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Crystallization, preliminary X-ray diffraction analysis and Patterson search of oxyhaemoglobin I from the wolf (*Chrysocyon brachiurus*)

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Oxyhaemoglobin I isolated from the Brazilian wolf *Chrysocyon brachiurus* has been crystallized and X-ray diffraction data has been collected to 2.06 Å resolution using a synchrotron-radiation source. Crystals were determined to belong to the space group $P2_12_12_1$ and preliminary structural analysis revealed the presence of one tetramer in the asymmetric unit. The structure was determined using standard molecular-replacement techniques and is currently being refined using maximum-likelihood protocols. This is the first haemoglobin isolated from a member of the Canidae family to be crystallized and it will provide further insights in the comparative biochemistry of vertebrate haemoglobins.

1. Introduction

Haemoglobin is the standard model for studies of allosteric control in proteins. Systematic studies of haemoglobin structures led to the two-state model proposed by Monod *et al.* (1965). According to this model, there are two possible states, the T state with low oxygen affinity and the R state with higher oxygen affinity. Crystallographic studies by Perutz and colleagues have established that the two states are structurally different (Perutz, 1972), with the sharpest differences being a relatively simple displacement of the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers (Baldwin & Chothia, 1979). However, more recently several authors have proposed the existence of other conformational states (Jayaraman *et al.*, 1995; Huang *et al.*, 1996; Mercedes da Silva *et al.*, 1992; Smith *et al.*, 1991).

Structural studies of mammalian haemoglobins started in the 1960s with the work of Perutz. However, crystallographic studies of haemoglobins isolated from a number of animal classes still need to be performed. Furthermore, even for the well studied mammalian haemoglobins, most of the available structural information is focused on human haemoglobins. For instance, there is still no haemoglobin structure available for the Canidae family. In the present work, we report the crystallization and the preliminary X-ray diffraction analysis of a haemoglobin isolated from the wolf *Chrysocyon brachiurus*, an endangered species native to Brazil, as part of our continuing efforts to obtain further insights into allosteric control in proteins.

2. Materials and methods

2.1. Purification and crystallization

Blood from adult specimens of *C. brachiurus* was collected using heparinized syringes. Red blood cells were washed six times by centrifugation in large volumes of 50 m*M* Tris–HCl buffer pH 8.5 containing 1.7% NaCl and 1 m*M* EDTA. Haemolysis was accomplished using the same buffer but without sodium chloride. The cell debris were removed by centrifugation (1100*g* for 2 h) and filtration (Millipore membrane, pore size 1.6 µm). Organic phosphates and other small molecules were removed by filtration through Sephadex G-25 and several passages through Amberlite MB-1 (mixed-bed resin). Haemoglobin I was purified by ion-exchange chromatography using DEAE–Sephadex and a linear gradient between 50 m*M* Tris–HCl pH 9.0 and bis–Tris 50 m*M* pH 6.5.

The purified haemoglobin was deionized using Amberlite MB-1 as described above, concentrated and stored in liquid nitrogen until use. Haemoglobin concentration was estimated using the molar extinction coefficients of human HbAo (Riggs, 1981).

The haemoglobin used in the crystallization experiments was dissolved in water. Crystals of haemoglobin from *C. brachiurus* were obtained under several different crystallization conditions following sparse-matrix methods (Jancarik & Kim, 1991) and using the hanging-drop vapour-diffusion technique. The best crystals were obtained after one week of growth from drops in which 7 µl haemoglobin solution (12 mg ml^{-1}) was mixed with an equal volume of 30% polyethylene glycol 1500

Table 1
Detailed X-ray diffraction statistics for synchrotron data from a crystal of oxyhaemoglobin I isolated from *C. brachiurus*.

Resolution (Å)	Number of independent reflections	Completeness for all data (%)	Redundancy	R_{merge}† for all data (%)
100.00–4.59	3174	81.6	2.79	2.9
4.59–3.64	3442	93.4	2.71	2.9
3.64–3.18	3493	95.6	2.61	3.8
3.18–2.89	3498	95.9	2.65	4.9
2.89–2.68	3472	96.4	2.65	6.5
2.68–2.52	3476	96.0	2.65	7.9
2.52–2.40	3434	95.9	2.65	9.2
2.40–2.29	3433	96.0	2.64	11.1
2.29–2.21	3433	95.4	2.64	13.0
2.21–2.13	3359	94.5	2.62	16.4
2.13–2.06	3360	93.8	2.60	20.4
All reflections	37574	94.1	2.66	9.0

† $R_{merge} = 100 \times \sum_{hkl}[\sum_i(|I_{hkl,i} - \langle I_{hkl}\rangle|)]/\sum_{hkl,i}(I_{hkl})$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with indices h, k and l, and $\langle I_{hkl}\rangle$ is the mean intensity of that reflection.

reservoir solution. A crystal was mounted in a borosilicate glass capillary tube for X-ray data collection.

