



## THE THERMOSTABILITY OF HAEMOGLOBINS FROM THE HOT-SPRING FISH, *OREOCHROMIS ALCALICUS GRAHAMI*: COMPARISONS WITH ANTARCTIC AND TEMPERATE SPECIES

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**Abstract**—1. The thermostability of haemoglobin was measured in three species of fish living at different environmental temperatures (*ET*).

2. The time (min) for 50% denaturation ( $T_{1/2d}$ ) of the haemoglobin at 2 mg ml<sup>-1</sup> in 50 mM phosphate buffer, pH 7.3 was 46.4 ± 1.7 for *Oreochromis alcalicus grahami* (*ET*, 35 to 42°C), 43.1 ± 1.9 for *Oreochromis niloticus* (*ET*, 18 to 26°C) and 19.2 ± 0.3 for the Antarctic teleost, *Notothenia coriiceps* (*ET*, -1.5 to 1°C) (Mean ± SEM, *N* = 5-7 preparations). In contrast,  $T_{1/2d}$  for haemoglobin from birds and mammals is usually in excess of 500 min.

3. These results suggest that the lower thermostability of haemoglobins in fish relative to birds and mammals is not solely a function of differences in body temperature.

**Key Word Index:** Haemoglobin; Lake Magadi; tilapia; *Oreochromis alcalicus grahami*; *Oreochromis niloticus*; *Notothenia coriiceps*; blood; temperature

### INTRODUCTION

Although fish occur at -1.9°C to greater than 43°C, no one species can tolerate such a wide range of temperatures. As the vast majority of fish are ectotherms, temperature has an all pervasive influence on the functioning and stability of their structural molecules, especially proteins. Numerous studies have shown a correlation between the resistance of proteins to thermal denaturation and environmental temperature (Alexandrov, 1977; Johnston and Walesby, 1977; Low and Somero, 1976).

The thermostability of haemoglobins has been the focus of several studies in fish and other vertebrates (Tondo *et al.*, 1980; Borgese *et al.*, 1982; Harrington *et al.*, 1982; Warren and Wells, 1988). Tondo *et al.* (1980) established that mammalian, avian and reptilian haemoglobins were considerably more stable at higher temperatures than either amphibian or fish haemoglobins and the differences appear, in part, to be correlated to the thermal environment in which these molecules function. Among the fishes, Antarctic

teleosts represent one thermal extreme (down to -1.86°C) whilst the other may be represented by the geothermal hot-spring fish, *Oreochromis alcalicus grahami*, which lives at temperatures in excess of 40°C (Coe, 1966; Johnston *et al.*, 1994).

The haemoglobins of *O. a. grahami* are exposed to temperatures similar or greater than those encountered by mammals and birds. Therefore a question of interest was whether the thermostability of *O. a. grahami* haemoglobins was greater than those of other fish species and more similar to that of mammals and birds. Comparisons were made with the temperate teleost, *Oreochromis niloticus*, and the Antarctic teleost, *Notothenia coriiceps*.

### MATERIALS AND METHODS

#### *Experimental animals*

*Oreochromis alcalicus grahami* (mean body mass 8.9 ± 0.4 g, mean ± SD) were collected from Lake Magadi, Kenya, in locations close to geothermal hot-spring vents. The water temperature in these areas was between 36-38°C, although in some locations where fish were living the water temperature exceeded 43°C (Johnston *et al.*, 1994). The fish were netted and transported back to the University of

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Nairobi where they were kept in tanks filled with water from Lake Magadi.

*Oreochromis niloticus* [L.] (mean body mass  $11.6 \pm 2.8$  g) were collected from the Sagana River Tilapia Hatchery, Kenya which is supplied with water at about 21°C.

*Notothenia coriiceps* [Nybelin] (mean body mass  $78.1 \pm 9.6$  g) were caught around Signy Island, Antarctica, transported to the UK, and maintained at 0 to 1°C in a cold aquarium facility at the Gatty Marine Laboratory, University of St. Andrews, Scotland.

