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# SUBCELLULAR LOCALIZATION AND BIOCHEMICAL PROPERTIES OF THE ENZYMES OF CARBAMOYL PHOSPHATE AND UREA SYNTHESIS IN THE BATRACHOIDID FISHES *OPSANUS BETA*, *OPSANUS TAU* AND *PORICHTHYS NOTATUS*

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## Summary

**The subcellular localization and biochemical properties of the enzymes of carbamoyl phosphate and urea synthesis were examined in three representatives of fishes of the family Batrachoididae, the gulf toadfish (*Opsanus beta*), the oyster toadfish (*Opsanus tau*) and the plainfin midshipman (*Porichthys notatus*). The primary objective of the study was to compare the biochemical characteristics of these fishes, which represent a range between ammoniotelism and ureotelism (*O. beta* being facultatively ureotelic), with previous patterns observed for an ammoniotelic teleost (*Micropterus salmoides*, the largemouth bass) and an obligate ureogenic elasmobranch (*Squalus acanthias*, the dogfish shark). The present study documents the expression of mitochondrial carbamoyl phosphate synthetase (CPSase) III and cytosolic CPSase II (and its associated enzymes of pyrimidine synthesis, dihydroorotase and aspartate carbamoyltransferase) in the livers of all three batrachoidid species. Both mitochondrial and cytosolic activities of arginase were present in the livers of all three species, as were cytosolic glutamine synthetase and argininosuccinate synthetase and lyase. However, *O. beta* also showed mitochondrial glutamine synthetase activity and higher total hepatic levels of glutamine synthetase than either *O. tau* or *P. notatus*. Taken together, these observations confirm that the arrangement of these enzymes in the batrachoidid fishes has greater similarity to that of *M. salmoides* than to that of *S. acanthias*. However, differences within the family appear to coincide with the different nitrogen excretion strategies. *O. tau* and *P. notatus* are primarily ammoniotelic and most closely resemble the ammoniotelic *M. salmoides*, whereas ureotelism in *O. beta* is correlated with the presence of a mitochondrial glutamine synthetase and the ability to induce higher total glutamine synthetase activities than *O. tau* or *P. notatus*. Additionally, isolated mitochondria from *O. beta* were able to generate citrulline from glutamine, whereas those from *O. tau* were not. Also in contrast to *S. acanthias*, glutamine synthetase activities in the mitochondria of *O. beta* are consistently lower than those of CPSase III. This and other kinetic observations lend support to the hypothesis that glutamine synthetase may be an important regulatory control point in determining rates of ureogenesis in *O. beta*.**

Key words: ureogenesis, carbamoyl phosphate synthetase, glutamine synthetase, *Opsanus beta, Opsanus tau, Porichthys notatus,* enzyme compartmentation.

## Introduction

Although most fish species are ammoniotelic and synthesize and excrete little urea, in some species all the enzymes of the urea cycle are present in the liver and significant ureogenesis occurs (Mommsen and Walsh, 1991, 1992; Wood, 1993; Anderson, 1995). Marine elasmobranchs (sharks, skates and rays) synthesize and retain urea in their tissues at high concentrations (e.g. $0.4\,mol\,l^{-1}$) for the purpose of osmoregulation (Perlman and Goldstein, 1988) and thus they can be viewed as being obligately ureogenic, as are mammals. However, the enzymology of the urea cycle in the liver of the spiny dogfish (*Squalus acanthias*), a representative marine elasmobranch, has been relatively well characterized and has been shown to differ from that of mammalian species in several respects (Anderson, 1991; Campbell and Anderson, 1991). The most notable difference is that whereas in mammalian species carbamoyl phosphate

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is formed directly from ammonia in mitochondria in a reaction catalyzed by carbamoyl phosphate synthetase (CPSase) I, in *S. acanthias*, ammonia is first incorporated in mitochondria into glutamine, which is then utilized for carbamoyl phosphate formation (Anderson and Casey, 1984). This is accomplished as a result of the co-localization of high levels of glutamine synthetase and a CPSase III in the mitochondrial matrix (Casey and Anderson, 1982). The properties and structure of CPSase III are very similar to those of CPSase I, except that glutamine rather than ammonia serves as the nitrogen-donating substrate (Anderson, 1981; Casey and Anderson, 1983). Glutamine- and *N*-acetyl-L-glutamate-dependent CPSase III in invertebrates and fish is considered to be the evolutionary precursor to the ammonia- and *N*-acetyl-L-glutamate-dependent CPSase I present in ureotelic terrestrial vertebrates and mammalian species; the function of both is related to urea synthesis (Anderson, 1995; Hong *et al.* 1994). As a result of the high levels of both of these enzymes in the matrix, isolated mitochondria efficiently synthesize citrulline from either glutamine or glutamate plus ammonia in the presence of ornithine, with succinate as the energy source (Anderson and Casey, 1984). Arginase, as well as glutamine synthetase, is localized in the mitochondrial matrix; in the liver of mammalian species, both of these enzymes are localized in the cytosol (Casey and Anderson, 1985). The absence of glutamine synthetase in the cytosol of elasmobranchs apparently precludes its availability for glutamine-dependent carbamoyl phosphate formation in the liver as the first step in pyrimidine nucleotide biosynthesis; unlike mammalian enzymes, the CPSase that catalyzes this reaction (CPSase II) and the other enzyme activities of the pyrimidine pathway are not expressed in *S. acanthias* (Anderson, 1989). CPSase II in vertebrate species, like CPSase III, utilizes glutamine as the nitrogen-donating substrate but, unlike either CPSase I or III, does not require *N*-acetyl-L-glutamate (AcGlu) for activity and is localized in the cytosol (Evans, 1986).

Considerably less is known about urea cycle function and enzymology in teleost fishes. CPSase III, glutamine synthetase and the other urea cycle enzymes are present in the liver of largemouth bass (*Micropterus salmoides*), a freshwater teleost, although at much lower levels than in elasmobranch liver (Anderson, 1976; Cao *et al.* 1991). In contrast to sharks, however, glutamine synthetase is localized in the cytosol, and CPSase II and the other enzymes of the pyrimidine nucleotide pathway are present in the liver (in the cytosol). Isolated *M. salmoides* liver mitochondria are apparently unable to synthesize citrulline either from glutamate plus ammonia (as expected, since glutamine synthetase is localized in the cytosol) or from glutamine (an unexpected result); also unlike shark liver mitochondria, glutamine does not support respiration by isolated *M. salmoides* liver mitochondria. Since these species are presumably primarily ammoniotelic, like most other freshwater teleosts, the function of the full complement of urea cycle enzyme activities, albeit at relatively low levels, and the significance of the absence of mitochondrial glutamine synthetase with respect to the function of mitochondrial glutamine-dependent CPSase III activity and urea cycle activity are unclear.

In contrast to *M. salmoides*, recent studies have clearly demonstrated active ureogenesis in the marine teleost *Opsanus beta* (gulf toadfish), which correlates with the activities of CPSase III, glutamine synthetase and the other urea cycle enzymes in liver at levels comparable to those found in elasmobranchs (Mommsen and Walsh, 1989; Walsh *et al.* 1994). However, the function of the urea cycle in this species has not been definitively established. *O. beta* is thought to be primarily ammoniotelic, becoming facultatively ureotelic when exposed to stressful environmental situations, e.g. high ammonia concentrations, crowding, confinement or extended exposure to air (Walsh *et al.* 1990, 1994). Urea synthesis does not play a primary role in osmoregulation or regulation of acid–base balance (Walsh *et al.* 1989, 1990; Barber and Walsh, 1993) in *O. beta*. The onset of ureogenesis in *O. beta* is accompanied or preceded by an increase in glutamine synthetase activity in liver, but with little change in the level of CPSase III or other urea cycle enzymes. When fully induced, ureogenesis occurs at rates comparable to those observed in elasmobranchs (Walsh *et al.* 1994). Liver arginase and glutamine synthetase have been reported to be localized in the mitochondria, analogous to the situation in elasmobranchs (Mommsen and Walsh, 1989). In contrast, glutamine synthetase was reported in the cytosol in the closely related oyster toadfish (*O. tau*). This may be related to the observation by Mommsen and Walsh (1989) that the rate of urea synthesis by isolated hepatocytes from *O. tau* was only 7 % of that observed for isolated hepatocytes from *O. beta* and Read's (1971) preliminary observations that this species is ammoniotelic.