2.2. X-ray data collection and processing

X-ray diffraction data were collected at a wavelength of 1.37 Å using a synchrotron-radiation source (Station PCr, Laboratório Nacional de Luz Síncrotron, LNLS, Campinas, Brazil) and a 30 cm MAR imaging-plate detector (MAR Research) at room temperature. The crystal-to-detector distance was set to 160.0 mm. Using an oscillation range of 0.7°, 90 images were collected and the raw X-ray diffraction data were processed to 2.06 Å resolution using *DENZO* and scaled using *SCALEPACK* (Otwinowski, 1993).

Autoindexing procedures, combined with analysis of the X-ray diffraction pattern and averaging of equivalent intensities, were used in the characterization of the Laue symmetry.

2.3. Molecular replacement

The crystal structure of the haemoglobin isolated from *C. brachiurus* was determined by standard molecular-replacement methods using the program *AMoRe* (Navaza, 1994). The atomic coordinates of the human oxyhaemoglobin tetramer deposited in the PDB (Shaanan, 1983; accession code 1hho) were used as a search model. All water and phosphate molecules were removed from the search model and the temperature factors for all atoms were set to 20 Å^2, but the haem groups were kept in the model. The atomic coordinates for the search model were translated to set their centre of gravity at the origin and were then rotated so that the principal axes of inertia of the search model lie parallel to the orthogonal axes.

Cross-rotation functions were calculated in the resolution ranges 10.0–4.5, 8.0–3.0 and 6.0–3.0 Å, with a sampling step of 2.5°, using the program *AMoRe* (Navaza, 1994). These calculations were carried out with an integration radius of 25 Å. The rotation which generated the highest correlation coefficient (CC) was applied to the search model and used in the subsequent translation-function computations, based on data in the same resolution range. Translation functions were computed in the resolution range 10.0–4.5 Å for space groups $P222$, $P222_1$, $P2_12_12$ and $P2_12_12_1$ in order to confirm the space group.

The best solution model was selected on the basis of the magnitude of the R factor and correlation coefficient.

3. Results and discussion

Crystals of haemoglobin isolated from *C. brachiurus* suitable for X-ray diffraction experiments have average dimensions of about 1.5 × 0.7 × 0.5 mm. The crystal has a primitive orthorhombic Laue lattice, with unit-cell parameters $a = 54.2$ (1), $b = 87.7$ (1), $c = 133.4$ (1) Å. Assuming the asymmetric unit content to be one tetramer of molecular weight 64.5 kDa, the V_m value is 2.46 Å^3 Da^{-1} (Matthews, 1968). Assuming a value of 0.74 cm^3 g^{-1} for the protein partial specific volume, the calculated solvent content in the crystal is 50.0% and the calculated crystal density is 1.18 g cm^{-3}. Detailed data-collection statistics for the synchrotron data are given in Table 1. The intensities $h00$, $0k0$ and $00l$ were collected; however, they all have intensities bellow $1\sigma(I)$, which indicates the presence of three screw axes.

The rotation search for different resolution ranges (10.0–4.5, 8.0–3.0 and 6.0–3.0 Å) produced correlation coefficients between 17.7 and 59.6%. The highest magnitude of the correlation function was obtained in the resolution range 10.0–4.5 Å for the Euler angles $\alpha = 89.6$, $\beta = 79.2$, $\gamma = 173.3°$. The results of the translation search are listed in Table 2. The highest correlation coefficient and the lowest R factor were obtained for the space group $P2_12_12_1$.

Table 2
Fractional coordinates (T_X, T_Y, T_Z) after translation-function computation for all possible space groups which belong to the Laue group primitive orthorhombic.

The values in parentheses are those found for the second peaks.

Space group	T_x	T_y	T_z	CC (%)	R factor
$P222$	0.3696	0.0820	0.4676	29.0 (28.7)	51.9 (52.9)
$P222_1$	0.3452	0.0106	0.3922	19.0 (17.9)	54.7 (55.9)
$P2_12_12$	0.3442	0.0643	0.1638	17.7 (16.6)	55.1 (55.7)
$P2_12_12_1$	0.0919	0.0096	0.4143	57.6 (39.7)	42.5 (49.0)

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