Biochem. J. (1989) 261, 523–529 (Printed in Great Britain)

Glutamine-dependent carbamoyl-phosphate synthetase and other enzyme activities related to the pyrimidine pathway in spleen of *Squalus acanthias* (spiny dogfish)

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The first two steps of urea synthesis in liver of marine elasmobranchs involve formation of glutamine from ammonia and of carbamoyl phosphate from glutamine, catalysed by glutamine synthetase and carbamoyl-phosphate synthetase, respectively [Anderson & Casey (1984) J. Biol. Chem. **259**, 456–462]; both of these enzymes are localized exclusively in the mitochondrial matrix. The objective of this study was to establish the enzymology of carbamoyl phosphate formation and utilization for pyrimidine nucleotide biosynthesis in *Squalus acanthias* (spiny dogfish), a representative elasmobranch. Aspartate carbamoyltransferase could not be detected in liver of dogfish. Spleen extracts, however, had glutamine-dependent carbamoyl-phosphate synthetase, aspartate carbamoyltransferase, dihydro-orotase, and glutamine synthetase activities, all localized in the cytosol; dihydro-orotate dehydrogenase, orotate phosphoribosyltransferase, and orotidine-5′-decarboxylase activities were also present. Except for glutamine synthetase, the levels of all activities were very low. The carbamoyl-phosphate synthetase activity is inhibited by UTP and is activated by 5-phosphoribosyl 1-pyrophosphate. The first three enzyme activities of the pyrimidine pathway were eluted in distinctly different positions during gel filtration chromatography under a number of different conditions; although complete proteolysis of inter-domain regions of a multifunctional complex during extraction cannot be excluded, the evidence suggests that in dogfish, in contrast to mammalian species, these three enzymes of the pyrimidine pathway exist as individual polypeptide chains. These results: (1) establish that dogfish express two different glutamine-dependent carbamoyl-phosphate synthetase activities, (2) confirm the report [Smith, Ritter & Campbell (1987) J. Biol. Chem. **262**, 198–202] that dogfish express two different glutamine synthetases, and (3) provide indirect evidence that glutamine may not be available in liver for biosynthetic reactions other than urea formation.

INTRODUCTION

Carbamoyl-phosphate synthetase catalyses formation of carbamoyl phosphate, which is utilized as the initial precursor for two major metabolic pathways, one leading to biosynthesis of pyrimidine nucleotides and the other to arginine and/or urea (Ratner, 1973). Mammalian ureotelic species possess two carbamoyl-phosphate synthetases, an ammonia- and acetyl-glutamate-dependent carbamoyl-phosphate synthetase located exclusively in the mitochondrial matrix in liver and a glutamine-dependent carbamoyl-phosphate synthetase located in the cytosol of hepatic and extra-hepatic tissues. The function of the mitochondrial ammonia-dependent carbamoyl-phosphate synthetase is related to the urea cycle and detoxification of ammonia; the enzyme is subject to regulation by alteration of the levels of the enzyme and of the allosteric effector acetyl-glutamate, which is required for activity (Meijer & Hensgens, 1982; Powers-Lee & Meister, 1988). The glutamine-dependent carbamoyl-phosphate synthetase, which catalyses carbamoyl phosphate formation for the purpose of pyrimidine nuceotide biosynthesis in the cytosol, is subject to feedback inhibition by UTP, is activated by 5-phosphoribosyl 1-pyrophosphate (PRPP), and is part of a multifunctional complex in which carbamoyl-phosphate synthetase, aspartate carbamoyltransferase and dihydro-orotase activities (which catalyse the first three steps unique to the pyrimidine nucleotide pathway) are located on a single polypeptide chain (Evans, 1986; Makoff & Radford, 1978; Mori *et al.*, 1975; Jones, 1980).

We have identified several features of urea synthesis in marine elasmobranchs [sharks, skates, and rays, which synthesize and retain high levels of urea as well as trimethylamine *N*-oxide (TMAO) and other amines for the purpose of osmoregulation (Goldstein & Forster, 1970; Smith, 1936)] that are distinctly different from the urea cycle pathway in mammalian ureotelic species. The first step of ammonia assimilation in liver mitochondria of *Squalus acanthias* (spiny dogfish), a representative elasmobranch, is formation of glutamine, which then serves as the nitrogen-donating substrate for carbamoyl phosphate formation (Anderson & Casey, 1984). This is accomplished as a result of the localization of high levels of glutamine synthetase exclusively in the mitochondrial matrix in liver and the presence of a unique glutamine- and acetylglutamate-dependent carbamoyl-phosphate synthetase in the liver, also in the mitochondrial matrix (Anderson, 1980, 1981; Casey & Anderson, 1982, 1985; Shankar & Anderson, 1985). The properties of the glutamine synthetase are similar to those of mammalian

Abbreviations used: PRPP, 5-phosphoribosyl 1-pyrophosphate; TMAO, trimethylamine *N*-oxide; DTT, dithiothreitol.

and avian glutamine synthetases, except that the enzyme is activated by halogen anions and has an unusually low K_m for ammonia. The properties of the glutamine- and acetylglutamate-dependent carbamoyl-phosphate synthetase are very similar to those of the mitochondrial ammonia- and acetylglutamate-dependent carbamoyl-phosphate synthetase found in mammalian ureotelic species, except for the fact that glutamine rather than ammonia serves as the nitrogen-donating substrate for carbamoyl phosphate formation.

