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Research Article

Soluble malate dehydrogenase of *Geophagus brasiliensis* (Cichlidae, Perciformes): Isolated isoforms and kinetics properties

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Abstract

Kinetic properties and thermal stabilities of *Geophagus brasiliensis* skeletal muscle unfractionated malate dehydrogenase (MDH, EC 1.1.1.37) and its isolated isoforms were analyzed to examine a possible *sMDH-B** locus duplication in a fixation process influenced by genetic drift. Two optimal pHs were detected: 7.5 for AB1 unfractionated muscle phenotype and its B1 isoform, and 8.0 for AB1B2 unfractionated muscle phenotype, A and B2 isoforms. While *G. brasiliensis* A isoform could be characterized as thermostable, the duplicated B isoform cannot be assumed as thermolabile. K_m values for isolated B2 isoforms were 1.6 times lower than for B1. A duplication event in progress best explains the electrophoretic six-band pattern detected in *G. brasiliensis,* which would be caused by genetic drift.

Key words: gene duplication, sMDH, substrate concentration, temperature, pH isoforms.

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Introduction

The malate dehydrogenase system (MDH, EC 1.1.1.37; Malate: NAD oxidoreductase) is represented by two major forms in vertebrates and invertebrates: soluble (sMDH) and mitochondrial (mMDH). The two forms differ in their electrophoretic mobility (Markert and Moller, 1959), kinetic behaviour (Banaszak and Bradshaw, 1975), amino acid composition (McAlister-Henn, 1988), antigenic properties (Kitto and Lewis, 1967) and are controlled by separated gene loci (Davidson and Cortner, 1967; Bailey *et al.*, 1969, 1970; Whitt, 1970). Two gene loci, *sMDH-A** and *sMDH-B**, encode sMDH in most studied fish and amphibian species (Schwantes and Schwantes, 1977, 1982 a,b; De Luca *et al.*, 1983; Coppes *et al.*, 1987; Fenerich-Verani *et al.*, 1990; Monteiro *et al.*, 1991, 1998; Farias and Almeida-Val, 1992; Lin and Somero, 1995a,b; Caraciolo *et al.*, 1996, Aquino-Silva *et al.*, 1997; 1998; 2003, Lin *et al.*, 2002)

The subtropical teleost *Geophagus brasiliensis* (Perciformes), studied by Monteiro *et al.* (1991, 1998), showed a six-banded pattern for sMDH in 84% of the specimens. To explain this pattern three hypotheses have been suggested, (i) a duplication event in processing their *sMDH-B** locus, (ii) the presence of three loci *sMDH-A**, *sMDH- B1**; and *sMDH-B2** with a null allele within *B2**, and (iii) overdominance of a variant allele **119* at *sMDH-B**. In a previous paper, Aquino-Silva *et al.* (1998) analyzed the effect of temperature and pH on unfractionated sMDH liver, muscle and heart of *Geophagus* in order to examine their *K*m (OAA), tissue specificities by Klebes method and thermostability tests. These tests have resulted in similar responses for both B-isoforms, in both sMDH phenotypes, suggesting a recent locus duplication for *sMDH-B**. To verify this hypothesis, the present paper describes optimum pH, thermal stability, and *K*m (OAA) of total *Geophagus* sMDH and isolated isoforms.

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Material and Methods

Tissue preparation

Using a net, forty-one specimens of *G. brasiliensis* were collected from the Monjolinho Reservoir at the Federal University of São Carlos, State of São Paulo, Brazil. Annual temperature at the *G. brasiliensis* capture site ranges from 12.3 °C - 23.0 °C. White muscle from each individual was dissected immediately after being captured and kept at -20 °C. A small piece of each tissue was homogenized (w/v) in a 50 mM phosphate buffer, pH 7.0 using a Potter-Elvejhem tissue homogenizer and then centrifuged

at 19,000 x g for 30 min at 4 °C in a Sorvall RC5B centrifuge. Crude extract were used for electrophoretic and spectrophotometric analyses.

