Expression of Carbamoyl-phosphate Synthetase III mRNA during the Early Stages of Development and in Muscle of Adult Rainbow Trout (Oncorhynchus mykiss)*

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It has been reported that the activities of the urea cycle-related enzymes ornithine carbamoyltransferase and carbamoyl-phosphate synthetase III (CPSase III) are induced during early life stages of ammocotenic rainbow trout (Oncorhynchus mykiss), suggesting that the urea cycle may play a physiological role in early development in teleost fish (Wright, P. A., Felskie, A., and Anderson, P. M. (1995) J. Exp. Biol. 198, 127–135).

CPSase III cDNA prepared from embryo mRNA was sequenced, confirming the existence of the CPSase III gene in trout and its expression. The deduced amino acid sequence of the CPSase III is homologous to other CPSases.

Supporting evidence for the expression of CPSase III activity in trout embryos was obtained by demonstrating expression of CPSase III mRNA as early as day 3 post-fertilization, reaching a maximum at 10–14 days, declining to a minimum at day 70, and then increasing to a relatively constant level from days 90 to 110 (relative to total RNA). Unexpectedly, in tissues of adult and fingerling trout, CPSase III mRNA was found to be present in muscle but not in other tissues, including liver. This finding was confirmed by assay of extracts, which showed CPSase III and ornithine carbamoyltransferase activity in muscle but not in other tissues. The pyrimidine nucleotide pathway-related CPSase II mRNA was expressed in all tissues.

Most teleost fishes are ammonotelic in terms of nitrogen excretion and, until recently, a functional urea cycle was not known to exist in teleosts (for reviews, see Refs. 1–3). However, interest in the urea cycle and expression of urea cycle enzymes in teleost fishes has increased in recent years for two reasons: 1) reports of a functional urea cycle in several teleost fishes, a freshwater air-breathing catfish (Heteropneustes fossilis) (4, 5), an alkaline lake-adapted tilapia (Oreochromis alcalicus grahami) (6), and the marine toadfishes Opsanus tau and Opsanus beta (7); and 2) documentation of a urea cycle-related carbamoyl-phosphate synthetase (CPSase) in liver of largemouth bass (Micropterus salmoides) (8–10), trout (Oncorhynchus mykiss) free embryos (11), liver and kidney of the Indian air-breathing catfish (H. fossilis) (12), and liver of the marine toadfishes and midshipman (Porichthys notatus) (7, 13, 14).

Three classes of CPSases are known (15). In ureotelic mammalian and amphibian species, the first step of the urea cycle is catalyzed by CPSase I, which utilizes only ammonia as the nitrogen-donating substrate, requires AGA as an allosteric activator for activity, and is localized exclusively in the mitochondrial matrix in liver and small intestine. Carbamoyl phosphate formation for pyrimidine nucleotide biosynthesis in all vertebrates is catalyzed by CPSase II, which utilizes glutamine as the physiologically significant nitrogen-donating substrate, does not require AGA for activity (and activity is not affected by the presence of AGA), is subject to allosteric inhibition by UTP, and is localized in the cytosol of many tissues as part of a multifunctional enzyme (commonly referred to as CAD) that includes the activities of the next two steps of the pathway, aspartate transcarbamoylase and dihydroorotase.

CPSase III is found in invertebrates (16, 17), elasmobranch fishes (sharks and rays) (18), and, as noted above, in some teleost fishes. The properties and function of CPSase II are very much like those of the urea cycle-related CPSase I, except that glutamine is utilized as the nitrogen-donating substrate instead of ammonia (9, 19). The sequence of the CPSase III cDNA from spiny dogfish shark (a representative elasmobranch) has been reported and is homologous to other CPSases, sharing highest similarity in amino acid sequence to rat, human, and frog CPSase I (70–75% identity) and less to CPSase II (about 50% identity) (20). The glutamine- and AGA-dependent CPSase III is thought to be the evolutionary precursor to the ammonia- and AGA-dependent CPSase I of ureotelic mammalian and amphibian species (1, 7, 15, 20, 21). It has been well established that the function of CPSase III in elasmobranch fishes is related to urea synthesis for the purpose of osmoregulation (18, 22, 23). Although the presence of the urea cycle-related CPSase III has been documented in a few teleost fishes as noted above, its function (and the function of the urea cycle in general) is not known, and, it has not been definitively established if the gene for CPSase III is present in all teleost fishes, although this is likely to be the case (3).