This study was initiated to obtain information that would help to define more clearly the distinguishing biochemical properties and strategies of carbamoyl phosphate and urea synthesis in fish. The specific objective was to establish whether the distribution of liver CPSase III, CPSase II and related enzymes in the facultatively ureogenic marine teleost toadfishes is analogous either to that in the ammoniotelic freshwater teleost *M. salmoides* or to that in the obligate ureo-osmotic marine elasmobranch *S. acanthias*. Preliminary observations with the ammoniotelic (T. P. Mommsen, personal communication) confamilial species *Porichthys notatus* (plainfin midshipman) are also reported. The results indicate that the batrachoidid fish are similar to *M. salmoides*, but that arginase activity in both toadfish species and *P. notatus* and glutamine synthetase activity in *O. beta* are present in liver in both the cytosol and mitochondria. Furthermore, the maximum glutamine synthetase activity in isolated mitochondria from *O. beta* is lower than the CPSase III activity, suggesting that cytosolic glutamine must be recruited for maximal rates of ureogenesis. The results are discussed in the context of the role of ureogenesis in toadfish and in teleosts in general.

## Materials and methods

Mature gulf toadfish (*Opsanus beta* Goode and Bean) were collected by roller trawler in South Biscayne Bay, Florida, during the late spring and early summer of 1993 and the late spring of 1994. For experiments that were carried out in Duluth, the fish were held in running seawater aquaria for 1 or 2 days and then shipped by overnight air express in sea water at ambient temperature to Duluth, where they were maintained in static artificial seawater aquaria until used for experiments within 1–3 days. Mature oyster toadfish (*Opsanus tau* L.) were provided by the Marine Biological Laboratory (Woods Hole, Massachusetts) and shipped to Duluth, where they were maintained as described above. *Porichthys notatus* Girard were collected from tidal flats around Bodega Bay in California or San Juan Island in Washington and shipped to Duluth, where they were maintained as described above. For experiments that were carried out in Miami, *O. beta* were kept in flowing sea water without feeding, as previously described (Walsh *et al.* 1994).

Protein was determined by the dye-binding method using a reagent kit from Bio-Rad. Most biochemicals and chromatographic media were obtained from Sigma Chemical Co.; radioisotopes were from Research Products International Corp.

### *Subcellular fractionation*

Subcellular fractionation was carried out as described previously with minor modifications (Cao *et al.* 1991). All steps were carried out at 4 °C. Freshly excised livers (pooled from 3–5 fish, approximate mass 4 g) were minced and suspended in 5.5 volumes of fractionating buffer (0.25 $mmol\,l^{-1}$ sucrose, 0.5 $mmol\,l^{-1}$ EDTA, 5 $mmol\,l^{-1}$ potassium phosphate, 30 $mmol\,l^{-1}$ Hepes buffer, pH 7.4, and 1 $mmol\,l^{-1}$ dithiothreitol, DTT). The suspension was homogenized by using a motor-driven Potter–Elvehjem glass homogenizer with a loose-fitting Teflon pestle; homogenization was accomplished by using four or five stroke cycles at a relatively low speed. The homogenate was centrifuged at 125 ***g*** for 10 min to remove unbroken cells and other debris. The supernatant was decanted and saved. The loose pellet was suspended in 3.5 volumes of fractionation buffer, homogenized a second time as above, and centrifuged at 125 ***g*** for 10 min. The supernatant was decanted and combined with the first supernatant; the loose pellet (debris fraction) was saved and treated as indicated below. The combined supernatants were centrifuged at 14 600 ***g*** for 10 min to give a well-defined and firm pellet (mitochondrial fraction). The supernatant from this centrifugation step is the soluble fraction. The mitochondrial pellet was washed once with 9 volumes of fractionation medium. The washed mitochondrial pellet and the pelleted debris fraction were each suspended in 9 volumes of assay buffer (0.05 $mol\,l^{-1}$ Hepes, pH 7.5, 0.05 $mol\,l^{-1}$ KCl, 0.5 $mmol\,l^{-1}$ EDTA and 1 $mmol\,l^{-1}$ DTT) containing 0.1 % Triton X-100, sonicated to facilitate breakage of mitochondria and other organelles, and centrifuged at 14 600 ***g*** for 10 min. A portion (20 ml) of each of the supernatants from this centrifugation (mitochondrial and debris fractions, respectively) and the supernatant obtained from the initial high-speed centrifugation step (soluble fraction) were passed through a Sephadex G-25 column (2 cm×22 cm) equilibrated with assay buffer. Fractions containing most of the protein were pooled; the protein concentration was determined before and after this gel filtration chromatography step to adjust enzyme activities for dilution. These three different pooled fractions were used immediately for enzyme assays; the sum of enzyme units in the three fractions was used to determine the units of enzyme activity per gram liver. As noted in the Results section, in the case of *O. tau*, homogenization as outlined above gave a homogenate in which most of the mitochondria sedimented in the initial low-speed centrifugation step. Homogenization using a Tissumizer (15 s, intermediate speed) as previously described (Anderson and Casey, 1984) overcame this problem. For this species, however, the liver was initially suspended in 9 volumes of homogenization buffer and a second extraction was not carried out.

### *Enzyme and protein assays*

On the assumption that both argininosuccinate synthetase and lyase were present, their activities were determined as a pair by measuring citrulline- and ATP-dependent formation of [$^{14}$C]fumarate from [$^{14}$C]aspartate, as previously described, except that the reaction was carried out for 10 and 20 min (Cao *et al.* 1991). Reaction mixtures for arginase contained 0.06 $mol\,l^{-1}$ glycine, pH 9.7, 4.8 $mmol\,l^{-1}$ $MnCl_2$, 15.2 $mmol\,l^{-1}$ L-[guanido-$^{14}$C]arginine (400 000 cts $min^{-1}$) and extract in a final volume of 0.25 ml; the reaction was terminated after 10 and 20 min and [$^{14}$C]urea was determined as described previously (Casey and Anderson, 1982; Rüegg and Russell, 1980). To be certain that the assay was conducted at a sufficiently high arginine concentration to give near zero-order reaction kinetics, the assays of both the cytosolic and mitochondrial fractions were carried out at 10, 20 and 40 $mmol\,l^{-1}$ [$^{14}$C]arginine; essentially the same results were obtained. Aspartate carbamoyltransferase activity was determined by measuring the amount of [$^{14}$C]carbamoylaspartate formed from [$^{14}$C]aspartate after reactions lasting for 30 and 60 min, as previously described (Anderson, 1989). Dihydro-orotase, glutamine synthetase (reaction times of 0, 10 and 20 min), lactate dehydrogenase and glutamate dehydrogenase (the reaction mixture contained 0.2 $mol\,l^{-1}$ $NH_4Cl$, 0.1 $mmol\,l^{-1}$ NADH, 0.01 $mol\,l^{-1}$ $\alpha$-ketoglutarate, 0.035 $mol\,l^{-1}$ Hepes, pH 7.4, 0.035 $mol\,l^{-1}$ KCl, 0.35 $mmol\,l^{-1}$ EDTA and 0.75 $mmol\,l^{-1}$ DTT) were measured as previously described (Anderson, 1989; Shankar and Anderson, 1985; Casey and Anderson, 1982). The reaction mixture for ornithine carbamoyltransferase contained 10 $mmol\,l^{-1}$ ornithine, 5 $mmol\,l^{-1}$ carbamoyl phosphate, 0.05 $mol\,l^{-1}$ KCl, 0.05 $mol\,l^{-1}$ Hepes, pH 7.4, 0.5 $mmol\,l^{-1}$ DTT, 0.25 $mmol\,l^{-1}$ EDTA and extract in a final volume of 0.5 ml; the reaction was terminated and protein precipitated after 10 and 20 min by addition of 75 $\mu$l of 2 $mol\,l^{-1}$ $HClO_4$. The citrulline concentration in the supernatant obtained after

centrifugation was determined as previously described (Xiong and Anderson, 1989).

