Purification and crystallization of haemoglobin from donkey (*Equus asinus*)

D. Balasundaresan, K. Saraboji, and M.N. Ponnuswamy*

Department of Crystallography and Biophysics, University of Madras, Guindy Campus, Chennai 600025, India

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Abstract

Haemoglobin acts as an important protein for oxygen carrier in all living beings. Purification of donkey haemoglobin was done using PEG 4000 as a separating medium. Crystallization was achieved using hanging drop vapor diffusion method using 2.8 M phosphate buffer, pH 6.8. Data collection was done using mar345 image plate detector system. The crystals grown under 2.8 M phosphate buffer are monoclinic with space group C2 and cell dimensions *a* = 107.664 Å, *b* = 63.084 Å, *c* = 54.042 Å, and *β* = 111.747°.

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Haemoglobin is a tetrameric protein that carries oxygen from the lungs to the tissues and carbon dioxide from the tissues back to lungs. For efficient functioning, haemoglobin needs to bind oxygen tightly in the oxygen-rich atmosphere of the lungs and be able to release oxygen rapidly in the relatively oxygen-poor environment of the tissues. The process happens in a most elegant and intricately coordinated way. The active site in each subunit consists of an iron atom, bound to four pyrrole nitrogen atoms of a porphyrin and to *Ne* of His (F8), with the reversibly bound oxygen occupying the sixth co-ordination position at the iron [1–3].

According to two-state model for allosteric enzymes [4] haemoglobin is in equilibrium between a low-affinity, tense (T) form and a relaxed (R) high-affinity one, each having a distinct quaternary structure, thus the allosteric properties and oxygen transport characteristics of haemoglobin have attracted considerable study. Many papers have been published on the elucidation of quaternary structure–function relationships of haemoglobin [5–10].

The amino acid identity between donkey and horse haemoglobin is 98% for α-subunit and the β-subunit is conserved in all *Equus* species [11] from a sequence comparison. We initiated the three-dimensional structure of donkey haemoglobin to study the quaternary structure–function relationship and to compare it with horse haemoglobin. This paper reports the purification, crystallization, and preliminary analysis of donkey haemoglobin at 3.0 Å resolution.

Materials and methods

**Isolation and purification.** Purification of donkey haemoglobin was done as described by Lu et al. [12]. Fresh whole blood obtained from donkey was mixed with 2 g EDTA to avoid blood clotting. RBCs were isolated from whole blood by centrifugation at 5000 rpm for 20 min, washed three times with two volumes of 0.9% saline, and hemolyzed by adding three volumes of triple distilled water. The hemolyzed solution was centrifuged at 15,000 rpm for 1 h to yield Hb solution free of cell debris. The Hb solution was mixed with stock solutions of PEG 4000 and K2HPO4/NaH2PO4 to obtain a solution containing 12.5% PEG 4000, 12.5% phosphate, pH 10, with the addition of 12.5% NaOH.

After phase separation, the top phase was withdrawn and added to NaH2PO4 to a final composition of 12.5% PEG 4000, 12.5% phosphate, pH 7.0. After mixing and phase separation, the bottom phase containing Hb solution was dialyzed with triple distilled water and lyophylized. The Hb solution gives single zone upon electrophoresis.

**Crystallization.** Haemoglobin was crystallized by hanging-drop vapor diffusion method at room temperature from drops containing 10 µl protein solution (80 mg/ml in water) and 4 µl reservoir solution,
equilibrated against 1 ml reservoir solution (2.8 M phosphate buffer, pH 6.8). Good quality crystals were used for data collection (Fig. 1).

Data collection and processing. The crystals were mounted in 0.5 mm Lindmann tubes and the data were collected using mar345 image plate detector system. A dataset for 180°C176 (1°C176 oscillation frames, each exposed for 240 s) up to 3.0°C23 Å resolution was collected with a crystal-to-detector distance of 100 mm at 293 K. All the data were indexed, integrated, merged, and scaled using DENZO and SCALEPACK from the HKL program package [13].

Results and discussion

Haemoglobin crystals obtained by vapor diffusion method (hanging-drop) were of size 0.20 × 0.15 × 0.02 mm. The crystals were grown within 10 days. Crystals of haemoglobin grown under 2.8 M phosphate buffer, pH 6.8, are monoclinic with space group C2 and cell dimensions a = 107.664 Å, b = 63.084 Å, c = 54.042 Å, and β = 111.747°. Evaluation of crystal packing parameter [14] indicated that the lattice can accommodate two molecules (α1β1 dimer) per asymmetric unit (V_m (Å³/Da) −2.26) with a solvent content of 53.5%.

The structure solution was obtained by molecular replacement method using aquamet horse haemoglobin (PDB id: 2MHB) as starting model, using the program AMoRe [15] implemented in CCP4 suite (Collaborative Computational Project, No. 4, 1994). The solution resulted in a conventional crystallographic R-factor of 35.1% and correlation co-efficient of 72.1% with two molecules in an asymmetric unit. Further refinement of the structure and model building to the electron density map using program O [16] is underway.

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References


Fig. 1. A single crystal of haemoglobin.