CPSase III activity cannot be detected in extracts of liver from many adult teleost species (10, 13). As noted above, largemouth bass, the toadfishes, midshipman, the Indian catfish, H. fossilis, and the alkaline lake-adapted tilapia are exceptions. Rainbow trout (O. mykiss), a species that has been shown to be a typical ammonotelic fish that is very susceptible...
to ammonia toxicity (24, 25), is an example of a teleost species where CPSase III activity cannot be detected in adult liver (11). However, we have recently shown that ornithine carbamoyltransferase and CPSase III activities are induced during early life stages, induction beginning at hatch, reaching a peak at 60 days post-fertilization, and then declining (11). These results together with other studies have suggested that expression of CPSase III and the urea cycle during early life stages may be characteristic for all ammonotelic teleosts, the function perhaps being related to detoxification of ammonia (24, 25), is an example of a teleost species stages may be characteristic for all ammonotelic teleosts, the function perhaps being related to detoxification of ammonia (24, 25), is an example of a teleost species. Consequently, it is quite possible that CPSase III was also expressed earlier than the time of hatch, but activity was not detectable. In addition, due to the very low level of activity that was observed, the possibility of artifacts resulting in misidentification of the type of CPSase could not be completely excluded (11). The purpose of this study was to determine if CPSase III mRNA is expressed during early life stages in trout embryos. The sequence of rainbow trout CPSase III cDNA was determined, thus confirming the existence of the CPSase III gene in trout and its expression. The sequence of a CPSase III cDNA from any teleost species has not been previously reported. Supporting evidence for the expression of CPSase III in trout embryos was obtained by demonstrating expression of CPSase III mRNA. In addition, the unexpected finding of the presence of CPSase III mRNA and CPSase III activity in muscle but not in other tissues, including liver, is reported.

MATERIALS AND METHODS

Fertilized rainbow trout (O. mykiss (Walbaum)) embryos and adult and fingering trout were obtained from Blue Springs Hatchery (Hanover, Ontario). Embryos were maintained in continuous-flow incubating troughs (7–13 °C) for the needed length of time (water pH 8.0). Fertilized trout eggs (embryos, 7–8 °C) at 3, 10, 21, and 29 days post-fertilization and trout embryos (free embryos with the yolk sac removed; 7–10 °C) at 40, 50, and 60 days post-fertilization, alevis (yolk sac completely absorbed by 62 days post-fertilization; 10–11 °C) at 70, 82, 90, 99, and 110 days post-fertilization, and freshly excised liver, intestine, spleen, kidney, and muscle tissues from fingering and adult fish were immediately frozen in liquid nitrogen and stored at −70 °C until needed.

RNA Isolation, cDNA Synthesis, and Analysis

The sequences of primers used in the PCR, for cDNA synthesis, and for sequencing by primer walking are listed in the table below. Table I

| Primer | Location | Orientation | Nucleotide sequence |
|--------|----------|-------------|---------------------|
| 1      | 438–444  | (QAGEDFY)   | Left                |
| 2      | 793–800  | (MKSVGEM)   | Right               |
| 3      | 630–636  | (WKEVEYE)   | Left                |
| 4      | 763–769  | (FEPSLFDY)  | Right               |
| 5      | 701–710  | (QYALPGLSE) | Left                |
| 6      | 992–998  | (EFDFCWA)   | Right               |
| 7      | 816–822  | (MVDENCV)   | Right               |
| 8      | 1057–1064| (MGQLPNY)   | Right               |
| 9      | 952–958  | (WPAQTNY)   | Right               |
| 10     | 882–888  | (MKNIADH)   | Right               |
| 11     | 788–795  | (EIGSSMKS)  | Left                |
| 12     | 859–866  | (LHSGVTVD)  | Right               |
| 13     | 840–847  | (LHGELAVP)  | Right               |
| 14     | 904–911  | (LLLKAQD(T7)) | Right             |
| 15     | 793–800  | (MKSVGEVM)  | Right               |
| 16     | 782–788  | (FLRSTK)    | Left                |
| 17     | 882–888  | (MKNIADH(T7)) | Right             |

RNA Isolation, cDNA Synthesis, and Analysis and Isolation of PCR Products—Poly(A)+ RNA was isolated from whole trout embryos at 8 days post-fertilization and freshly excised adult liver samples that had been frozen in liquid nitrogen and stored at −70 °C using the FastTrack kit (Invitrogen Corporation, San Diego, CA) according to the directions supplied. The guanidinium-based buffer was used for the embryos because the large amount of yolk did not disrupt well in the normal lysis buffer.

