

Muscle as the Primary Site of Urea Cycle Enzyme Activity in an Alkaline Lake-adapted Tilapia, *Oreochromis alcalicus grahami**

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The tilapia fish *Oreochromis alcalicus grahami* from Kenya has adapted to living in waters at pH 10.5 by excreting the end product of nitrogen metabolism as urea rather than as ammonia directly across the gills as occurs in most fish. The level of activity in liver of the first enzyme in the urea cycle pathway, carbamoyl-phosphate synthetase III (CPSase III), is too low to account for the observed high rates of urea excretion. We report here the surprising finding that CPSase III and all other urea cycle enzyme activities are present in muscle of this species at levels more than sufficient to account for the rate of urea excretion; in addition, the basic kinetic properties of the CPSase III appear to be different from those of other known type III CPSases. The sequence of the CPSase III cDNA is reported as well as the finding that glutamine synthetase activity is present in liver but not in muscle. This unusual form of adaptation may have occurred because of the apparent impossibility of packaging the needed amount of urea cycle enzymes in liver.

Most teleostean fishes are ammonotelic, excreting nitrogen wastes across their gills as ammonia (1, 2). An exception is a tilapia (*Oreochromis alcalicus grahami*) from Lake Magadi in Kenya, which has adapted to conditions of high pH (10–10.5) by using ureotelism as a means of nitrogen excretion (3–5). However, the level of activity in liver of the first enzyme in the urea cycle pathway, carbamoyl-phosphate synthetase III (CPSase III),¹ is too low to account for the observed high rates of urea excretion (3–5). We report here the surprising finding that CPSase III and all other urea cycle enzyme activities are present in muscle of this alkaline lake-adapted tilapia at levels more than sufficient to account for the rate of urea excretion; in

addition, the basic kinetic properties of the CPSase III appear to be different from those of other known type III CPSases. The sequence of the CPSase III cDNA is reported as well as the finding that GSase activity is present in liver but not in muscle.

In ureotelic terrestrial vertebrates the complete urea cycle pathway is confined to liver and the first step is catalyzed by mitochondrial CPSase I, which requires the allosteric effector AGA for activity and utilizes only ammonia as the nitrogen-donating substrate; CPSase I is thought to have evolved from CPSase III (6). CPSase III, found in invertebrates and some fish, differs from CPSase I by the fact that it can utilize glutamine as the nitrogen-donating substrate (6). In liver of the two species of teleost fishes where expression of ureotelism has been well documented, CPSase III is localized exclusively in the mitochondrial matrix, as occurs in ureotelic terrestrial vertebrates, but in contrast to ureotelic terrestrial vertebrates, GSase is also localized entirely or partially in the mitochondria (7–11). CPSase III and ornithine carbamoyltransferase, which catalyzes the second step of the urea cycle, activities have recently been reported in muscle of several different species of teleostean fishes (12–15), but the levels of CPSase III activity are extremely low (*e.g.* 0.5–2 nmol/min/g of muscle), and the physiological significance, if any, has not been established. These observations prompted us to examine muscle as a site for urea cycle activity in the tilapia.

EXPERIMENTAL PROCEDURES

Lake Magadi tilapia (*O. a. grahami*) were captured by seine net from Fish Springs Lagoon in Lake Magadi, Kenya in February 1997. Following a 20-min transport time to an on-site laboratory, fish were anesthetized in metomidate, and tissues were rapidly dissected. For studies of urea cycle enzymes, tissues were flash-frozen in liquid nitrogen, transported to North America in a dry shipper, and kept at –70 °C until use.

Extracts for measuring units/gram of tissue of urea cycle enzymes in frozen samples (Table I) were prepared and assayed as described previously (7, 12), except tissue samples were smaller (0.2 g), and the ATP-regenerating system was not used in the CPSase assay.