The first step of ammonia detoxification in liver of avian species also involves glutamine formation catalysed by glutamine synthetase localized in the mitochondrial matrix, but the glutamine formed exits the mitochondria and serves as the nitrogen-donating substrate for several steps in the purine biosynthetic pathway in the cytosol, resulting in formation and excretion of uric acid (Campbell *et al.*, 1987). In these species glutamine would, therefore, be available in the cytosol of hepatic tissue for pyrimidine nucleotide biosynthesis.

Except for one indirect report from our laboratory of apparent glutamine-dependent, acetylglutamate-independent carbamoyl-phosphate synthetase activity in some marine teleosts (Anderson, 1980), little is known about the source of carbamoyl phosphate for pyrimidine nucleotide biosynthesis in elasmobranchs or any species of fish. This question is of particular interest in elasmobranch liver because of the glutamine-dependent carbamoyl phosphate synthesis in the mitochondria. The purpose of the study reported here was to establish the nature of the initial steps of pyrimidine nucleotide biosynthesis in spiny dogfish. Several possibilities could be considered: pyrimidine nucleotide biosynthesis occurs only in extrahepatic tissues involving a different glutamine-dependent carbamoyl-phosphate synthetase analogous to the glutamine-dependent carbamoyl-phosphate synthetase in mammalian species and a cytosolic glutamine synthetase; carbamoyl phosphate formed from glutamine in the mitochondrial matrix of liver cells can exit the mitochondria and be utilized for pyrimidine nucleotide biosynthesis; or, a portion of the glutamine formed in the mitochondria in liver can exit to the cytosol and serve as substrate for another glutamine-dependent carbamoyl-phosphate synthetase analogous to that of mammalian species. Our results suggest that at least the first steps of the pyrimidine nucleotide pathway are absent from the liver but that glutamine synthetase, a different glutamine-dependent carbamoyl-phosphate synthetase analogous to the pyrimidine-related carbamoyl-phosphate synthetase in mammalian species, aspartate carbamoyltransferase and dihydro-orotase activities are present in the cytosol of spleen.

Portions of this work were presented at the 1989 Annual Meeting of the American Society for Biochemistry and Molecular Biology in San Francisco (Anderson, 1988).

EXPERIMENTAL PROCEDURES

These studies were carried out at Friday Harbor Laboratories, University of Washington. Spiny dogfish were captured by trawling in the waters near San Juan Island and were held in a large circular holding tank provided with a constant supply of fresh seawater. Radioisotopes were purchased from Research Products International Corp. Most biochemicals and chromatography media were purchased from Sigma Chemical Co.

Subcellular fractionation

All steps were carried out at 4 °C. Freshly excised spleen (~ 15 g) from spiny dogfish was minced and suspended in 8 vol. of isolation medium [0.25 M-sucrose, 0.15 M-KCl, 0.3 M-urea, 0.15 M-TMAO, 1 mM-EGTA, 0.02 M-Hepes, pH 7.5 (Anderson & Casey, 1984)]. The suspension was homogenized for 20 s with a Tissumizer (Tekmar model SDT 1810 equipped with a model 18N probe and a model TR5T speed controller) at a reduced speed corresponding to a setting of 30 on the speed controller. The homogenate was centrifuged at 40 ***g*** for 10 min to remove unbroken cells and debris (the sedimented fraction was not enriched in any of the enzyme activities of interest). Half of the supernatant was utilized for assay of all of the enzymes of interest (total units). The other half of the supernatant was centrifuged at 14500 ***g*** for 10 min and the resulting supernatant was removed from the well-defined pellet (mitochondria plus nuclei and, perhaps, other organelles). The mitochondrial pellet was washed by resuspending in isolation medium and centrifuging at 14500 ***g*** for 10 min. The washed pellet was then suspended in assay buffer (0.05 M-Hepes, pH 7.6, 0.1 M-KCl, 0.5 mM-EDTA and 1 mM-DTT) and the resulting suspension was sonicated to break up the mitochondria and other organelles. The supernatant obtained from the initial centrifugation at 14500 ***g*** for 10 min was centrifuged at 100000 ***g*** for 90 min. The resulting supernatant (soluble fraction) was removed and the pellet (particulate fraction) was resuspended in assay buffer. All fractions (total units, mitochondrial, soluble and particulate) were equilibrated with assay buffer and low molecular mass metabolites were removed at the same time by passing 20 ml of each through a 90 ml column of Sephadex G-25 equilibrated with assay buffer. The centre of the eluted protein peak was collected to minimize dilution, and the protein concentration was determined before and after gel filtration to adjust for dilution. These pooled fractions were used immediately for enzyme assays.

