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Phylogenetic aspects of carbamoyl phosphate synthetase in lungfish: A transitional enzyme in transitional fishes

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ABSTRACT

Carbamoyl phosphate synthetase (CPS) catalyses the formation of carbamoyl phosphate from glutamine or ammonia, bicarbonate and ATP. There are three different isoforms of CPS that play vital roles in two metabolic pathways, pyrimidine biosynthesis (CPS II) and arginine/urea biosynthesis (CPS I and CPS III). Gene duplication has been proposed as the evolutionary mechanism creating this gene family with CPS II likely giving rise to the CPS I/III clade. In the evolutionary history of this gene family it is still undetermined when CPS I diverged from CPS III on the path to terrestriality in the vertebrates. Transitional organisms such as lungfishes are of particular interest because they are capable of respiring via gills and with lungs and therefore can be found in both aquatic and terrestrial environments. Notably, enzymatic characterization of the mitochondrial CPS isoforms in this transitional group has not led to clear conclusions. In order to determine which CPS isoform is present in transitional animals, we examined partial sequences for liver CPS amplified from five species of lungfish, and a larger fragment of CPS from one lungfish species (*Protopterus annectens*) and compared them to CPS isoforms from other fish and mammals. Enzyme activities for *P. annectens* liver were also examined. While enzyme activities did not yield a clear distinction between isoforms (virtually equal activities were obtained for either CPS I or III), CPS sequences from the lungfishes formed a monophyletic clade within the CPS I clade and separate from the CPS III clade of other vertebrates. This finding implies that the mitochondrial isoform of CPS in lungfish is derived from CPS I and is likely to have a physiological function similar to CPS I. This finding is important because it supports the hypothesis that lungfish employ a urea cycle similar to terrestrial air-breathing vertebrates.

1. Introduction

Carbamoyl phosphate synthetase (CPS) is an essential enzyme found in all classes of organisms and is involved in pyrimidine biosynthesis and the ornithine–urea cycle (O-UC)/arginine biosynthesis (Anderson, 1995a; Holden et al., 1999). The evolution of the CPS gene family provides a novel way to examine questions related to nitrogen metabolism as vertebrates transitioned from an aquatic to a terrestrial lifestyle. Nitrogen metabolism in fishes has two primary end products, ammonia and urea (Griffith, 1991; Wood, 1993; Wright, 1995; Walsh, 1998). Ammonia toxicity is a biological consequence of nitrogen metabolism that both aquatic and terrestrial organisms must overcome (Korsgaard et al., 1995; Anderson, 2001; Ip et al., 2001). Aquatic organisms avoid ammonia toxicity by excreting excess ammonia to the external aqueous environment (Korsgaard et al., 1995; Ip et al., 2001; Wilkie 2002; Weihrauch et al., 2009), whereas many terrestrial vertebrates metabolize ammonia to urea via the O-UC and then excrete urea (Mommsen and Walsh, 1992; Janis and Farmer, 1999; Ip et al., 2001; Randall and Tsui, 2002; Ip et al., 2004a,b; Sayer, 2005). Ammonia is utilized during the formation of carbamoyl phosphate via CPS directly (or indirectly via glutamine synthetase (GS)) for both of the pathways.

Three different isoforms of CPS have been described: CPS II (EC 6.3.5.5) is involved in pyrimidine synthesis, while the other two isoforms, CPS I (EC 6.3.4.16) and CPS III (EC 6.3.5.5), are involved with nitrogen metabolism via the O-UC. All isoforms result in the production of carbamoyl phosphate, but they differ in their nitrogen donor substrate, allosteric effectors, co-factors and their cellular location (Table 1). CPS I and III have been described in animals and are involved in the O-UC (Hong et al., 1994; Anderson, 1995a,b; 2001). Both CPS I and III are localized to the mitochondrial matrix and require N-acetyl glutamate (NAG) as a co-factor, but CPS I utilizes ammonia as a nitrogen donor while CPS III utilizes glutamine (Hong et al., 1994; Anderson, 1995a,b; van den Hoff et al., 1995). While enzymatic studies have described CPS I mainly in terrestrial vertebrates, CPS III has been described mainly in invertebrates and fishes, including the coelacanth (Brown and Brown, 1967; Goldstein et al. 1973; Mommsen

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Table 1
Differences in the CPS isoforms found in vertebrates.
Modified from Anderson, 1995a.

