDNASIS.

Sequence of a CPSase II cDNA segment

Toadfish intestine cDNA was used as a template in the PCRs for generating CPSase-II-specific fragments. CPSase-II-

Table 1. *Sequence of primers*

Primer	Location	Orientation	Nucleotide sequence
1	1005–1013 (MDIYELENP)	Antisense	NGGGTTRTCSAGRTCYTAYATYTCCA
2	786–794 (MVDENCVGF)	Sense	ATGGTGGAYGARAACTGTGTKGGSTTYGA
3	955–961 (EFDWCAV)	Antisense	ARVGCRCACCARTCRAAYTC
4	874–883 [LGFSDKQIAM(T7)]	Antisense	GGAACCTAATACGACTCACTATAGGGTAATGCT ATCTGCTTGTCTGAAAAACCCAG
5	600–606 (WKEIEYE)	Sense	TGGAAIGARRTIGARTAYGA
6	282–289 (IMKCDVDI)	Sense	ATHATGAARTGYGAYGAYGTNGAYAT
7	353–360 (QQMWISKQ)	Antisense	TGYTTNSWDATCCACATYTGYTG
8	786–793 (EIGSSMKS)	Sense	AAATAGGTAGTTCCATGAAGAG
9	902–909 [LLLMAKQD(T7)]	Antisense	GGAACCTAATACGACTCACTATAGGGATCCTGT TTGGCCATCAGCAGCAA
10	796–804 (HTLKPASDE)	Sense	CACAACATTAAGCCTGTTTCTGAGAAG
11	289–296 (IRKDLYAN)	Sense	ATCCGTAAGGACCTGTACGCTAAC
12	344–349 [SILASL(T7)]	Antisense	GGAACCTAATACGACTCACTATAGGGACAGAGA GGCCAGGATGGA
13	151–158 (GMEQEYTI)	Sense	GGCATGGAGCAAGAGTACACCATT
14	254–261 [VASFQPKP(T7)]	Antisense	GGAACCTAATACGACTCACTATAGGGGGGCTT AGGGTCAAATGAGGCGAC

The sequences of primers used in polymerase chain reactions (PCRs), for cDNA synthesis and for sequencing by primer walking are listed.

The location of carbamoyl phosphate synthetase (CPSase) primers 1–5 and 10 is given as the corresponding shark CPSase II amino acid sequence (Korte et al., 1997). The locations in β-actin sequences (primers 6, 7, 11 and 12) are based on sequences indicated in the text.

T7 represents the promoter sequence that binds T7 polymerase.

The locations of glutamine synthetase (GSase) sequences (primers 13 and 14) are based on the toadfish GSase amino acid sequence (Walsh et al., 1999).

The locations of primers 8 and 9 are given as the toadfish CPSase III amino acid sequence.

specific consensus primers 1 and 2 (40 pmol of each) (Table 1) were used for the first-time PCR, together with 1 µl of cDNA in a 50 µl standard reaction mixture (Hong et al., 1994). The DNA thermal cycler was programmed as follows: 94 °C (denaturation), 30 s; 55 °C (annealing), 30 s; and 72 °C (extension), 90 s; this cycle was repeated 35 times. The PCR reactions resulted in several products when analyzed by electrophoresis on 2 % agarose gel in TAE (standard Tris acetate–EDTA buffer) buffer (detected by ethidium bromide staining). A second-time PCR was then carried out with primer 2 and nested CPSase consensus primer 3 (Table 1) using 1 µl of PCR product from the first-time PCR reaction mixture. The thermal cycler was programmed as for the first-time PCR. A major band of approximately 525 base pairs (bp), the expected size, was observed by electrophoresis. The products were isolated from the electrophoresis gel using β-Agarase I (New England Biolabs, Beverly, MA, USA) and were sequenced using a Thermo Sequenase kit (Amersham Life Science, Inc., Cleveland, OH, USA) with [^{33}P]ddNTP as the radioactive label. The sequence reactions were analyzed with Long Ranger gel (FMC Bioproducts). Primers 2 and 3 were used for sequencing. Additional 5′ sequence was obtained using a similar approach with toadfish CPSase II sequence-specific primer 4 (which had been designed for synthesis of CPSase-II-specific probe, as noted below) and CPSase consensus primer 5.

Sequence of CPSase III cDNA

The procedure for determining the sequence of toadfish CPSase III cDNA was virtually identical to that described by Korte et al. (1997), except that the initial consensus primer pair was as described by Hong et al. (1994).

