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For further information about this article or if you need reprints, please contact:

Moahmmad Reza Dayer
Department of Biology,
Faculty of Science,
Shahid Chamran University,
Ahvaz, Iran

Tel: +98611-3331045
Fax: +98611-3331045

Temperature Tolerant Hemoglobin Variant of *Barbus sharpeyi*

¹Maryam Nekoei, ²Moahmmad Reza Dayer,
¹Behzad Laame-Rad and ³Mohammad Saaid Dayer

Hemoglobin is a tetrameric protein of $\alpha_2\beta_2$ subunits. The main function of hemoglobin is to pick up oxygen from surrounding environment. Since oxygen solubility in water is very low the availability of dissolved oxygen is affect by different factors e.g., temperature, salinity and pH. Temperature increment accounted as a more threatening factor for fish in regions with hot climate. In the present work, through conducting different experiment and using different methods such as circular dichroism, differential scanning calorimetry we decided to study hemoglobin stability against temperatures. *Barbus sharpeyi* was used as a model habitat in hot climate. Our results show that *Barbus sharpeyi* hemoglobin shows high melting high transition temperature of 68°C which tolerate against thermal denaturation for long period. The wide transition range of 56-68°C indicates that B.S. can adapt and survive in hot climate unexpectedly.

Key words: *Barbus sharpeyi*, hemoglobin, circular dichroism, differential scanning calorimetry

¹Department of Biology, Faculty of Science, Al-Zahra University, Tehran, Iran

²Department of Biology, Faculty of Science, Shahid Chamran University, Ahvaz, Iran

³Department of Parasitology and Medical Entomology, Tarbiat Modares University, Tehran, Iran

INTRODUCTION

Hemoglobin is a respiratory protein found in vertebrate erythrocytes that takes part in biological activities such as oxygen and CO₂ transport, H₂O₂ dispersion, electron transfer (Mojtahedi *et al.*, 2008; Brittain, 2002; Clementi *et al.*, 2007), oxygen storage and buffering (Baumann and Dragon, 2005) with good performance. However, the most important function of hemoglobin remains to be oxygen transportation. Hemoglobins obtained from different kinds of animals show structure-function relationships which enable their adaptation to unfavorable environmental conditions such as low oxygen pressure (Bonini-Domingos *et al.*, 2007). This relationship provides an interesting example of molecular mechanism acquired during animal evolution that helps animal to meet its need for oxygen (Clementi *et al.*, 1994). Unlike Antarctic ice fish, Channichthyidae which lack hemoglobin as an oxygen carrier, various species of fish kingdom obtain oxygen with aid of hemoglobin as an oxygen carrier (Sidell and O'Brien, 2006). Therefore, hemoglobin in these animals is expected to binds enough oxygen even under harsh and threatening conditions. This implies that fish hemoglobin may have experienced a high degree of evolutionary structural changes conferring sophisticated regulatory mechanisms as per fish circumstances. Oxygen solubility in water is only about 3.5 mg 100 mL⁻¹ and yet influenced by different environmental variables e.g., temperature, pH, salinity, ionic composition and environmental pollutants. Henceforth aquatic habitats differ from oxygen availability point of view. Oxygen deficiency poses a serious problem on fish survival and cause fish to exert different mechanism for adaptation such as synthesis of a wide spectrum of hemoglobins (hemoglobin isoforms) using different genes and evolving their gas exchanging organs (Di Prisco *et al.*, 2000). The first mechanism is adapted by some temperate fish species such as *Barbus grypus* (Dayer *et al.*, 2011). This species possesses different hemoglobin isoforms with different oxygen affinity that enable oxygen capture at various oxygen levels. Another possible mechanism that may function especially in warm climate is the presence of thermally stable variant of hemoglobin that functions at high temperature (Clementi *et al.*, 1994; Val and de Almeida-Val, 1988; Val, 1986). *Barbus sharpeyi* (Gunther, 1874), the most resistant fish to hot climate in Kuzestan Province of Iran, having a tetrameric hemoglobin and a single electrophoretic band is believed to use this kind of mechanism to resist against the hot climate (Mukhaysin and Jawad, 2012; Mohammadi and Marammazi, 2000). The present work aimed to answer two important questions in relation

to temperature resistance of B.S. hemoglobin. 1) What are structural characteristics of B.S. hemoglobins and how these characteristics confer tolerance against the hot climate in Khuzestan? 2) Are there structural bases for interdependence of the body temperature (T_b) and transition temperature (T_c) for hemoglobin?

MATERIALS AND METHODS

Materials: All materials used were of analytical grade and were purchased from sigma chemical Co.