Gel filtration chromatography of spleen extracts

All steps were carried out at 4 °C. Extracts were prepared by suspending spleen (~ 15 g) that had been rapidly excised from spiny dogfish and minced in 3 vol. of extraction buffer containing 0.05 M-Hepes, pH 7.5, 0.1 M-KCl, 0.5 mM-EDTA, 2 mM-DTT, 0.2 mM-phenylmethanesulphonyl fluoride, 0.2 mM-benzamidine, 0.02 mg of trypsin inhibitor/ml, and either 0.01 M-glutamine or 0.02 M-ATP, 0.024 M-$MgCl_2$, and 0.01 M-$NaHCO_3$; the suspension was homogenized with a Tissumizer as described above followed by sonication at half power for 30 s with a Heat Systems–Ultrasonics Inc. model W-375 sonifier equipped with a 0.5 inch (12.7 mm) probe. The extract was centrifuged at 14000 ***g*** for 12 min and a 5–10 ml aliquot of the resulting supernatant was immediately subjected to gel filtration chromatography on either a Sephacryl S-300 column (2.2 cm × 47 cm) or Sephadex G-200 column (2.2 cm × 20 cm or 2.2 cm × 44 cm) equilibrated with the same solution employed as the extract buffer. (In most experiments 20 ml of the extract supernatant was first passed through a 90 ml column of Sephadex G-25 equilibrated with the appropriate extract buffer; a 10 ml aliquot collected from the centre of the

eluted protein peak was used for subsequent chromatography.) Elution was carried out as rapidly as possible; a constant flow rate of ~ 0.3 ml/min was maintained by utilizing a peristaltic pump, and fractions of ~ 4.2 ml were collected.

The molecular size of the eluted proteins was estimated from the elution volumes by comparison with the elution volumes of proteins of known molecular mass as described by Andrews (1965). The reference proteins and their M_r values were thyroglobulin (660000), apoferritin (443000), amylase (200000), alcohol dehydrogenase (150000), bovine serum albumin (67500) and carbonic anhydrase (29000).

Enzyme and protein assays

Glutamine synthetase (γ-glutamyl transferase and biosynthetic activities), lactate dehydrogenase, glutamate dehydrogenase and cytochrome oxidase activities and protein were measured as previously described (Shankar & Anderson, 1985; Casey & Anderson, 1982).

Carbamoyl-phosphate synthetase activity was determined by measuring the [^{14}C]carbamoyl phosphate formed from [^{14}C]bicarbonate after reaction for 45 min at 26 °C as previously described (Anderson *et al.*, 1970; Anderson, 1980); the standard reaction mixture contained 0.01 M-glutamine, 0.02 M-ATP, 0.024 M-$MgCl_2$, 5 mM-$H^{14}CO_3^-$ (2×10^8 c.p.m.), 0.4 mM-PRPP, 0.06 M-KCl, and 0.03 M-Hepes buffer, pH 7.6, in a final volume of 1 ml.

Aspartate carbamoyltransferase activity was determined by measuring the amount of [^{14}C]carbamoyl aspartate formed from [^{14}C]aspartate after reaction for 45 min at 26 °C. The standard reaction mixture contained 2 mM-[^{14}C]aspartate (3×10^5 c.p.m.), 2.5 mM-carbamoyl phosphate, 0.08 M-KCl and 0.04 M-Hepes, pH 7.6, in a final volume of 0.25 ml. Reaction was stopped by addition of 25 μl of 2 M-HCl. The acidified reaction mixture was applied to a small column (0.5 cm × 5 cm) of Dowex 50W X8 (H^+) and the [^{14}C]carbamoyl aspartate was eluted with 3 ml of water and the eluted radioactivity measured with a scintillation counter.

Dihyhdro-orotase was assayed by measuring the amount of carbamoyl aspartate produced after 2 h of reaction at 26 °C (Smith *et al.*, 1980; Christopherson & Jones, 1979). The reaction mixture contained 2 mM-dihydro-orotate and 0.2 M-Tris buffer, pH 8.7, in a final volume of 0.5 ml. Reaction was terminated by addition of 75 μl of 2 M-$HClO_4$; the protein precipitate was removed by centrifugation and the carbamoyl aspartate present in 0.5 ml of the supernatant was determined colorimetrically as described by Powers & Pierson (1980).

