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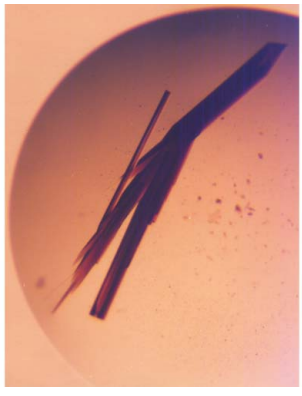
Purification, crystallization and preliminary crystallographic study of low oxygen-affinity haemoglobin from cat (*Felis silvestris catus*) in two different crystal forms

Haemoglobin is a metalloprotein which plays a major role in the transportation of oxygen from the lungs to tissues and of carbon dioxide back to the lungs. The present work reports the preliminary crystallographic study of low oxygen-affinity haemoglobin from cat in different crystal forms. Cat blood was collected, purified by anion-exchange chromatography and crystallized in two different conditions by the hanging-drop vapour-diffusion method under unbuffered low-salt and buffered high-salt concentrations using PEG 3350 as a precipitant. Intensity data were collected using MAR345 and MAR345dtb image-plate detector systems. Cat haemoglobin crystallizes in monoclinic and orthorhombic crystal forms with one and two whole biological molecules ($\alpha_2\beta_2$), respectively, in the asymmetric unit.

1. Introduction

Haemoglobins from mammals can be classified into two groups: high and low oxygen-affinity species. Mammals with high oxygen-affinity haemoglobin include humans and dogs and the haemoglobin α- and β-chains comprise 141 and 146 amino-acid residues, respectively, whereas species with low oxygen-affinity haemoglobin such as cattle, goats, sheep, buffalo and cats have 141 and 145 amino-acid residues in the haemoglobin α- and β-chains, respectively. Sequence comparison of both the α- and β-chains showed that the absence of histidine at the second position in the β-chain is likely to be responsible for low oxygen affinity in cat haemoglobin (Bunn, 1971; Perutz & Imai, 1980). The sequence identity of human and cat haemoglobins is 85% and 84% in the α- and β-subunits, respectively.

The MWC (Monod–Wyman–Changeux) model elucidated the existence of the two conformational states of haemoglobin, namely the low-affinity T state and the fully liganded high-affinity R state, which have distinct quaternary structures (Monod *et al.*, 1965). Subsequently, many haemoglobin structures have been shown to adopt different conformational states such as R2, RR2 and R3 that arise from alterations of the crystallization conditions (Silva *et al.*, 1992; Mueser *et al.*, 2000; Safo & Abraham, 2001, 2005). In order to study the different conformational states of low oxygen-affinity haemoglobin, an effort has been made to crystallize a sample from cat using unbuffered low-salt and buffered high-salt conditions.



2. Experimental procedure

2.1. Isolation and purification

Fresh cat blood was collected from the Government Veterinary Hospital, Tindivanam, Tamilnadu, India and subsequently treated with 2 g EDTA to avoid clotting. Red blood cells (RBC) were isolated from the whole blood by centrifugation at 1500*g* for 20 min. Isolated RBC were washed three times with two volumes of 0.9%(*w*/*v*) saline solution and haemolyzed by the addition of three times the volume of triple-distilled water. After 1 h, the haemolyzed solution was centrifuged at 5000*g* for 1 h, yielding cell-free haemoglobin solution as the supernatant. The haemoglobin solution was

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

Values in parentheses are for the highest resolution shell.

	Monoclinic form		Orthorhombic form	
	Room temperature	Cryotemperature	Room temperature	Cryotemperature
X-ray source	Cu $K\alpha$	Cu $K\alpha$	Cu $K\alpha$	Cu $K\alpha$
Wavelength (Å)	1.5418	1.5418	1.5418	1.5418
Temperature (K)	293	100	293	100
Oscillation angle (°)	1	1	1	1
Exposure time (min)	7	1	5	1
Space group	$P2_1$	$P2_1$	$P2_12_12_1$	$P2_12_12_1$
No. of crystals used	1	1	1	1
Crystal dimensions (mm)	0.7 × 0.3 × 0.2	0.5 × 0.4 × 0.3	0.5 × 0.6 × 0.7	0.4 × 0.5 × 0.6
Crystal-to-detector distance (mm)	150	135	100	135
Unit-cell parameters (Å,°)	a = 56.045, b = 74.195, c = 72.272, β = 102.803	a = 55.507, b = 73.217, c = 70.380, β = 103.381	a = 86.022, b = 96.465, c = 146.345	a = 84.788, b = 94.053, c = 142.252
Resolution range (Å)	30.0–2.2 (2.28–2.20)	30.0–2.0 (2.07–2.00)	30.0–2.9 (3.00–2.90)	30.0–2.4 (2.48–2.40)
Observed reflections	72197	89964	99416	152319
Unique reflections	28427	35212	26800	44340
Matthews coefficient (V_M) (Å^3 Da^{-1})	2.35	2.23	2.44	2.28
Solvent content (%)	47.73	44.95	49.54	45.98
No. of molecules in ASU	1	1	2	2
R_{merge}† (%)	6.85 (30.36)	7.39 (32.37)	9.80 (26.48)	8.09 (31.26)
Average redundancy	2.46 (2.50)	2.50 (2.55)	3.65 (3.14)	3.39 (3.37)
Completeness	95.2 (93.7)	93.8 (92.2)	96.0 (98.5)	97.3 (94.2)
Average $I/\sigma(I)$	6.4 (1.2)	5.1 (1.2)	4.1 (1.4)	3.8 (1.2)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