Carbamoyl phosphate synthetase activity was determined by measuring the [$^{14}$C]carbamoyl phosphate formed from [$^{14}$C]bicarbonate after reactions lasting for 45 or 60 min, as previously described (Anderson *et al.* 1970; Anderson, 1980); the standard reaction mixture contained 0.02 mol l$^{-1}$ ATP, 0.024 mol l$^{-1}$ $MgCl_2$, 5 mmol l$^{-1}$ [$^{14}$C]bicarbonate ($2\times10^7$ cts min$^{-1}$), 0.05 mol l$^{-1}$ Hepes, pH 7.5, 0.05 mol l$^{-1}$ KCl, 0.2 mmol l$^{-1}$ EDTA, 0.4 mmol l$^{-1}$ DTT and either 10 mmol l$^{-1}$ glutamine or 100 mmol l$^{-1}$ $NH_4Cl$ in the presence or absence of 0.5 mmol l$^{-1}$ *N*-acetylglutamate or 1 mmol l$^{-1}$ UTP.

All enzyme assays were carried out at 26 °C.

### *Citrulline synthesis*

Mitochondria were isolated essentially as described above, except that the minced liver was initially suspended in 9 volumes of fractionation buffer (0.25 mol l$^{-1}$ sucrose, 0.5 mmol l$^{-1}$ EGTA, 0.03 mol l$^{-1}$ Hepes, pH 7.5, 5 mmol l$^{-1}$ potassium phosphate) and the 'debris' pellet obtained after the first low-speed centrifugation step was not extracted a second time. Homogenization was accomplished using a Potter–Elvehjem homogenizer (*O. beta*) or a Tissumizer (*O. tau*) as described above. The washed mitochondrial pellet was suspended in fractionation buffer containing 5 mg ml$^{-1}$ bovine serum albumin (approximately equivalent to 0.3 ml g$^{-1}$ of liver from which the mitochondria were isolated). The respiratory viability of the isolated mitochondria was monitored as previously described at 26 °C (Anderson and Casey, 1984; Anderson, 1986*a*,*b*; Cao *et al.* 1991). The standard assay buffer (final concentration in the 4 ml reaction cell, which included 0.2 ml of the mitochondrial suspension) contained 0.088 mol l$^{-1}$ sucrose, 0.175 mmol l$^{-1}$ EGTA, 0.036 mol l$^{-1}$ Hepes, pH 7.5, 6 mmol l$^{-1}$ potassium phosphate, 0.09 mol l$^{-1}$ KCl, 6 mmol l$^{-1}$ $NaHCO_3$, 2 mmol l$^{-1}$ $MgCl_2$, 0.15 mmol l$^{-1}$ ADP and 10 mmol l$^{-1}$ sodium succinate (or other energy source). The respiratory control ratios using succinate as an energy source were greater than 5 for *O. beta* and 3.5 for *O. tau*.

The rates of citrulline synthesis at 26 °C were determined as previously described (Anderson and Casey, 1984; Anderson, 1986*a*,*b*; Cao *et al.* 1991). The standard reaction mixture contained 0.088 mol l$^{-1}$ sucrose, 0.175 mmol l$^{-1}$ EGTA, 0.038 mol l$^{-1}$ Hepes, pH 7.5, 6 mmol l$^{-1}$ potassium phosphate, 0.09 mol l$^{-1}$ KCl, 5 mmol l$^{-1}$ [$^{14}$C]bicarbonate ($5\times10^7$ cts min$^{-1}$), 0.05 ml of mitochondrial suspension, potential nitrogen-donating substrates as indicated and 10 mmol l$^{-1}$ ornithine (when present) in a volume of 0.5 ml at 26 °C. [$^{14}$C]Citrulline formation was determined by measuring the ornithine-dependent acid-stable radioactivity formed with time.

### *Glutamine synthetase and CPSase III activity in isolated mitochondria*

Mitochondria were isolated as described above for studies of citrulline synthesis. The washed mitochondrial pellet obtained after the final centrifugation was suspended in 0.05 mol l$^{-1}$ Hepes, pH 7.6, containing 0.05 mol l$^{-1}$ KCl and 0.1 % Triton X-100 (5 ml g$^{-1}$ of liver used for the isolation) and subjected to brief homogenization and sonication to solubilize the mitochondria. A small volume (0.1 ml) was used in the standard assays for CPSase III and glutamine synthetase. Values for the $K_m$ for different substrates for CPSase III were determined using this preparation of CPSase III and the standard assay described above, except that the concentration of the substrate was varied. In all cases, the substrate being varied was absent in control assays to confirm that a detectable concentration of the substrate was not present in the mitochondrial extract. The ratio of transferase to biosynthetic activity of mitochondrial glutamine synthetase was determined as previously described using glutamine synthetase from a mitochondrial extract partially purified by gel filtration chromatography on a column of Sephacryl S-300 equilibrated with 0.05 mol l$^{-1}$ Hepes buffer, pH 7.5, containing 0.15 mol l$^{-1}$ KCl and 5 % ethylene glycol. Glutamine synthetase activity was located by both the standard transferase assay and by measuring ADP formation in the biosynthetic direction as previously described (Shankar and Anderson, 1985). The ratio of the biosynthetic rate to the transferase rate in the four fractions corresponding to the peak of glutamine synthetase activity was 0.050, which is close to the value of 0.067 obtained for *S. acanthias* glutamine synthetase (Shankar and Anderson, 1985).

### *Gel filtration chromatography of the soluble fraction on Sephacryl S-300*

Samples were prepared by homogenization as described for the isolation of mitochondria for citrulline synthesis, except that only 5 volumes of fractionation buffer was used and the fractionation buffer also contained 1 mmol l$^{-1}$ DTT, 0.02 mg ml$^{-1}$ phenylmethylsulphonyl fluoride, 0.02 mg ml$^{-1}$ benzamidine and 0.015 mg ml$^{-1}$ trypsin inhibitor. The initial centrifugation was at 14 600 ***g*** for 10 min to give the soluble fraction (supernatant) directly. A portion of the supernatant was immediately added to a Sephacryl S-300 gel filtration column (2 cm×50 cm) equilibrated with a solution containing 0.1 mol l$^{-1}$ KCl, 0.05 mol l$^{-1}$ Hepes, pH 7.5, 0.5 mmol l$^{-1}$ EDTA, 0.015 mol l$^{-1}$ $MgCl_2$, 0.01 mol l$^{-1}$ ATP, 0.01 mol l$^{-1}$ $NaHCO_3$, 2 mmol l$^{-1}$ DTT, trypsin inhibitor (0.015 mg ml$^{-1}$) and 10 % glycerol. Elution was carried out at a rate of approximately 30 ml h$^{-1}$ and fractions of approximately 5 ml were collected. All steps were carried out at 4 °C. Enzyme assays were carried out immediately after elution was complete.