Hb purification: *Barbus sharpeyi* were collected from Khuzestan fish reproduction center. Blood samples were drawn from the caudal vein by heparinized syringes. Immediately after sampling, erythrocytes were centrifuged at 1000 × g for 5 min at 4°C and washed three times with isotonic 1.7% NaCl solution. The crude lysate was achieved by adding 1 vol. of ice-cold. Stroma was removed by centrifugation at 31000 × g for 30 min at 4°C. We used a Sephadex G-25 column (1.5 × 45 cm) that was equilibrated with a 100 mM phosphate buffer pH 7.0. Elution was carried out with a 0.1 M phosphate buffer pH 7.0 at a flow rate of 25 mL per h.

Circular dichroism experiments: Circular Dichroism (CD) spectroscopy is appropriate technique to study secondary and tertiary structures of proteins (Quddus and Ma, 2003). We used of circular dichroism to monitor structural changes of the proteins.

Ultraviolet (UV) CD spectra were measured with a Aviv 215 CD spectropolarimeter equipped with a temperature Peltier controller. Thermal unfolding of hemoglobin was studied between 25 and 67°C in a 0.1 cm-thick quartz cuvette with a 0.1 cm optical path length. The starting temperature of the hemoglobin solution was adjusted to 25°C and then (stepwise or gradually) increased. The sample was allowed to equilibrate for 1 min at each temperature point. Then, a full wavelength scan was performed in the UV region between 190 and 260 nm. For measurement of the α-helical content of the proteins 222±2 nm wavelength scans were Selected Because CD method is sensitive at 222 nm to α-chain content in globins (Greenfield, 1996; Ranjbar *et al.*, 2006). The fractional change in the ellipticity at 222±2 nm was calculated according to under Equation:

$$F_{abs} = \frac{[E_{abs}(T) - E_{25}]}{[E_{max} - E_{25}]}$$

where, E_{abs}(T) is the ellipticity at 222 nm at temperature T, E_{max} is the ellipticity at the maximum temperature (°C) used and E₂₅ is the ellipticity at 25°C (Jiang *et al.*, 2001).

Spectroscopic experiments: Absorption spectra were recorded with a Shimadzu model UV-3100 (Japan) spectrophotometer and a thermostatically controlled cell compartment with a Haak D8 water bath. Spectrophotometric measurements were studied between 30 and 80°C. The spectra were recorded after 3 at every temperature. The absorbance were recorded at 280 nm. The concentration of hemoglobin solutions in the experiments was 2.5 mg mL⁻¹.

DSC experiments: Differential scanning calorimetric measurement of B.S hemoglobins were carried out in an ultra-sensitive Scal-1 microcalorimeter (Moscow, Russia) with a 0.3 mL cell at a heating rate of 2 K min⁻¹. Temperature ranges of 20 to 80°C were selected for heat transfer record. At this stage of the experiment, the buffer baseline was subtracted and the data were normalized with respect to protein concentration. To prevent a possible degassing of the solution during the heating process, the pressure was maintained at 2 atmospheres during all DSC runs. This software enabled us to deconvolute C_p^{ex} (excess C_p) profile to a number of sub peaks the energetic domains that contributed to observed DSC profile. T_m is one of application DSC that study in different experiments (Chottanom and Srisa-Ard, 2011; Baimark, 2009) T_m is the transition temperature at C_{p,max} and it is a criterion of protein stability.

RESULTS

Figure 1 represents heat capacity changes in constant pressure for B.S. hemoglobin obtained from DSC experiment. In this graph the highest value of C_p belongs to the temperature midpoint or melting temperature of hemoglobin (T_m) which is calculated to be 68°C. Turbidity measurement at 630 nm (data not shown) shows that B.S. hemoglobin unlike other hemoglobin used remain stable for long time of up to 1 h which in turn indicate more stable conformation for B.S hemoglobins. There are miscellaneous works used T_m as an index for hemoglobin stability (Baimark and Srisuwan, 2012; Srisa-Ard *et al.*, 2008; Kumaresan *et al.*, 2011; N'Dri *et al.*, 2007). Figure 2 shows changes in absorbance of hemoglobins at the wavelengths of 280 nm versus temperature as other measure of structural alteration in B.S. hemoglobin. The maximum point of this thermal profile is defined as the critical point equals to protein melting point (T_m). Figure 3 is pulled out from the data of Fig. 2. Indeed this graph depicts the derivation of Fig. 2 and shown as dY/dX against temperature. This derivation was made to sharps the graph point showing T_m. The maximum point in Fig. 3 belongs to B.S. T_m. This finding is completely