removed carefully by suction. The sample was dialyzed in distilled water overnight, lyophilized and stored at 279 K.

The lyophilized cat haemoglobin sample was reconstituted with water and loaded onto a DEAE-Cellulose anion-exchange chromatographic column (15 × 1.5 cm) equilibrated with water. The column was initially eluted with water, followed by stepwise elution with various concentrations of sodium chloride solution. A single peak was obtained at 0.1 *M* sodium chloride (Neelagandan *et al.*, 2007; Balasubramanian *et al.*, 2009). The homogeneity of the purified cat haemoglobin was checked using native PAGE as shown in Fig. 1 (Davis, 1964). Two fractions were taken from the purified haemoglobin samples and dialyzed against water and phosphate buffer, respectively. The dialyzed samples were then lyophilized and stored at 279 K.

Figure 1
10% native PAGE showing purified (lane *a*) and partially purified (lane *b*) cat haemoglobin.

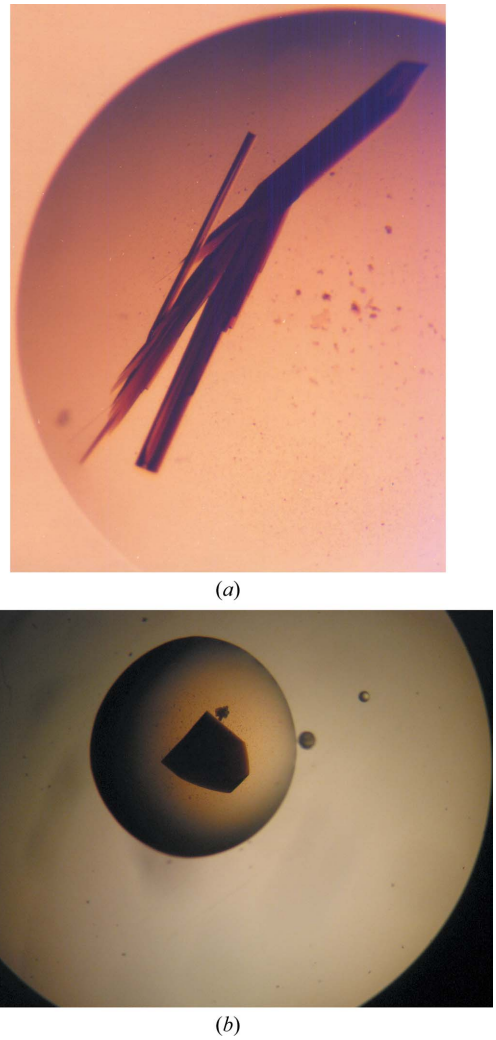
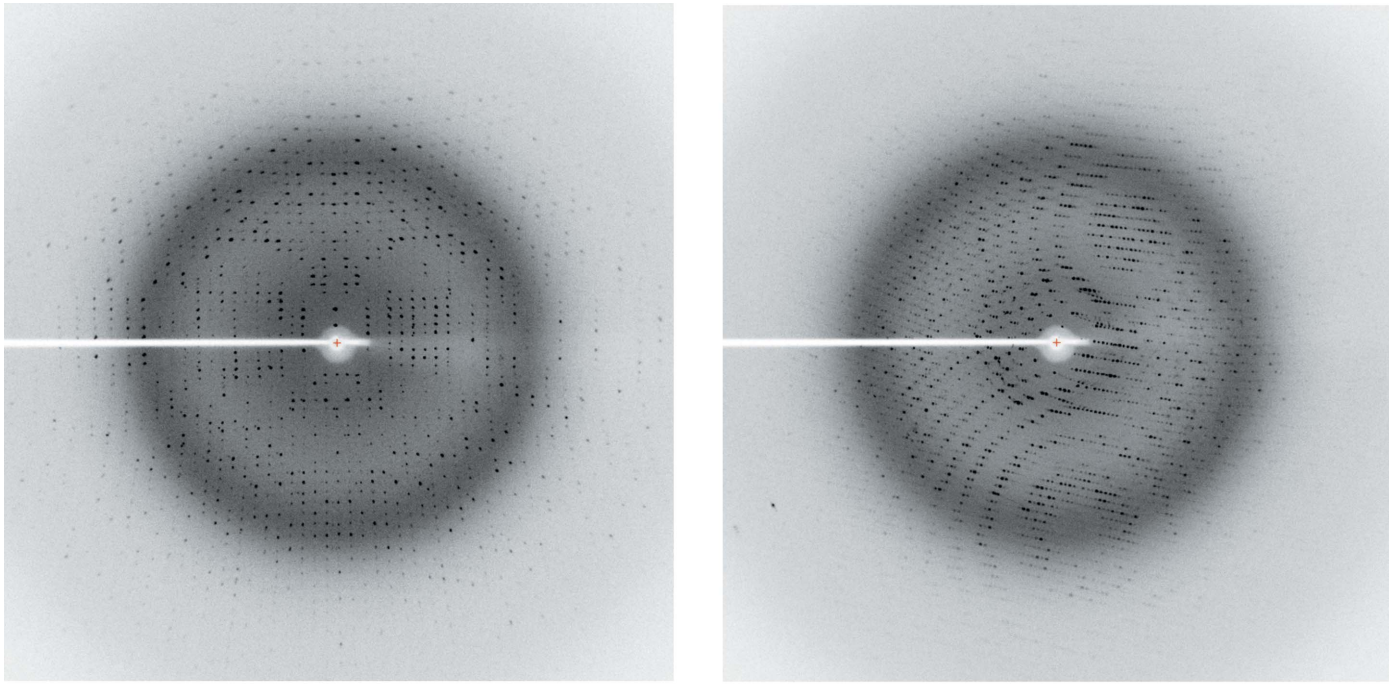


