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Received 27 September 2012

Accepted 13 December 2012

Purification, crystallization and preliminary crystallographic studies of haemoglobin from mongoose (*Helogale parvula*) in two different crystal forms induced by pH variation

Haemoglobin (Hb) is a respiratory pigment; it is a tetrameric protein that ferries oxygen from the lungs to tissues and transports carbon dioxide on the return journey. The oxygen affinity of haemoglobin is regulated by the concentration of oxygen surrounding it and several efforts have revealed the shapes of Hb in different states and with different functions. However, study of the molecular basis of Hbs from low-oxygen-affinity species is critically needed in order to increase the understanding of the mechanism behind oxygen adaptation. The present study reports the preliminary crystallographic study of low-oxygen-affinity haemoglobin from mongoose, a burrowing mammal. Haemoglobin from mongoose was purified by anion-exchange chromatography, crystallized using the hanging-drop vapour-diffusion method and diffraction data sets were collected from monoclinic (2.3 Å resolution) and orthorhombic (2.9 Å resolution) crystal forms obtained by pH variation. The monoclinic and orthorhombic asymmetric units contained half and a whole biological molecule, respectively.

1. Introduction

Haemoglobin (Hb) is the integral molecule for oxygen transport in erythrocytes; it is found throughout the animal kingdom and in nearly all vertebrates (Anthea *et al.*, 1993). The oxygen-transport mechanism of haemoglobin allows us to understand not only respiratory transport, but also the environmental adaptation of creatures with unique oxygen-transport characteristics (Wells, 1999). Hb is a tetrameric protein composed of two pairs of globin polypeptide chains ($\alpha 1\beta 1$ and $\alpha 2\beta 2$); each chain contains a haem group bonded to a central Fe^{2+} ion. The quaternary structure of the haemoglobin orchestrates its function by exhibiting a transformation between tense (T) and relaxed (R) states; these states can switch allosterically without intermediates in the two-state model (Baldwin & Chothia, 1979; Perutz, 1972; Perutz & Ten Eyck, 1972). Several subsequent crystallographic experiments revealed the existence of intermediate conformations between the R and T states (R2, RR2 and R3), primarily by screening crystals obtained under different crystallization conditions based on varying the ion concentration and pH (Smith & Simmons, 1994; Smith *et al.*, 1991; Silva *et al.*, 1992; Schumacher *et al.*, 1997; Safo & Abraham, 2005). Furthermore, it has been found that pH is a primary regulator of the oxygen affinity of haemoglobin (Bellingham *et al.*, 1971) and several crystallographic studies have been conducted to investigate specific conformational changes in haemoglobin as a function of pH (Kuwada *et al.*, 2011; Mazzarella *et al.*, 2006; Nieves-Marrero *et al.*, 2010; Robinson *et al.*, 2003).

Mammalian haemoglobins can be broadly divided into two groups: those with intrinsically high oxygen affinity (*e.g.* those from human, horse, rabbit, guinea pig, rat *etc.*) and those with low oxygen affinity and sensitivity, including those from goat, sheep, cow and cat (Bunn, 1971). The mongoose is a mammal that digs underground burrows and uses them as a shelter from predation and climatic changes. Mongooses also have a great tolerance towards snake venoms. In order to understand the behaviour of mongooses under such extreme conditions, crystallographic studies were initiated in the haemoglobin molecule, particularly to investigate the role of conformational changes in the oxygen-regulation process. Here, haemoglobin from

mongoose was purified and crystallization experiments were performed at different pH values under normal atmospheric conditions. This is the first structural study of a haemoglobin from a member of the *Herpestidae* family.

2. Materials and methods

2.1. Isolation and purification

Fresh whole blood from mongoose was collected and 20 ml of the blood was subsequently treated with 5 ml 0.9% (w/v) saline solution containing 0.5 g EDTA to avoid clotting. After qualitative treatment, the red blood cells (RBC) were isolated from the whole blood by centrifugation for 30 min at 8000 rev min⁻¹ and 277 K. The isolated RBC pellet was washed three times in two volumes of 0.9% (w/v) isotonic saline solution and haemolysed by the addition of three times the volume of triple-distilled water. The maximum yield was achieved after 90 min of haemolysation, and subsequent centrifugation at 10 000 rev min⁻¹ at 277 K for 30 min yielded cell-free haemoglobin solution as the supernatant. The haemoglobin solution was carefully removed by suction and extensively dialysed in distilled water for 15 h. Finally, the sample was lyophilized and stored at 279 K.

The lyophilized mongoose haemoglobin sample was reconstituted with distilled water and loaded onto a DEAE-Cellulose anion-exchange chromatographic column (10 × 1.5 cm) equilibrated with

Figure 1
Native PAGE showing purified mongoose Hb in lane 2 (molecular mass 66.5 kDa) and molecular-mass standards (labelled in kDa) in lane 1.

Figure 2
Crystals of mongoose Hb in (a) monoclinic and (b) orthorhombic crystal forms.

Table 1

Crystallographic data-collection statistics.

Values in parentheses are for the highest resolution shell.

