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Purification, Crystallization, Preliminary X-ray Diffraction and Molecular-Replacement Studies of White-Breasted Water hen (*Amaurornis Phoenicurus*) Haemoglobin

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Abstract

Haemoglobin is an interesting physiologically significant protein composed of specific functional prosthetic haem and globin moieties. In recent decades, there has been substantial interest in attempting to understand the structural basis and functional diversity of avian haemoglobins (Hbs). Towards this end, purification, crystallization, preliminary X-ray diffraction and molecular-replacement studies have been carried out on *Amaurornis phoenicurus* Hb. Crystals were grown by the hanging drop vapor-diffusion method using PEG 2000 and NaCl as precipitants. The crystals belonged to the primitive monoclinic system $P2_1$, with unit-cell parameters a = 65.33 Å, b = 93.14 Å, c = 98.54 Å, β = 100.48°; a complete data set was collected to a resolution of 2.6 Å. The Matthews coefficient of 2.30 $Å^3$ Da^{-1} for the crystal indicated the presence of two $\alpha_2\beta_2$ tetramers in the asymmetric unit.

Key words: White-breasted Water hen, Haemoglobin, Purification, Crystallization, Diffraction

1. Introduction

Hemoglobin, the major respiratory protein found in vertebrates, helps to carry oxygen from the lungs and it also transports oxygen to the tissues through blood capillaries[1]. Animals respond to their physiological demand for oxygen by possessing different pigments like Hemoglobin, Hemoerythrin and Hemocyanin[2,3] which greatly helps to increase the oxygen binding capacity through the prosthetic group-the metal ion associated with the protein. Birds are unique in their ability to fly which is highly energy consuming form of locomotion. The respiratory system of birds differs from that of mammals by uniquely adopting for very high oxygen consumption during flight[4]. The respiratory system of birds comprises mainly i) the lungs and ii) the nine interconnected thin walled air-sacs which fills larger part of the body[5], whereas in mammals the oxygen in the respiratory tract forms a much smaller proportion of the body's total oxygen store. The exchange area of air-capillaries is ten times larger in birds than in man. Also the avian RBC's are highly nucleated and larger than in mammals. But the avian RBC's has shorter half-life span which might be due to their high body temperature and metabolic rate. The interesting thing in birds during the condition of hypoxia is the cardiovascular adjustments arising from the large heart to body weight ratio and high stroke volume compared to mammals[6]. The ability of birds to maintain an efficient oxygen supply to the brain during severe hypoxia is an important adaptation contributing to their exceptional tolerance at extreme altitudes. The significant increase in cerebral blood flow during deep hypoxia at very low blood pCo2 indicates that the cerebral blood vessels in birds are sensitive to changes in carbon dioxide. Also it is found that the Avian Hbs contains more hydrophobic amino-acid residues than mammalian Hbs[7,8]. This observation is consistent with the presence of a higher content of hydrophobic residues in avian hemoglobin, its higher thermal stability and attainment of a tense (T) state. The conservation of hydrophobic domains in proteins such as the avian Hbs might have, in fact, required the stabilization of tertiary structure, in order to main-

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tain the function of the protein through a long period of evolution[8]. Thus, Hb of White-breasted water hen which lives near freshwater, ponds and pools were studied for understanding the mechanism of oxygen affinity. These birds also survive in muddy and polluted water where the oxygen levels were very low and temperature dependent. Hence to understand its oxygen affinity characteristics, attempts has been made to determine its crystal structure of haemoglobin. Here we report the purification, crystallization and preliminary x-ray diffraction statistics of waterhen haemoglobin were explained in detail.