The instructions for first-strand synthesis of cDNA from the poly(A)+ RNA provided with the Riboclon cDNA Synthesis System M-MLVH kit (Promega, Madison, WI) were followed. Oligo(dT) was used as primer for cDNA synthesis unless indicated otherwise, such as in the case of the 3′- and 5′-cDNA segments described below.

Total RNA for use in ribonuclease protection assays was extracted using Trizol Reagent (Life Technologies, Inc.) according to the instructions provided except a modified, high salt, RNA precipitation step was included (27). The RNA concentration was determined by absorbance at 260 nm and the samples were kept at −80 °C until needed.

Gel electrophoresis of the PCR reaction mixtures employed Nusieve 3:1 agarose (FMC Bioproducts, Rockland, ME) and ethidium bromide staining. Where necessary, the sample was purified from the gel by one of several standard procedures.

Strategy for Obtaining CPSase II- and CPSase III-specific cDNA Segments by the PCR—Consensus primers were designed on the basis of conserved sequences observed by alignment of several CPSase Is, IIs, and IIIs (20, 28). Primers for the PCR were either synthesized using a PCR-Mate 391 DNA Synthesizer (Applied Biosystems, Foster City, CA) or purchased from Integrated DNA Technologies (Corvalle, IA). All PCRs were carried out in a DNA Thermal Cycler (Perkin-Elmer).

Trout embryo cDNA was used as a template in the PCRs for generating CPSase III-specific DNA fragments, since previous studies had suggested that CPSase III activity was present in early life stages of trout development (11). Forty pmol each of primer 1 and 2 (Table I) were used for the first stage of nested PCR, along with 1 μl of cDNA in a 50-μl standard reaction mixture (20), except that in some cases 1.5 units of Taq DNA polymerase were used instead of 2.5 units. The DNA thermal cycler was programmed for standard touchdown PCR (29): the first cycle was 5 min at 94 °C (denaturation), 1 min at 55 °C (annealing), and 2 min at 72 °C (extension); the next two cycles were 30 s at 94 °C, 1 min at 54 °C, and 2 min at 72 °C; the annealing temperature was decreased using this pattern until the annealing temperature was 50 °C, and this cycle was then repeated for a total of 30 times. This stage of the PCR was repeated using 1 μl of product from the initial PCR using the same primers and programs. This resulted in several products when analyzed by gel electrophoresis. A second stage of PCR was then carried out with nested consensus primers 3 and 4 (Table I) using 1 μl of PCR product from the first stage amplification with primers 1 and 2. A very small amount of ~425-bp product, the expected size, was observed by gel electrophoresis. One μl of this PCR reaction mixture was then used for reamplification with primers 3 and 4 using a modified touchdown PCR program: 5 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C for the first cycle; for each succeeding cycle the time at 94 °C was only 30 s and the
annealing temperature was decreased by 1 °C until the annealing temperature was at 50 °C; this cycle was then repeated a total of 20 cycles. One major ~425-bp product was observed by electrophoresis, and was isolated as described above. This product was then cloned into a plasmid and several positive clones were sequenced. The derived amino acid sequence aligned in the instructions with the predicted amino acids homologous to the region bracketed by the selected nested consensus primers 3 and 4.

The PCR was then carried out using a specific primer (5) based on the sequence of the 425-bp fragment and a consensus primer (6) based on a region of highly conserved downstream sequence; this region brackets a purported "gap" unique to CPSase II (see Fig. 1 and "Results" and Fig. 2 and "Discussion"). Forty pmol of each of the above primers were used with 1 μl of trout embryo cDNA. The standard touchdown PCR conditions described above were used. The size of the major product obtained was ~900 bp, as expected. The derived amino acid sequence based on a partial sequence of this product showed high amino acid sequence identity to the other CPSases, and since the gap characteristic of CPSase II was not present, it was concluded that this fragment was derived from CPSase III cDNA.