Dihydro-orotate dehydrogenase, orotate phosphoribosyltransferase and orotidine 5′-phosphate decarboxylase were assayed by spectrophotometric procedures as described by Smith *et al.* (1980).

Appropriate controls (e.g., analysis at zero time and/or absence of a substrate) were included in all enzyme assays and product formation was shown to be linear with time. A unit of enzyme activity is defined as 1 μmol of product formed/min at 26 °C.

Immunoblotting

Immunoblotting of the soluble cytosol fraction of spleen, prepared as described under 'Subcellular fractionation' above, was carried out by Dr. Darwin D. Smith, as previously described (Smith *et al.*, 1987; Ritter *et al.*, 1987). Brain and liver tissue from dogfish were homogenized directly in the SDS/polyacrylamide-gel electrophoresis sample buffer.

RESULTS AND DISCUSSION

Absence of aspartate carbamoyltransferase activity from liver extracts

Radioactive as well as colorimetric assays under a number of different assay and extraction conditions (e.g., pH, buffer, presence or absence of MgATP, urea and TMAO, and variations in substrate concentration) were utilized in efforts to detect aspartate carbamoyltransferase in liver extracts. The limit of detection was ~ 0.001 units/g of tissue. No evidence for aspartate carbamoyltransferase activity could be obtained. We conclude on the basis of these studies that aspartate carbamoyltransferase activity is probably not expressed in liver tissue, which if correct implies that pyrimidine nucleotide biosynthesis *de novo* does not occur in hepatic tissue in these species.

Presence of enzyme activities catalysing the first three steps of pyrimidine nucleotide biosynthesis *de novo* in spleen

Activities of glutamine synthetase and the enzymes catalysing the first three reactions of the pyrimidine nucleotide biosynthetic pathway (glutamine-dependent carbamoyl-phosphate synthetase, aspartate carbamoyltransferase and dihydro-orotase) are present in spleen extract. The levels of activity of these enzymes and evidence that all four are present in the cytosol are shown in Table 1. The distribution of these enzymes after subcellular fractionation is similar to that of the cytosolic marker enzyme lactate dehydrogenase and is clearly not similar to that of the mitochondrial enzymes cytochrome oxidase and glutamate dehydrogenase. None of the activities were in the pellet after centrifugation at 100000 *g* for 90 min.

Campbell and co-workers have established that glutamine synthetase in brain tissue of dogfish is also localized in the cytosol and that the brain enzyme is an isoenzyme form of the liver enzyme; the subunit M_r values of the cytosolic brain and mitochondrial liver forms are 45000 and 47000, respectively (Smith *et al.*, 1987). Immunoblot analysis of the dogfish spleen glutamine synthetase showed that the electrophoretic mobility of the cytosolic spleen enzyme under denaturing conditions in the presence of SDS is identical to that of the cytosolic brain glutamine synthetase, and that the apparent subunit M_r (45000), like that of the brain enzyme, is less than that of the liver mitochondrial glutamine synthetase (Fig. 1). Kinetic studies of the biosynthetic reaction of the glutamine synthetase from spleen after gel filtration chromatography as described below were limited by the presence of an active ATPase activity, but the results clearly indicated that the spleen enzyme, like the liver enzyme, has a very low K_m for ammonia (Shankar & Anderson, 1985). Unlike the liver enzyme, however, the spleen enzyme was found to be quite stable and the molecular size estimated by gel filtration chromatography was not significantly affected by the presence of MgATP and glutamate. The level of glutamine synthetase biosynthetic activity in dogfish spleen (0.28 units/g of tissue) assayed at 26 °C is

Table 1. Presence of carbamoyl-phosphate synthetase, aspartate carbamoyltransferase, dihydro-orotase and glutamine synthetase in the cytosol of spiny dogfish spleen

Subcellular fractionation and enzyme assays were carried out as described in the text. Units are expressed as μmol/min for enzyme activity and as mg for protein.