Data statistics	Monoclinic form (pH 8.2)	Orthorhombic form (pH 7.0)
Source	Cu K α	Cu K α
Wavelength (Å)	1.5417	1.5417
Crystal dimensions (mm)	0.4 × 0.3 × 0.3	0.5 × 0.3 × 0.2
Rotation range per image (°)	1	1
Total rotation range (°)	180	136
Exposure time per image (s)	60	60
Space group	C2	P2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å, °)	$a = 86.03$, $b = 88.81$, $c = 55.63$, $\beta = 115.38$	$a = 55.92$, $b = 88.21$, $c = 129.16$
Mosaicity (°)	0.29	0.15
Resolution (Å)	30–2.3	30–2.9
No. of measured reflections	61615	89126
No. of unique reflections	16749	16199
Data completeness (%)	99.0 (100)	98.8 (100)
Multiplicity	3.7	5.5
$R_{\text{merge}}^{\dagger}$ (%)	7.6 (31.3)	14.2 (43.3)
$\langle I/\sigma(I) \rangle$	13.20 (4.0)	10.80 (4.0)
Overall B factor from Wilson plot (Å ²)	19.5	19.5

$$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

water (Knapp *et al.*, 1999). The column was initially eluted with water followed by a sodium chloride concentration gradient (0.1–1.0 M in 0.1 M steps) at a flow rate of 2 ml min⁻¹. The purified sample corresponding to a single peak was obtained at 0.2 M NaCl. The homogeneity of the purified mongoose haemoglobin was confirmed by 10% native PAGE as shown in Fig. 1 (Davis, 1964) and the concentration was estimated to be 20 mg ml⁻¹ using the Bradford absorption method at 595 nm (Bradford, 1976).

2.2. Crystallization

Initial crystallization screening was performed using different precipitants such as 2-methyl-2,4-pentanediol (MPD) and PEG (molecular mass in the range 400–8000) as well as by varying the protein concentration (5–20 mg ml⁻¹) and pH. Diffraction-quality crystals of mongoose haemoglobin were obtained in two different crystal forms by pH variation using the hanging-drop vapour-diffusion technique at 293 K. The monoclinic form crystals were obtained in a buffered condition from drops comprising 3 μ l protein solution (20 mg ml⁻¹) and 2 μ l reservoir solution composed of 25% PEG 3350, 50 mM Tris–HCl pH 8.2 equilibrated against 1 ml reservoir solution. The orthorhombic form crystals were obtained in an unbuffered condition from drops comprising 2 μ l protein solution (20 mg ml⁻¹) and 2 μ l reservoir solution consisting of 35% PEG equilibrated against 1 ml reservoir solution consisting of 35% PEG

Figure 3
X-ray diffraction patterns of mongoose Hb in (a) monoclinic and (b) orthorhombic crystal forms.

3350 without any buffer or additives; however, the pH of the unbuffered reservoir solution was found to be 7.0, which is close to the physiological pH of haemoglobin found in the peripheral tissues (Voet *et al.*, 2008). Microscopic images of the crystals are shown in Figs. 2(a) and 2(b).

2.3. Data collection and processing

Crystals of the monoclinic and orthorhombic forms were mounted in cryoloops and cryoprotected by rapid soaking in mother liquor supplemented with 20% (v/v) glycerol before flash-cooling in a stream of gaseous N₂ at 100 K. X-ray diffraction data were collected for both crystal forms at 100 K at the in-house G. N. Ramachandran X-ray facility using a MAR345dtb image-plate detector. All images were processed, scaled and merged using *XDS* (Kabsch, 1993). Data-collection and processing statistics are presented in Table 1.

3. Results and discussion

Various combinations of precipitant, salt concentration and pH have been used in the crystallization of haemoglobin from various species to produce different crystal forms (Balasubramanian *et al.*, 2009a,b; Kaushal *et al.*, 2008). In the present study, by altering the pH value, mongoose haemoglobin was crystallized at room temperature in two different crystal forms, monoclinic and orthorhombic, under alkaline (pH 8.2) and neutral pH (unbuffered) conditions, respectively. The crystals of the monoclinic and orthorhombic forms of mongoose Hb diffracted to 2.3 and 2.9 Å resolution, respectively (Figs. 3a and 3b). The crystal packing revealed that the orthorhombic form accommodates one whole biological molecule in the asymmetric unit with a solvent content of 50%, whereas the monoclinic form contains half a molecule in the form of a dimer, with a solvent content of 58% (Kantardjieff & Rupp, 2003; Matthews, 1968). Both crystal forms were solved by the molecular-replacement program *Phaser* using

rabbit haemoglobin (PDB entry 2rao; S. Sundaresan, P. Charles, K. Neelagandan & M. N. Ponnuswamy, unpublished work) as a starting model. The solution displayed good crystal packing without any steric clashes between symmetry-related molecules, with log-likelihood gains of 995 for the monoclinic form and 1567 for the orthorhombic form. Model building was performed by multiple sequence alignment of 199 mammalian sequences comprising 11 burrowing species. The starting model clearly resolves the alterations of 33 amino acids in the $\alpha 1\beta 1$ dimer. Further work to build the model and refine the structure is in progress using the molecular-graphics program *Coot* (Emsley & Cowtan, 2004) and *REFMAC5* (Murshudov *et al.*, 2011) through the *CCP4i* interface (Potterton *et al.*, 2003), respectively.

We thank the reviewers for their constructive comments. The Department of Biotechnology (DBT), Government of India, is gratefully acknowledged for financial assistance to create the in-house G. N. Ramachandran X-ray facility in CAS in Crystallography and Biophysics. KS gratefully acknowledges a Professor T. R. Rajagopalan memorial research grant, SASTRA University, India.

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