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Purification, crystallization and preliminary X-ray analysis of haemoglobin from ostrich (*Struthio camelus*)

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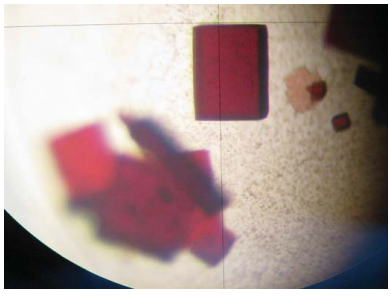
Haemoglobin is a tetrameric protein that carries oxygen from the lungs to tissues and carbon dioxide from tissues back to the lungs. The oxygen-binding properties of haemoglobin are regulated through the binding of allosteric effectors. The respiratory system of avian species is unique and complex in nature when compared with that of mammals. In avian species, inositol pentaphosphate (inositol-P_5) is present in the erythrocytes of the adult and is thought to be the major factor responsible for the relatively high oxygen affinity of the whole blood. The ostrich (*Struthio camelus*) is a large flightless bird which contains inositol tetrakisphosphate (inositol-P_4) in its erythrocytes and its whole blood oxygen affinity is higher. Efforts have been made to explore the structure–function relationship of ostrich haemoglobin. Ostrich haemoglobin was purified using ion-exchange chromatography. Haemoglobin crystals were grown by the hanging-drop vapour-diffusion method using PEG 3350 as the precipitant in 50 m*M* phosphate buffer pH 7.2. Data were collected using a MAR345 image-plate detector system. The crystals of ostrich haemoglobin diffracted to 2.2 Å resolution. They belonged to the orthorhombic space group $P2_12_12_1$ with one whole biological molecule in the asymmetric unit; the unit-cell parameters were a = 80.93, b = 81.68, c = 102.05 Å.

1. Introduction

Avian haemoglobins are functionally similar to mammalian haemoglobins. Avian species have non-elastic, small and rigid lungs that are connected by a number of thin-walled air sacs that cover a large part of the body cavity and help in the exchange of gases. The exchange of gases also occurs between air and the walls of the blood capillaries and is estimated to be ten times higher than in man. This kind of tenacity helps in the efficient functioning of the birds at elevated temperatures and high altitudes to provide the energy expenditure that is required during flight (Duncker, 1972). In most avian species inositol pentaphosphate is present in high concentrations in the erythrocytes of the adult and is responsible for the relatively high P_{50} of the whole blood in birds. Inositol pentaphosphate lowers the oxygen affinity of haemoglobin (giving a higher P_{50}) and is present in red blood cells to modify the haemoglobin function. The red blood cell of the adult ostrich contains an organic compound, namely inositol tetrakisphosphate, in higher concentrations and the red blood cell count is comparatively lower than those of other flightless avian species (Isaacks *et al.*, 1977). The three-dimensional structure determination of ostrich haemoglobin has been initiated in order to understand its structure–function relationship and can be compared with that of bar-headed goose, which flies at altitudes of about 9000 m and has higher oxygen affinity.



2. Experimental procedure

2.1. Isolation and purification

Fresh whole ostrich blood was collected from Livestock Research Station, Kattupakkam, Tamil Nadu, India and subsequently mixed

Table 1
Data-collection statistics for ostrich haemoglobin.

Values in parentheses are for the highest resolution shell.

X-ray source	Cu $K\alpha$
Wavelength (Å)	1.5418
Temperature (K)	100
Oscillation angle (°)	1
Crystal-to-detector distance (mm)	200
Resolution (Å)	29.60–2.2 (2.30–2.22)
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 80.93, b = 81.68, c = 102.05
Observed reflections	133277
Unique reflections	33356
Matthews coefficient (V_M; Å^3 Da^{-1})	2.59
Solvent content (%)	52.51
No. of molecules in ASU	1
R_{merge} (%)	6.31 (23.10)
Redundancy	3.19 (3.67)
Completeness	99.1 (93.5)
Average $I/\sigma(I)$	6.5 (1.7)

with 0.2 *M* EDTA solution to avoid clotting. Red blood cells were isolated from the whole blood by centrifugation at 1400*g* for 20 min and the recovered precipitant was washed three times with two volumes of 0.9%(*w*/*v*) saline and haemolyzed by the addition of three times the volume of distilled water. The haemolyzed solution was centrifuged at 5600*g* for 1 h, which yielded cell-free haemoglobin solution as the supernatant. The sample was applied onto an anion-exchange chromatography column using DEAE-cellulose as the column material; the chromatographic column was equilibrated with water (Knapp *et al.*, 1999). The salt-gradient elution was achieved using a NaCl gradient from 0.1 to 1.0 *M*. The haemoglobin was eluted at 0.1 *M* NaCl and was collected at a rate of 3 ml min^{-1}. Purified ostrich haemoglobin yielded a single band on 10% native PAGE (Davis, 1964). The purified haemoglobin along with the crude sample is shown in Fig. 1.