CPS isoform	Nitrogen substrate	Allosteric effector	Cellular location	Biochemical pathway
CPS I	Ammonia	N-acetylglutamate	Mitochondrial matrix	O-UC/arginine
CPS II	Glutamine	UTP (−), PRPP (+)	Cytosol	Pyrimidine biosynthesis
CPS III	Glutamine	N-acetylglutamate	Mitochondrial matrix	O-UC/arginine

and Walsh, 1989; Hong et al., 1994; Anderson, 1995a,b; Felskie et al., 1998; Kong et al., 1998; Anderson, 2001).

The proposed evolution of the CPS gene/protein family is that CPS I/CPS III in eukaryotes likely arose from a CPS II-like gene and CPS I arose from CPS III (Hong et al., 1994; Anderson, 1995a; van den Hoff et al., 1995). It has been proposed that the prokaryote CPS II formed from a fusion of a glutamine amidotransferase domain (GAT) and two synthetase domains (Hong et al., 1994; Anderson, 1995a; van den Hoff et al., 1995). In eukaryotes a mitochondrial signal and NAG binding site were added to CPS I/CPS III and later terrestrial vertebrates lost the glutamine binding site (Hong et al., 1994; Anderson, 1995a; van den Hoff et al., 1995). Phylogenetic studies of all CPS isoforms to date revealed that the CPS II isoform is basal to the CPS I/III clade and some authors have suggested multiple gene duplication events led to the diversification of the CPS isoforms (van den Hoff et al., 1995; Lawson et al., 1996; Zhou et al., 2000; Lindley et al., 2007). Hong et al. (1994) suggested that CPS III represents an evolutionary intermediate between CPS II and CPS I and an analysis of the evolution of urea synthesis in vertebrates by Mommsen and Walsh (1989) indicated that the origins of CPS I may have arisen within the lungfish group. Recent studies on O-UC enzyme activities in lungfishes described CPS III activity in *Protopterus dolloi*, *P. aethiopicus*, and *P. annectens* and those authors also showed that both glutamine and ammonia can act as the nitrogen substrate for CPS III, although the activity is much less when the nitrogen substrate utilized is ammonia (Chew et al., 2003; Loong et al., 2005). These findings led Loong et al. (2005) to suggest that CPS I was not derived from CPS III before the evolution of extant lungfishes. Notably, studies of lungfish CPS to date have relied exclusively on enzyme activity measurements.

The dipnoans or dual air-breathers represented by lungfishes are an ideal group for examining the evolution of nitrogen metabolism and specifically CPS evolution due to the unique aquatic and terrestrial environments that they inhabit. Lungfishes are seasonally exposed to dry periods where they no longer have access to water to excrete their excess nitrogenous wastes and they must find ways to reduce ammonia levels (Graham and Lee, 2004). Some lungfishes overcome this problem by decreasing overall ammonia production during their exposure to land and increasing their *de novo* synthesis of urea via the O-UC (Chew et al., 2004; Ip et al., 2004a, 2005; Loong et al., 2005). Increased urea production also has the added benefit of decreasing water loss (Griffith, 1991).

Lungfishes are members of the Sarcopterygii or lobe-finned fishes which are thought to be the first among the extant vertebrates to transition from water to land. There are only six extant species of lungfishes, represented by three families and three genera, all of which reside in fresh water (Graham, 1997; Brinkmann et al., 2004a; Nelson, 2006). Phylogenies created for extant lungfishes based on morphological characteristics, including skull structure, dentition, urogenital systems and external gills, place the Australian lungfish, *Neoceratodus forsteri*, as the most ancestral species (Miles, 1977; Marshall, 1987; Wake, 1987; Lundberg, 1993; Tokita et al., 2005). The South American lungfish, *Lepidosiren paradoxa* and the African lungfishes, the *Protopterus* species, are classified as the sister taxa and these two groups represent the most derived condition for the lungfishes (Miles, 1977; Marshall, 1987; Wake, 1987; Lundberg, 1993; Tokita et al., 2005). Molecular studies have also found similar familial relationships for living lungfishes, with Ceratodontidae being ancestral to Lepidosirenidae and Protopteridae, which are sister groups (Meyer and Dolven, 1992; Hedges et al., 1993; Zardoya and Meyer, 1996; Brinkmann et al., 2004a, 2004b; Tokita et al., 2005; Mallatt and Winchell, 2007).