Partial sequence of β-actin cDNA

Toadfish intestine cDNA was used as template in the PCRs for generating a toadfish β-actin DNA fragment. Actin consensus primers 6 and 7 (40 pmol of each) (Table 1) were used for the PCR, along with 1 µl of cDNA in a 50 µl standard reaction mixture (Hong et al., 1994). The DNA thermal cycler was programmed for standard touchdown PCR (Don et al., 1991): the first cycle was 5 min at 94 °C (denaturing), 30 s at 60 °C (annealing), and 30 s at 72 °C (extension); the next cycle was 30 s at 94 °C, 30 s at 59 °C, and 30 s at 72 °C; the annealing temperature was decreased using this pattern until the annealing temperature was 50 °C, and this cycle was then repeated 20 times. The PCR product was analyzed by agarose gel electrophoresis, and a single band of the expected size, approximately 230 bp, was observed and then isolated as described above. The product was sequenced as described above using primers 6 and 7 (Table 1).

RNA isolation for ribonuclease protection assays

Total RNA was extracted from approximately 0.5 g of tissue

using Trizol reagent (Gibco BRL Life Technologies, Grand Island, NY, USA) according to the instructions provided, except that a modified high-salt RNA precipitation step was included (Chomczynski and Mackey, 1995). RNA concentration was determined by absorbance at 260 nm, and the samples were kept at −70 °C until used.

Templates for preparing RNA probes for CPSase II, CPSase III, GSase and β-actin

CPSase III sequence-specific primers 8 and 9 (Table 1) were designed to give a template for preparing a probe for CPSase III in which the sequence included the CPSase II 'gap' area (Fig. 1). A T7 promoter was appended onto the 5′ end of primer 9 (antisense strand) as outlined in the instructions in the MAXIscript kit (see below). Standard PCR conditions were used with 40 pmol of each of these primers and 1 µl (approximately 1 ng) of a toadfish CPSase-III-specific template. This template was a fragment (approximately 4 kbp) of toadfish CPSase III cDNA obtained from a 3′ RACE (rapid amplification of cDNA ends) as described by Korte et al. (1997). The thermal cycler was programmed for 35 cycles of 30 s at 94 °C, 60 s at 58 °C, and 1 min at 72 °C. The major product of approximately 400 bp was isolated by electrophoresis. After precipitation with ethanol and sodium acetate, the sample was dissolved in a small volume of TE (standard Tris–EDTA buffer) buffer, pH 8, and the concentration was determined by electrophoresing a sample on another agarose gel and comparing the signal intensity of the product with that of standards.

Template for preparing the toadfish CPSase II probe was made in the same way using primers 10 and 4 (Table 1). The CPSase-II-specific product (approximately 525 bp) described above was used as template. The major PCR product using these primers was approximately 300 bp, as expected.

Template for preparing the toadfish β-actin probe was also made by PCR in a similar way, using primers (11 and 12) specific for the actin sequence with the purified 230 bp β-actin product described above as template. Standard PCR reaction conditions with 40 pmol of each of these primers and 1 µl (approximately 10 ng) of the purified toadfish β-actin product