Enzyme electrophoesis

Electrophoreses were carried out in horizontal gels containing 14% (w/v) corn starch prepared according to Val *et al.* (1981), using the pH 6.9 buffer system described by Whitt (1970). A voltage gradient of 5 V/cm was applied for 14-17 h at 4 °C. After electrophoresis, the starch gels were sliced lengthwise and the lower halves incubated in an MDH staining solution described by Monteiro *et al.* (1991). Nomenclature of sMDH gene loci, subunits and iso/allozymes was determined according to Shaklee *et al.* (1989). lsolation of sMDH isoforms was carried out by using an electrophoretic technique according to De Luca *et al.* (1983).

Determination of pH and temperature optima

For pH-activity relation studies, a 50 mM sodium phosphate buffer (pH 5.0-8.5) and a 0.2 mM Tris-HCI buffer (pH 9.0-9.5) was used and assays were carried out in a solution containing 0.33 mM oxaloacetate and 0.20 mM NADH. Crude extracts and isolated isoforms were tested for thermal stability by submitting each sample to 50 °C for 10-60 min in the absence of substrate or coenzyme. Subsequently the samples were cooled on ice and centrifuged at 19.000 g for 30 min at 4 °C. Their residual activities were examined at 25 °C in the direction of oxaloacetate reduction through the change in absorbance at 340 nm in a HP-8452A Diode Array Spectrophotometer using a temperature controlled cuvette holder. The assays were carried out in a 50 mM imidazole chloride buffer containing 0.33 mM oxaloacetate and 0.20 mM NADH. Controls were kept in an ice-water mixture.

Kinetic analysis

The kinetic parameters of sMDH (total and isolated isoforms) were determined at 20 °C by measuring the oxidation of NADH spectrophotometrically at 340 nm. The assays were carried out in a 50 mM imidazole chloride buffer containing 0.20 mM NADH and different concentrations of oxaloacetate. Oxamate at 10 mM was added to inhibit lactate dehydrogenase activity resulting from any pyruvate occurring in the assay medium. All assays were performed in triplicate and initiated by adding 10 µL of enzyme (crude extract) or 100 µL (isolated isoform) to 1.0 mL of assay medium. Apparent Michaelis-Menten constants (*K*m) were calculated by Lineweaver Burk method using double-reciprocal plots of velocity *vs.* substrate concentrations.

Statistical analysis

Allele frequencies were determined by Chi-square tests and analyzed for Hardy-Weinberg equilibrium. Statistical differences among optimum pH, thermal stability, and *K*m (OAA) values of total and isolated isoforms of *Geophagus* sMDH were determined by Student's T test with 95% confidence interval.

Results

Muscle extracts from 41 *G. brasiliensis* specimens confirmed both electrophoretic patterns for sMDH obtained previously by Monteiro *et al.* (1991, 1998) and Aquino-Silva *et al.* (1998) (Figure 1). Assuming Hardy-Weinberg equilibrium, the χ^2 value for allele frequencies (178.6; 1*d.f.* $p < 0.001$), indicates that these samples are not in equilibrium for this locus. A homogeneity χ^2 test (0.197 1*d.f.* $0.75 > p > 0.5$) showed that the phenotypic proportion between the two samples (Aquino-Silva *et al.*, 1998 and present sample) were maintained.

Comparisons of *Geophagus* skeletal muscle unfractionated sMDH and its isolated isoforms have shown similar pH optima for oxaloacetate reduction: AB1, A and B1 pH 7.5; AB1B2, B1 and B2 pH 8.0 (Figure 2A and B respectively). Statistical analyses of optimum pH values showed no significant difference between muscle extract AB1B2 and its isoforms ($p = 0.26$ for AB1B2 *vs.* B1; $p = 0.60$ for AB1B2 *vs.* B2 and $p = 0.60$ for B1 *vs.* B2).