With this background in mind, we performed phylogenetic analyses of a CPS fragment isolated from the liver of five of the six extant species of lungfishes in order to determine both the type of CPS isoform(s) present in lungfishes as well as the phylogenetic relationships of CPS with respect to lungfish. We also performed phylogenetic analyses on a larger fragment of CPS in *P. annectens* that spanned both the GAT domain and the synthetase domain.

2. Materials and methods

2.1. Tissue collection and preservation

P. dolloi, *P. annectens*, *P. aethiopicus* and *L. paradoxa* were imported from Africa to Singapore and were acclimatized in the laboratory for one month in plastic aquaria filled with water containing 2.3 mmol L^{-1} Na^{+}, 0.54 mmol L^{-1} K^{+}, 0.95 mmol L^{-1} Ca^{+2}, 0.08 mmol L^{-1} Mg^{+2}, 3.4 mmol L^{-1} Cl^{-} and 0.6 mmol L^{-1} HCO_3^{-}, at pH 7.0 and 25 °C (Loong et al., 2005). Water was changed daily, fish were fed frozen bloodworms during their acclimatization and a 12 h:12 h light:dark cycle was maintained. *N. forsteri* was imported to Macquarie University in Sydney, Australia from Queensland, Australia by Dr. Jean Joss and kept in a 1700 L fiberglass tank in fresh water (0 ppt) on a natural light cycle. Food was withdrawn 48 h prior to the experiment. Fish were euthanized by a blow to the head and liver tissue was excised and immediately placed in RNA*later*® (Ambion®) at a ratio of 4:1 RNA*later*®:tissue and then frozen and stored at −80 °C. Liver tissue of *P. annectens* was also saved for CPS enzyme activity assays and was flash frozen in liquid nitrogen.

2.2. CPS enzyme activity assay

Approximately 100 mg of liver was homogenized on ice in a 2.0 mL microcentrifuge tube using an IKA Ultra Turrax® T8 Homogenizer in 4× volume of homogenization buffer (20 mmol L^{-1} K_2HPO_4, 10 mmol L^{-1} HEPES, 0.5 mmol L^{-1} EDTA, 1 mmol L^{-1} dithiothreitol, in 50% glycerol, pH 7.5 at 25 °C). Homogenates were centrifuged for 5 min at 4 °C in an Eppendorf® 5415D centrifuge at 16,000 *g* and the supernatant was used directly. CPS enzyme activity for both CPS I and CPS III was assayed for *P. annectens* based on the colorimetric production of citrulline similar to the methods used by Mommsen and Walsh (1989) and Kajimura et al. (2006).

2.3. RNA extractions

Chaotropic RNA extractions were carried out from lungfish liver tissue (100 mg) using the methods of Whitehead and Crawford (2005). RNA was purified using the RNeasy® mini protocol (Qiagen) and quantified and the quality checked by using a NanoDrop ND-1000 spectrophotometer. RNA was re-precipitated by adding 0.1 volume of 3M sodium acetate followed by 2.5 volumes of 100 % ethanol and stored at –20 °C.

2.4. Amplification of small fragments of CPS gene in five species of lungfish

CPS primers "2" and "3" (I and J in Supplemental Table 1) designed by Korte et al. (1997) were used to amplify CPS product from hepatic RNA. A modified Qiagen OneStep RT-PCR protocol was utilized where both RT reaction and PCR reaction are performed in the same tube. Each One-Step RT-PCR reaction was performed in 25 μL and utilized