Enzyme	Units/g of tissue	Percentage of total recovered enzyme activity or protein		Percentage of extract units or protein recovered in fractions
		Mitochondrial fraction	Soluble fraction	
Glutamate dehydrogenase	1.8	66.8	1.8	78
Cytochrome oxidase	2.2	67.4	10.6	78
Lactate dehydrogenase	70.5	9.8	72.6	85
Glutamine synthetase	0.28	13.8	72.7	86
Carbamoyl-phosphate synthetase	0.0009	10.6	106.0	117
Aspartate carbamoyltransferase	0.022	9.0	62.8	73
Dihydro-orotase	0.04	12.2	72.3	85
Protein	123	15.3	106.5	99

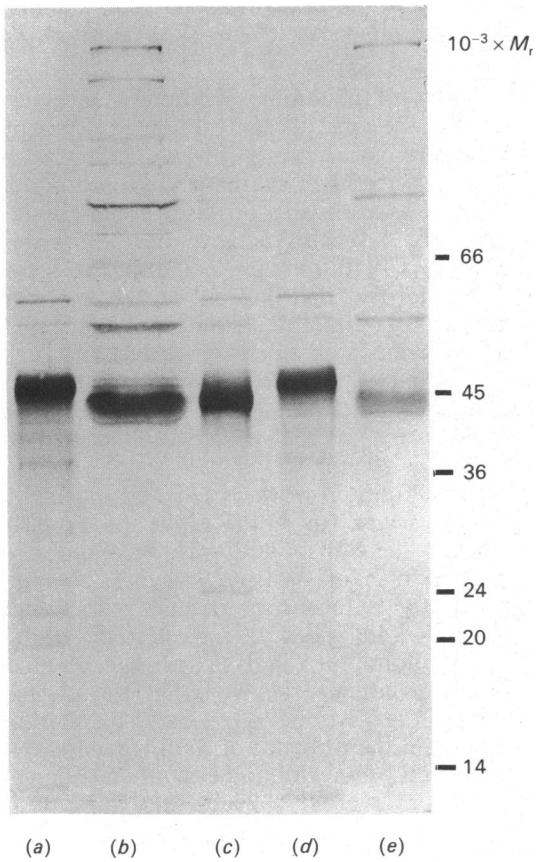

Fig. 1. Immunoblotting of glutamine synthetase from dogfish spleen, brain and liver

Immunoblot analysis was carried out as described in the Experimental procedures section. Lanes *a* and *d*, 40 μg of total liver homogenate protein; lanes *b* and *e*, 80 and 40 μg, respectively, of cytosolic spleen protein; lane *c*, 40 μg of total brain homogenate protein.

somewhat less than that reported previously for liver [1.34 units/g of tissue (Casey & Anderson, 1982; Shankar & Anderson, 1985)]) and is comparable to that reported by Smith *et al.* (1987) for dogfish brain and stingray brain and liver (0.54, 0.20, and 0.46 units/g of tissue, respectively, when assayed at 30 °C).

The total units of carbamoyl-phosphate synthetase activity increased by a small amount after subcellular fractionation, but even so the level of activity was extremely low (Table 1). The effect of MgATP concentration on carbamoyl-phosphate synthetase activity is shown in Fig. 2; at lower concentrations of MgATP the activity is subject to significant feedback inhibition by UTP and is markedly activated by PRPP. Ammonia can substitute for glutamine as the nitrogen-donating substrate, but the maximum velocity is about half of that obtained with glutamine and the apparent K_m is 12 mM, compared to 0.01 mM for glutamine. The presence of acetylglutamate had no effect on enzyme activity. Enzyme activity is quite labile; nearly all activity is lost within 24 h after gel filtration chromatography as described below. The kinetic properties of the spleen carbamoyl-phosphate synthetase are characteristic of the glutamine-dependent cytosolic carbamoyl-phosphate synthetase activities that function in the pyrimidine nucleotide biosynthetic pathway in mammalian species (Evans, 1986; Makoff & Radford, 1978; Mori *et al.*, 1975). These sults clearly indicate that dogfish do express another utamine-dependent carbamoyl-phosphate synthetase, ı addition to the mitochondrial glutamine-dependent arbamoyl-phosphate synthetase in liver, and that the ınction of the enzyme is related to pyrimidine nucleotide iosynthesis.

The level of aspartate carbamoyltransferase activity is also very low. The aspartate carbamoyltransferase activity is unusually labile (about half of the activity is lost within 24 h after gel filtration chromatography) and the apparent molecular size estimated by gel filtration chromatography is quite large (see below). The apparent K_m for carbamoyl phosphate is 4 μM, similar to that reported for other mammalian aspartate carbamoyltransferase activities; the apparent K_m value for aspartate appears to be very high (> 10 mM), but an accurate estimate could not be made because of apparent substrate inhibition at higher concentrations of aspartate.