A CPSase II-specific fragment of DNA was sought using 1 μl of trout embryo cDNA and 40 pmol each of primers 7 and 8 (consensus primers designed specifically for CPSase II, Table I; see "Discussion") using the modified touchdown conditions described above. A major product of ~720 bp was obtained and was partially sequenced. The sequence obtained was homologous to other CPSase sequences but was difficult to read in some areas, possibly due to the presence of two similar templates (e.g., corresponding to CPSase II and CPSase III cDNA). Subsequent PCRs with this cDNA and various consensus primers were not successful in obtaining a desired fragment. Consequently, cDNA prepared from poly(A) RNA isolated from adult trout liver was used because enzyme assays indicated the presence of CPSase II, but the absence of CPSase III, in liver extracts. A PCR with 40 pmol each of consensus primers 3 and 6 (Table I) using the standard touchdown conditions gave a major product of ~1100 bp. A hemi-reamplification of this product (1 μl) using consensus primer 3 and specific primer 9 (from sequence obtained from the 720-bp product, assuming that this product was derived from a CPSase II cDNA) yielded an expected 960-bp product. The PCR conditions used for reamplification were: 5 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C for 1 cycle; then 29 more cycles where the step at 94 °C was only 30 s. The major band at 960 bp was isolated and sequenced. The sequence was clearly homologous to corresponding sequence of other CPSases, but was not the same as that determined for the CPSase III-specific product obtained using embryonic cDNA as template, and was characterized by the presence of the gap described above. The sequence presented here represented the indicated region of the CPSase I cDNA.

Strategy for Sequencing the Remainder of the CPSase III cDNA—The cDNA needed to determine the sequence extending toward the 3′-end of the cDNA was obtained by 3′-rapid amplification of cDNA ends PCR (30) with cDNA made from a lock-docking primer (31). The first strand of cDNA was made from trout embryo poly(A) RNA template and primer 10 using the EberCycler (Ambion). A nested PCR using the EberCycler was then carried out with modifications for amplifying long sequences of cDNA (20) using specific primers 5 and 11. Gel electrophoresis showed several products ranging in size from a few hundred base pairs to several kilobase pairs. A hemi-reamplification using 1 μl of the above reaction mixture and 50 pmol each of primers 11 and 12 and the long range conditions, except the annealing step was increased from 54 °C to 58 °C, gave a major product of ~3.5 kilobases as well as several smaller ones. The 3.5-kilobase pair product was removed from the gel and used for sequencing by primer walking. Both strands were sequenced.

The cDNA needed to determine the sequence extending to the 5′-end was obtained using the Marathon cDNA Amplification kit (Clontech, Palo Alto, CA). An initial PCR was carried out using a 50-fold dilution of cDNA, prepared from trout embryo poly(A) RNA as described using the manufacturer’s instructions, and 10 pmol of AP1 primer (from kit) and primer 13 (based on specific sequence data). Standard PCR reaction components were used with 1.25 units of Taq polymerase and 0.075 units of Pfu DNA polymerase (Stratagene). The cycling conditions were: 1 cycle of 3 min at 94 °C and 5 min at 60 °C, then 29 cycles of 0.5 min at 94 °C followed by 1 min at 68 °C. One μl of the reaction was then used as template for a reamplification using 40 pmol each of primer AP2 (from kit) and primer 14, a nested specific primer. The cycling conditions were as described above except 25 cycles were used instead of 29. This resulted in a major product of ~2.5 kilobase pairs which was isolated from agarose gels and both strands were sequenced by primer walking. Sequence analyses utilized version 7 of GCG sequence analysis software from the Wisconsin Genetics Computer Group.

Probes for Ribonuclease Protection Assays—The template for preparing the CPSase III probe was made using specific primers 12 and 15 (Table I); the T7 promoter was included on the 5′-end of primer 15 as outlined in the instructions for the MAXIscript Kit (Ambion, Austin, TX). Standard PCR conditions with 40 pmol of each of these primers and ~0.05 ng of the CPSase III-specific ~900-bp product, described above, as template were used. The thermal cycler was programmed for 1 cycle of 3 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C; then 29 more cycles with only a 30-s denaturation step. The major product of ~460 bp was excised from the gel. The CPSase II probe was prepared in a similar manner using primers 16 and 17 (Table I) and the CPSase II-specific 960-bp product described above as template. The major PCR product using these primers was ~300 bp, as expected.

RNA probes were synthesized from these templates using [α-32P]UTP (10 mCi/ml, 8000 Ci/mmole, DuPont NEN) and purified according to instructions provided with the MAXIscript kit.

Ribonuclease Protection Assays—The RPA II kit (Ambion) was used to perform the ribonuclease protection assays. The instructions provided with the kit were followed unless described differently. For CPSase II and CPSase III, 50 and 100 μg of total RNA, respectively, and 100,000 cpm of the appropriate probe were used for each sample, except as indicated otherwise. After overnight incubation of RNA samples with the probe at 43 °C, the RNase A digestion step was carried out using 200 μl of a 1:100 dilution of solution R (concentrated RNase A and RNase T1). The reaction mixtures were incubated at 14 °C for 40 min. 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-mm thick) at 250 volts for 60 min. Gels were exposed to x-ray film at ~80 °C as described in the RPA II protocol.