Thermal stability of unfractionated sMDH from skeletal muscle extracts is shown in Figure 3A. The half-lives of AB1 and AB1B2 muscle extracts were about 54 and 5 min respectively and the differences were significant ($p = 0.01$). To determine more precisely the thermal stability of the A, B1 and B2 isoforms, partially purified isoforms

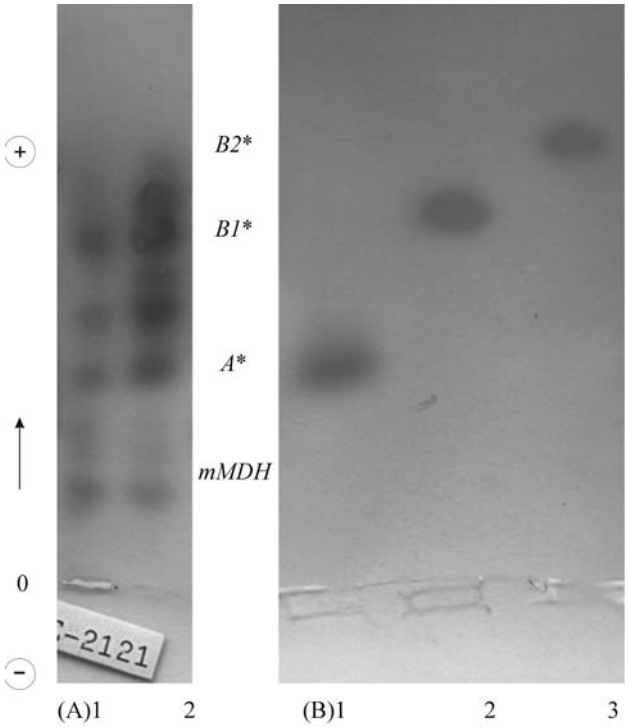

Figure 1 - (A) Malate dehydrogenase from skeletal muscle extracts of *Geophagus brasiliensis*: (1) *AB1** (2) *AB1B2** (B) *s*MDH isolated isoforms: (1) A, (2) B1 and (3) B2. mMDH mitochondrial form; O - origin.

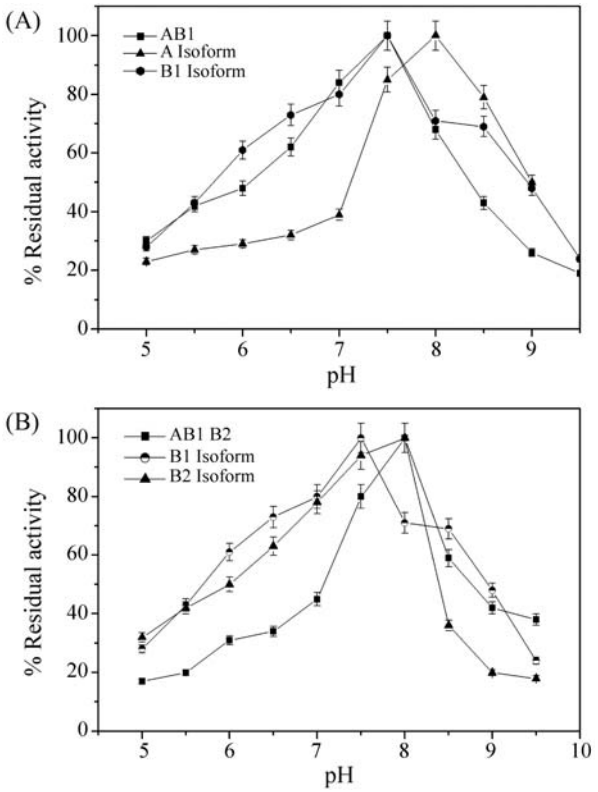

Figure 2 - (A) Optimal pH of sMDH from skeletal muscle of *G. brasiliensis* AB1 phenotype and its isoforms. (B) optimal pH of sMDH from skeletal muscle of *G. brasiliensis* AB1B2 phenotype and its isoforms. Error bars are 95% confidence intervals for each pH determination.