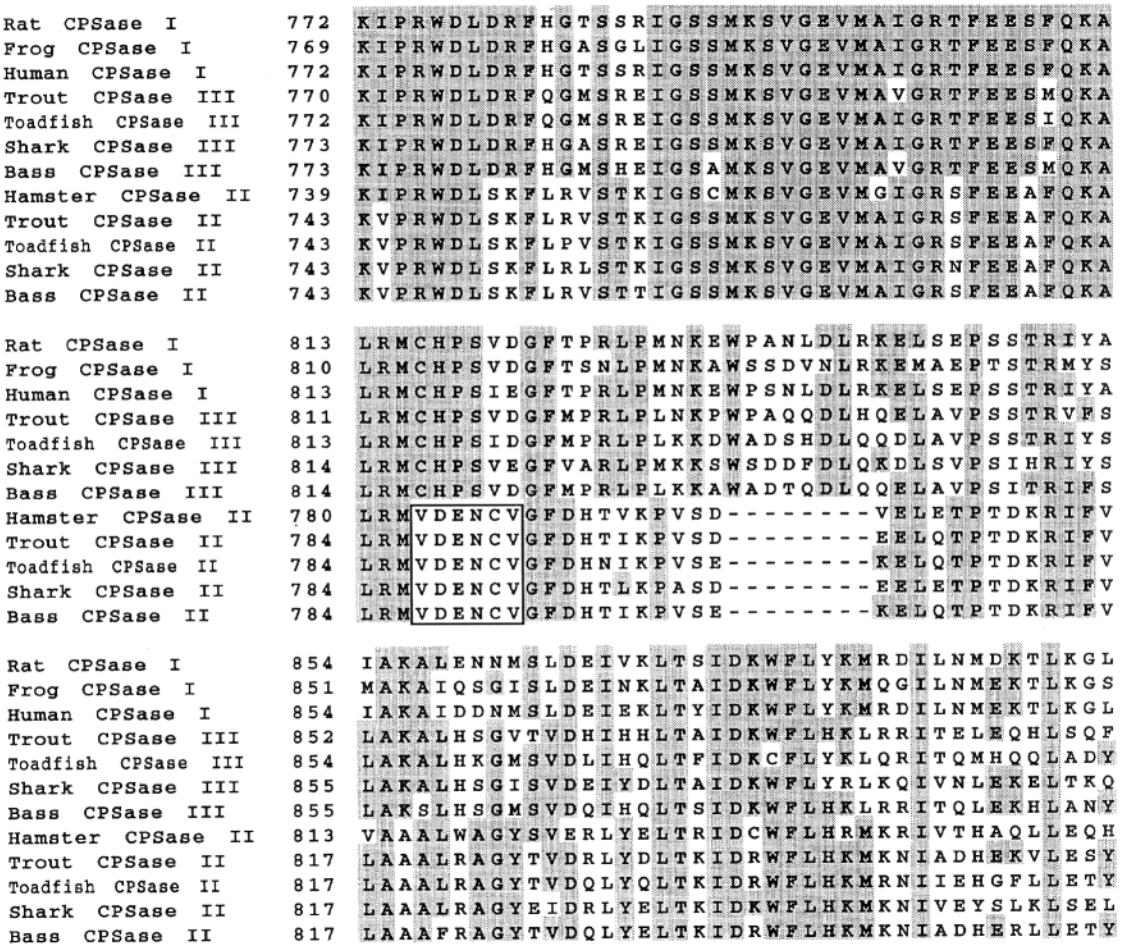

Fig. 1. Alignment of the partial deduced amino acid sequences of selected carbamoyl phosphate synthethases (CPSases). The amino acid residues of the trout, bass and toadfish CPSase IIs are arbitrarily assigned the same numbers as shark CPSase II since the complete amino acid sequences are not known. Identical residues are shaded. The CPSase II 'gap' region is indicated by dashes. The boxed sequences represent putative conserved sequences unique to the CPSase IIs.

as template were used. The thermal cycler was programmed for standard touchdown PCR as described above. The major product was approximately 190 bp, as expected.

Toadfish GSase sequence-specific primers 13 and 14 were used to amplify by PCR a 340 bp segment of toadfish GSase cDNA; PCR conditions were 94 °C for 1 min, 30 cycles of 94 °C for 1 min and 68 °C for 3 min, and ending with a 68 °C extension for 3 min. The product was isolated and used as template for making the RNA probe for GSase mRNA.

Biotin-labeled RNA probes for CPSase II, CPSase III, GSase and actin

Biotin-labeled RNA probes complementary to the cDNA templates with attached T7 promoter described above were prepared with biotin-cytosine triphosphate (CTP) using Brightstar Biotinscript and MAXIscript *in vitro* transcription kits (Ambion Inc., Austin, TX, USA) following the instructions in the kits. After synthesis, the probes were purified by polyacrylamide gel electrophoresis to remove incomplete products, as suggested by the manufacturer. The probes were precipitated by ethanol, dissolved in nuclease-free water, and the concentration was determined by absorbance at 260 nm. The probes were divided into small samples and kept at −70 °C until use. Alternatively, probes were prepared using the MAXIscript transcription kit with CTP instead of biotin-CTP and were then labeled after isolation with psoralen-biotin using a BrightStar psoralen-biotin labeling kit (Ambion Inc.); the labeled probes were stored in labeling buffer at −70 °C.