### *Chromatographic and electrophoretic characterization of arginase*

Arginase, in either the soluble or mitochondrial fractions obtained from isolation of mitochondria to be utilized for citrulline synthesis, as described above, was partially purified by anion exchange chromatography on a TSK-gel Toyopearl

DEAE-650 M column (0.9 cm×15 cm). A portion (10 ml) of the soluble fraction was passed through a Sephadex G-25 column (2 cm×20 cm) equilibrated with 0.01 mol $l^{-1}$ Hepes, pH 7.6, containing 0.01 mol $l^{-1}$ KCl. The fractions containing protein were pooled and applied to the DEAE column. The column was washed with this same buffer until material absorbing at 280 nm was no longer being eluted. Proteins on the column, including arginase, were then eluted with a linear gradient of increasing KCl concentration (0.01 mol $l^{-1}$ to 0.3 mol $l^{-1}$, 200 ml total volume containing 0.01 mol $l^{-1}$ Hepes, pH 7.6). A portion (0.8 ml) of the suspended mitochondrial fraction was diluted to 10 ml with fractionation buffer and sonicated to break the mitochondria. After centrifugation at 14 000 $g$ for 10 min, the supernatant (approximately 10 ml) was subjected to anion exchange chromatography as described above.

After each DEAE chromatography step, fractions containing high arginase activity were pooled, mixed with glycerol to give a concentration of 10 %, and stored at −20 ˚C. The two different fractions (mitochondrial and soluble) were subjected to polyacrylamide gel electrophoresis (PAGE) in an SE 600 Hoefer slab gel apparatus. The 1.5 mm thick gel was prepared using 4 % total acrylamide (3.3 % crosslinking bisacrylamide) polymerized in 0.375 mol $l^{-1}$ Tris/chloride buffer, pH 8.8, with 0.05 % ammonium persulphate and 0.05 % TEMED. A stacking gel was not used. The reservoir buffer was 0.025 mol $l^{-1}$ Tris and 0.192 mol $l^{-1}$ glycine (pH 8.3). Current was applied for 45 min before adding the sample to reduce the concentration of ammonium persulphate. Each sample (200 $\mu$l, diluted with Tris/chloride, pH 8.8, to give a concentration of 0.28 mol $l^{-1}$) was applied to a 1.8 cm wide slot and subjected to electrophoresis for 2 h at 60 mA (constant current). The two separate lanes were cut from the gel slab, divided into 0.5 cm wide sections and assayed directly for arginase activity as described above (37 ˚C for 1 h).

### *Nitrogen excretion in* Opsanus tau

Although the oyster toadfish (*O. tau*) is thought to be ammoniotelic and minimally ureotelic on the basis of short-term (<2 h) *in vivo* measurements and *in vitro* studies (Read, 1971; Mommsen and Walsh, 1989), it has been suggested that environmental conditions may induce ureotelism (Griffith, 1991). Therefore, nitrogen excretion rates were measured in long-term (48 h) confined (stressful) conditions as described for *O. beta* by Walsh *et al.* (1994). Total liver glutamine synthetase activity was also measured at the end of this 48 h period.

## Results

### *Subcellular distribution of enzymes in* Opsanus beta

The results from three different subcellular fractionation experiments are shown in Table 1. Although some mitochondrial breakage apparently occurred in experiment 1 (as shown by the presence of significant levels of the mitochondrial marker enzyme glutamate dehydrogenase as well as other mitochondrial enzyme activities in the soluble fraction), the urea cycle enzymes CPSase and ornithine carbamoyltransferase are clearly localized in the mitochondria while argininosuccinate synthetase and lyase activities are localized in the soluble fraction, as expected. However, a significant proportion of arginase (approximately 50 %) and glutamine synthetase (approximately 60 %) activities were unexpectedly found in the soluble fraction. Glutamine synthetase activity associated with the mitochondrial fraction was not solubilized by repeated washing with isolation mixture containing an additional 0.15 mol $l^{-1}$ KCl, suggesting that the presence of the enzyme in the mitochondrial fraction is not due to non-specific ionic interactions with mitochondria. Assays of arginase using the same conditions described by Mommsen and Walsh (1989) gave the same results, as did assays using

Table 1. *Enzyme activities in different subcellular fractions of* Opsanus beta *liver*

| Enzyme | Activity ($\mu$mol min$^{-1}$ g$^{-1}$ liver) | | | Percentage of total enzyme activity in each fraction: Debris | | | Mitochondrial fraction | | | Soluble fraction | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Experiment | | | Experiment | | | Experiment | | | Experiment | | |
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Lactate dehydrogenase | 8.2 | 4.3 | 9.0 | 9 | 20 | 15 | 5 | 7 | 5 | 85 | 74 | 80 |
| Argininosuccinate synthetase and lyase | 0.66 | 0.38 | 0.75 | 1 | <1 | 1 | 2 | <1 | <1 | 97 | >90 | 99 |
| Dihydro-orotase | 0.05 | 0.14 | 0.10 | 1 | 7 | 11 | 7 | 9 | 9 | 92 | 83 | 79 |
| Aspartate carbamoyltransferase | 0.07 | 0.09 | 0.13 | 5 | 11 | 2 | 5 | 9 | 5 | 90 | 80 | 93 |
| Glutamate dehydrogenase | 2.7 | 10.2 | 6.2 | 10 | 15 | 24 | 67 | 83 | 71 | 22 | 3 | 5 |
| Ornithine carbamoyltransferase | 36.5 | 65 | 115.5 | 17 | 20 | 27 | 52 | 71 | 65 | 31 | 9 | 8 |
| Glutamine synthetase | 3.1 | 4.7 | 13.1 | 7 | 14 | 19 | 19 | 29 | 37 | 73 | 58 | 43 |
| Arginase | 8.2 | 10.9 | 17.9 | 9 | 16 | 21 | 29 | 38 | 44 | 62 | 46 | 35 |
| Carbamoyl phosphate synthetase | 0.24 | 0.46 | 0.8 | 19 | 23 | 29 | 63 | 71 | 64 | 17 | 5 | 7 |

Subcellular fractionation and enzyme assays were carried out as described in the text.

Experiment 3 was the same as experiments 1 and 2, except that an effort had been made to ‘confine’ the fish for several days before analysis.

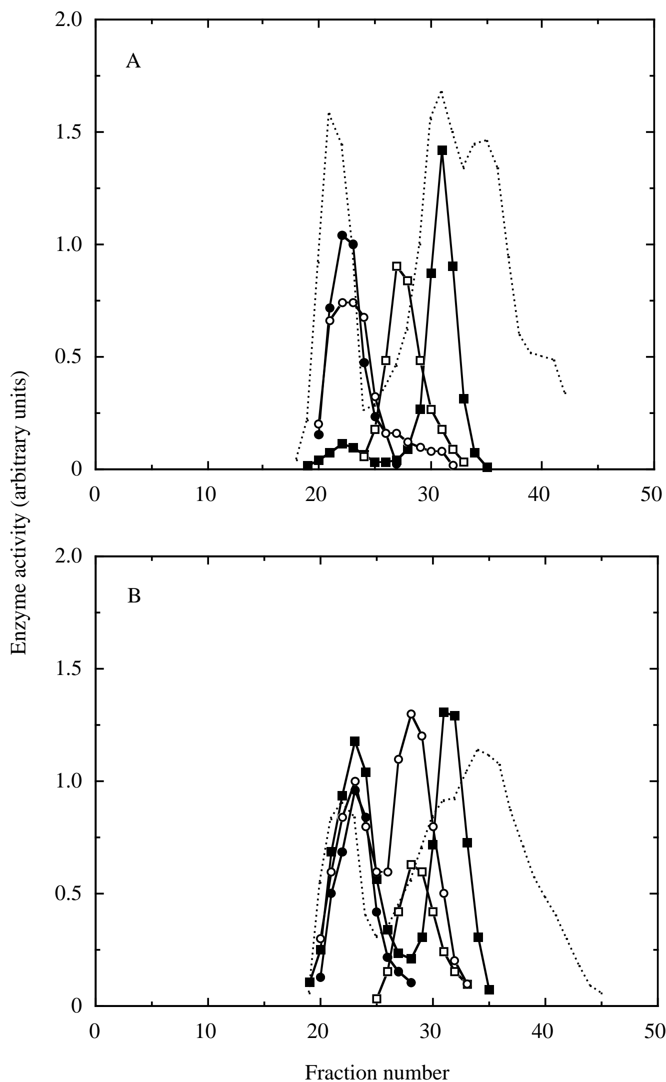


Fig. 1. A typical result of gel filtration chromatography of the soluble fraction obtained from *Opsanus beta* (A) or *O. tau* (B) liver on Sephacryl S-300. Preparation of the soluble fraction, gel filtration chromatography and enzyme assays were carried out as described in the text. ■, carbamoyl phosphate synthetase (CPSase) (glutamine and *N*-acetylglutamate present in assay mix); □, glutamine synthetase; ●, aspartate carbamoyltransferase; ○, dihydro-orotase; dotted line, protein.