In contrast to the carbamoyl-phosphate synthetase

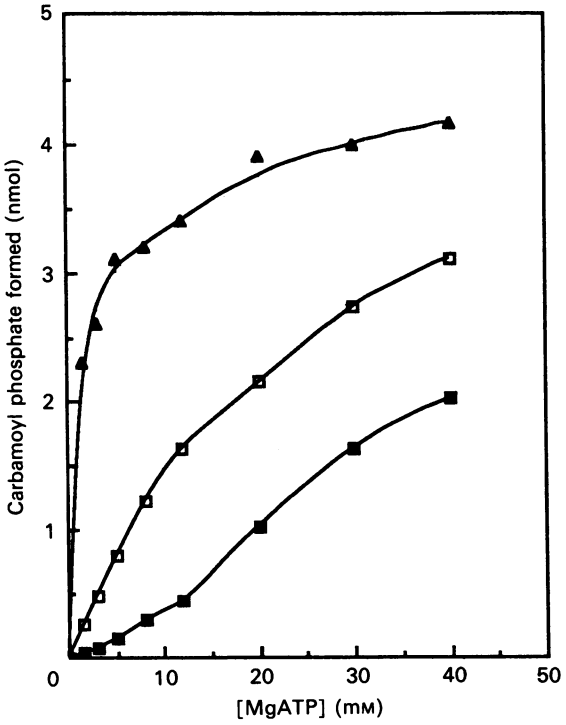

Fig. 2. Effect of ATP concentration on carbamoyl-phosphate synthetase activity in the presence and absence of UTP or PRPP

Carbamoyl-phosphate synthetase activity was assayed as described in the Experimental procedures section, except the reaction mixtures contained 4 mM-$MgCl_2$/ATP as indicated with $MgCl_2$ present in equimolar concentration in the absence (□) or presence of either 0.2 mM-PRPP (▲) or 1 mM-UTP (■).

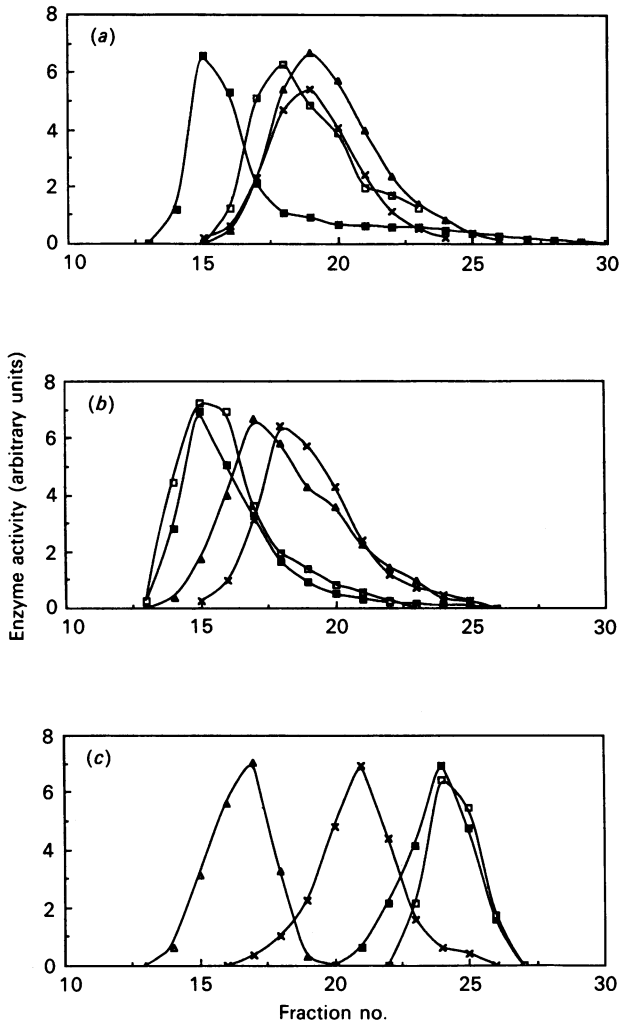

Fig. 3. Gel filtration chromatography of extracts of dogfish spleen

Preparation of extracts, gel filtration chromatography, and enzyme assays were carried out as described in the Experimental procedures section. Panels (*a*) and (*b*), gel filtration chromatography on Sephadex G-200 (2.2 cm × 44 cm) in the presence of glutamine (*a*) or MgATP plus bicarbonate (*b*). ■, Aspartate carbamoyltransferase; □, dihydro-orotase; ▲, carbamoyl-phosphate synthetase; ×, glutamine synthetase. Enzyme activity is expressed as arbitrary units. Panel (*c*): gel filtration chromatography on Sephacryl S-300 (2.2 cm × 47 cm) in the presence of glutamine. △, Dihydro-orotate dehydrogenase; ×, glutamine synthetase; ■, orotate phosphoribosyltransferase; □, orotidine 5′-monophosphate decarboxylase.

and aspartate carbamoyltransferase activities, the dihydro-orotase activity is quite stable. However, the level of activity was also very low and it was not possible to determine the kinetic properties of the enzyme using the assay procedure that was available at the time and location of this study. One unusual feature of this enzyme that was identified, however, was apparent association induced by the presence of MgATP (see below).