Ribonuclease protection assays

CPSase III and CPSase II mRNAs were measured by ribonuclease protection assays (RPAs) using an RPA II ribonuclease protection assay kit (Ambion Inc.) following the instructions provided with the kit. After overnight incubation of the RNA samples with the probe at 43 °C, the ribonuclease (RNAase) digestion step was carried out using 200 µl of a 1:100 dilution of solution R (concentrated RNAase A and RNAase T1). The reaction mixtures were incubated at 37 °C for 30 min (4 °C for analysis with samples that included β-actin). The undigested RNA–RNA duplexes were precipitated and resuspended in gel loading buffer and subjected to electrophoresis through 5 % denaturing polyacrylamide gels (16 cm×18 cm×0.75 cm thick) at 250 V for 60 min. GSase mRNA was measured in the same way, except that an RPA III ribonuclease protection assay kit was used, and RNAase digestion was carried out at 26 °C.

Undigested duplex mRNA from the polyacrylamide gels was transferred to a positively charged nylon membrane (Ambion Inc.) using a PolyBlot electrotransfer system (American Bionetic, Inc., Hayward, CA, USA) or a mini trans blot electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA). After transfer, the membranes were baked at 80 °C for 15 min.

The BrightStar BioDetect detection kit (Ambion Inc.) was used for the detection of the biotin-labeled probes. The instructions provided with the kit were followed. After finishing all the washing steps, the membranes were kept at room temperature for 2 h and then exposed to X-ray film for various times as needed to give optimal exposure. The intensity of the images was determined using a Bio-Rad image-analysis system (Gel Doc 1000) with accompanying software (version 2.1) for quantification.

Western analysis

SDS–PAGE was performed on crude homogenates (1:3 tissue:homogenization buffer, centrifuged at 16 000 ***g***) of liver from confined and unconfined toadfish. The gels were then subjected to western analysis using rabbit antibodies to pure chicken GSase (Smith et al., 1983) (antibody generously supplied by Dr J. W. Campbell, Rice University) according to the methods described by Walsh (1996). Proteins were transferred from gels to Immobilon-P PVDF membranes (Millipore) in a Hoefer TE 77 Semiphor transfer unit with a transfer buffer of 25 mmol l^{-1} Tris, 192 mmol l^{-1} glycine, pH 8.3, 15 % methanol, at 35 mA for 45 min. Membranes were incubated with rabbit anti-chicken GSase diluted 1:20 000 for 1 h and visualized by an enhanced chemiluminescence system (ECL, Amersham) following the manufacturer's directions using 20 mmol l^{-1} Tris/HCl, pH 7.6, 137 mmol l^{-1} NaCl, 0.5 % Tween-20 and a horseradish-peroxidase-conjugated anti-rabbit immunoglobulin diluted 1:7500 as the second antibody. Antibody signal was quantified by standard densitometric scanning. Portions of the same crude homogenates were used to determine GSase transferase activity, as described by Walsh (1996), and antibody intensity correlated significantly with GSase activity (see legend to Fig. 6).

Results

Sequences of CPSase III, CPSase II and β-actin

The amino acid sequence deduced from the partial sequence of the cDNA for CPSase II along with the corresponding portion of the CPSase III sequence is shown in Fig. 1 in alignment with the corresponding portions of other known CPSase Is and CPSase IIIs and several CPSase IIs. The amino acid sequence of this portion of toadfish CPSase II is clearly homologous to that of other known CPSases, is not the same as that determined for the corresponding region of toadfish CPSase III, and is characterized by a 'gap' region of eight amino acid residues that appears to be characteristic of other eukaryotic CPSase IIs (corresponding to shark CPSase III amino acid sequence 773–895) (Korte et al., 1997; Kong et al., 1998). Thus, it was concluded that this sequence represents the expected portion of CPSase II cDNA.

A 4725-nucleotide cDNA sequence containing an open reading frame (ORF) from +70 to +4581 coding for CPSase III was obtained. A portion of the deduced amino acid sequence corresponding to shark CPSase III amino acid sequence 773–895 is shown in the alignment in Fig. 1. The deduced amino acid sequence has 1504 residues with a calculated molecular mass of 165 092, which is very close to the molecular mass of other type III CPSases. The first 37 residues are