Subcellular localization studies (Table II) of GSase, glutaminase, and arginase were conducted on-site using the isolation and assay methods previously described (7), except homogenates were centrifuged at 4 °C at 82 × *g* for 5 min to pellet the debris fraction and the resulting supernatant at 13,800 × *g* for 5 min to pellet the mitochondrial fraction. Glutaminase was assayed as described previously (16). Lactate dehydrogenase and glutamate dehydrogenase were assayed as cytosolic and mitochondrial marker enzymes, respectively, as described previously (7).

Muscle CPSase III was partially purified and its molecular weight estimated by gel filtration chromatography. Muscle tissue (0.6 g) stored at –80 °C was homogenized with 2.5 ml of 0.1 M HEPES buffer, pH 7.5, containing 1 mM dithiothreitol, 0.1 M KCl, 0.1 mM EDTA, and 15% glycerol, sonicated, and centrifuged as described previously (13). The supernatant was applied to a Sephacryl S-300 column (0.8 × 25 cm)

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF119250.

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¹ The abbreviations used are: CPSase, carbamoyl-phosphate synthetase; GSase, glutamine synthetase; AGA, *N*-acetyl-L-glutamate; PAGE, polyacrylamide gel electrophoresis.

TABLE I

Urea cycle enzymes in muscle and liver of *O. a. grahami*

Values are means \pm S.E. ($n = 3$). OCTase, ornithine carbonyltransferase; ASSase, argininosuccinate synthetase; ASLase, argininosuccinate lyase.

	Muscle	Liver
	$\mu\text{mol}/\text{min}/\text{g tissue}$	
CPSase		
Ammonia as substrate	0.19 \pm 0.02	0.06 \pm 0.02
Glutamine as substrate	0.17 \pm 0.02	0.04 \pm 0.01
OCTase	4.37 \pm 1.10	3.0 \pm 0.5
ASSase/ASLase	0.19 \pm 0.06	0.01 \pm 0.01
Arginase	38 \pm 12	51.0 \pm 5.7
GSase (γ -glutamyltransferase)	0.31 \pm 0.01	6.2 \pm 2.2

equilibrated with the same buffer used to prepare extract and eluted at 4 °C in 1.2-ml fractions. The peak of enzyme activity (in three or four fractions) was pooled and used for the kinetic studies. The molecular size of the CPSase was estimated by comparison of the elution volume with the elution volumes of cyanase and alcohol dehydrogenase (molecular weights of 170,000 and 150,000, respectively), which had been added to the extract before placing on the column.

Kinetic studies of the CPSase III (Table III) were conducted on enzyme from muscle extracts that had been partially purified as noted above. The reaction mixtures contained 10 mM glutamine or 10 mM NH_4Cl , 25 mM MgCl_2 , 20 mM ATP, 5 mM $^{14}\text{C}[\text{HCO}_3^-]$ (1×10^6 cpm), 50 mM Hepes buffer, pH 7.6, 50 mM KCl, and 0.8 mM AGA where indicated. K_m and V_{max} values were determined by measuring rates at different concentrations of ATP (5 mM excess MgCl_2), AGA, NH_4Cl , or glutamine, respectively, and fitting the data to the Lineweaver-Burk equation for double-reciprocal plots of rate versus concentration with weighting to the fourth power; correlation coefficients were >0.97 . Where the plots were not linear, the concentration that gave a half-maximal rate is cited for K_m .

SDS-PAGE was carried out essentially as described previously (17). For sequencing, the protein bands were blotted to a polyvinylidene difluoride membrane and the N-terminal sequence of the tilapia muscle band with the same mobility as rat CPSase I was determined by the Mayo Protein Core Facility (Rochester, MN).

The sequence of the CPSase III cDNA was determined following previously described procedures for RNA isolation (from tilapia muscle), cDNA synthesis, analysis and isolation of PCR products, strategy for obtaining CPSase III-specific cDNA segments by PCR using consensus primers and sequence analysis of these segments, the strategy for sequencing the remainder of the CPSase III cDNA by primer walking, and sequence analysis (12, 13). Numbering of amino acid residues of the deduced CPSase III amino acid sequence begins with the initial methionine residue of the entire translated product, *i.e.* including the mitochondrial targeting sequence.

