

**EVOLUTIONARY IMPLICATIONS AND KINETIC PROPERTIES
OF VITAMIN-C SYNTHESIZING ENZYME (GLO) FROM
SOUTH AMERICAN LUNGFISH (*LEPIDOSIREN PARADOXA*).**

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Abstract

The ability of vitamin C synthesis in vertebrates has been described in reptiles, birds and some mammalian groups. Such ability is well established in fish belonging to “primitive” groups as the Dipnoans and Crossopterygians. Thus, the ancestors of Sarcopterygians, Actinopterygians, and other groups of modern vertebrates shared the genetic information for synthesis of the enzyme L-gulonolactone oxidase (GLO), which catalyses vitamin C synthesis. According to literature, the properties of the enzyme GLO from Chrossopterygians correspond to those of the enzyme occurring in amphibians, birds, and mammals, although the occurrence of GLO among modern Actinopterygians (Teleosteans) is still under debate. The South American lungfish resembles modern vertebrates (amphibians and reptiles), regardless of its primitive character among Teleostomi. The present paper describes the properties of the enzyme GLO present in the liver and kidney of the South American lungfish, *Lepidosiren paradoxa*, and compares those properties with the homologous GLO occurring in other vertebrate groups.

Introduction

L-gulonolactone oxidase (GLO, E.C. 1.1.3.8) catalyzes the final step of the L-ascorbic acid synthesis in the kidney of vertebrates such as amphibians and reptiles, or in the liver of some mammals and higher order of birds (Charterjee, 1973). Most fishes, a few avian groups and the primates lost the ability of synthesizing GLO and require a dietary supplement of L-ascorbic acid (Charterjee, 1973; Birney et al., 1979). Data on the ontogenetic development of GLO in vertebrates are scarce and fragmentary (Jenness et al., 1984). Thus, the ability to synthesize ascorbic acid and tissue distribution of GLO varies phylogenetically among vertebrates. As teleosts are unable to synthesize vitamin C, Charterjee (1973) suggested that tetrapods developed the pathway for synthesizing ascorbic acid *de novo*. Dykhuizen et al. (1980) reported that the Australian lungfish, *Neoceratodus forsteri*, is able to synthesize ascorbic acid in the kidney and, consequently, the appearance of the information for GLO was prior to the arousal of land vertebrates. As many authors agree, the Chrossopterygian, the amphibians, the reptiles, and the Actinopterygian groups all seem to have arisen from a common ancestor some time before the middle Devonian (375 MYA). Since GLO activity was confirmed in all the above-mentioned groups, except by the Actinopterygian, it is likely that the common ancestor could synthesize vitamin C. The absence of this ability in fishes and anthropoid primates suggests that the information for such enzyme was lost after evolutionary divergence of those groups.

During the last decade, many authors have disagreed about fish ability to synthesize vitamin C (reviewed in Dabrowski, 1994). Such discussions have helped to improve the methodology to assay the enzyme and describe its kinetic properties with more accuracy, which may help establish the homology between the enzymes in several vertebrate groups.

In the present paper we report, for the first time, the activity levels of GLO in South American lungfish (*Lepidosiren paradoxa*) and compare its kinetic properties with other species belonging to other vertebrate groups: a mammalian species – the hamster *Rattus norvegicus*; a reptilian - the Amazon aquatic turtle *Podocnemis expansa*; and an amphibian – the common frog *Bufo marinus*.

Materials and Methods

Tissue homogenate –liver and kidney of 4 lungfishes (*Lepidosiren paradoxa*), liver of 4 rats (*Rattus norvegicus*), kidney of 3 frogs (*Bufo marinus*) and kidney of 3 Amazonian aquatic turtles (*Podocnemis expansa*) were excised from the animals, after they were anesthetized with ether. Tissue was washed twice with 0,85% NaCl solution in the proportion (1:4 - w:v) and homogenized in 50mM potassium phosphate buffer (pH 7.0). The extract was centrifuged at 20.000xg, 4°C at a centrifuge Sorvall RC-5B for 30 min. Supernatant was used for enzyme assays.