identified as the mitochondrial targeting signal sequence by comparison with other CPSase Is and IIIs; the sequence exhibits little similarity to the corresponding sequence in other CPSase Is or IIIs, but is composed of basic amino acid residues and lacks acidic amino acid residues, which is characteristic of peptide sequences that signal import into mitochondria (von Heijne et al., 1989; Hartl et al., 1989). The amino acid sequence is homologous with sequences of other CPSases, with 82, 83, 84 and 75 % identity to CPSase III from trout, tilapia, bass and shark, respectively (accession numbers 1518088, 119250, 2245664 and 2133791, respectively), 69, 71 and 72 % identity to CPSase I from human, rat and frog, respectively (accession numbers 4033707, 117492 and 2118284, respectively), 52, 50 and 49 % identity to CPSase II from shark, hamster and human pyrimidine-pathway-related-CAD (a multifunctional single polypeptide chain that includes the first three enzyme activities of the pyrimidine pathway in higher eukaryotes, i.e. CPSase II, aspartate transcarbamoylase and dihydro-orotase) CPSase II, respectively (accession numbers 3024509, 131696 and 1709955, respectively), and 38 % identity to *Escherichia coli* CPSase II (accession numbers 115627 and 115621). Like other CPSases, the toadfish CPSase III can be divided into a glutaminase domain (Phe38–Ile405) and a synthetase domain (Ser422–Gly1504) with a linker region (Val406–Val421) in between. Cys292 in the glutaminase domain can be readily identified from sequence alignment with other glutamine-dependent CPSases as the cysteine residue required for utilization of the amide nitrogen of glutamine as substrate *via* formation of a γ-glutamyl thioester intermediate. Also, like other CPSases, the toadfish CPSase III synthetase domain has two homologous regions (Gly428–Ser818 and Gly980–Ser1352), with 26 % identity and 24 % additional similarity in the aligned sequences. The two cysteine residues (1327 and 1337) in the C-terminal region of the synthetase domain identified as apparently distinguishing conserved features of acetylglutamate-dependent CPSases are conserved in this acetylglutamate-dependent CPSase III. Other major conserved residues in other CPSases which have been identified as mechanistically functional are also present in the toadfish CPSase III (Hong et al., 1994; Anderson, 1995b).

The base sequence (coding strand) of the portion of β-actin cDNA that was sequenced was as follows: AAGTGTGATG-TGGACATCCGTAAGGACCTGTACGCTAACACTGTGC-TTTCCGGAGGTACCACTATGTACCCTGGTATTGCTGA-CAGGATGCAGAAGGAAATCACAACCCTGGCCCCATC-CACCATGAAGATTAAGATTATTGCCCCACCTGAGCGT-AAATCATCCGTCTGGATTGGAGGCTCCATCCTGGCCT-CTCTG. The deduced amino acid sequence based on the reading frame beginning at the first codon (AAG) is as follows: KCDVDIRKDLYANTVLSGGTTMYPGIADRMQKEITAL-APSTMKIKIITPPERKSSVWIGGSILASL. This amino acid sequence is highly conserved, being identical to the corresponding portion of the sequence for several other β-actins (K284–L349); see sequences found under GenBank accession numbers AAA49197 (grass carp), CAA24528 (rat) and AAC59891 (pufferfish).

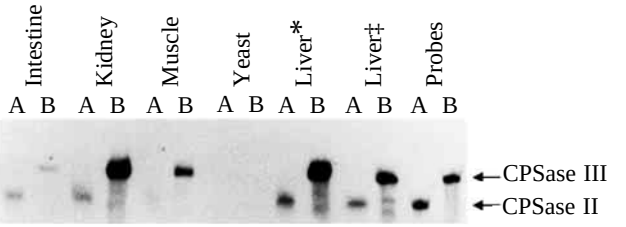

Fig. 2. Expression of carbamoyl phosphate synthethase II (CPSase II) and CPSase III mRNA in four different tissues of gulf toadfish (*Opsanus beta*). mRNA was detected using ribonuclease protection assays (RPAs) as described in Materials and methods. Lanes A and B correspond to CPSase II and CPSase III probes, respectively. Lanes A and B were loaded with sample that originally contained 100 and 30 μg of total RNA, respectively, except as follows: Liver*, 80 and 20 μg, respectively; Liver‡, 60 and 10 μg, respectively; Probes, biotin-labeled probes loaded with 100 μg of yeast RNA, without the RNAase step.

Expression of CPSase III and II mRNA in several toadfish tissues

Biotin-labeled RNA probes complementary to toadfish CPSase II and CPSase III sequences, respectively, were used to detect CPSase II and CPSase III mRNA by the RPA. The results are shown in Fig. 2. A constant amount of total RNA (30 μg for CPSase III, 100 μg for CPSase II) was loaded onto each lane, so the apparent differences in CPSase II and CPSase III mRNA expression are relative to the total amount of RNA. As noted in Fig. 2, CPSase III mRNA is expressed in liver, as expected, but also in kidney, in muscle and, at lower levels, in intestine. CPSase II mRNA was also expressed in all tissues analyzed, but at lower levels than CPSase III, particularly in muscle, noting that over three times as much RNA was loaded onto the gel for CPSase II analysis as for CPSase III analysis. These differences are not due to differences in the specific activities of the two probes, which were essentially the same, and are probably not due to electrophoresis loading problems, since the same results were obtained when repeated several times and the intensities varied as expected when different amounts of RNA were loaded onto the gels (e.g, Fig. 2, lanes labeled liver* and liver‡).

