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 (CP-Sase III), is too low to account for the observed high rates of urea excretion. We report here the surprising finding that CP-Sase 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 CP-Sase III appear to be different from those of other known type III CP-Sases. The sequence of the CP-Sase 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 (CP-Sase III), is too low to account for the observed high rates of urea excretion (3–5). We report here the surprising finding that CP-Sase 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 CP-Sase III appear to be different from those of other known type III CP-Sases. The sequence of the CP-Sase 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.

Muscle CP-Sase 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 (11). The supernatant was applied to a Sephacryl S-300 column (0.8 × 25 cm)
TABLE I
Urea cycle enzymes in muscle and liver of O. a. grahami

|                      | Muscle | Liver |
|----------------------|--------|-------|
| CPSase               | µmol/min/g tissue | µmol/min/g tissue |
| Ammonia as substrate | 0.19 ± 0.02 | 0.06 ± 0.02 |
| Glutamine as substrate | 0.17 ± 0.02 | 0.04 ± 0.01 |
| OCTase               | 4.37 ± 1.10 | 3.0 ± 0.5 |
| ASSase/ASLase        | 0.19 ± 0.06 | 0.01 ± 0.01 |
| Arginase             | 38 ± 12 | 51.0 ± 5.7 |
| GSase (γ-glutamyltransferase) | 0.31 ± 0.01 | 6.2 ± 2.2 |

Values are means ± S.E. (n = 3). OCTase, ornithine carbamoyltransferase; ASSase, argininosuccinate synthetase; ASLase, argininosuccinate lyase.

Two P. J. Walsh, unpublished data.

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 ~5% of the transerase activity (7, 20), the biosynthetic GSase activity in liver and muscle together (~0.02 µmol/min/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 Vmax with ammonia as substrate is 10% or less than the Vmax 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 Km for the other decreases), and 4) the Km for glutamine is 0.1–0.2 mM (21, 22). In contrast, for the tilapia CPSase III assayed with 20 mM MgATP: 1) the Vmax with ammonia as substrate is greater than the Vmax with glutamine, 2) AGA is not required for activity with either ammonia or glutamine as nitrogen-donating substrate and the presence of AGA increases Vmax only slightly. 3) AGA does not affect the apparent Km for glutamine (or ammonia), and 4) the Km for glutamine is quite high. Similar results are obtained at 1 mM MgATP, except that the Km for ammonia in the absence of AGA is lower (1.8 mM). The Km 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 ureosomatic spiny dogfish shark (Squalus acanthias, a representative ureosomatic 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 (Km = 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 (Heteropneustes fossilus) (24), which has invited speculation that CPSase I and CPSase III activities may both be present in H. fossilus; like all other CPSases, K+ is required for activity (Km = 0.01 mM), pH optimum is 7.8; Km 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 ~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
of glutaminase, which is localized in the mitochondria as ex- localized predominantly in the cytosol in this tilapia. The level of which is quite variable in teleosts (2, 7, 27), appears to be step of the urea cycle pathway and the subcellular localization same as rat CPSase I.

III/g of tilapia muscle if staining of the tilapia CPSase III is the CPSase III) indicated that there is at least 0.7 mg of CPSase 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 sub- jected to SDS-PAGE, the intensity of this protein band corre- lated 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 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, ad- aptation 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 ≈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.

### Table II

| Enzyme       | Liver     | Percent of total |
|--------------|-----------|------------------|
|              | Debris    | Soluble          | Mitochondrial |
|              | μmol/min/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

| Substrate | K<sub>m</sub> (glutamine) | V<sub>max</sub> (glutamine) |
|-----------|--------------------------|---------------------------|
| -AGA      | 1.5 mm                    | 0.019 μmol/40 min          |
| +AGA      | 1.9 mm                    | 0.023 μmol/40 min          |
| Substrate | K<sub>m</sub> (ammonia)   | V<sub>max</sub> (ammonia)  |
| Glutamine | 5.6 mm                    | 0.037 μmol/40 min          |
| Ammonia   | 5.2 mm                    | 0.041 μmol/40 min          |
| AGA       | 0.7 mm (half-maximal)     | 0.2 mm (half-maximal)     |
|           | 0.7 mm (half-maximal)     | 0.2 mm (half-maximal)     |

### 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.
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