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Crystallization of sheep (*Ovis aries*) and goat (*Capra hircus*) haemoglobins under unbuffered low-salt conditions

K. Neelagandan, Pon. Sathya Moorthy, M. Balasubramanian and M. N. Ponnuswamy*

Centre of Advanced Study in Crystallography and Biophysics, University of Madras, Guindy Campus, Chennai 600 025, India

Correspondence e-mail: mnpsy@hotmail.com

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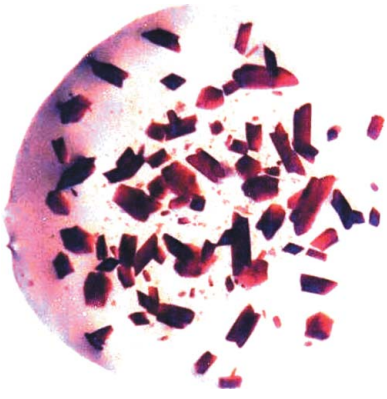
Haemoglobin is a tetrameric protein that plays a vital role in the transport of oxygen from the lungs to the tissues and of carbon dioxide back to the lungs. Even though a large amount of work has already been performed in this area, the study of the haemoglobin structures of avian and mammalian species is rather incomplete. Efforts are being made to understand the salient features of the species mentioned above. Here, whole blood plasma was collected from sheep and goat and purified by anion-exchange chromatography; the haemoglobins were crystallized by the hanging-drop vapour-diffusion method under unbuffered low-salt conditions using PEG 3350 as a precipitant. Data collection was carried out using a MAR345 image-plate detector system. Sheep haemoglobin crystallizes in the orthorhombic space group $P2_12_12_1$ with one whole biological molecule ($\alpha_2\beta_2$) in the asymmetric unit, with unit-cell parameters $a = 60.231$, $b = 70.695$, $c = 131.479$ Å. In contrast, goat haemoglobin crystallizes in the triclinic system with two biological molecules ($\alpha_2\beta_2$) in the unit cell. The unit-cell parameters are $a = 53.103$, $b = 69.382$, $c = 96.098$ Å, $\alpha = 110.867$, $\beta = 91.133$, $\gamma = 109.437^\circ$.

1. Introduction

Mammalian haemoglobins can be broadly classified into two groups: those with an intrinsically high oxygen affinity and those with low oxygen affinity. Those with a higher affinity for oxygen include that of humans and contain 141 and 146 amino-acid residues in the α- and β-chains, whereas the lower affinity group, such as those of sheep and goat, have 141 and 145 amino-acid residues in the α- and β-chains, respectively. When the sequences of the α- and β-chains of sheep and goat haemoglobins are compared with the human sequences, an absence of the histidine residue at the second position of the β-chain is found. This probably hampers oxygen affinity, leading to low oxygen affinity in the sheep and goat haemoglobins (Bunn, 1971; Perutz & Imai, 1980). The amino-acid identity of human haemoglobin to those of sheep and goat is 86% for the α-subunits and 82% and 80% for the β-subunits, respectively. The Monod–Wyman–Changeux model reveals two conformational states, namely the low-affinity T state (tensed) and the high-affinity R state (relaxed), which have discrete quaternary structures (Monod *et al.*, 1965). Many more research publications have revealed further R2, RR2 and R3 structural conformations of liganded haemoglobins (Silva *et al.*, 1992; Mueser *et al.*, 2000; Safo & Abraham, 2001, 2005). Different crystal forms have been found to occur on alteration of the crystallization conditions. Screening of crystals grown under different crystallization conditions reveals the existence of energetically accessible structures of haemoglobin in solution. To explore the possibility of obtaining new crystal forms, an attempt was made to crystallize sheep and goat haemoglobins using unbuffered very low salt conditions under normal atmospheric conditions.