Extracts, Enzyme Assays, and Subcellular Fractionation—Rainbow trout, fingerlings (25–40 g) and adults (~1 kg), were obtained from Blue Spring Fish Farm (Hanover, Ontario), Alma Research Station (Alma, Ontario), or Wild Spring Fish Farm (Sandstone, Minnesota). Freshly excised tissues (~1.5 g) were minced, added to 4 volumes of extract buffer (0.05 M Hepes, pH 7.5, 0.05 M KCl, 1 mM dithiothreitol, and 0.5 mM EDTA), and homogenized with a Tekmar Tissumizer (SDT1810 with SDT100EN probe) for 30 s. The homogenate was subjected to brief sonication and then centrifuged at 14,500 × g for 10 min. An aliquot of the supernatant (~2 ml) was passed through a 11-cm column of Sephadex G-25 equilibrated with extract buffer; the majority of the protein and eluted as a concentration maximum for the enzyme assays. The protein concentration was measured before and after the gel filtration chromatography step in order to adjust for dilution for calculating units/g tissue.

Ornithine carbamyltransferase, glutamine synthetase, arginase, glutamate dehydrogenase, argininosuccinate synthase and argininosuccinate lyase together, dihydroorotase, aspartate transcarbamoylase, and other dehydrogenase activities were measured as described previously (14). CPSase activity was assayed by a modification of the procedure described by Anderson et al. (32) and Anderson (13). The standard reaction mixture contained 20 mM ATP, 25 mM MgCl2, 25 mM phosphoenolpyruvate, 2 units of pyruvate kinase, 5 mM [14C]bicarbonate (3 × 106 cpm), 20 mM glutamine, 1.7 mM AGA, 1.7 mM UTP, 0.04 mM Hepes, pH 7.6, 0.04 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, and extract in a final volume of 0.3 ml. Reaction was initiated by adding extract (0.09 ml). After 30 min at 26 °C, the reaction was stopped by adding 40 μl of freshly prepared 2 M NH4OH in 0.5 M NaOH containing 0.5 mM carbamoyl phosphate. After 5 min, 0.1 ml of 4 M NH4Cl, pH 8.7, was added and the mixture was placed in a boiling water bath for 15 min in an exhaust hood. After cooling, the sample was added to a small column containing 2.2 cc of Dowex 1 × 8 (OH–; 50–100 mesh) anion exchange resin. The sample was washed into the resin with 0.5 ml of 0.5 mM urea. [14C]Urea that had been formed from the [14C]carbamyl phosphate as a result of heating with NH4OH was then eluted from the column with 5 ml of 0.5 mM urea. The radioactivity in the 5 ml of eluate was measured with a liquid scintillation spectrometer. Reactions were carried out in duplicate and each assay series for each sample also included controls in which the reaction was carried out in the absence of the reaction mixture and where the reaction was stopped at zero time. In addition, control experiments were included in which a small volume (0.01 ml) of concentrated extract prepared from liver of spiny dogfish (Squalus acanthias) was included with and without trout tissue extract as a positive control and to confirm that the extracts were not inhibitory to CPSase activity. A unit of enzyme activity is defined as 1 μmol of
product formed per min. Subcellular fractionation of freshly excised liver tissue into mitochondrial and cytosolic fractions was carried out as described previously (33).

RESULTS

Sequence of a Fragment of CPSase II—Comparison of the alignments of all known CPSase Is and IIIs with CPSase II from hamster, slime mold, and shark suggests that a characteristic feature of CPSase IIIs is the absence of amino acid residues corresponding to the shark CPSase III amino acid sequence 834–841 (Fig. 1). We refer here to this as a gap in CPSase II. If this relationship were true for trout CPSase II and CPSase III, isolation and sequencing of a corresponding fragment of trout cDNA that included this region would establish if the fragment represented a sequence of CPSase II or III. As shown in Fig. 1 and as noted under “Materials and Methods,” this gap was, in fact, found to be present in trout CPSase II and this was helpful in identifying products obtained by the PCR using consensus primers as sequences of CPSase II or CPSase III.

Sequence of Trout CPSase III cDNA—The derived amino acid sequence of trout CPSase III is shown in Fig. 2. The open reading frame was identified by comparing the derived amino acid sequence with that of other CPSases, including CPSase III from spiny dogfish (Fig. 2) and CPSase I from rat, human, and frog (20). The nucleotide sequence is available from GenBank under accession number U65893.