Gel filtration chromatography of spleen extracts

The elution profiles of the four cytosolic enzyme activities described in the previous section obtained when extracts of spleen prepared with either glutamine or MgATP and bicarbonate present in both the extract and elution buffers were subjected to gel filtration chromatography on Sephadex G-200 (2.2 cm × 44 cm column) are shown in Fig. 3. Dihydro-orotase elutes in nearly the same position as aspartate carbamoyltransferase when MgATP and bicarbonate are present, but significantly later and just ahead of glutamine synthetase when glutamine is present and MgATP and bicarbonate are absent. Carbamoyl-phosphate synthetase also elutes at a somewhat earlier position when ATP and bicarbonate are present, but glutamine synthetase is not significantly affected. Similar results were obtained when chromatography was carried out using Sephacryl S-300 or the shorter (2.2 cm × 20 cm) Sephadex G-200 column (to minimize elution time). Estimation of the molecular size of these enzymes based upon their elution position from the Sephacryl column relative to proteins of known M_r gave values of 2.9×10^5 (3×10^5), 3.8×10^5 (6.4×10^5), 4.8×10^5 (10×10^5), and 10×10^5 (10×10^5) for glutamine synthetase, carbamoyl-phosphate synthetase, dihydro-orotase, and aspartate carbamoyltransferase, respectively (values in parentheses represent values obtained when MgATP and bicarbonate are present instead of glutamine). Because of the large volumes of extract added to the columns these values are subject to considerable

error, but the estimates do indicate that these enzymes, particularly aspartate carbamoyltransferase, elute as fairly high-M_r entities.

The elution profiles suggest that carbamoyl-phosphate synthetase, aspartate carbamoyltransferase, and dihydro-orotase exist as separate polypeptide chains rather than as three enzyme activities associated with a single polypeptide chain as found in mammalian tissues. An effort was made to minimize proteolysis (protease inhibitors, rapid analysis) and there was no evidence of association in the elution profiles when either glutamine or ATP and bicarbonate were present. The single multifunctional polypeptide chain possessing the three activities from hamster cells has an M_r of 240000, associates to give oligomers of various sizes (predominantly hexamers) and can be cleaved by proteolytic enzymes to give enzymically active fragments, suggesting that different regions of the polypeptide are folded into separate domains, each domain representing one of the enzyme activities. Partial proteolysis of the multifunctional complex can be carried out to give a fragment with aspartate carbamoyltransferase activity that has a M_r of 60000, which associates to a trimer and under some circumstances can undergo considerable further aggregation; partial proteolysis can also yield a fragment with dihydro-orotase activity that has an M_r of 44000 and which associates to a dimer (Evans, 1986; Grayson *et al.*, 1985; Grayson & Evans, 1983; Davidson *et al.*, 1981; Kelly *et al.*, 1986). On the basis of these observations, the different elution volumes and the apparently large size of the enzymes from dogfish spleen could be due to specific association of each of the enzymically active individual domains representing each enzyme activity after proteolysis of a multifunctional complex. However, no evidence for association of these three activities was obtained under several different conditions (presence of either glutamine or MgATP plus bicarbonate in the extraction and elution buffers; presence or absence of protease inhibitors). The observation that the apparent molecular sizes of dihydro-orotase and carbamoyl-phosphate synthetase are increased when MgATP and bicarbonate are present (presumably representing specific association), and the three enzyme activities clearly remain separate entities, would appear to reflect specific properties of individual proteins (the position of the aspartate carbamoyltransferase elution profile remains unchanged when MgATP and bicarbonate are present during gel filtration chromatography on Sephacryl S-300, indicating that even though dihydro-orotase elutes in a position nearly identical to that of aspartate carbamoyltransferase under these conditions, it is probably not complexed with the aspartate carbamoyltransferase). Carrey (1986) reported that the presence of MgATP significantly reduces the susceptibility of the mammalian multifunctional enzyme to proteolysis. Even without the presence of protease inhibitors these three activities have been reported to copurify as a single entity when purified from mouse spleen or rat liver, suggesting that the activities remain associated under nondenaturing conditions even if proteolysis occurs in the regions between the domains representing the three different enzyme activities (Hoogenraad *et al.*, 1971; Mori & Tatibana, 1973). Mukherjee *et al.* (1988) recently reported that these three enzymes apparently do exist as separate polypeptide chains in *Leishmania donavani*, a lower eucaryote; Tampitag & O'Sullivan (1986) and Aoki & Oya (1987) have reported similar observations in protozoan parasites.