2. Experimental procedure

2.1. Isolation and purification

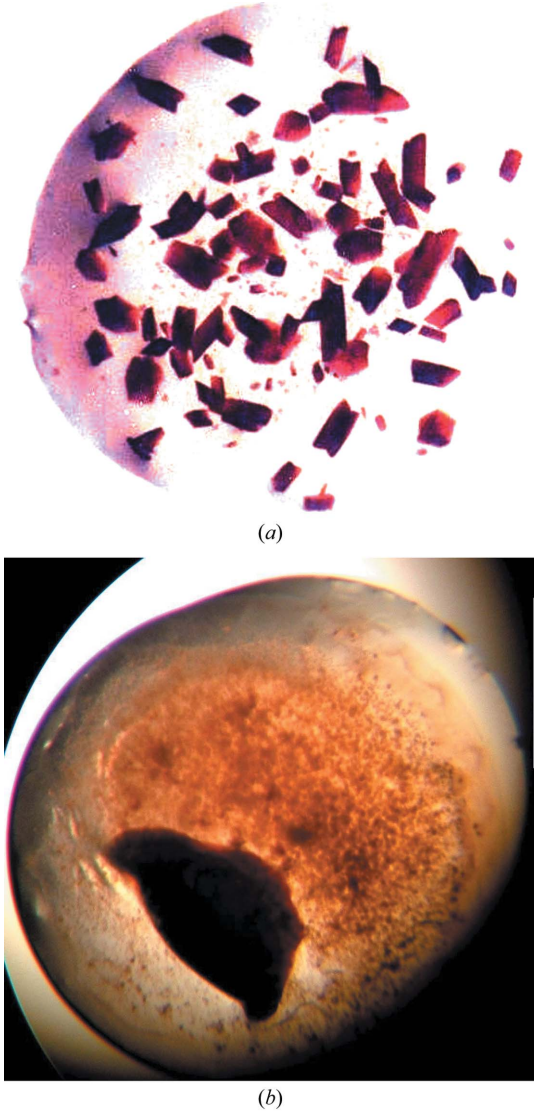
Fresh whole blood from sheep and goats was collected from a slaughterhouse and subsequently treated with 2 g EDTA to avoid

clotting. Red blood cells (RBC) were isolated from the whole blood by centrifugation at 1398*g* for 20 min. Isolated RBC were washed three times with two volumes of 0.9%(*w*/*v*) saline solution and haemolyzed by addition of three times the volume of distilled water. Subsequent centrifugation at 5590*g* for 1 h yielded cell-free haemoglobin solution as supernatant. Haemoglobin solution was removed cautiously by suction and dialyzed against distilled water for 48 h, changing the distilled water twice per day.

The sample was then loaded onto a DEAE-cellulose anion-exchange chromatography column (15 × 1.5 cm) equilibrated with water at physiological pH (Knapp *et al.*, 1999). The column was eluted with water, followed by stepwise elution with various concentrations of sodium chloride solution. A single peak obtained at 0.1 *M* NaCl was collected at a rate of 3 ml min^{-1}. Purified sheep and goat haemoglobins yielded a single band on SDS–PAGE (Laemmli, 1970).

2.2. Crystallization

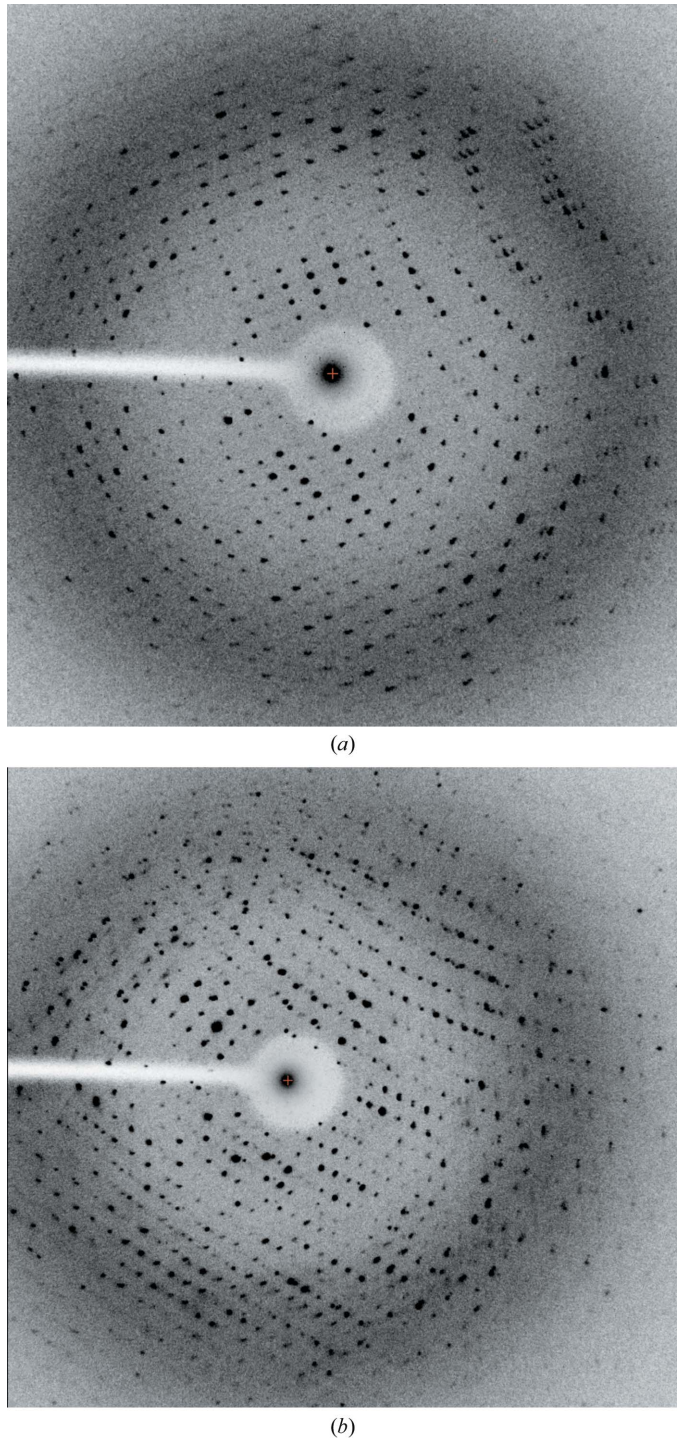
Sheep and goat haemoglobins were crystallized at room temperature under unbuffered low-salt conditions using the hanging-drop vapour-diffusion method. Lyophilized sheep and goat haemoglobins were dissolved in water and their concentrations were estimated to be 35 and 40 mg ml^{-1}, respectively, using the Bradford absorption method at 595 nm (Bradford, 1976). The samples were screened for crystallization using various protein concentrations and varying the precipitants in the range PEG 400–10 000. Diffraction-quality crystals of sheep haemoglobin were obtained by equilibrating 2 µl of 20% PEG 3350 (Sigma) and 2 µl haemoglobin solution against 1 ml reservoir solution (20% PEG 3350). Crystals of goat haemoglobin were obtained by equilibrating 2 µl of 20% PEG 3350 and 3 µl haemoglobin solution against 1 ml reservoir solution (25% PEG