2. Experimental Section

2.1. Isolation and Purification

Fresh whole blood from white breasted water hen was collected transferred immediately to 0.01% EDTA to avoid clotting and stored at 4°C. Red blood cells (RBC) were isolated from blood by centrifugation at 1,398 g for 20 min at 4°C. Isolated RBC were washed thrice with two volumes of 0.9% (w/v) saline solution and haemolyzed by addition of three volume of Millipore water. Subsequent centrifugation at 5,590 g for 1 h yielded cell-free haemoglobin solution as supernatant. The isolated protein was extensively dialyzed against distilled water for 24 h to remove trace salts and then the sample was loaded onto a DEAE-cellulose anion-exchange chromatography column (15-1.5 cm) equilibrated with water at physiological pH[9]. The column was eluted with water, followed by stepwise elution with various concentration of sodium chloride solution. A single peak obtained at 0.1 M NaCl was collected at a rate of 2 mL·min^{-1}. A small portion of the sample was used to check for protein content using Bradford assay[10]. Purified protein was further analyzed by electrophoresis technique were used to assess its purity in 12% SDS-PAGE & 10% NATIVE-PAGE[11] followed by the Coomassie Blue & silver-staining method [Fig. 1(a, b)].

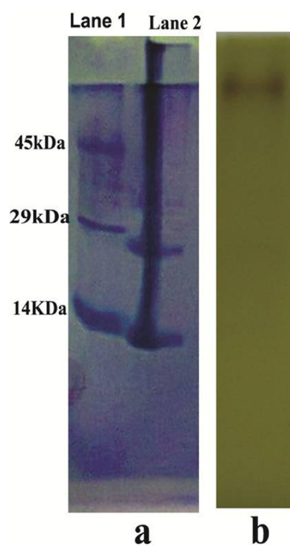

Fig. 1. (a) 15% SDS-PAGE gel stained with Coomassie Blue, lane 1 standard Molecular weight Marker, lane 2 white breasted water hen haemolysate Hb. (b) 10% native-page gel stained by silver staining method.

2.2. Crystallization and X-ray Data Collection

Crystals were grown by the hanging-drop vapor diffusion method using 6 μL droplets consisting of equal volumes of 20 mg·mL^{-1} protein solution and reservoir solution consisting of 35% (w/v) polyethylene glycol (PEG) 2000, 0.5 M NaCl. Crystals suitable for X-ray diffraction grew within 5 days at 291 *K* (Fig. 2). The Hb crystals were mounted in a cryoloop and data were collected at Cryo temperature using a MAR345 imaging plate at the Central Leather Research Institute (CLRI), Chennai. A total of 179 frames were collected at 291 *K* a crystal-to-detector distance of 100 mm, an oscillation angle of 1° and an exposure time of 300 s per image; the crystal diffracted to a maximum resolution of 2.6 Å (Fig. 2). Intensity measurements were processed and analyzed using iMOSFLM[12]. The data-collection, processing statistics are summarized in Table 1

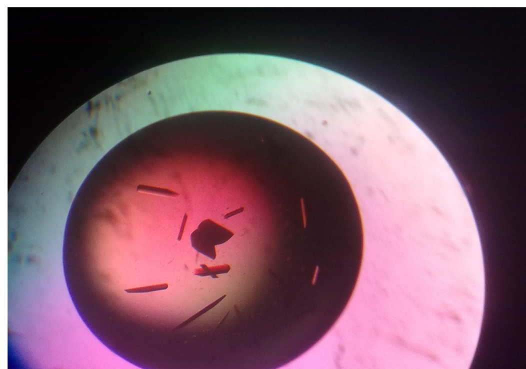

Fig. 2. Three dimensional single crystals

Table 1. X-ray data-collection and processing statistics

X-ray source	-	Cu$K\alpha$
Wavelength (Å)	-	1.5418
Temperature (K)	-	100
Oscillation (°)	-	1
Exposure time (s)	-	300
Detector distance (mm)	-	100
Resolution range (Å)	-	18.63-2.6
†R_{merge} (%)	-	11(31)
Space group	-	$P2_1$
Unit cell dimensions (Å, °)	-	a = 65.33
		b = 93.14
		c = 98.54
		β = 100.48
Asymmetric unit	-	$\alpha_2\beta_2$ tetramer
Completeness (%)	-	97.6 (98.8)
Mean {I/σ(I)}	-	6.2 (3.1)
Multiplicity	-	2.9 (3.1)
No. of observations	-	100626
No. of unique reflections	-	34912
V_M (A^3Da^{-1})	-	2.30
Solvent content (%)	-	46.63