different concentrations of arginine (data not shown). The activities expressed as units per gram liver of the urea cycle enzymes are generally comparable to the values reported by Mommsen and Walsh (1989).

Aspartate carbamoyltransferase and dihydro-orotase activities are present in the liver and are localized in the soluble fraction. The presence of CPSase II activity (undetectable by direct assay because of the high level of CPSase III activity) in the soluble fraction was established by gel filtration chromatography on Sephacryl S-300. As shown in Fig. 1A, two peaks of CPSase activity are present. As noted in Table 3 and Fig. 1A, the peak of CPSase activity that eluted first is characteristic of CPSase II (high activity with ammonia, little effect of acetylglutamate, significant inhibition by UTP, some activation by phosphoribosylpyrophosphate (PRPP), high molecular mass and co-elution with dihydro-orotase and aspartate carbamoyltransferase activities). The second peak of CPSase activity is characteristic of CPSase III activity (significant activation by acetylglutamate, little effect of UTP, molecular mass of about 160 kDa). The apparently low level of CPSase II activity relative to CPSase III activity reflects the very high level of CPSase III activity in *O. beta*. CPSase III activity is presumably that which has been released from broken mitochondria. Two peaks of dihydro-orotase activity are present, one eluting with CPSase II (presumably as a multifunctional protein) and the other (variable amounts from one experiment to another) later in the same region as glutamine synthetase.

### *Nitrogen excretion in* Opsanus tau

Over a 48 h period, *O. tau* excretion rates were 6.34± 2.88 $\mu$mol N 100 g$^{-1}$ h$^{-1}$ (mean ± S.E.M.; *N*=3) for ammonia and 1.45±1.16 $\mu$mol N 100 g$^{-1}$ h$^{-1}$ (*N*=3) for urea, representing 81.4 % and 18.6 %, respectively, of the total nitrogen excretion. At the end of the 48 h period, hepatic glutamine synthetase activities were 1.41±0.13 $\mu$mol min$^{-1}$ g$^{-1}$ (*N*=3).

### *Subcellular distribution of enzymes in* Opsanus tau

The results of three different subcellular fractionation experiments are shown in Table 2. Most mitochondria apparently sedimented during the initial low-speed centrifugation and were in the debris fraction when liver was homogenized with a Potter–Elvehjem homogenizer, as described for *O. beta* (note the large enrichment of glutamate dehydrogenase, ornithine carbamoyltransferase and CPSase activities in the debris fractions in experiments 1 and 2). Nevertheless, most glutamine synthetase was associated with the soluble fraction, as expected, but a significant proportion of the arginase activity was also present in the soluble fraction. Homogenization with a Tissumizer appears to overcome the problem of the mitochondria sedimenting at low speed, and the distribution of mitochondrial marker enzymes (experiment 3) is similar to that obtained with *O. beta* (Table 1), *M. salmoides* (Cao *et al.* 1991) and *S. acanthias* (Casey and Anderson, 1982). The results in experiment 3 confirm the expected localization of glutamine synthetase in the soluble fraction and the observation of a significant fraction of arginase in the soluble fraction. The average levels of glutamine synthetase, CPSase and ornithine carbamoyltransferase per gram liver are lower in *O. tau* than in *O. beta*, whereas the level of arginase activity is higher. As with *O. beta*, CPSase II, aspartate carbamoyltransferase and dihydro-orotase are present in the liver, are localized in the soluble fraction and co-elute during gel filtration chromatography on Sepharose S-300 (Tables 2, 3; Fig. 1B). A significant second peak of dihydro-orotase activity was also apparent. The aspartate carbamoyltransferase and dihydro-orotase activities per gram liver are about the same as those

Table 2. *Enzyme activities in different subcellular fractions of* Opsanus tau *liver*

| Enzyme | Activity ($\mu$mol min$^{-1}$ g$^{-1}$ liver) | | | Percentage of total enzyme activity in each fraction: Debris | | | Mitochondrial fraction | | | Soluble fraction | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Experiment | | | Experiment | | | Experiment | | | Experiment | | |
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Lactate dehydrogenase | 8.8 | 10.4 | 7.5 | 19 | 24 | 27 | 5 | 11 | 7 | 76 | 65 | 65 |
| Argininosuccinate synthetase and lyase | 0.18 | 0.44 | 0.22 | 8 | 11 | 9 | 4 | 2 | <1 | 88 | 86 | 91 |
| Dihydro-orotase | 0.10 | 0.11 | -- | 9 | 13 | -- | 3 | 10 | -- | 88 | 76 | -- |
| Aspartate carbamoyltransferase | 0.05 | 0.08 | 0.08 | 12 | 6 | 12 | 0.7 | <1 | <1 | 87 | 94 | 88 |
| Glutamate dehydrogenase | 5.8 | 1.73 | 1.1 | 67 | 59 | 31 | 37 | 36 | 68 | <1 | 5 | 1 |
| Ornithine carbamoyltransferase | 14.8 | 20.8 | 29.2 | 58 | 57 | 37 | 35 | 40 | 57 | 8 | 3 | 6 |
| Glutamine synthetase | 0.9 | 1.0 | 2.1 | 18 | 13 | 12 | 10 | 5 | 4 | 72 | 82 | 84 |
| Arginase | 23.1 | 32.8 | 31.5 | 25 | 24 | 37 | 25 | 36 | 26 | 50 | 40 | 37 |
| Carbamoyl phosphate synthetase | 0.12 | 0.1 | 0.23 | 73 | 56 | 36 | 24 | 41 | 62 | 3 | 3 | 2 |

Subcellular fractionation and enzyme assays were carried out as described in the text.
Homogenization was accomplished with a Potter–Elvehjem homogenizer (experiments 1 and 2) or a Tissumizer (experiment 3).

found in *O. beta* (Table 1) and in *M. salmoides* (Cao *et al.* 1991).

### *Chromatographic and electrophoretic properties of arginase from* Opsanus beta

If arginase is present in both the mitochondria and the cytosol, it might be expected that the properties of each would be different and that this could be detected by chromatographic or electrophoretic methods. For example, at the very least, a mitochondrial import signal peptide on the mitochondrial arginase may be missing. However, the cytosolic and mitochondrial arginase activities eluted in essentially the same position during ion exchange column chromatography (Fig. 2A,B) and had essentially the same electrophoretic mobility during non-denaturing PAGE (Fig. 3).

