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Purification, crystallization and preliminary X-ray diffraction studies on avian haemoglobin from pigeon (*Columba livia*)

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Haemoglobin is a physiologically significant metalloprotein that is involved in the exchange of gases for sustaining life. The respiratory system of birds is unique and complex compared with that of mammals. Many investigations of avian haemoglobins have revealed the presence of inositol pentaphosphate (IP5), a principal allosteric effector that is involved in regulation of their function. Structural investigations of avian haemoglobins are presently not adequate to explain their function. Efforts have been made in this direction in order to understand the oxygen-binding affinity involved in adapting to hypoxia in avian haemoglobins. Fresh whole blood was collected from pigeon (*Columba livia*) and purified using a DEAE cellulose anion-exchange chromatographic column. Crystallization of pigeon haemoglobin was accomplished using the hanging-drop vapour-diffusion method using PEG 3350 as a precipitant in 50 m*M* sodium acetate buffer pH 5.5 with 1 *M* NaCl. Data collection was carried out using a MAR345 image-plate detector system. The crystals diffracted to 2 Å resolution. Pigeon haemoglobin crystallizes in a triclinic space group, with two whole biological molecules in the asymmetric unit and with unit-cell parameters $a = 55.005$, $b = 65.528$, $c = 104.370$ Å, $\alpha = 78.742$, $\beta = 89.819$, $\gamma = 65.320^\circ$.

1. Introduction

Birds fly long distances for food, mates and shelter. Since ancient times, pigeons have been used for messenger services owing to their social behaviour with humans. Active flapping flight requires maximum energy provision to the flight muscles and their active function requires an efficient circulatory system (Milsom *et al.*, 1973). The respiratory system of birds is distinctive and complex, with small inelastic rigid lungs connected by thin-walled air sacs that exchange gases during respiration. The exchange of gases in the air sacs is ten times greater than that in mammals and helps in efficient function at elevated temperatures and high altitudes to provide the energy expenditure required during flight (Islam, 1990). Comparative investigation of avian and mammalian haemoglobins revealed the presence of inositol pentaphosphate (IP5) and 2,3-disphosphoglycerate (DPG), respectively, as the principal allosteric effectors in regulating their functions (Ajloo *et al.*, 2002). The presence of haemoglobin components such as 2,3-DPG ATP or IP5 with high intrinsic properties, low concentration and weak interaction with organic phosphate aids in regulating oxygen affinity during migration at high altitudes.

Bar-headed goose haemoglobin and greylag goose haemoglobin are avian haemoglobins which have been studied in both the relaxed (R) state and the tense (T) state. Comparison of the sequence of bar-headed goose haemoglobin with that of greylag goose haemoglobin yields 97 and 99% identity for the α and β chains, respectively, with the substitution of four residues (Zhang *et al.*, 1996). The sequence identity of pigeon haemoglobin to bar-headed goose haemoglobin and greylag goose haemoglobin is 81 and 83% for the α chain and 91 and 93% for the β chain, respectively. Perutz (1983) hypothetically suggested that the residue replacements αPro119→Ala and

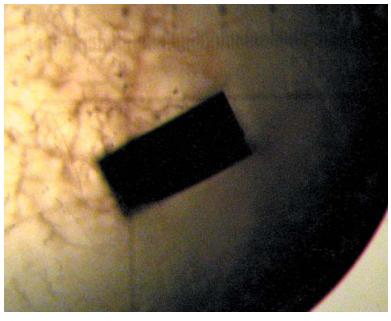

Table 1
Data-collection and data-processing statistics for pigeon haemoglobin.

Values in parentheses are for the highest resolution shell.