(*a*)

(*b*)

Figure 1
Crystals of (*a*) sheep and (*b*) goat haemoglobin.



(*a*)

(*b*)

Figure 2
Diffraction patterns of (*a*) sheep and (*b*) goat haemoglobin.

Table 1
Data-collection and data-processing statistics.

	Sheep haemoglobin	Goat haemoglobin
X-ray source	Cu $K\alpha$	Cu $K\alpha$
Wavelength (Å)	1.5417	1.5417
Oscillation angle (°)	1	1
No. of frames collected	120	180
Exposure time (min)	5	5
Space group	$P2_12_12_1$	$P1$
No. of crystals used	1	1
Crystal-to-detector distance (mm)	100	100
Unit-cell parameters (Å, °)	a = 60.231, b = 70.695, c = 131.479	a = 53.103, b = 69.382, c = 96.098, α = 110.867, β = 91.133, γ = 109.437
Resolution range (Å)	30–2.7	30–2.7
No. of reflections	49414	57637
No. of unique reflections	13253	31524
$R_{\rm merge}$ (%)	13.8	14
Average redundancy	3.1	1.8
Completeness	95.7	90.38
$\langle I/\sigma(I)\rangle$	3..9	3.7

3350). Fig. 1 shows the crystals of sheep and goat haemoglobins obtained.

2.3. Data collection and processing

Good-quality crystals of sheep and goat haemoglobin were mounted in 1 mm quartz capillary tubes with mother liquor at both ends of the tube to avoid damage. Data collection was carried out using a MAR345 image-plate detector system at the Central Leather Research Institute (CLRI), Chennai, India. Diffraction patterns of sheep and goat hemoglobin are shown in Figs. 2(*a*) and 2(*b*), respectively. Data-collection and processing statistics are listed in Table 1. The data sets collected were indexed, integrated, merged and scaled using the *AUTOMAR* and *SCALEPACK* software packages (Bartels & Klein, 2003).

3. Results and discussion

Sheep haemoglobin crystallizes in a different crystal form compared with other haemoglobin samples reported under low-salt conditions. Goat haemoglobin crystallizes in a triclinic space group, a unique feature in mammalian species. Crystal-packing parameters reveal that sheep haemoglobin contains one molecule in the asymmetric unit with a solvent content of 43%, whereas goat haemoglobin accommodates two ($\alpha_2\beta_2$) molecules in the form of a dimer, with a solvent content of 43.17% (Matthews, 1968).

Attempts are being made to solve the structures of sheep and goat haemoglobins using the molecular-replacement method with carbonmonoxy haemoglobin as a starting model (PDB code 1g08) in *AMoRe* and *MOLREP* implemented in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Further work is in progress.

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