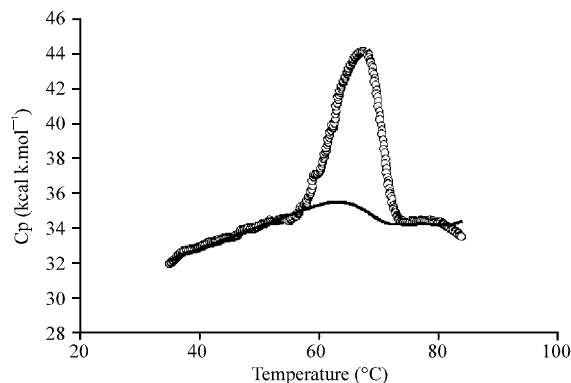


Fig. 1: Plot of C_p (heat capacity at constant pressure) against temperature. The peak maximum indicates the hemoglobin melting temperature, T_m. The data used here is obtained from DSC experiments. Hemoglobin concentration of 2 mg mL⁻¹ and 50 mM phosphate buffer, pH 7 was used

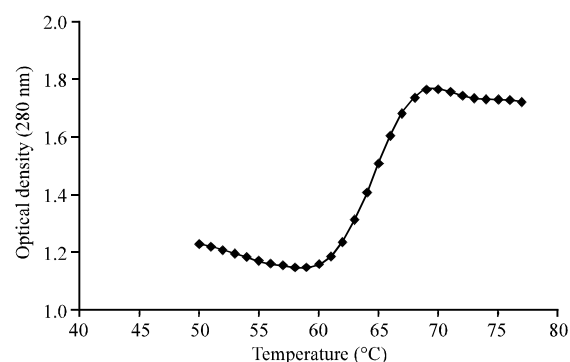


Fig. 2: Changes in hemoglobins optical density at 280 nm against temperature. Hemoglobin concentration of 4 mg mL⁻¹ and 50 mM phosphate buffer, pH 7 was used

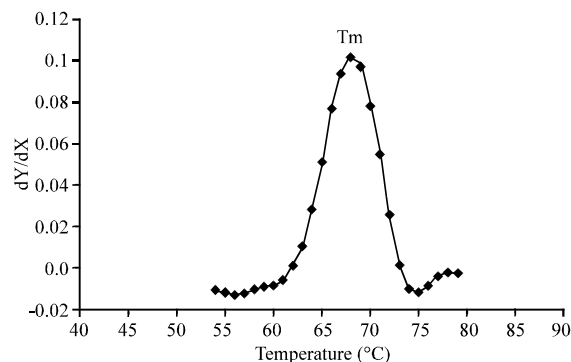


Fig. 3: Differential plot of the data presented in Fig. 2 illustrated as dY/dX against temperature to magnify B.S. hemoglobin melting temperature, T_m

agreeing with DSC findings shown in Fig. 1 form B.S. melting temperature of 68°C. Figure 4 shows the effect

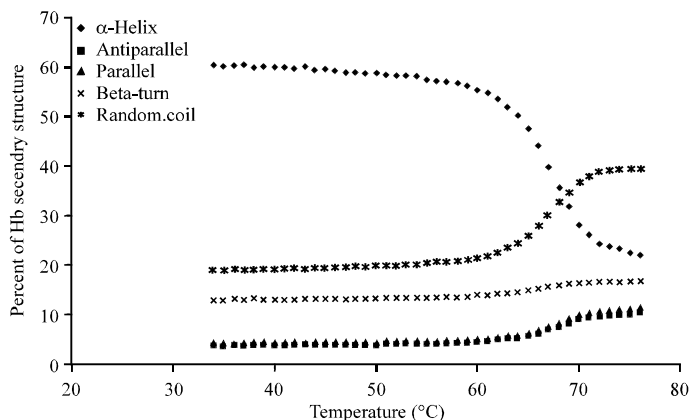


Fig. 4: Change in hemoglobin secondary structure with temperature (in %). The data were obtained using circular dichroism method. Hemoglobin concentration of 2 mg mL⁻¹ and 50 mM phosphate buffer with pH 7 was used

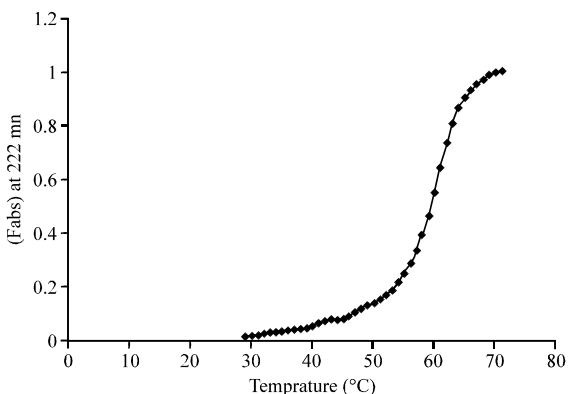


Fig. 5: Fractional change in circular dichroism ellipticity at 222 nm with temperature increment for B.S. hemoglobin. Hemoglobin concentration of 2 mg mL⁻¹ and 50 mM phosphate buffer with pH 7 was used