The derived amino acid sequence has 1,518 residues (including the mitochondrial targeting signal sequence, amino acid residues 1–35), with a calculated molecular weight of 166,577. This sequence has 77% identity to CPSase III from spiny dogfish shark (the only other known CPSase III sequence) (20), 74, 72, and 73% identity to CPSase I from rat (34), human (35), and frog (21), respectively, and 54, 41, 39, and 56% identity to the published CPSase II sequences from hamster (28, 36), Drosophila (37), Dictyostelium (38), and shark (39), respectively.

Like other CPSases (15, 40, 41), trout CPSase III (Fig. 2) can be divided into a glutaminase domain (36–404) and a synthetase domain (422–1518) with a linker region (405–421) in between. In the glutaminase domain, Cys-291 can be identified by sequence alignment with other glutamine-dependent CPSases as the cysteine residue required for formation of the γ-glutamylthioester intermediate, a common feature in the mechanism of all amidotransferases and required for glutamine-dependent CPSase activity (15, 42). Also like other CPSases, the trout CPSase III synthetase domain contains two homologous halves (confirmed by dot matrix analysis; 43); the N-terminal half extends from Leu-426 to Leu-825 and the C-terminal half from Ala-951 to Ser-1350. Alignment analysis of these two halves shows 22% identity in the amino acid sequences. Two specific cysteine residues (1325 and 1335) in the C-terminal half of the synthetase domain have been identified as apparently distinguishing and conserved features of the AGA-dependent CPSases (20); both of these cysteine residues are conserved in the trout CPSase III. Other conserved amino acid sequences and individual amino acid residues identified in shark CPSase III that are common to CPSases in general and which have been identified as being mechanistically important are also present in the trout CPSase III (20).

Expression of CPSase III mRNA in Trout—32P-Labeled probes complementary to trout CPSase III and CPSase II sequences, respectively, were used to detect CPSase III and CPSase II mRNA by the ribonuclease protection assay. The results are shown in Figs. 3 and 4. A constant amount of total RNA was loaded onto each lane for electrophoresis, so the apparent changes in CPSase III and CPSase II mRNA expression relate to an amount relative to the total RNA. The more common approach of measuring expression relative to a housekeeping gene, even if a characterized housekeeping gene was available for trout, was not considered to be any more useful for the purposes of these studies involving developing tissues than measuring expression relative to the total RNA. As noted in Fig. 3, CPSase III mRNA was expressed very early after fertilization, reaching a maximum relative to total RNA at 10–14 days, declining to a minimum at day 70, and then increasing to a relatively constant level from days 90 to 110. Expression of CPSase II followed a similar pattern, except that the decline between days 10–14 and days 90–110 was not as marked. CPSase II mRNA was expressed in all tissues of trout fingerlings analyzed, but, surprisingly, CPSase III mRNA was expressed only in muscle (Fig. 4); similar results were obtained.

FIG. 1. Alignment of the partial amino acid sequences of all known AGA-dependent CPSase Is and IIIs and three AGA-independent CPSase IIIs, illustrating a putative characteristic gap in the CPSase IIIs. The amino acid residues of the trout CPSase II are arbitrarily assigned the same numbers as shark CPSase II since the complete amino acid sequence is not known. The boxed sequences represent putative conserved sequences unique to the CPSase IIIs.
Fig. 2. Aligned deduced amino acid sequences of trout CPSase III and shark (spiny dogfish, S. acanthias) CPSase III. Identical residues are indicated by shaded amino acids. The sequences comprising the mitochondrial signal sequence, glutaminase domain, and synthase domain are indicated by 4. The conserved cysteine residue essential for glutamine-dependent activity is identified by 2. The two cysteine residues uniquely conserved in AGA-dependent CPSases I and III (20) are identified by ç.
with trout adult tissues (data not shown).

**Presence of CPSase III Activity in Adult Trout Tissue**—As shown in Table II, CPSase II activity is present in liver (glutamine-dependent activity inhibited by UTP but not activated by AGA), but there was no evidence of CPSase III activity (no activation by AGA). This was confirmed by the results of subcellular fractionation studies showing that virtually all of the CPSase activity is localized in the cytosol and that activity retains its characteristic signature as that of a CPSase II (Table III). No CPSase activity was detectable in intestinal tissue and only CPSase II activity was present in kidney extracts; in contrast, low levels of CPSase III activity were present in muscle extracts (Table II). The presence of ornithine carbamoyltransferase activity paralleled the distribution of CPSase III activity. Similar results were obtained with trout fingerlings (data not shown).