Expression of CPSase III mRNA in liver of stressed and unstressed toadfish

Total RNA was isolated from liver from stressed (confined) and unstressed (control) toadfish (N=5 in each group), and CPSase III mRNA expression was measured by RPAs using the biotin-labeled RNA probe complementary to a segment of toadfish CPSase III mRNA. The same amount of total RNA was loaded onto each lane for electrophoresis, and CPSase III mRNA expression was measured relative to β-actin as a housekeeping gene to minimize loading errors. The results shown in Fig. 3 indicate that expression of CPSase III mRNA appears to be considerably increased in the stressed compared with unstressed toadfish, both in terms of absolute values and relative to β-actin. This change was quantitatively assessed by

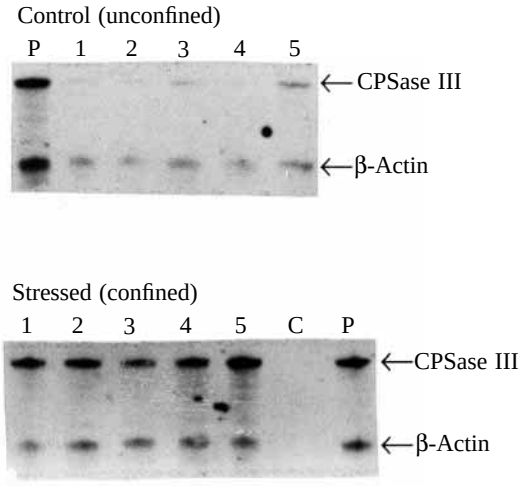

Fig. 3. Expression of carbamoyl phosphate synthethase III (CPSase III) mRNA in liver of stressed and control toadfish (*Opsanus beta*). mRNA was detected using ribonuclease protection assays (RPAs) as described in Materials and methods. Each lane was loaded with sample that originally contained 25 μg of total RNA (lane C, yeast RNA). Lanes P correspond to biotin-labeled CPSase III and actin probes loaded with 25 μg of yeast RNA without the RNAase step.

image analysis of the CPSase III and β-actin bands (Table 2), the results indicating that the ratio of CPSase III mRNA to β-actin mRNA increases approximately fivefold in the stressed toadfish compared with the controls. Since these results were somewhat unexpected given the fact that the same treatment does not affect CPSase activity (Julsrud et al., 1998; Walsh and Milligan, 1995), an additional experiment (Table 2) was conducted using a different probe (labeled with psoralen-biotin) to confirm these results; in this case, a nearly 10-fold increase in CPSase III mRNA was observed.

Expression of CPSase III mRNA in muscle of stressed and unstressed toadfish

The same strategy used for analysis of mRNA expression in liver was used to determine whether there were changes in expression of CPSase III mRNA in muscle relative to the expression of β-actin mRNA. The results are shown in Fig. 4 and in Table 2. In contrast to liver, the ratio of CPSase III mRNA relative to that of β-actin mRNA did not change when toadfish were subject to stress compared with controls.

Table 2. *Expression of CPSase III and GSase mRNA relative to β-actin mRNA in liver and muscle of toadfish*

	CPSase III mRNA/β-actin mRNA	
Experiment	Control	Confined
CPSase, liver		
Experiment 1	0.23±0.06	1.13±0.27*
Experiment 2	0.27±0.11	2.67±0.85*
CPSase, muscle		
Experiment 1	0.47±0.10	0.38±0.09
GSase, liver		
Experiment 1	0.16±0.02	0.80±0.19*

*Significantly different from the control value (P<0.05).
Values are means ± 1 S.E.M. (N=5).
CPSase, carbamoyl phosphate synthetase; GSase, glutamine synthetase.