Dihydro-orotate dehydrogenase, orotate phosphoribosyltransferase, and orotidine 5′-decarboxylase

The activities of these enzymes, which catalyse the last three steps of the pyrimidine nucleotide pathway to give UMP, were also found to be present in spleen extracts, but the levels of activity were too low for accurate estimation of tissue activity or determination of their subcellular distribution using the colorimetric assay methods available at the time and location of this study. Dihydro-orotate dehydrogenase activity could be assayed in the subcellular fractions prepared as outlined in Table 1 and was found to be associated only with the particulate fractions, about 50% in the 'mitochondria' fraction and 25% in the 'microsomal' fraction. In mammalian tissues this enzyme is associated with the mitochondrial outer membrane, but is readily solubilized; the other two enzymes are localized in the cytosol (Keppler & Holstege, 1982; Jones, 1980). The presence of these enzyme activities are clearly indicated in the elution profile obtained from gel filtration chromatography on Sephacryl S-300 (Fig. 3). Dihydro-orotate dehydrogenase activity is excluded from Sephacryl S-300, indicating a species of high molecular mass, which could represent a mitochondrial fragment obtained after sonication. The other two enzyme activities appear to elute together and at a position corresponding to an M_r of about 150000. These two enzymes exist as a multifunctional complex involving a single polypeptide chain having an M_r of about 50000 in mammalian species, but which can associate to a dimer (Keppler & Holstege, 1982).

Conclusion

These results clearly establish that spiny dogfish express two different glutamine-dependent carbamoyl-phosphate synthetases. As indicated by our previous studies, the function of the mitochondrial glutamine-dependent carbamoyl-phosphate synthetase in liver is related to urea synthesis, and this enzyme is present at high concentrations. The extremely low level of carbamoyl-phosphate synthetase activity reported in spleen in this study is localized in the cytosol and the function of this enzyme is apparently clearly related to formation of carbamoyl phosphate for pyrimidine nucleotide biosynthesis. The kinetic properties of this carbamoyl-phosphate synthetase and the subcellular localization of the carbamoyl-phosphate synthetase and other enzymes of the pyrimidine nucleotide biosynthetic pathway in dogfish spleen appear to be analogous to that of mammalian species. Unlike mammalian species, however, the first three enzymes of the pathway do not appear to be associated as a multifunctional complex consisting of a single polypeptide chain.

Our results also confirm the observation by Smith *et al.* (1987) of existence of a cytosolic glutamine synthetase in extrahepatic tissue that is different from the mitochondrial glutamine synthetase in the liver. The absence of aspartate carbamoyltransferase activity in the liver suggests that pyrimidine nucleotide synthesis *de novo*, and the glutamine-dependent carbamoyl-phosphate synthetase related to the pyrimidine nucleotide pathway, may exist only in extrahepatic tissues and that the liver would be dependent on salvage pathways and/or dietary

sources for nucleotide formation in these species. [These activities are also present in testis at levels comparable to spleen (results not shown).] This would be consistent with the specific localization of liver glutamine synthetase in mitochondria; in contrast to avian species, the glutamine formed in the mitochondria may all be utilized for urea synthesis and, therefore, would not be available as a substrate in the cytosol for pyrimidine nucleotide biosynthesis (or other pathways involving glutamine as a source of nitrogen in reactions catalysed by an appropriate amidotransferase). These observations indicating that glutamine may not be available in liver for reactions other than mitochondrial carbamoyl phosphate formation related to the urea cycle and the observation that glutamine is absent from serum in elasmobranchs (Leech *et al.*, 1979) may have a bearing on reports that sharks apparently have an unusually low incidence of neoplasms (Bodine *et al.*, 1985; Lee & Langer, 1983). Glutamine is a major source of nitrogen and is a significant source of energy for tumour growth (Quesada *et al.*, 1988; Moreadith & Lehninger, 1984).

Glutamine- and acetylglutamate-dependent carbamoyl-phosphate synthetase is also present in liver of largemouth bass (*Micropterus salmoides*), a freshwater teleost, but at much lower levels than in marine elasmobranchs (Anderson, 1976, 1980; Casey & Anderson, 1983). In contrast to dogfish, however, aspartate carbamoyltransferase activity is present in liver of bass (Anderson, 1976), and it will, therefore, be of interest to determine whether the function of the glutamine- and acetylglutamate-dependent carbamoyl-phosphate synthetase in bass is related to pyrimidine nucleotide biosynthesis.

This work was supported by National Science Foundation Research Grant DCB 8608090. The author would like to express appreciation to the University of Washington, Friday Harbor Laboratories, for use of their facilities, to Drs. J. W. Campell and D. D. Smith, Jr., for carrying out the immunoblot analysis, to Drs. D. R. Evans and C. A. Luer for helpful discussions, and to Carol Anderson for excellent technical assistance.

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Received 29 December 1988/24 February 1989; accepted 1 March 1989