**DISCUSSION**

A difficulty in using consensus primers for the PCR as an approach to specifically amplify CPSase-specific cDNA is that highly conserved regions used to design the consensus primers are common to both CPSase III and CPSase II. If mRNA for both CPSases is present in a tissue, it may be difficult to determine if a product is that of one or the other or both. Alignment analysis revealed a short segment of 8 amino acids (which may or may not be contiguous as shown in Fig. 1) in the known AGA-dependent CPSase IIIIs and Is that is absent in shark and hamster CPSase II (Fig. 1). Use of consensus primers bracketing this region would be expected to give products of different length, thus providing a tentative identification of the product of the PCR as CPSase III or CPSase II. This approach was used successfully in the study described here with trout; using cDNA prepared from mRNA isolated from embryonic tissue, a PCR product was obtained that was identified as CPSase III by the fact that the 8-amino acid segment described above was present in the sequence and that the size was larger than would be expected if the segment had been absent. Likewise, we were able to obtain a CPSase II PCR product when cDNA prepared from liver mRNA was used as template; this was confirmed by the size of the product and by the sequence, which showed that the 8-amino acid sequence described above was not present.

With the additional CPSase III sequence reported here we have been able to begin identifying conserved sequences unique to CPSase IIs or to AGA-dependent CPSase IIIIs and Is. These efforts suggest that it may be possible to design consensus primers specific for CPSase III and to selectively amplify CPSase III in the presence of CPSase II cDNA. We have been able to accomplish this with cDNA prepared from largemouth bass and gulf toadfish liver mRNA. As noted under "Materials and Methods," this approach was used with partial success in this study to obtain amplification of CPSase II-specific cDNA. The region from 787 to 793 (trout CPSase II amino acid sequence) represents a conserved sequence in the CPSase IIIs shown in Fig. 1 that does not appear to occur in the CPSase Is and IIIIs; primer 7 was designed as a consensus primer with potential specificity for CPSase II based on this sequence. Thus, despite the high degree of identity and similarity in sequence of the different types of CPSases, specific amplification of the DNA of one or the other type of CPSase appears to be possible and will likely be more specifically accomplished when additional sequences of CPSase III or I become available for comparison. This may provide an approach for determining if the CPSase III gene is present in the many teleost species where CPSase III activity appears to be absent.

The only unusual feature of the sequence of the trout CPSase III is that the sequence is longer due to additional amino acids at the C-terminal end than other CPSase IIIIs or Is. Similar additional sequence is also present at the C-terminal of CPSase III cDNA from two other teleost species we have sequenced.4

Previous studies indicated that CPSase III activity was expressed during the early stages of development of trout, but the level of activity observed was very low and barely detectable; transient expression of relatively high levels of ornithine carbamoyltransferase between 40 and 110 days post-fertilization was also observed, which supported the data indicating expression of CPSase III activity (11). In that study it was also reported that CPSase III activity could not be detected in adult trout liver, suggesting that expression of the urea cycle in general, and CPSase III in particular, may occur only during early life stages of development. The results presented here support the report of Wright et al. (11) that CPSase III is expressed in the early stages of development of trout and show that CPSase III mRNA is also expressed in trout embryos. CPSase III mRNA is expressed very early in development (a few days post-fertilization) and is highest in trout embryos at 10–14 days post-fertilization (relative to total RNA). These results together with the previous report: 1) of transient expression of unusually high levels of ornithine carbamoyltransferase activity and of CPSase III activity between days 40 and 110 post-fertilization, and 2) that urea accumulates to over 2

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4 W. L. Salo and P. M. Anderson, unpublished observations.
ms before hatching (11) suggest that expression of the urea cycle during the early stages of development is physiologically significant.

Although the highest levels of CPSase III activity measured were very low and activity before day 40, if present, was probably too low to detect, the maximum levels of ornithine carbamoyltransferase activity was easily detected and there appeared to be little ornithine carbamoyltransferase expression until day 40 (11). If CPSase III activity follows a similar pattern, i.e. there is little CPSase III expression before day 40, these results suggest that translation may lag considerably behind transcription or that CPSase III mRNA is degraded at a much greater rate than CPSase III. On the other hand, urea synthesis occurs early in embryos (11), suggesting that CPSase III and ornithine carbamoyltransferase activities are also expressed early or the urea is arising from other sources.

Although the decline to a minimum at day 70 does not necessarily represent a decline in absolute amounts of CPSase III mRNA, a consideration may be that the physiological function of CPSase III early in development (~10–50 days post-fertilization) may be different than that expressed late in development (>60 days post-fertilization) or in adult tissues (i.e. muscle). In situ studies of embryonic tissues currently in progress may provide additional information relative to specificity of organ expression at different stages of development.