RESULTS

As shown in Table I, all five urea cycle enzymes are present in both liver and muscle, but except for arginase the levels are higher in muscle; this is particularly true for CPSase III, which appears to be rate-limiting on the basis of maximal activity. The levels of activity in muscle are surprisingly high, comparable with the levels of these enzymes in liver of ureosmotic elasmobranchs (18) and the ureogenic teleost gulf toadfish (*Opsanus beta*) (7, 19). Liver and muscle represent ≈ 2 and 60% of total mass, respectively,² so the total units of CPSase III in muscle are ≈ 130 times that of liver. The rate of urea excretion in tilapia has been reported to be about 0.07 $\mu\text{mol}/\text{min}/\text{g}$ of fish (3–5). Assuming saturating concentrations of substrate for CPSase III, this rate can be readily sustained by the level of either ammonia- or glutamine-dependent CPSase III ($\approx 0.11 \mu\text{mol}/\text{min}/\text{g}$ of fish), but the level of CPSase III in liver ($<0.001 \mu\text{mol}/\text{min}/\text{g}$ of fish) is not nearly sufficient to account for this rate of urea excretion.

GSase γ -glutamyltransferase activity is present in liver, but there is very little in muscle where high levels of CPSase III are

localized (Table I). In addition, the GSase in liver is localized in the cytosol (Table II), analogous to largemouth bass (*Micropterus salmoides*) where the liver mitochondrial glutamine-dependent CPSase III appears to have little or no function (13). Since GSase biosynthetic activity is $\approx 5\%$ of the transferase activity (7, 20), the biosynthetic GSase activity in liver and muscle together ($\approx 0.02 \mu\text{mol}/\text{min}/\text{g}$ of fish) is not sufficient to support urea synthesis if urea-related carbamoyl phosphate formation required glutamine.

Based on these results, we examined the properties of tilapia CPSase III to determine its ability to use ammonia directly as nitrogen-donating substrate. The general kinetic properties of the muscle CPSase III are summarized in Table III. In other type III CPSases: 1) the V_{max} with ammonia as substrate is 10% or less than the V_{max} with glutamine as substrate, 2) AGA under normal assay conditions is required for activity, 3) the binding of glutamine and AGA are synergistic (*i.e.* as the concentration of one is increased, the K_m for the other decreases), and 4) the K_m for glutamine is 0.1–0.2 mM (21, 22). In contrast, for the tilapia CPSase III assayed with 20 mM MgATP: 1) the V_{max} with ammonia as substrate is greater than the V_{max} with glutamine, 2) AGA is not required for activity with either ammonia or glutamine as nitrogen-donating substrate and the presence of AGA increases V_{max} only slightly, 3) AGA does not affect the apparent K_m for glutamine (or ammonia), and 4) the K_m for glutamine is quite high. Similar results are obtained at 1 mM MgATP, except that the K_m for ammonia in the absence of AGA is lower (1.8 mM). The K_m values of 2–5 mM for ammonia are comparable with those observed for the ammonia-dependent activity of other type III (21, 22) and type I (23) CPSases. Like the ureosmotic spiny dogfish shark (*Squalus acanthias*, a representative ureosmotic elasmobranch) CPSase III (21) and type I CPSases (23), however, AGA does affect MgATP binding and, therefore, activity at physiological concentrations of ATP. Marked sigmoid kinetics (nonlinear double-reciprocal plots of rate versus MgATP concentration) are observed when AGA is absent (half-maximal rate obtained at 0.7 mM MgATP), but hyperbolic Michaelis-Menten kinetics (linear double reciprocal plots of rate versus MgATP concentration) with glutamine ($K_m = 0.2$ mM) as substrate or greatly reduced sigmoid kinetics with ammonia (half-maximal rate obtained at 0.2 mM) as substrate are observed when AGA is saturating. Other properties of the tilapia CPSase III include: activities with glutamine and ammonia are not additive as is observed for the ammonia- and glutamine-dependent CPSase activities in liver extracts from an Indian catfish (*Heteroptneustes fossilis*) (24), which has invited speculation that CPSase I and CPSase III activities may both be present in *H. fossilis*; like all other CPSases, K^+ is required for activity ($K_m = 0.01$ M); pH optimum is 7.8; K_m for AGA is <0.01 mM; activity is not inhibited by UTP, a negative allosteric effector for the pyrimidine pathway-related CPSase II (6); asparagine cannot replace glutamine as substrate. When subjected to gel filtration chromatography, CPSase activity eluted between alcohol dehydrogenase and cyanase, corresponding to a maximum molecular weight of $\approx 160,000$. This estimated value is typical of all type I and type III CPSases (6) and is in close agreement with the molecular weight of 160,760 calculated from the amino acid sequence (without the mitochondrial leader sequence), indicating that the CPSase III exists as a monomer under the described gel filtration chromatography conditions.