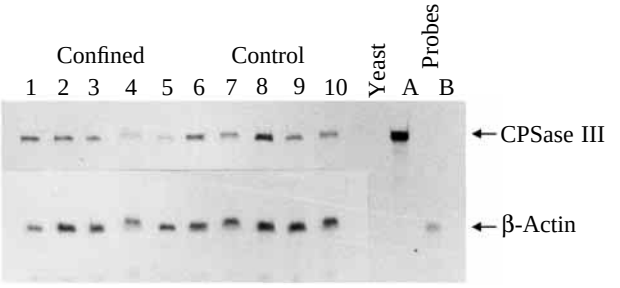

Fig. 4. Expression of carbamoyl phosphate synthethase III (CPSase III) mRNA in muscle of stressed and control toadfish (*Opsanus beta*). mRNA was detected using ribonuclease protection assays (RPAs) as described in Materials and methods. Lanes 1–5 correspond to stressed (confined) toadfish and lanes 6–10 to unstressed (control) toadfish. Each lane was loaded with sample that originally contained 25 μg of total RNA (Yeast, yeast RNA). Lanes A and B correspond to biotin-labeled CPSase III and actin probes, respectively, loaded with 25 μg of yeast RNA without the RNAase step.

Expression of GSase mRNA and protein in liver of stressed and unstressed toadfish

As shown in Fig. 5 and in Table 2, GSase mRNA level in liver also increased (fivefold) when toadfish were confined compared with controls. This increase in GSase mRNA was accompanied by an approximately eightfold increase in levels of GSase protein, as shown in Fig. 6.

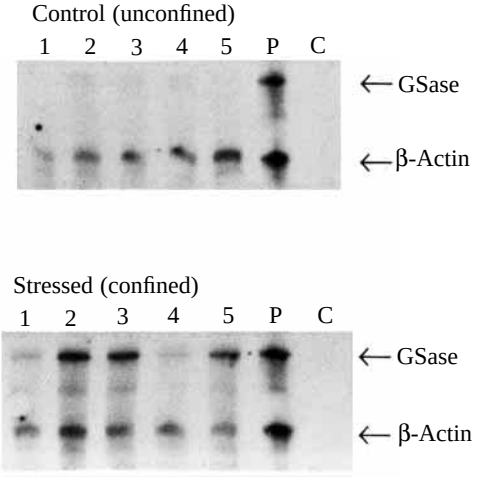

Fig. 5. Expression of glutamine synthetase (GSase) mRNA in liver of stressed and control toadfish (*Opsanus beta*). mRNA was detected using ribonuclease protection assays (RPAs) as described in Materials and methods. Each lane was loaded with sample that originally contained 20 μg of total RNA (lane C, yeast RNA). Lanes P correspond to biotin-labeled GSase and β-actin probes loaded with 25 μg of yeast RNA without the RNAase step.

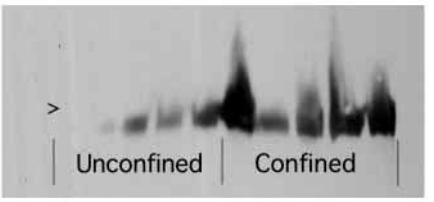

Fig. 6. Western blots showing level of expression of glutamine synthetase (GSase) in liver of stressed and control toadfish (*Opsanus beta*) using chicken GSase antibody. Total protein is equivalent in all lanes (0.5 mg), and > indicates migration of a 46 kDa standard. Antibody signal intensity (relative absorbance units mm^{-2}, *y*) correlates highly with independent measurements of GSase activity (units g^{-1} liver, *x*) (y=9.93x–3.84, r^2=0.93). Intensity was significantly different between treatments (unconfined 3.02±2.08 relative absorbance units mm^{-2}; confined 25.4±6.40 relative absorbance units mm^{-2}, means ±1 S.E.M., N=5 per treatment, P<0.05).

Discussion

While there is growing evidence that the expression of urea cycle enzymes may be a general feature of the early life history stages of teleost fish (Wright et al., 1995; Chadwick and Wright, 1999), the gulf toadfish *O. beta* is a relatively unusual teleost with full expression of urea cycle enzymes in the adult phase. The activity of CPSase III, an important diagnostic enzyme for the presence of a fully functional urea cycle, has been documented for the mitochondria of liver (with lower activities in muscle, kidney and intestine) of gulf toadfish in several detailed studies (Anderson and Walsh, 1995; Julsrud et al., 1998; Wood et al., 1995). The present study confirms that there is indeed a CPSase III gene present in this species (Fig. 1). As expected, the toadfish CPSase III has a mitochondrial targeting signal and functional domains typical of CPSase III enzymes. In further agreement with previous studies of enzyme activities, CPSase III mRNA is expressed in liver, muscle, kidney and intestine (Fig. 2).