#### Blood collection and processing

Blood was collected from stunned and pithed fish by cardiac puncture using heparinised 1 ml syringes. Due to the small body masses of *O. a. grahami* and *O. niloticus*, blood collected from 2–5 fish was pooled. The blood was centrifuged in 1.5 ml microcentrifuge tubes for 3 min at (5000 *g*) and the plasma discarded. The packed red blood cells from the *Oreochromis* species and *N. coriiceps* were re-suspended with 0.9% saline and 1.3% saline respectively, and then re-centrifuged. This process was repeated three times and following the final wash the erythrocytes were lysed by the addition of ice-cold distilled water. The cellular debris was pelleted, leaving a haemoglobin solution which was diluted to 4 mg ml<sup>-1</sup> with distilled water.

#### Heat denaturation

The stock solution of haemoglobin was diluted with an equal volume of 0.1 M phosphate buffer (pH 7.3) and dispensed into 1.5 ml microcentrifuge tubes in duplicate. The tubes were capped, one tube immediately placed into an ice slurry to act as a control, and others placed into a water bath at 50°C. Samples were removed from the water bath at regular intervals and placed into the ice slurry to prevent further thermal denaturation. The heat-denatured haemoglobin was collected by centrifugation and the haemoglobin concentration of the supernatant determined by the Drabkin's cyanmethaemoglobin method using a Sigma kit (No. 525). The haemoglobin remaining in the supernatant was expressed as a percentage of the control and plotted against time. From individual runs, the time for 50% denaturation ( $T_{1/2d}$ ) was determined by interpolation and the means  $\pm$  SEM for each species calculated (a method comparable with previous studies, Borgese *et al.*, 1982; Warren and Wells, 1988). Students *t*-tests were used to determine any statistical differences between the  $T_{1/2d}$  values. Exponential equations were fitted to the data and the decay constants for each species derived (i.e.  $Y = A_0 e^{-K_1 t}$ ; where *Y* is % haemo-

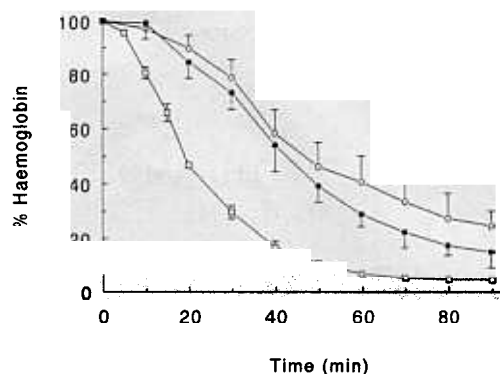


Fig. 1. The effect of incubation at 50°C on the % denaturation of haemoglobins from the hot-spring fish, *Oreochromis alcalicus grahami* (○) the tropical fish, *Oreochromis niloticus* (●), and the antarctic teleost, *Notothenia coriiceps* (□).

globin,  $A_0 =$  constant,  $t =$  time and  $-K_1$  the decay constant). A straight line was also fitted to the natural log of % haemoglobin *vs* time and analysis of covariance used to determine any statistical difference between the species.

#### RESULTS

The effects of incubation at 50°C on the gross unfolding of haemoglobin in dilute solution (2 mg ml<sup>-1</sup> in 0.05 M phosphate buffer, pH 7.3) for the three species studied is shown in Fig. 1. The haemoglobin from *N. coriiceps* was significantly more susceptible to heat denaturation than haemoglobins from *O. a. grahami* and *O. niloticus*. The time to 50% precipitation ( $T_{1/2d}$ ) for *N. coriiceps* was  $19.2 \pm 0.3$  min ( $N = 5$ ) compared to  $43.1 \pm 1.9$  min ( $N = 6$ ) for *O. niloticus* and  $46.3 \pm 1.7$  min ( $N = 7$ ) for *O. a. grahami*. There was no significant difference in the  $T_{1/2d}$  for the two *Oreochromis* species.

The exponential equations fitted to the data and correlation coefficients are shown in Table 1. Analysis of covariance on straight lines fitted to natural log of % haemoglobin *vs* time produced similar conclusions to statistical analysis of  $T_{1/2d}$ . There was a significant difference between *N. coriiceps* and the two *Oreochromis* species ( $P > 0.05$ ), but no significant difference between *O. a. grahami* and *O. niloticus*.