The amino acid sequence of the tilapia CPSase III is homologous to other CPSases, *e.g.* 86, 71, and 50% identity with CPSase III from largemouth bass (13), CPSase I from rat (25), and shark CPSase II (26), respectively. The sequence has the same domain structure as other CPSases and the same highly

² P. J. Walsh, unpublished data.

TABLE II
Enzyme activities in different subcellular fractions of *O. a. grahami* liver

Values are means \pm S.E. ($n = 3$). LDHase, lactate dehydrogenase; GDHase, glutamate dehydrogenase; GLNase, glutaminase.

Enzyme	Liver	Percent of total		
		Debris	Soluble	Mitochondrial
	$\mu\text{mol}/\text{min}/\text{g}$			
LDHase	22.5 ± 6.0	9.3 ± 2.6	86.2 ± 3.8	4.5 ± 1.4
GDHase	11.3 ± 2.2	17.2 ± 5.0	13.6 ± 3.7	69.2 ± 2.0
GSase	6.2 ± 2.2	5.6 ± 1.0	93.5 ± 1.5	0.9 ± 0.6
GLNase	1.9 ± 0.4	32.3 ± 2.2	1.9 ± 0.8	65.9 ± 1.4
Arginase	50.9 ± 5.7	22.2 ± 3.0	67.5 ± 3.5	10.3 ± 5.3

TABLE III
Summary of kinetic properties of *O. a. grahami* CPSase III

	-AGA	+AGA
K_m (glutamine)	1.5 mM	1.9 mM
K_m (ammonia)	5.6 mM	5.2 mM
K_m (MgATP)		
Glutamine as substrate	0.7 mM (half-maximal)	0.2 mM
Ammonia as substrate	0.7 mM (half-maximal)	0.2 mM (half-maximal)
V_{\max} (glutamine)	0.019 $\mu\text{mol}/40$ min	0.023 $\mu\text{mol}/40$ min
V_{\max} (ammonia)	0.037 $\mu\text{mol}/40$ min	0.041 $\mu\text{mol}/40$ min

conserved histidine residues that have been shown to have an important mechanistic function (6). The open reading frame codes for 1,535 amino acid residues, the first 37 of which correspond to an expected mitochondrial signal sequence which, when removed, would yield a mature protein with the N-terminal sequence FSVKTQTAHL; the sequence also has the expected cysteine residues required for activity with glutamine (Cys-298) and binding of AGA (Cys-1352 and Cys-1362) (6).