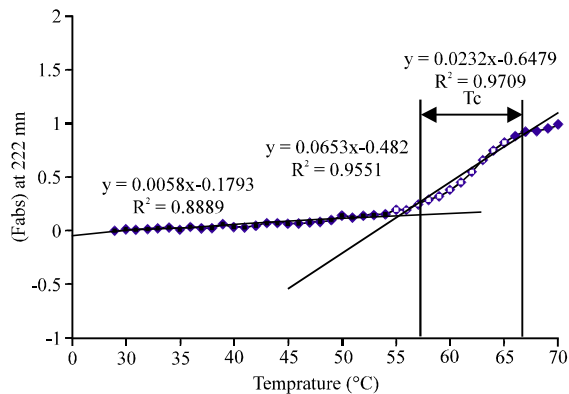


Fig. 6: Plot of fractional ellipticity at 222 nm showing critical temperature, T_c for B.S. hemoglobin. Hemoglobin concentration of 2 mg mL⁻¹ and 50 mM phosphate buffer, pH 7 was used

increasing temperature on hemoglobin secondary structures as percent in contrast to the total secondary structure content. The secondary structures of α -helix, anti parallel, parallel, beta-tern and random coil were calculated using Aviv software (www.avivbiomedical.com/circular.php). As it obvious the main secondary structure of hemoglobin in its native state is α -helix structure. Upon heat treatment this structure is decreased and the random coil instead increased to the same degree. However other secondary structures of anti parallel, parallel and beta-tern structures not significantly changed. Figure 5 indicate the fractional change in B.S. hemoglobin CD ellipticity at 222 nm. This curve give us the maximum transition temperature (T_c) of 68°C. Figure 6 was plotted so as to determine transition

temperature ranges that appear to be ranged from 56-68°C. The cross part of the best-fitting lines were regarded as the beginning and end of the accelerated transition temperature range (T_c) as previously shown by Digel *et al.* (2006).

DISCUSSION

Protein stability at high temperature (60-70°C) is primarily due to higher interaction between hydrophobic groups of protein (Bull and Breese, 1973; Irbäck and Sanderlin, 2000; Wagschal *et al.*, 1999; Kumar *et al.*, 2000; Chothia *et al.*, 1976). The hydrophobic properties of protein are considered as a factor stabilized the tertiary structure of protein (Bigelow and Channon, 1976; Chothia *et al.*, 1976). Perutz (1983) had shown that

protected hydrophobic domain insure protein thermal stability during evolution and maintain their performance in thermophilic conditions. The melting temperature of 68°C that reported here for B.S. (Fig. 1-6) indicates high thermal stability for B.S. hemoglobins tertiary structure. This thermal stability ultimately enabled *Barbus sharpeyi* to habitat the extreme conditions of higher temperature in Khuzestan Province. As Turi *et al.* (1981) proposed that proteins as active and functional units of living organisms answered to the environmental stimuli via changing their three dimensional conformation, we postulate this logic may be responsible for B.S. resistance for such a harsh conditions. Digel *et al.* (2006) calculated T_c for *Ornithorhynchus anatinus* (with body temperature 31-33) and *Tachyglossus aculeatus* (with body temperature 31-33) to be 34°C in contrast to our finding for B.S. indicates the more stability of B.S. hemoglobin. Based on these findings Digel *et al.* (2006) expressed that "Nature knows by protein structure where body temperature must be set". Transition temperature (T_c) for B.S. as depicted in Fig. 6 is calculated in the range 56-68°C (Kinderlerer *et al.*, 1973). Considering these temperature range we can conclude that B.S. with its stable hemoglobin can adapt hot climate and pick up dissolved oxygen and survive easily.

CONCLUSION

Present findings confirm that B.S. erythrocytes contain more compact and more stable conformation of tetrameric hemoglobin with higher transition temperatures when contrasted to human hemoglobin. Unlike *Barbus grypus* which uses the synthesis of different hemoglobin isoforms as a mechanism of adaptation. *Barbus sharpeyi* employs a temperature resistant form of hemoglobin of highly enough oxygen affinity to survive the hot climate of Khuzestan Province in Iran.

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