Table 3. *Effect of substrates, UTP, phosphoribosylpyrophosphate and* N*-acetylglutamate on CPSase activity in the two different peaks of CPSase activity obtained during gel filtration chromatography of the soluble fractions shown in Fig. 1*

| Components in reaction mixture | [$^{14}$C]carbamoylphosphate formed (nmol): *Opsanus beta* Peak 1 | *Opsanus beta* Peak 2 | *Opsanus tau* Peak 1 | *Opsanus tau* Peak 2 |
|---|---|---|---|---|
| Glutamine | 3.4 | 14.0 | 4.2 | 0.9 |
| Glutamine + acetylglutamate | 4.0 | 55.2 | 4.2 | 2.0 |
| Glutamine + UTP | 0.7 | 9.2 | 0.1 | -- |
| Glutamine + phosphoribosyl-pyrophosphate | 3.5 | 11.5 | 5.0 | -- |
| Ammonia | 2.6 | 0.8 | 3.5 | -- |
| Ammonia + acetylglutamate | 2.6 | 1.7 | 4.0 | -- |

The standard assay mixture was used for measuring CPSase activity except that glutamine, acetylglutamate, UTP, phosphoribosylpyrophosphate and ammonia were absent or present as indicated.

### *Citrulline synthesis by isolated mitochondria*

Isolated mitochondria from *O. beta* are capable of catalyzing citrulline synthesis from bicarbonate, ornithine and glutamine, using succinate as an energy source, reflecting the presence of CPSase III and ornithine carbamoyltransferase in the mitochondria. However, as shown in Fig. 4, little citrulline synthesis occurred when glutamate plus ammonia was utilized as the nitrogen-donating substrate in place of glutamine; an unexpected result given the existence of glutamine synthetase activity in the mitochondria. Considerable variation was observed from one mitochondrial preparation to another with respect to the rate and time course of citrulline synthesis from glutamine, but in all cases there was little or no synthesis from glutamate plus ammonia. The data in Table 1 indicate that the maximal total units of biosynthetic glutamine synthetase activity (calculated from the transferase rates) range from 52 to 82 % of the CPSase III activity, with mitochondrial percentages being correspondingly lower. A ratio of mitochondrial glutamine synthetase to CPSase III of less than 1 was confirmed by experiments in which mitochondria were isolated as described in the Materials and methods section and CPSase III and glutamine synthetase activities were assayed directly. In these experiments, glutamine synthetase activity (biosynthetic) was 63±7 % (*N*=3) of the CPSase III activity. The total glutamine synthetase activities in these preparations was higher than for the preparations shown in Table 1, reflecting the likelihood that these fish were stressed (Walsh *et al.* 1994); nonetheless, maximal rates of glutamine synthetase were still lower than those of CPSase III.

The state 3 rates of respiration (nmol $O_2$ consumed min$^{-1}$ mg$^{-1}$ protein) of isolated mitochondria from two experiments

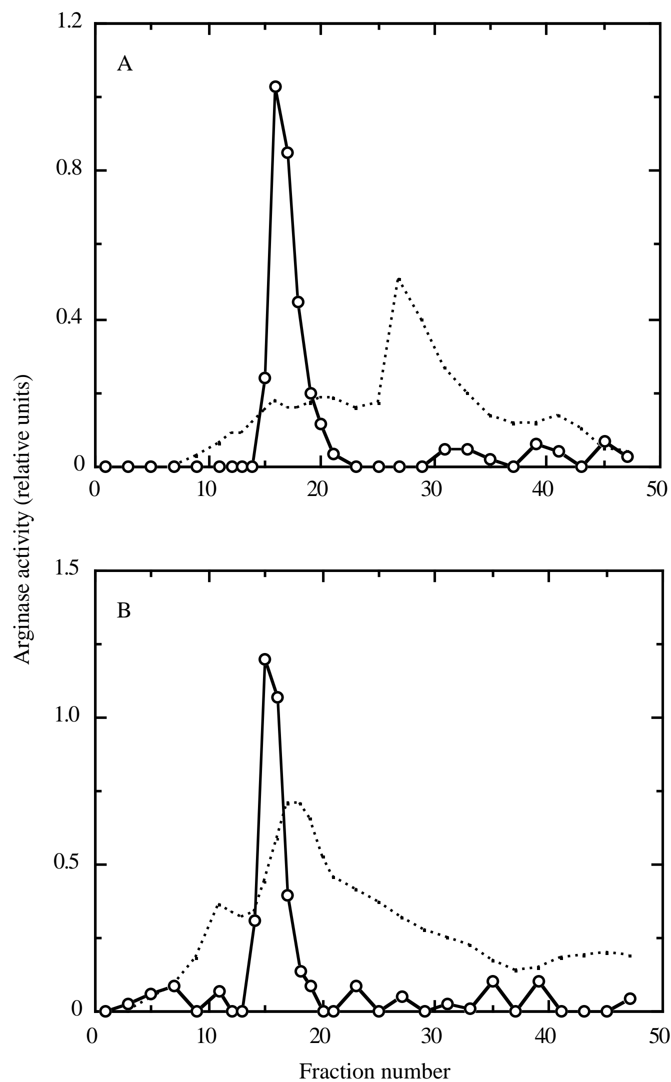


Fig. 2. Representative examples of DEAE ion exchange column chromatography of arginase in mitochondrial and cytosolic fractions obtained from *O. beta* liver. (A) Mitochondrial fraction; (B) soluble fraction. ○, arginase; dotted line, protein.

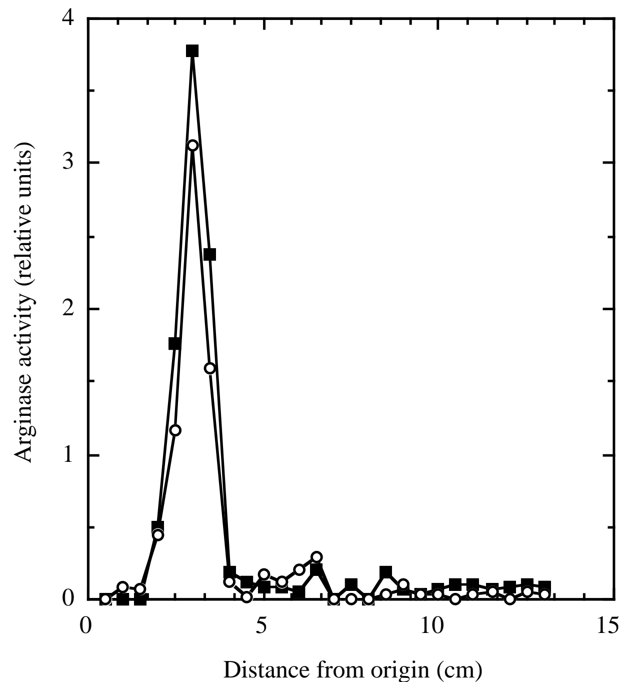


Fig. 3. Polyacrylamide gel electrophoresis of arginase obtained after DEAE ion exchange column chromatography. ■, mitochondrial fraction; ○, soluble fraction.