Table 1. Exponential equations ( $y = A_0 e^{-K_1 t}$ ); where  $y =$  % haemoglobin,  $t =$  time (min), and  $-K_1 =$  decay constant and correlation coefficients ( $r$ )

Species	Exponential equation	Correlation coef.
	$y = 124.9 e^{-0.022t}$	
	$y = 115.6 e^{-0.017t}$	
	$y = 101.5 e^{-0.040t}$	

## DISCUSSION

Borgese *et al.* (1982) categorised the thermostability of fish haemoglobins at 50°C into four types. At pH 7.0, type I, II and III haemoglobin all had times for 50% denaturation ( $T_{1/2}d$ ) greater than 50 min. Type IV haemoglobin had  $T_{1/2}d$  values that were less than 50 min. Borgese *et al.* (1982) and Warren and Wells (1988) found considerable variation in haemoglobin  $T_{1/2}d$  values between different genera of fish. The haemoglobins of *O. a. grahami*, *O. niloticus*, and *N. coriiceps* all had  $T_{1/2}d$  values less than 50 min and appear to fit into the Type IV category. In this study the Antarctic fish, *N. coriiceps* was the most sensitive to heat denaturation, 50% of the haemoglobin was denatured after 19 min. Warren and Wells (1988) also found that the haemoglobins from the Antarctic fish *Pagothenia borchgrevinki* and *Gymnodraco acuticeps* fitted the Type IV category having  $T_{1/2}d$  values at pH 7.0 of approximately 17 min and 7.7 min, respectively. The two *Oreochromis* species would also appear to have a type IV haemoglobin but with an increased  $T_{1/2}d$  (43 and 46 min). Although *O. a. grahami* is found in water 20°C warmer than *O. niloticus* their haemoglobins unfolded at a similar rate at 50°C, suggesting similar levels of structural stability.

Johnston and Walesby (1977) found a correlation between the half-life of denaturation ( $T_{1/2}d$ ) of myofibrillar ATPase activity in dilute suspension at 37°C (0.05 mg ml<sup>-1</sup>) and environmental temperature (ET). Values for  $T_{1/2}d$  varied from around 1–2 min in several antarctic fish, to 12 to 48 min in Mediterranean species (ET, 12–25°C), and were about 270 min in *Tilapia nigra* (ET, 20 to 30°C) and 550 min in *O. a. grahami* from Lake Magadi (ET, 35 to 42°C). Such measurements of  $T_{1/2}d$  are not a function of differences in the thermostability of the ATPase enzyme *in vivo*, where proteins are present in much higher concentrations, but rather reflect interspecific variations in the numbers and strengths of the weak bonds stabilising protein tertiary structure. It has been suggested that enzymes of cold-adapted fish benefit from a more flexible structure at low temperatures. For example, the conformational changes involved in the binding and releasing of ligands may be more favourable if fewer weak bonds need to be broken. There may have been a trade off during evolution between cell temperature, the stabilisation of protein tertiary structure and the activation energy of enzyme reactions (Johnston and Walesby, 1977; Low and Somero, 1976; Somero, 1991). At high body temperatures more rigid protein tertiary structures are required in the face of higher average kinetic energies. In the case of myosin, the free energy

of activation ( $\Delta G^\ddagger$ ) of the actin-activated ATPase activity is positively correlated with environmental temperature (Johnston and Walesby, 1977). The -SH groups of myosin from Antarctic fish react more quickly with DTNB than myosin from tropical species, which suggests that the former has a more 'open' molecular structure (Johnston *et al.*, 1975). Similar relationships between thermodynamic activation energies, susceptibility to denaturation and indices of molecular 'openness' have been reported for M<sub>4</sub>-lactate dehydrogenase and pyruvate kinases from a range of vertebrates (Low and Somero, 1976; 1991). A distinction should be made between the loss of particular functional properties of a protein due to heat denaturation and the gross unfolding of protein structure which accompanies its precipitation from solution, as in the case of haemoglobins in the present study. The latter is likely to provide a much poorer index of how functional properties vary with temperature, and may account for the similar values obtained for  $T_{1/2}d$  in *O. niloticus* and *O. andersoni* in spite of a 20°C difference in body temperature.