X-ray source	Cu $K\alpha$
Wavelength (Å)	1.5417
Oscillation angle (°)	1
No. of frames collected	180
Exposure time (s)	420
Space group	$P1$
No. of crystals used	1
Crystal-to-detector distance (mm)	120
Unit-cell parameters (Å, °)	a = 55.005, b = 65.528, c = 104.370, α = 78.742, β = 89.819, γ = 65.320
Resolution range (Å)	30–2.0 (2.07–2.0)
No. of reflections	1559970
No. of unique reflections	79185
$R_{\rm merge}$ (%)†	6.83
Average redundancy	1.75 (1.71)
Completeness (%)	92.0 (89.8)
$\langle I/\sigma(I)\rangle$	6.1 (1.4)
Mosaicity (°)	0.050

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the measured intensity of reflection I and $\langle I(hkl)\rangle$ is the mean intensity.

βLeu55→Ser in the interface region of bar-headed goose haemoglobin and pigeon haemoglobin, respectively, play a prime role in abolishing van der Waals interactions between proline and leucine, which in turn enhances the oxygen affinity by loosening the central cavity. The flights of birds are classified into two types: (i) a rapid flapping of wings during searching or chasing and (ii) a gliding motion during migration at high altitudes without flapping of wings. Pigeons are renowned for their rapid flight; they fly over long distances with an average speed of 96 km h^{-1} without halting. In view of the abovementioned properties, an attempt has been made to crystallize pigeon haemoglobin in order to explore its function.

2. Experimental procedures

2.1. Isolation and purification

Fresh whole pigeon blood was collected from the slaughterhouse and subsequently treated with 2 g EDTA to avoid clotting. Red blood cells (RBCs) were isolated from the whole blood by centrifugation at 1400g for 20 min. Isolated RBCs were washed three times with two volumes of 0.9%(w/v) saline solution and haemolyzed by the addition of three volumes of distilled water. Subsequent centrifugation at 5600g for 1 h yielded cell-free haemoglobin solution as the supernatant. The dialyzed sample was loaded onto a DEAE-cellulose anion-exchange chromatography column equilibrated with water (Knapp *et al.*, 1999). The column was initially eluted with water, followed by a stepwise increase in NaCl concentration; a sample corresponding to the single peak obtained at 0.1 M NaCl was collected at a rate of 3 ml min^{-1}. The homogeneity of the purified pigeon haemoglobin was confirmed by 10% native PAGE as shown in Fig. 1 (Davis, 1964).

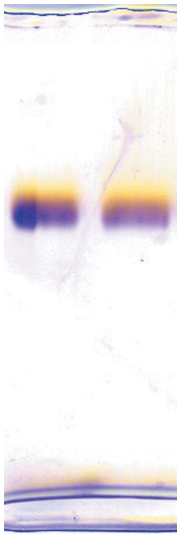

Figure 1
10% native PAGE of purified pigeon haemoglobin.

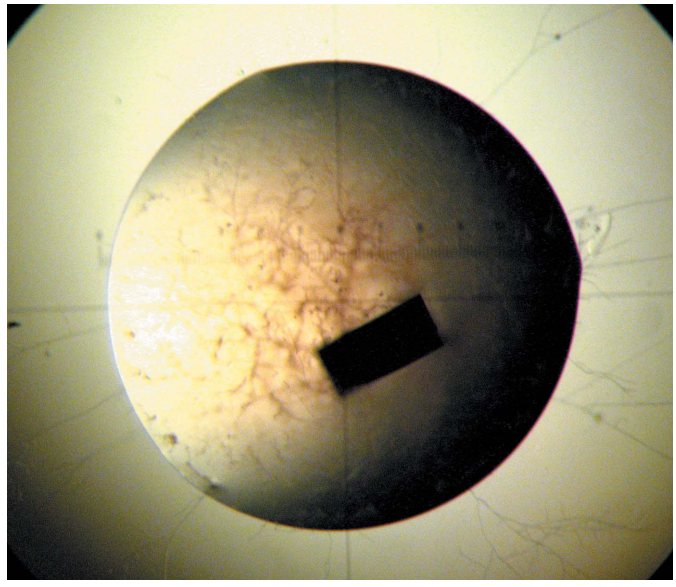

Figure 2
Single crystal of pigeon haemoglobin.