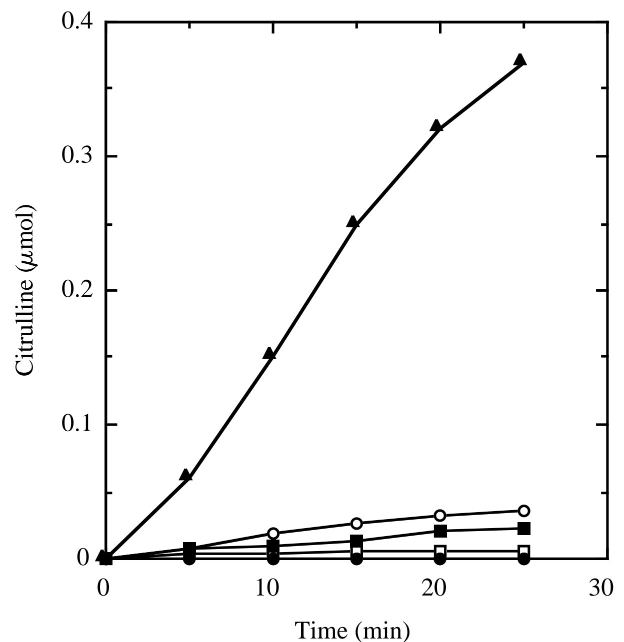


Fig. 4. Time course of citrulline synthesis by isolated *O. beta* mitochondria. Mitochondria (0.75 mg protein) were incubated at 26 °C in the standard reaction mixture (0.5 ml) containing 5 mmol $l^{-1}$ glutamine (▲), 1 mmol $l^{-1}$ $NH_4Cl$ plus 5 mmol $l^{-1}$ glutamate (○), 1 mmol $l^{-1}$ $NH_4Cl$ (■), 5 mmol $l^{-1}$ glutamate (□) or no additions (●), as indicated. Citrulline concentration at the indicated times was measured as described in the text. Values are means of duplicate measurements.

with 3–4 livers of *O. beta* with different substrates (10 mmol $l^{-1}$) were 21.1±2.2 (succinate), 9.2±1.3 (glutamate), 11.8±0.5 (malate), 10.8±2.8 (glutamine) and 16.3±1.2 (glutamate plus malate).

Efforts to demonstrate citrulline synthesis from either glutamine or glutamate plus ammonia by isolated mitochondria from *O. tau* were unsuccessful, despite comparable respiration rates and respiratory control ratios to those of isolated mitochondria from *O. beta*. Synthesis from glutamate plus ammonia would not be expected because of the absence of glutamine synthetase in mitochondria. Respiration rates of isolated *O. tau* mitochondria on various substrates were similar to profiles for *O. beta* mitochondria (data not shown).

### *Kinetic properties of* Opsanus beta *CPSase III*

The mitochondrial CPSase was confirmed to be a CPSase

Table 4. *Enzyme activities in different subcellular fractions of* Porichthys notatus *liver*

| Enzyme | Activity ($\mu$mol min$^{-1}$ g$^{-1}$ liver) | Percentage of total enzyme activity in each fraction: Debris | Mitochondrial fraction | Soluble fraction |
|---|---|---|---|---|
| Lactate dehydrogenase | 6.2 | 17 | 3 | 80 |
| Argininosuccinate synthetase and lyase | 0.04 | 17 | <1 | 83 |
| Dihydro-orotase | 0.02 | 22 | <1 | 78 |
| Aspartate carbamoyltransferase | 0.17 | 5 | 1 | 93 |
| Glutamate dehydrogenase | 8.2 | 15 | 77 | 8 |
| Ornithine carbamoyltransferase | 10.1 | 19 | 67 | 15 |
| Glutamine synthetase | 1.3 | 8 | 6 | 86 |
| Arginase | 8.4 | 26 | 38 | 35 |
| Carbamoyl phosphate synthetase | 0.02 | 21 | 58 | 24 |

Subcellular fractionation and enzyme assays were carried out as described in the text.

III: activity with either ammonia or glutamine required the presence of acetylglutamate (AcGlu) and maximum activity with ammonia was about 14 % of that obtained with glutamine. The apparent $K_m$ values (in mmol l$^{-1}$) were 1.1 for bicarbonate (in the presence of 3 mmol l$^{-1}$ AcGlu), 0.14 for glutamine, 0.03 for AcGlu (in the presence of 20 mmol l$^{-1}$ glutamine) and 4 for $NH_3/NH_4^+$. The $K_m$ values for glutamine and AcGlu were dependent on the concentration of the other, i.e. as the concentration of one increased, the $K_m$ for the other decreased (data not shown).

### *Preliminary observations with* Porichthys notatus

The results of a single subcellular fractionation of livers from *P. notatus* are shown in Table 4. The results are similar to those obtained for *O. tau*, except that the activities of CPSase, argininosuccinate synthetase and lyase were much lower. The presence of CPSase III in the mitochondrial fraction and CPSase II in the soluble fraction was confirmed by assaying under different conditions (Table 5). The CPSase (III) associated with the mitochondrial fraction was not significantly affected by UTP, was significantly stimulated by AcGlu and had little activity with ammonia. The CPSase (II) associated with the soluble fraction was inhibited by UTP, was not greatly stimulated by AcGlu and was almost fully active in the presence of high concentrations of ammonia. The presence of CPSase II, dihydro-orotase and aspartate carbamoyltransferase in the soluble fraction was confirmed by gel filtration chromatography on Sephacryl S-300; the results were very similar to those obtained for *O. tau* (Fig. 1B) (data not shown), including two peaks of dihydro-orotase activity.

Table 5. *Effect of nitrogen-donating substrate, UTP and acetylglutamine on CPSase activity in mitochondrial and cytosolic fractions from liver of* Porichthys notatus

| Components in reaction mixture | Activity (nmol min$^{-1}$ g$^{-1}$ liver): Mitochondrial fraction | Soluble fraction |
|---|---|---|
| Glutamine | 0.2 | 2.9 |
| Glutamine + acetylglutamate | 9.9 | 4.0 |
| Glutamine + UTP | 0.3 | 0.3 |
| Glutamine + acetylglutamate + UTP | 9.6 | 1.5 |
| Ammonia | 0.1 | 2.2 |
| Ammonia + acetylglutamate | 1.1 | 2.8 |
| Ammonia + UTP | 0.4 | 0.2 |
| Ammonia + acetylglutamate + UTP | 1.0 | 0.7 |

The standard assay mixture was used for measuring CPSase activity except glutamine, ammonium chloride, acetylglutamate and UTP were present as indicated.

## Discussion

The compartmentation of CPSase III, CPSase II their and associated enzymes in the liver of the batrachoidid fishes *O. beta, O. tau* and *P. notatus* is generally analogous to the compartmentation of these enzymes in *M. salmoides*. CPSase II, aspartate carbamoyltransferase and dihydro-orotase activities, catalyzing the first three steps of the pyrimidine pathway, are present in the liver and are localized in the cytosol along with glutamine synthetase activity. CPSase III is localized in the mitochondria, along with ornithine carbamoyltransferase, and argininosuccinate synthetase and lyase are localized in the cytosol, as expected. In *S. acanthias*, glutamine synthetase is localized exclusively in the mitochondrial matrix in the liver, and the enzymes catalyzing pyrimidine nucleotide biosynthesis are present only in extra-hepatic tissue where glutamine synthetase is localized in the cytosol. Given that these batrachoidid fishes and *M. salmoides* represent a broad range of teleosts with respect to patterns of nitrogen metabolism and excretion, and that CPSase II activity has been reported in the liver of other teleost species (Anderson, 1980; Cao *et al.* 1991), in contrast to the situation in elasmobranchs, it is likely that the hepatic co-expression of CPSase III and CPSase II is a general teleost characteristic.

However, CPSase III activity may be absent or present at very low levels in most ammoniotelic teleost fishes.