CPSase III mRNA concentration increased five- to tenfold in gulf toadfish liver following 48 h of confinement (Fig. 3; Table 2), a treatment that causes toadfish to switch to nearly complete ureotely by a sharp reduction in the amount of ammonia excreted (for a review, see Walsh, 1997). However, this result was somewhat surprising in that prior studies have shown that the maximal *in vitro* activity of CPSase III (and presumably CPSase III protein concentration) in liver does not change during confinement (Walsh et al., 1994; Walsh and Milligan, 1995; Julsrud et al., 1998) and that any necessary upregulation of CPSase III activity could be accounted for *in vivo* by the doubling of the concentration of *N*-acetylglutamate, a positive allosteric effector of CPSase III, as reported by Julsrud et al. (1998). If non-variant maximal enzyme activities indeed reflect a constant CPSase III protein concentration, then we speculate that the large increase in CPSase III mRNA content helps to offset an increased degradation of CPSase III protein, as part of the general proteolytic effects of cortisol-induced gluconeogenesis from amino acids (Mommsen et al., 1999). In this view, the increase in CPSase III mRNA content would essentially maintain the threshold level of CPSase III activity needed for continued function of the urea cycle. This interpretation should be viewed as preliminary since our experimental design examined neither the fine-scale time course of CPSase III mRNA expression nor the possibility of post-translational modifications to CPSase III. The development of CPSase-III-specific antibodies for quantification of CPSase III protein, which has been complicated by the similar amino acid sequence of CPSase II, will be useful in future testing of this interpretation.

The role of CPSase III mRNA and enzyme activity in non-hepatic tissues (muscle, kidney and intestine) in gulf toadfish is still unknown. Recent studies in an obligate ureotelic teleost, the Lake Magadi tilapia (*Oreochromis alcalicus grahami*, now revised to *Alcolapia grahami*, Seegers et al., 1999), have demonstrated very low hepatic CPSase III activity, with the bulk of the total fish CPSase III activity occurring in the muscle (Lindley et al., 1999). These authors speculate that, since the Lake Magadi tilapia has such amazingly high rates of urea excretion (rates on a par with those of similarly sized mammals), muscle expression of the enzyme is needed to keep liver size reasonable relative to the need for a streamlined and laterally compressed piscine body plan (Lindley et al., 1999). This extraordinary need for extra-hepatic CPSase III activity does not appear to be the case for gulf toadfish, in that moderate levels of CPSase III activity in the liver can account for whole-animal urea production/excretion rates (Walsh, 1997). Further studies might focus on other roles for extra-hepatic CPSase III expression in toadfish, perhaps in relation to the cycling and transport of specific urea-cycle-related metabolites.

CPSase I in amphibians and mammals does not require glutamine as its substrate, but instead feeds ammonia nitrogen directly into carbamoyl phosphate and urea synthesis (for a review, see Anderson, 1995b). In these species, glutamine synthetase occupies a ‘fail-safe’ role, converting any ammonia not used by the urea cycle to a temporary pool of the less toxic glutamine. This pathway architecture is believed to result in a lower average ammonia concentration in these species (Meijer, 1995). However, since glutamine is the primary nitrogen-donating substrate for fish CPSase III, GSase plays a more pivotal role in fish urea synthesis. Indeed, in gulf toadfish, GSase activation is believed to be a critical step in the transition from ammoniotely to ureotely (Walsh, 1997). The current study documents that the increase in GSase activity seen during confinement-activated ureogenesis in toadfish is reflected in a similar increase in GSase protein content (Fig. 6), an observation consistent with studies of purified toadfish GSase which indicate no obvious allosteric modulators (Walsh, 1996). Furthermore, it appears that the increase in GSase protein concentration is accounted for, at least in part, by an increase in concentration of GSase mRNA (Fig. 5; Table 2). Since the increase in GSase activity during confinement is linked to the surge in plasma cortisol level at 2 h post-confinement (Hopkins et al., 1995), one obvious candidate for further mechanistic study is the role of cortisol in transcriptional regulation. In this regard, a putative

glucocorticoid-responsive element (GRE) in the 5′ flanking region of the gulf toadfish GSase gene has recently been discovered (P. J. Walsh, unpublished data). In addition, for the further study of both CPSase III and GSase mRNA dynamics, it will be important to follow mRNA concentrations on a finer time scale, particularly in the initial hours following the cortisol surge, and *in vitro* systems (e.g. hepatocytes) may be particularly useful for these studies.

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