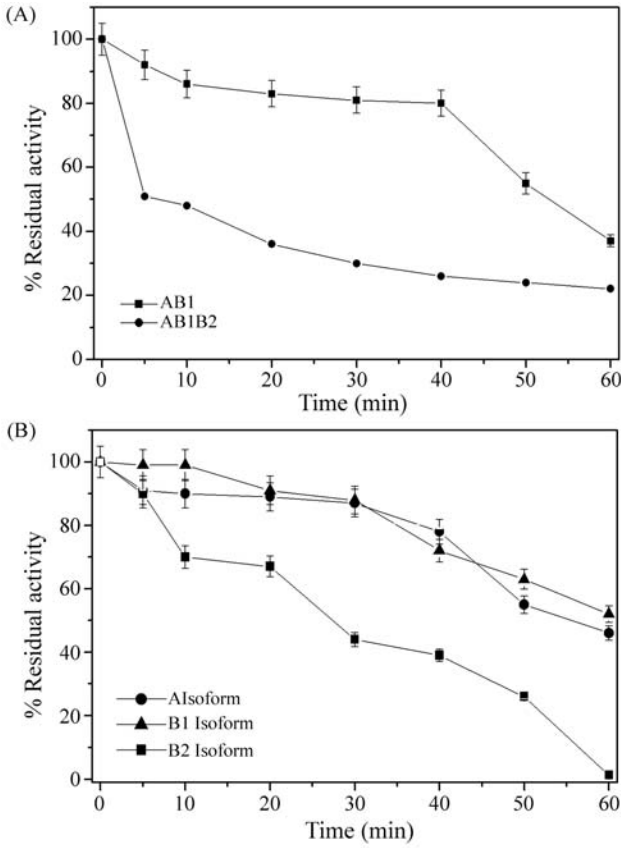

Figure 3 - (A) Thermal inactivation of MDH from AB1 and AB1B2 phenotype of *G.brasiliensis*. (B) Thermal inactivation of sMDH from A, B1 and B2 isoforms. Error bars are 95% confidence intervals for each temperature determination.

were also submitted to thermostability tests (Figure 3B). The half-lives of A, B1 and B2 isoforms were about 60 min for A and 5 min for B2. The B1 isoform did not show half-life during the tests., but no significant differences were observed among all the half-life values ($p = 0.47$ for A2 *vs.* B1; $p = 0.97$ for A2 *vs.* B2; $p = 0.76$ for B1 *vs.* B2).

The responses of both crude extract phenotypes and their respective predominant isoforms to increasing oxaloacetate concentrations are shown in Figure 4 (A and B). Optimal substrate concentrations differed between isoforms (A and B1 = 0.03 mM L^{-1} and B2 = 0.02 mM L^{-1}) but not between crude extracts (AB1 and AB1B2 = 0.03 mM L^{-1}). The *K*m (OAA) value (Figure 5) obtained for B1 isolated isoform was 1.6 times higher than that of the B2 isoform (0.024 mM L^{-1}x 0.015 mM L^{-1}). Statistical analyses revealed significant differences when AB1 *vs.* B1 isoform ($p = 0.001$) and AB1B2 *vs.* B2 isoform ($p = 0.078$) were compared.

Discussion

A pair of duplicated genes may represent two kinds of homology: orthology or paralogy. Duplicated genes related by speciation are orthologous and genes related by regional events or duplication of a single gene, *e.g.* through ploidy changes are paralogous (Ferris and Whitt, 1977, 1978, 1979; Ferris, 1984). The homologies of duplicated genes can be inferred from measurements of pH optima, heat stability and other kinetic properties like optimum substrate concentration or *K*m values.

In general, specific activity, thermostability and other kinetic enzyme properties vary with pH fluctuation. De Luca *et al.* (1983), studying adaptative features of sMDH in the subtropical fish *Astyanax fasciatus* showed that its isoforms have different pH optima for oxaloacetate reduction. In contrast, in comparative studies of sMDH paralogs of four species of Pacific barracudas adapted to different temperatures, Lin and Somero (1995b) obtained similar optima pH (7.6 and 7.7) for both isoforms in the direction of oxaloacetate reduction,. For *Geophagus* the optimum pH obtained with muscle extracts for the AB1 phenotype and B1 isoform (pH 7.5), was significantly different from the one obtained for AB1B2 phenotype, A and B2 isoforms (pH 8.0).