The subcellular localization of arginase in all three species and of glutamine synthetase in the highly ureogenic *O. beta* appears to be much more plastic than initially reported, however (Mommsen and Walsh, 1989). Significant activities of arginase were found in the cytosol, as well as in the mitochondria, of all three species examined (Tables 1, 2 and 4). The same results were obtained even when the methods used by Mommsen and Walsh (1989), which had indicated primarily mitochondrial arginase in both *O. beta* and *O. tau*, were repeated exactly (data not shown). The present results clearly indicate that urea can potentially be generated by arginase activity within both the cytoplasmic compartment (analogous to the situation in ureotelic terrestrial vertebrates, where arginase is localized in the cytosol) and the mitochondrial compartment (analogous to the situation in *S. acanthias*, where arginase is localized exclusively in the mitochondrial matrix) of batrachoidid fishes. Although Cao *et al.* (1991) concluded that arginase in the liver of *M. salmoides* was a mitochondrial enzyme, a higher percentage of the total arginase activity was present in the soluble fraction than that of the mitochondrial marker enzymes glutamate dehydrogenase or ornithine carbamoyltransferase, suggesting that arginase may actually be present in both the cytosol and the mitochondria of these species as well. The preliminary characterization studies described here suggest that there is little difference in size or charge between the mitochondrial and cytosolic arginases from *O. beta*. Campbell and Anderson (1991) have reviewed the apparently eclectic nature of the subcellular localization of arginase in different species and in different organs of the same species, pointing out that its subcellular localization probably reflects the efficiency of its metabolic role, which, in the case of the species described here, may be multifunctional. A detailed investigation of the structure and properties of the mitochondrial and cytosolic arginases in these fishes will be essential for an understanding of the significance of the presence of arginase activity in both compartments and its specific function in each.

The present study provides additional support for the suggestion that hepatic glutamine synthetase plays a central role in regulatory ureogenesis in batrachoidid fishes (Mommsen and Walsh, 1991; Walsh *et al.* 1994), with both glutamine synthetase activity and subcellular localization apparently being important characteristics. Both *O. tau* and *P. notatus* appear to be primarily ammoniotelic species (this study and T. P. Mommsen, personal communication, respectively), with urea contributing less than 20 % of the total nitrogen excreted. This relatively low level of urea excretion is typical of other teleost fishes, where the small amount of urea excreted can also be derived from non-urea-cycle pathways (Campbell and Anderson, 1991; Wood, 1993; Anderson, 1995). Correlated with ammoniotelism in both of these species are lower hepatic levels of glutamine synthetase ($<2\,\mu mol\,min^{-1}\,g^{-1}$ liver) and the localization of this activity in the cytosol (Tables 2 and 4). Additionally, *O. tau* did not exhibit a switch-over to ureotelism or an elevation in hepatic glutamine synthetase activity during 48 h of confinement (this study), as is observed for *O. beta* (Walsh *et al.* 1994). Similarly, lower levels of hepatic glutamine synthetase localized in the cytosol are observed in *M. salmoides*. In contrast, in *O. beta* the capability for ureogenesis is correlated with the presence of a mitochondrial form of glutamine synthetase (representing 30–40 % of the total hepatic glutamine synthetase activity), and highly active ureogenesis is correlated with the ability to induce an increase in the levels of total hepatic glutamine synthetase activity, which are an order of magnitude higher than those found in *O. tau* (Table 1, experiment 3 *versus* experiments 1 and 2, and Walsh *et al.* 1994), *P. notatus* (Tables 2, 4) or *M. salmoides* (Cao *et al.* 1991). The observation that *O. beta* also has a cytosolic form of glutamine synthetase like those of *O. tau, P. notatus* and *M. salmoides* may reflect the requirement for glutamine as a nitrogen-donating substrate for CPSase II and probably other amidotransferase activities in the cytosol. In contrast, the absence of a hepatic cytosolic glutamine synthetase in elasmobranchs appears to correlate with the absence of CPSase II (and probably other amidotransferases) in the liver.

Further aspects of this study provide insights into the possible mechanisms of regulation of ureogenesis in *O. beta* in relation to glutamine synthetase activity. Maximal rates of CPSase III activity (Tables 1, 3), citrulline synthesis from $5\,mmol\,l^{-1}$ glutamine by isolated mitochondria (Fig. 4) and ureogenesis by isolated mitochondria supplied with $5\,mmol\,l^{-1}$ glutamine (Barber and Walsh, 1993) appear to be more than adequate to support observed *in vivo* rates of ureogenesis (Barber and Walsh, 1993). These *in vitro* conditions, however, all bypassed the requirement for glutamine synthetase. Our results indicate that (per gram liver) the maximal biosynthetic rates of mitochondrial glutamine synthetase are significantly lower than the maximal biosynthetic rate of CPSase III and that the total hepatic glutamine synthetase activity is about the same as, or a little lower than, the total activity of CPSase III. Furthermore, the calculated maximal rates of mitochondrial glutamine synthetase are typically about equal to, or slightly higher than, the observed rates of ureogenesis *in vivo*. The actual rate of glutamine synthesis in mitochondria *in vivo* is probably lower than the maximal rates considered here, however, for at least two reasons. By analogy with the *S. acanthias* glutamine synthetase and other glutamine synthetases (Shankar and Anderson, 1985), the $K_m$ for glutamate for the *O. beta* enzyme is probably quite high ($>10\,mmol\,l^{-1}$) and it is likely that the enzyme is not saturated *in vivo*. An additional consideration may be that significant glutaminase activity is apparently present in the liver mitochondrial fraction ($0.1\,\mu mol\,min^{-1}\,g^{-1}$ liver, P. M. Anderson and P. J. Walsh, unpublished observation) and this may have an impact on glutamine availability for CPSase III activity. Taken together, these observations suggest that glutamine supply for CPSase III activity and ureogenesis may require supplementation by cytosolic glutamine synthetase and may be rate-limiting. The observation that the onset of

ureogenesis induced by stress is accompanied by a significant increase in total hepatic glutamine synthetase activity, with little change in other urea cycle enzymes, is consistent with this possibility. The $K_m$ for bicarbonate calculated for urea synthesis by isolated hepatocytes is $1.3\,mmol\,l^{-1}$ (Walsh *et al.* 1989), which is essentially the same as the $K_m$ of CPSase III for bicarbonate (this study), suggesting that CPSase III or a step preceding carbamoyl phosphate formation represents a rate-limiting step in ureogenesis. It appears from these considerations that the availability of glutamine would be the limiting factor.

If substantiated by further studies, the limitation of ureogenesis by the rate of glutamine synthesis in toadfishes also stands in contrast to the apparent situation in *S. acanthias*, where hepatic mitochondrial glutamine synthetase is localized in the mitochondria at levels that are considerably higher than the levels of CPSase III activity and appear to be adequate to support maximal rates of CPSase III function and ureogenesis (Casey and Anderson, 1982; Anderson and Casey, 1984; Shankar and Anderson, 1985). This may explain why isolated mitochondria from *S. acanthias* catalyze the formation of citrulline from glutamate plus ammonia at a rate only a little lower than that obtained with glutamine (Anderson and Casey, 1984), but isolated mitochondria from *O. beta* apparently do not. If mitochondrial glutamine levels limit ureogenesis in batrachoidid fish, the mechanisms and rates of transport of glutamine into the mitochondria, including species comparisons within the family and with elasmobranchs, and a possible role for glutaminase activity in the regulation of ureogenesis represent fruitful directions for future research. In addition, elucidation of the possible contributions of variations in the mitochondrial concentrations of AcGlu, the kinetic properties of the mitochondrial glutamine synthetase and the steady-state concentrations of mitochondrial glutamate would be helpful in understanding the mechanisms of regulation of ureogenesis in *O. beta.*

The CPSase III from *O. beta* appears to have kinetic properties very similar to the CPSase III from both *S. acanthias* and *M. salmoides* (Anderson, 1981; Casey and Anderson, 1983). The results persented here firmly establish that the AcGlu-dependent mitochondrial CPSase in the toadfishes is in fact a CPSase III, and further support the view that, where present, AcGlu-dependent CPSase activity in fish is characteristically due to CPSase III and not to CPSase I (Mommsen and Walsh, 1989; Anderson, 1995).

In summary, the biochemical properties of carbamoyl phosphate and urea synthesis in the batrachoidid fishes are similar to those in the teleost *M. salmoides*, representing a strategy that is probably characteristic of teleosts in general and which differs from that of the elasmobranch fishes. As a group, this family offers unique opportunities for obtaining further insights into the regulation and evolution of ureogenesis in fishes.

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