CPSase III from shark and smallmouth bass have turnover rates of ≈ 0.2 $\mu\text{mol}/\text{min}/\text{mg}$ of protein at 26 °C with glutamine as substrate (21, 22). If the turnover rate of the tilapia CPSase III is similar, the presence of 0.2 unit of activity/g of muscle would correspond to ≈ 1 mg of CPSase/g of muscle, which should be detectable in crude extracts by SDS-PAGE. As shown in Fig. 1, a protein band with the same mobility as rat CPSase I can be readily detected in tilapia, but not in trout, which has very little CPSase III activity (12). The identity of this band as CPSase III was confirmed by showing that the sequence of the first 10 amino acid residues of the protein in this protein band was identical to the N-terminal sequence predicted for the mature protein after removal of the mitochondrial signal sequence as noted above, which also indicates that the enzyme is probably localized in the mitochondria. When aliquots of fractions obtained after gel filtration chromatography were subjected to SDS-PAGE, the intensity of this protein band correlated exactly with the units of CPSase III activity. Quantitation of the intensities of the two bands (rat CPSase I and tilapia CPSase III) indicated that there is at least 0.7 mg of CPSase III/g of tilapia muscle if staining of the tilapia CPSase III is the same as rat CPSase I.

As noted in Table II, liver arginase, which catalyzes the last step of the urea cycle pathway and the subcellular localization of which is quite variable in teleosts (2, 7, 27), appears to be localized predominantly in the cytosol in this tilapia. The level of glutaminase, which is localized in the mitochondria as expected, is significantly higher (≈ 20 times) than in *O. beta*, which appears to utilize glutamine as substrate for the liver mitochondrial CPSase III (7).

DISCUSSION

This is the first report of the presence of all five urea cycle enzymes in muscle of fish or muscle of any species. The high

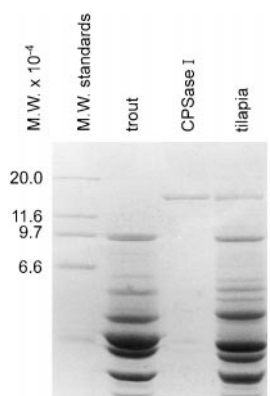


FIG. 1. SDS-PAGE of purified rat CPSase I and of trout and tilapia extracts of muscle. Lane 1, molecular weight standards. The amount of protein applied was 0.34 mg of rat CPSase I and that amount of trout and tilapia extract, which corresponded to 0.5 mg of muscle. The arrow indicates the bands for CPSase.

levels of all urea cycle enzymes in tilapia muscle reported here identify muscle as the likely major site for urea formation in this fish. This finding highlights the possible significance of recent reports of low levels of CPSase III and ornithine carbamoyltransferase activities in muscle of several other species of fish (12–15). The presence in muscle of a urea cycle pathway that is normally expressed at high levels only under specific environmental (3–5, 7–11) or life cycle (12) circumstances may be a common characteristic of fish.

The properties of the tilapia CPSase III differ from other type III CPSases, most notably by the facts that the enzyme has substantial activity in the absence of AGA and that ammonia appears to be as good a nitrogen-donating substrate as glutamine. The latter would be consistent with the very low level of GSase activity in muscle and insufficient levels in liver to provide glutamine for the CPSase III in muscle. Thus, adaptation may also include kinetic changes that favor ammonia as the primary substrate.

At the observed urea excretion rate, a 100-g tilapia fish would require ≈ 25 mg of CPSase III/g of liver if CPSase III was located only in liver. CPSase I in liver of ureotelic mammalian species accounts for $\approx 25\%$ of the total protein in the mitochondrial matrix and this is equivalent to only 5 mg of CPSase I/g of liver (28). If the CPSase III is located in the muscle, however, then the 50 mg CPSase III would be distributed in 60 g of tissue, or about 0.8 mg/g of muscle, close to the value reported here. We speculate that it may be impossible to achieve the observed high rate of urea excretion by packaging the required amount of urea cycle enzymes in the mitochondrial matrix of the small liver in this species. Because these fish require a laterally compressed body plan for efficient swimming, it is unlikely that additional ureogenesis through increased liver mass could be accommodated by the visceral cavity. Thus, it appears that adaptation to ureogenesis in this species includes a change in organ localization of the urea cycle enzymes.

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