Differences in thermostability properties between paralogous isoforms were not detected in *G. brasiliensis*, which is completely different from results described for some other fish where *sMDH-A** encodes a thermostable and *sMDH-B** a thermolabile isoform (Schwantes and

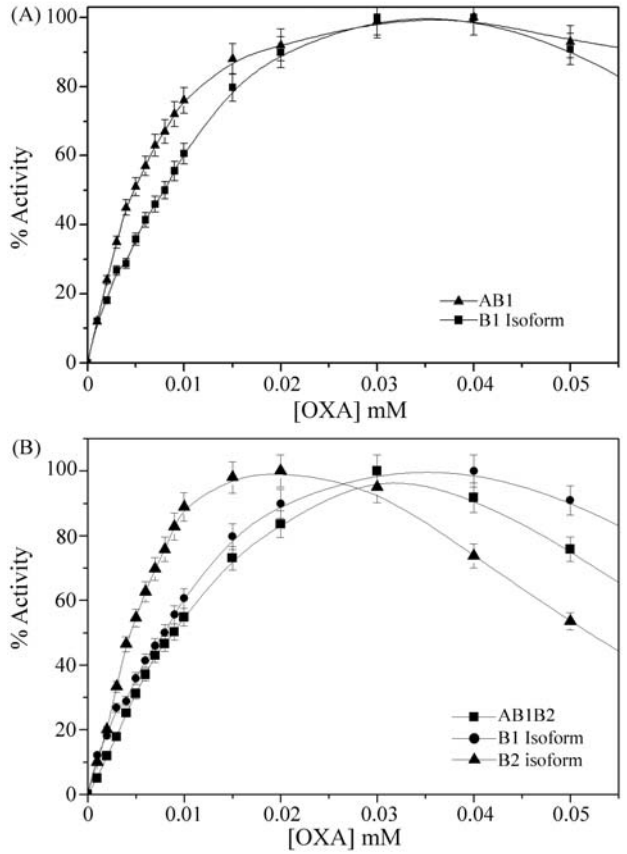

Figure 4 - (A) MDH saturation curves (oxaloacetate reduction) from *G. brasiliensis* AB1 phenotype and B1 isoform (B) MDH saturation curves (oxaloacetate reduction) from *G. brasiliensis* AB1B2 phenotype and its isoforms (B1 and B2). Error bars are 95% confidence intervals for each determination.

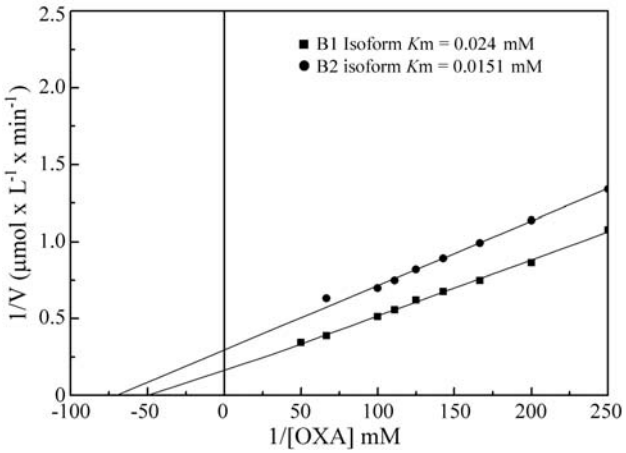

Figure 5 - *K*m curves for sMDH B1 and B2 isoforms,of *G. brasiliensis;* r = 0.999 and 0.998 respectively.

Schwantes, 1982a,b; De Luca *et al.*, 1983; Coppes *et al.*, 1987; Farias and Almeida-Val, 1992; Lin and Somero, 1995a,b; Caraciolo *et al.*, 1996; Lin *et al.*,2002; Aquino-Silva *et al.*, 1998).

Comparative studies on thermostable and thermolabile sMDH isoforms of barracudas from different physiological temperature ranges revealed similar kinetic properties for orthologs and varying ones for paralogs (Lin and Somero, 1995a,b). According to these authors, the absence of a thermolabile isoform in equatorial barracuda (*Sphyraena ensis*) could occur through two mechanisms, gene loss (or loss of a functional form of the gene) encoding this isoform, or repression of gene transcription at high temperatures. Thus, if the thermolabile isoform is not required physiologically, the gene that encodes this isoform may effectively be lost. However, Farias and Almeida-Val (1992) and Caraciolo *et al.* (1996) studying sMDH of Amazon fishes, detected a thermolabile locus product. Farias and Almeida-Val (1992) also showed a recent duplication of *sMDH-B** in Amazon cichlids. Schwantes and Schwantes (1982a), studying the adaptative features of sMDH loci in a temperate estuarine fish *Leiostomus xanthurus* (habitat temperature range 5-30 °C), showed that A and B subunits occur in different tissues at different levels, possibly reflecting environmental temperature fluctuations. On the other hand, Lin and Somero (1995a,b) studying the eurythermal species *Gillichthys mirabilis* (habitat temperature range 9-38 °C), showed that the two sMDH's isoforms vary in ratio seasonally and as a result of acclimation. According to these authors, the findings suggest that temperature can affect one or more of the processes that establish sMDH isozyme ratios, such gene transcription or protein degradation.