The haemoglobins of fish do vary in their thermostability at 50°C, the skate (*Raja erinacea*) has a  $T_{1/2}d$  inactivation of greater than 500 min (similar to that of man), while that of the goosefish (*Lophius americanus*) is less than 20 min (Borgese *et al.*, 1982) however this does not appear to be correlated with environmental temperature. The  $T_{1/2}d$  of the skate (*Raja erinacea*) haemoglobin and that of the dogfish (*Mustelus canis*) may be related to factors other than temperature such as the need to stabilise the molecule in the face of high concentrations of urea in their blood (Borgese *et al.*, 1982).

The water in the hot-springs of Lake Magadi is very unusual, having a pH of 10, a bicarbonate concentration of 200 mM, as well as a temperature of more than 37°C (Randall *et al.*, 1989). *O. a. grahami* has adapted to this environment, and in doing so changed the chemistry of its plasma, for example having a lower concentration of ammonia in the plasma but an increased urea concentration (Randall *et al.*, 1989). These changes in the composition of the plasma may have influenced the stability of the haemoglobins in *O. a. grahami*.

Previous comparative studies on the thermostability of haemoglobins have concluded that the differences that occur between the vertebrate classes are chiefly a function of body temperature, with the birds and mammals and their correspondingly high body temperatures generally having the most stable haemoglobins (Tondo *et al.*, 1980). Despite *O. a. grahami* having a body temperature similar or greater than birds and mammals, the thermostability of its haemoglobins are considerably less. Therefore,

it is apparent that having a high body temperature does not necessitate a highly thermostable haemoglobin.

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#### REFERENCES

- Alexandrov V. Y. (1977) Cells, molecules and temperature. Conformational Flexibility of macromolecules and ecological adaption. *Ecol. Stud.* pp. 330, Vol. 21. Springer-Verlag, Berlin.
- Borgese T. A., Harrington J., Borgese J. M. and Nagel R. L. (1982) Thermostability of fish haemoglobins. *Comp. Biochem. Physiol.* **72B**, 7–11.
- Coe M. J. (1966) The biology of *Tilapia grahami* Boulenger in Lake Magadi, Kenya. *Acta Tropica* **24**, 146–177.
- Harrington J. P. (1982) Thermostability of salmon haemoglobins. *Comp. Biochem. Physiol.* **73B**, 919–922.
- Johnston I. A., Frearson N. and Goldspink G. (1973) The effects of environmental temperature on the properties of myofibrillar adenosine triphosphatase from various species of fish. *Biochem. J.* **133**, 735–738.
- Johnston I. A., Walesby N. J., Davison W. and Goldspink G. (1975) Temperature adaptation in myosin of antarctic fish. *Nature* **254**, 74–75.
- Johnston I. A. and Walesby N. J. (1977) Molecular mechanisms of temperature in fish myofibrillar adenosine triphosphates. *J. Comp. Physiol.* **119**, 195–206.
- Johnston I. A., Guderley H., Franklin C. E., Crockford T. and Kamunde C. (1994) Respiration rates of isolated muscle mitochondria from fish living at temperatures ranging from  $-1^{\circ}\text{C}$  in Antarctica to  $43^{\circ}\text{C}$  in a geothermal hot spring. *J. Exp. Biol.* (In press).
- Low P. S. and Somero G. N. (1976) Adaptation of muscle pyruvate kinases to environmental temperature and pressure. *J. Exp. Zool.* **198**, 1–12.
- Randall D. J., Wood C. M., Perry S. F., Bergman H., Maloij G. M. O., Mommsen T. P. and Wright P. A. (1989) Urea excretion as a strategy for survival in a fish living in a very alkaline environment. *Nature* **337**, 165–166.
- Somero G. N. (1991) Biochemical mechanisms of cold adaptation and sterothermality in Antarctic fish. In *Biology of Antarctic Fish* (Edited by G. di Prisco, B. Maresia and B. Tota) pp. 238–247. Springer-Verlag, Berlin.
- Tondo C. V., Mendez H. M., and Reischl E. (1980) Thermostability of haemoglobins in homeothermic and nonhomeothermic vertebrates. *Comp. Biochem. Physiol.* **66B**, 151–154.
- Warren L. M. and Wells R. M. G. (1988) Thermostability of haemoglobins from Antarctic fish. *Polar Biol.* **9**, 101–106.