2.2. Crystallization

The purified haemoglobin was crystallized at 293 K using the hanging-drop vapour-diffusion method. The concentration was estimated to be 20 mg ml^{-1} using the Bradford absorption method at 595 nm (Bradford, 1976). The sample was screened for crystallization using varying concentrations of PEGs from molecular weight 2000 to 8000 as precipitants. Good diffraction-quality crystals of ostrich haemoglobin were obtained by equilibrating 3 µl haemoglobin solution and 3 µl reservoir solution consisting of 30% PEG 3350 (Sigma) in 50 m*M* phosphate buffer pH 7.2 against 1 ml reservoir solution. Fig. 2 shows crystals of ostrich haemoglobin obtained within a day.

Figure 1
10% native PAGE. Lane 1, crude sample. Lane 2, purified haemoglobin.

2.3. Data collection and processing

A crystal of ostrich haemoglobin of dimensions 0.4 × 0.4 × 0.2 mm was mounted in a cryo-loop with glycerol as a cryoprotectant and the intensity data were collected at 100 K on a MAR345dtb imaging plate. The diffraction pattern of an ostrich haemoglobin crystal is shown in Fig. 3. The data were indexed, integrated, merged and scaled using the *AUTOMAR* and *SCALEPACK* software packages (Bartels & Klein, 2003).

3. Results and discussion

Data-collection details for the ostrich haemoglobin crystal are listed in Table 1. Evaluation of the crystal-packing parameter indicated that the lattice can accommodate one whole biological molecule in the asymmetric unit, with a solvent content of 52.51% (Matthews, 1968). Structure solution was carried out by the molecular-replacement method with greylag goose oxyhaemoglobin as a starting model (PDB code 1faw; Liang *et al.*, 2001) using the program *Phaser*

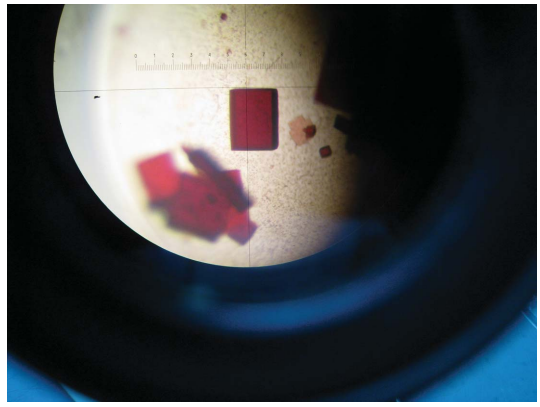


Figure 2
Crystals of ostrich haemoglobin (one division on the scale represents 0.01 mm).

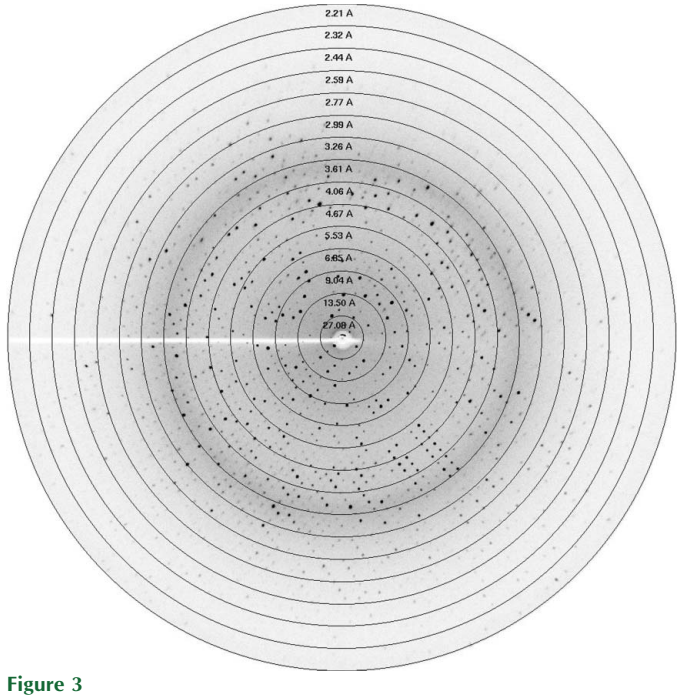


Figure 3
Diffraction pattern of ostrich haemoglobin along with resolution rings.

implemented in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Further work to model and refine the structure is in progress.

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