Enzyme assays – The increase in the absorbance of DCIP (2,6-Dichlorophenol-indophenol) in the assay mixture was followed at 600 nm at 25°C in a spectrophotometer Genesys 2 Spectronic. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the reduction of 1 µmol of DCIP per min. The extinction coefficient of DCIP at pH 7.0 was taken as 14.5 mM. A cuvette with a 1-cm light path contained 0.16 mM DCIP, 0.16mM PMS (phenazine methosulfate), 200 mM potassium phosphate buffer, 400 mM L-gulono-γ-lactone, 20 µl of enzyme solution (tissue extract), and 50mM phosphate buffer (pH 7.0) in a final volume of 1.0 ml and assayed for enzyme activity as described previously by Sugisawa et al. (1995). The blank contained all solutions, except L-gulono-γ-lactone. The reaction started with the addition of substrate and the enzyme activity was measured as the initial reduction rate of DCIP. Three enzyme reactions were done for each individual and situation (substrate, temperature, and pH) and the values are expressed as means ± SEM. Enzyme activity values were converted to µmoles of substrate (L-gulono-γ-lactone) oxidized and transformed in ascorbic acid per hour and per gram of fresh tissue. Lineweaver-Burk equation was applied after obtaining saturation plots and Km and Vmax were obtained by the equations of the linear regressions (95% confidence intervals).

Results and Discussion

Gulono Lactone Oxidase (GLO) maximum activity values (µmoles L-Gulono-γ-lactone.g⁻¹.h⁻¹), Km (mM of L-gulono-γ-lactone) and saturation curves from *Lepidosiren paradoxa*'s liver and kidney homogenates, *Rattus norvegicus*' liver, *Bufo marinus*'s kidney, and *Podocnemis expansa*'s kidney are presented in table 1 and figure 1.

Table I – Maximum activities ($\mu\text{moles L-Gulono-}\gamma\text{-lactone.g}^{-1}.\text{h}^{-1}$) and K_m (mM) values obtained for liver and kidney homogenates of the four studied species.

<i>Lepidosiren paradoxa</i> (liver) n=4	<i>Lepidosiren paradoxa</i> (kidney) n=4	<i>Rattus norvegicus</i> (liver) n=4	<i>Bufo marinus</i> (kidney) n=3	<i>Podocnemis expansa</i> (kidney) n=3
235,5 ± 21,00	274,5 ± 29,19	229,9 ± 10,20	213,00 ± 45,79	192,6 ± 16,21
35,72	35,60	13,18	68,54	63,02

*Data are expressed in Means±SEM

** K_m values obtained by Lineweaver-Burk equation.

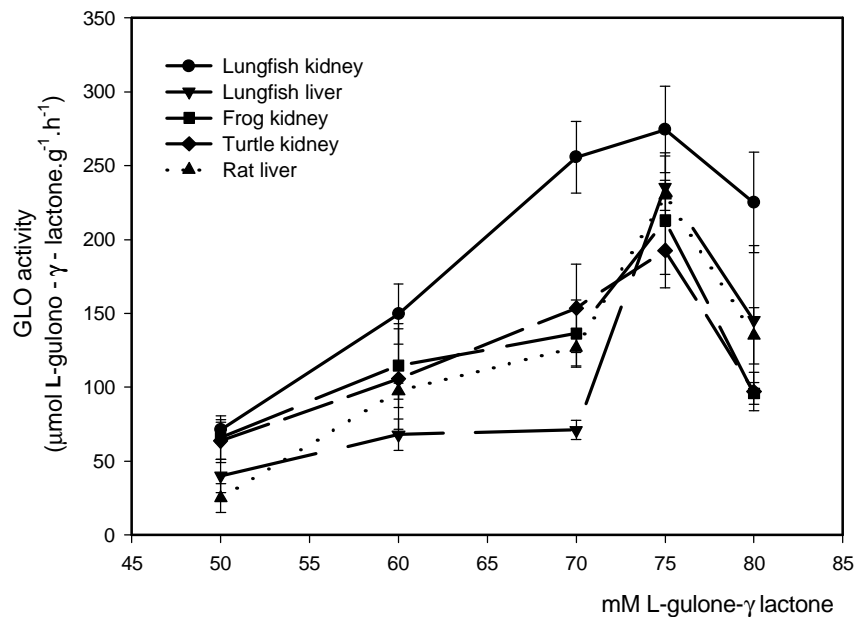


Figure 1. Substrate saturation plots for GLO levels in liver and kidney of *Lepidosiren paradoxa* compared with the other analyzed species.