Figure 2
Crystals of (*a*) monoclinic and (*b*) orthorhombic forms of cat haemoglobin.



(*a*) (*b*)

Figure 3
Diffraction patterns of (*a*) monoclinic and (*b*) orthorhombic forms of cat haemoglobin.

2.2. Crystallization

Cat haemoglobin was crystallized at room temperature under two different conditions, unbuffered low-salt and buffered high-salt, using the hanging-drop vapour-diffusion method. Lyophilized cat haemoglobin powder was dissolved in water and buffer; the concentration was estimated as 40 mg ml^{-1} using the Bradford absorption method at 595 nm (Bradford, 1976). The crystallization conditions of the haemoglobin were optimized by varying the concentration of protein and using different precipitants such as MPD and PEGs in the range 400–10 000.

Under unbuffered low-salt conditions, diffraction-quality crystals were obtained by the hanging-drop vapour-diffusion method from a drop containing 2 µl protein solution and 2 µl 50% PEG 3350 in water equilibrated against 1 ml reservoir containing the same solution. This condition yielded a monoclinic crystal form of cat haemoglobin.

Under buffered high-salt conditions, diffraction-quality crystals were obtained by the hanging-drop vapour-diffusion method from a drop containing 2 µl protein solution and 2 µl 40% PEG 3350 in 50 m*M* phosphate buffer pH 6.7 with 1 *M* NaCl equilibrated against 1 ml reservoir containing the same solution. This condition yielded an orthorhombic crystal form of cat haemoglobin. The crystals were obtained after a week and are shown in Figs. 2(*a*) and 2(*b*).

2.3. Data collection and processing

Crystals were mounted in a cryoloop and data were collected at cryotemperature using a MAR345dtb imaging plate at the in-house G. N. Ramachandran X-ray facility. Data were also collected for both crystal forms at room temperature using a MAR345 imaging plate at the Central Leather Research Institute (CLRI), Chennai. The diffraction patterns are shown in Figs. 3(*a*) and 3(*b*). Data-collection and data-processing statistics are presented in Table 1. The data sets were indexed, integrated, merged and scaled using the *AUTOMAR* and *SCALEPACK* software packages (Bartels & Klein, 2003).

3. Results and discussion

Cat haemoglobin was crystallized at room temperature under unbuffered low-salt and buffered high-salt conditions. Two different crystal forms were obtained.

The monoclinic crystal ($P2_1$) has a whole biological molecule in the asymmetric unit, with solvent contents of 44.95% and 47.73% at cryotemperature and room temperature, respectively, whereas the orthorhombic crystal ($P2_12_12_1$) has two whole biological molecules in the asymmetric unit, with solvent contents of 45.98% and 49.54% at cryotemperature and room temperature, respectively (Matthews, 1968).

Initial phase determination for both forms was attempted using the molecular-replacement method with bovine deoxyhaemoglobin as a starting model (PDB code 1hda; Perutz *et al.*, 1993) in *AMoRe* and *Phaser* as implemented in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Further work to solve, model and refine the structure is under progress.

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