†$R_{merge} = \Sigma_{hkl}\ \Sigma i^{|Ii(hkl) - \{I(hkl)\}|} / \Sigma_{hkl}\ \Sigma_i^{Ii(hkl)}$, where $Ii(hkl)$ is the ith measured intensity of reflection hkl and $\{I(hkl)\}$ is the mean intensity.
Values in the parentheses are for the highest resolution shell.

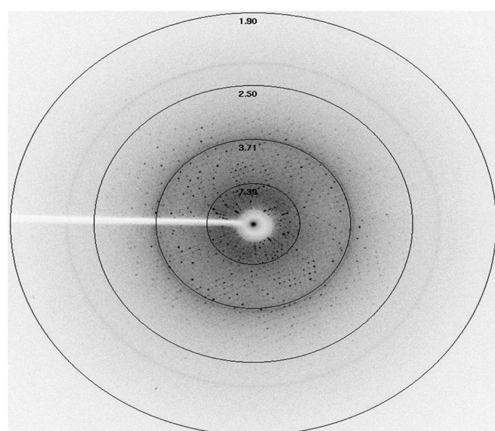

Fig. 3. X-ray diffraction pattern of white breasted water hen hemoglobin

and diffraction patterns of white breasted water hen hemoglobin is shown in Fig. 3.

3. Results and Discussion

Diffraction-quality crystals were grown using the hanging-drop vapor diffusion method from a reservoir solution containing 35% (w/v) PEG 2000 and sodium chloride as precipitants. Ward and coworkers did not find any major differences between the structures of deoxy haemoglobin A obtained using high salt and low-salt crystallization conditions[13]. The majority of the observed reflections were indexed in the primitive monoclinic space group $P2_1$, with unit-cell parameters a = 65.33 Å, b = 93.14 Å, c = 98.54 Å, β = 100.48°. A Matthews coefficient (Vm) of 2.30 $Å^3Da^{-1}$[16] and a solvent content of 46.63% were obtained assuming the presence of an $\alpha_2\beta_2$ tetramer in the asymmetric unit. A complete data set was collected to a resolution of 2.8 Å. A total of 100626 measured reflections were merged into 34 912 unique reflections with an R_{merge} of 11%. The mean I/σ (I) and overall completeness of the merged data set were 6.2 and 97.6%. Coordinates of liganded and unliganded avian Hbs were used as initial search model for molecular replacement, since the best solution was obtained from the deoxy form of human hemoglobin (PDB ID: 4HHB)[14]. Initial refinement was carried out in REFMAC[15] implemented in the CCP4 suite[16]; a randomly selected 5% of the total reflections were used for cross-validation[17]. Several rounds of refinement resulted in R_{factor} and R_{free} values of 0.27 and 0.33, respectively. 2mFo-Fc electron density was observed between the distal histidine and the Fe haem of all subunits, suggesting liganded (oxy or aquomet) haemoglobin. However, the complete amino-acid sequence of white breasted water hen Hb has yet to be determined, so mutating the corresponding amino acids based on the electron density map is not presently possible. Model building and final refinement of white breasted water hen Hb will be carried out after the complete amino-acid sequence has been determined.

4. Conclusions

However, the complete amino acid sequence is yet to be done. Hence attempt has been made to find the initial phases towards structure determination using human deoxy Hb as template. For molecular replacement, *AMORE* was used to find the rotational and translational matrices. Refinements were carried out with

REFMAC, and the present R_{factor} and R_{free} were to 0.27 and 0.33, respectively. As the Amino acid sequence is not known, modeling exact residues using $2F_o$-F_c and F_o-F_c may mislead. The complete structure can be arrived if amino acid sequencing has been done or high resolution data obtained.

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