The enzyme saturation curves and kinetic properties of all analyzed species are similar. GLO activities were similar in the kidney and liver in lungfish, suggesting that ascorbic acid synthesis occurs in both tissues. K_m values were lower in lungfish tissues, compared with turtle and frog kidney and higher than the K_m values of mammal liver. Enzyme K_m values represent the inverse of enzyme-substrate affinity and, therefore, it may be considered as the enzyme functional ability for product synthesis. Based on higher activities at all substrate levels (Figure 1) and low K_m values (Table 1) we can suggest that (excepting rat liver) lungfish kidney GLO is a very efficient enzyme compared to the other vertebrates analyzed.

The importance of retaining an efficient site to produce vitamin C in lungfish is probably related to its life style and respiration mode. During the dry season this animal remains burrowed into the mud and its oxygen demand may decrease. Low metabolic rate may remain in vital organs, such as kidney, heart and brain. Animal arousal involves an increase in metabolic levels, oxidative metabolism and, consequently, an increase in the amount of oxygen reactive species due to the return of high levels of air intake. Synthesizing antioxidants such as vitamin C should help to avoid possible tissue or cellular damage in both kidney and liver. Whether one gene or duplicate genes encode for GLO, still remains under investigation.

Enzyme thermostability was tested after tissue extract exposure to different temperatures during 5 min, using 75 mM potassium phosphate buffer. Activities were measured as above described and results are presented as enzyme activity levels obtained at the different tested temperatures (Figure 2). While expecting a higher sensitivity in rat liver enzyme, due to the fact that this species is endothermic, the GLO from lungfish liver and turtle kidney were affected by temperature more rapidly than the other groups.

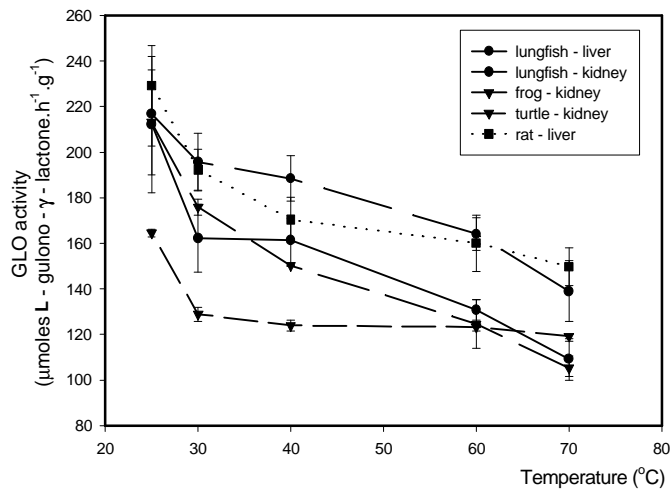


Figure 2. Temperature effects on maximum activity levels of the enzyme GLO from kidney and liver of *Lepidosiren paradoxa* compared with the other analyzed species.

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Results are presented in figure 3. Higher activities were obtained at pH 7.0 for all analyzed species and tissues. All enzymes presented the same reaction to pH variation indicating a good degree of homology between them.

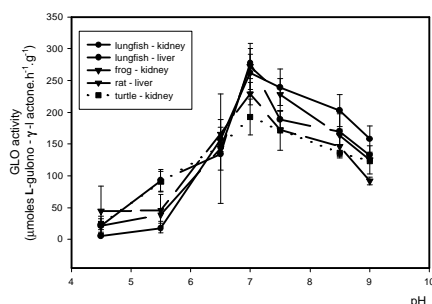


Figure 3. pH effects on maximum activity levels of the enzyme GLO from kidney and liver of *Lepidosiren paradoxa* compared with the other analyzed species.

The homology between GLO enzymes from lungfish and other vertebrates can be suggested from the above-described data. To date, there was no evidence that Dipnoi possessed the genetic information for GLO enzyme in the liver. Liver GLO have appeared only in eutherian mammals (Dykhuisen et al., 1980). These authors were unable to detect ascorbic acid formation in the liver of the Australian lungfish *Neoceratodus* and in the liver of Amphibian. Thus, this work is the first to describe the presence of GLO in two different organs (kidney and liver) of a primitive vertebrate, indicating that this information is prior to terrestrial vertebrate differentiation. The existence of more than one ancient loci encoding for the enzyme GLO deserves now new investigations.

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