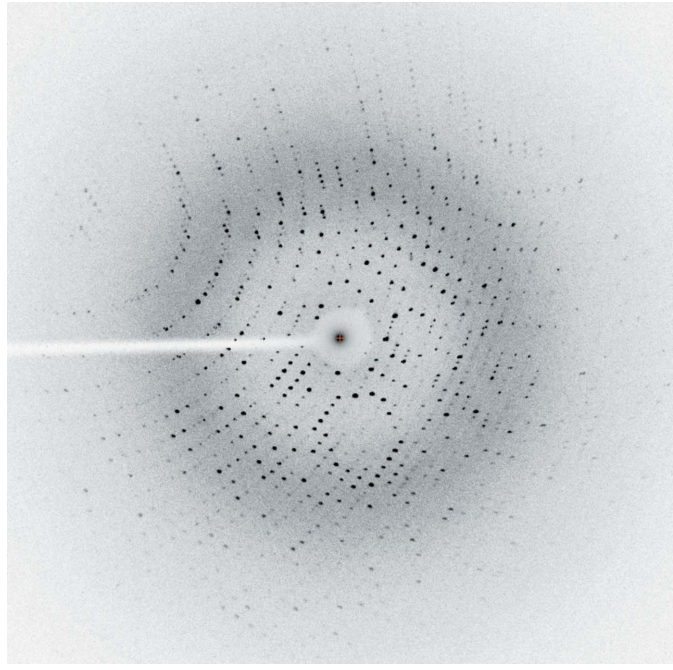

Figure 3
Diffraction pattern of pigeon haemoglobin.

2.2. Crystallization

The purified pigeon haemoglobin was dialyzed against 50 m*M* sodium acetate buffer pH 5.5 with 1 *M* NaCl for 48 h at 277 K, changing the dialyzing solution twice per day. The dialyzed sample was lyophilized and the concentration was estimated as 45 mg ml^{-1} using the Bradford absorption method at 595 nm (Bradford, 1976). The sample was screened with various precipitants (PEG 400–20 000). The sample was crystallized at room temperature with the same buffer using the hanging-drop vapour-diffusion method. Diffraction-quality crystals were obtained by equilibrating 2 µl protein solution and 2 µl of a well solution containing 30% PEG 3350 in 50 m*M* sodium acetate buffer pH 5.5 with 1 *M* NaCl against 1 ml well solution. Crystals were obtained within a week and are shown in Fig. 2. The dimensions of the crystal were 0.5 × 0.3 × 0.3 mm.

2.3. Data collection and processing

The pigeon haemoglobin crystal was mounted in a sealed quartz capillary tube containing mother liquor at both ends of the tube to reduce radiation damage. Data collection was carried out using a MAR345 image-plate detector system at the Central Leather Research Institute (CLRI), Chennai, India. The X-ray diffraction pattern of pigeon haemoglobin is shown in Fig. 3. Data-collection and processing statistics are listed in Table 1. The collected data sets were indexed, integrated, merged and scaled using *AUTOMAR* and *SCALEPACK* (Bartels & Klein, 2003).

3. Results and discussion

The pigeon haemoglobin crystals belonged to the triclinic space group *P*1, with unit-cell parameters a = 55.005, b = 65.528, c = 104.370 Å, α = 78.742, β = 89.819, γ = 65.320°. The crystal packing of pigeon haemoglobin in the presence of buffer at pH 5.5 with 1 *M* NaCl salt concentration accommodates two haemoglobin molecules ($\alpha 2\beta 2$) in the asymmetric unit, with a solvent content of 43.44% (Matthews, 1968).

The pigeon haemoglobin structure was solved by the molecular-replacement method with greylag goose oxyhaemoglobin as a starting model (PDB code 1faw; Liang *et al.*, 2001) using *Phaser* implemented in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Further model building and refinement are under way.

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