Thermostability tests realized with AB1 muscle extract, the tissue in which Klebes tests showed the presence of two A subunits for one of B1 (Aquino-Silva *et al.* 1998), showed an enzyme half-life at 54 min of incubation. However, when the isolated A and B1 isoforms were incubated, their half-lives were 60 min for the A isoform, whereas the B1 isoform did not show half-life during the tests. When themostability tests with AB1B2 muscle extract were performed, the tissue in which Klebes tests showed the presence of eight A subunits for one of B1 and one of B2 (Aquino-Silva *et al.*, 1998) the half-life was obtained within 5 min. The isolated A and B1 isoforms showed similar results obtained for the AB1 phenotype, and the isolated B2 isoform showed half-life within 30 min. According to Lin and Somero (1995b), some characteristics are conserved among orthologs of each sMDH isoform. Comparisons of orthologs with respect to themostability have shown that while the A isoform of *Geophagus* could be characterized as a thermostable isoform (like in *L. xanthurus* determined by Schwantes and Schwantes, 1982 a,b), in *A. fasciatus* determined by De Luca *et al.*, 1983, and in *S. idiates* by Lin *et al*, 2002), the possibly duplicated B isoform could not be characterized as thermolabile.

*K*m value conservation is interpreted as reflecting selection for the retention of the catalytic and regulatory capacities of enzymes and it is manifest through adaptations to changes in pressure, osmotic conditions, pH, as well as

temperature (Hochacka and Somero, 1984). In the present paper, *K*m (OAA) values obtained for the A isolated isoform were 2.5 times higher than for B1 and 4.0 times higher than for the B2 isoform (0.06 mM x 0.024 mM x 0.015 mM). The values obtained for the isolated B1 and B2 isoforms were 1.6 higher for B1 than for B2 the isoform (0.024 mM x 0.015 mM).

*K*m (OAA) values for B1 and B2 are comparable to other species. Consequently, the presence of a null allele proposed by Monteiro *et al.* (1991, 1998) is not probable. Considering the hypothesis of overdominance proposed by the same authors, the coefficient of selection and the segregational load (0.73 and 0.42, respectively according to Aquino-Silva *et al.* 1998) obtained for this allele also makes this hypotesis improbable. In addition to this, allele frequencies indicated that these samples are not in equilibrium for this locus.

Three different biological functions have been proposed for sMDH: gluconeogenesis, lipogenesis and malate-aspartate shuttle during aerobic glucolysis. Since these processes occur differently in various tissues, this would explain the different activities of the multiple forms of MDH. Their presence or absence would be due to different physiological and metabolic roles. According to Coppes *et al.* (1987), white muscle malate is oxidized by the B isoform to yield oxaloacetate with reduction of NAD. When temperatures decrease, the metabolic activities are reduced and oxaloacetate would be utilized in hexose synthesis or in transaminations. When temperatures increase, metabolic activity also increases and oxaloacetate would be reduced to malate by the A isoform, yielding NAD, which could be used by glyceraldehyde-3-phosphate dehydrogenase, favoring the glycolytic direction. If in the perciform *G. brasiliensis* the B2 isoform has arisen via gene duplication it would broaden this metabolic pathway (since it has a lower *K*m value and consequently a higher substrate affinity than the B1 isoform) by increasing the efficiency in the malate-aspartate shuttle which would involve the *B** locus.

According to our previous paper (Aquino-Silva *et al.* 1998), the first hypothesis proposed by Monteiro *et al.* (1991, 1998), *viz.* a duplication event in progress, better explains the complex electrophoretic pattern detected at *G. brasiliensis,* which would be influenced by genetic drift.

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