The results reported here indicate that the CPSase activity reported by Chiu et al. (44) in liver and kidney is actually due to the presence of pyrimidine nucleotide pathway-related CPSase II and not a urea cycle-related CPSase as assumed. A surprising finding, however, was the expression of CPSase III mRNA in muscle of adult trout, but not in any of the other tissues examined. This finding was confirmed by direct enzyme assays. The presence of significant levels of ornithine carbamoyltransferase in muscle, but not in other tissues, is consistent with these results. This is the first report of the presence of a urea cycle-related CPSase in muscle. In mammalian species the only tissue besides liver that has both CPSase I and ornithine carbamoyltransferase activity is the intestinal mucosa (45, 46). However, argininosuccinate synthase and argininosuccinate lyase are not present in the intestinal mucosa, and the citrulline formed is transported to other tissues for conversion to urea (47–49). A similar pathway may occur in trout with muscle, since although the levels of argininosuccinate synthase and argininosuccinate lyase in liver are low, the levels of activity are higher than in muscle. Although the level of CPSase III activity in muscle is very low, muscle comprises >50% of the body mass, thus providing the possibility of a significant level of total CPSase III and ornithine carbamoyltransferase activity. Chiu et al. (44) also reported much higher levels of ornithine carbamoyltransferase in muscle than in liver or kidney. Their studies with whole animals provided evidence of urea synthesis occurring early in embryos (11), suggesting that CPSase III and ornithine carbamoyltransferase activities are also expressed early or the urea is arising from other sources.

![Fig. 3. Expression of CPSase III and CPSase II mRNA in trout embryos (3–29 days post-fertilization), free embryos (40–60 days post-fertilization), and alevis (70–110 days post-fertilization). mRNA was detected by ribonuclease protection assays as described under "Materials and Methods." Lanes A and B correspond to CPSase III and CPSase II probes, respectively.](https://example.com/fig3)

**Table II**

| Enzyme activities in adult trout tissues | Liver | Muscle | Intestine | Kidney |
|----------------------------------------|------|-------|-----------|-------|
| CPSase                                 |      |       |           |       |
| + Gln                                  |      |       |           |       |
| + Gln + AGA                            |      |       |           |       |
| + Gln + AGA + UTP                      |      |       |           |       |
| Ornithine carbamoyltransferase         |      |       |           |       |
| Argininosuccinate synthase/argininosuccinate lyase |      |       |           |       |
| Arginase                               |      |       |           |       |
| Glutamine synthetase                   |      |       |           |       |
| Aspartate transcarbamoylase            |      |       |           |       |
| Dihydroorotase                         |      |       |           |       |

*Below level of detection.

![Fig. 4. Expression of CPSase III and CPSase II mRNA in five different tissues of trout fingerlings. mRNA was detected by ribonuclease protection assays as described under "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 50 and 100 μg of total RNA, respectively, except as follows. Muscle, 5 and 15 μg, respectively (CPSase II probe only); Muscle', 10 and 30 μg, respectively (CPSase III probe only); Yeast, 100 μg of yeast RNA, 32P-probes, RNase step not included.](https://example.com/fig4)
Subcellular distribution of CPSase III in adult trout liver

| Enzyme                        | Percent of total activity | umole/min/g tissue |
|-------------------------------|--------------------------|-------------------|
| CPSase                        |                          |                   |
| + Glu                        | 5.4                      | 94.6              |
| + Glu + AGA                  | 5.4                      | 94.6              |
| + Glu + AGA + UTP            | 6.7                      | 93.3              |
| Arginase                     | 79.2                     | 21.8              |
| Glutamine synthetase         | 10.2                     | 89.8              |
| Glutamate dehydrogenase      | 84.6                     | 15.4              |
| Lactate dehydrogenase        | 10.0                     | 90.0              |

Total of units of activity in mitochondrial and soluble fractions.

cycle activity by showing that [14C]ornithine was converted into [14C]arginine. The results here suggest that a physiologically significant urea cycle is present in adult trout, at least the first two steps of the urea cycle probably occur predominately in muscle.

The presence of CPSase III in muscle is apparently not unique to trout, since we have recently found CPSase III activity in muscle extracts of several teleost species. Although the function is not known, the report here of CPSase III and ornithine carbamoyltransferase activity in muscle represents a consideration that must be taken into account in future studies of the physiological significance of citrulline and/or urea synthesis in fish.






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