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Crystallization, preliminary X-ray analysis and molecular-replacement solution of the carboxy form of haemoglobin I from the fish *Brycon cephalus*

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Haemoglobin, the 'honorary enzyme' [Brunori (1999), *Trends Biochem. Sci.* **24**, 158–161], constitutes a prime prototype for allosteric models. Here, the crystallization and preliminary X-ray analysis of haemoglobin I from the South American fish *Brycon cephalus* are reported. X-ray diffraction data have been collected to 2.5 Å resolution using synchrotron radiation (LNLS). Crystals were determined to belong to the space group $P6_122$ and preliminary structural analysis revealed the presence of one dimer ($\alpha\beta$) in the asymmetric unit. The structure was determined using standard molecular-replacement techniques.

1. Introduction

Haemoglobins constitute a pool of proteins with varying and interesting structural and functional properties, especially when we focus on two large animal groups: reptiles and fishes. The presence of isoforms meets a wide spectrum of environmental and physiological needs (Perutz, 1996; DeYoung *et al.*, 1994; Smarra *et al.*, 1997, 1999, 2000; Fadel *et al.*, 2000).

Oxygen transport is adjusted according to physiological needs by allosteric control exerted by heterotropic effectors: mainly phosphates, but also chloride, protons, carbon dioxide, bicarbonate, urea and other chemical entities, depending on the source. Protons bind when haemoglobin (Hb) downloads oxygen at the tissue level (the Haldane effect) and simultaneously produce a decrease in oxygen affinity: the Bohr effect (Fago *et al.*, 1999; Seixas *et al.*, 1999). The present work was performed on haemoglobin I (Hb-I) from the South American teleost freshwater fish *B. cephalus*. Structural studies on fish haemoglobins can provide further evidence to establish new conformational states and allosteric control. A preliminary study on the functional properties of this haemoglobin revealed an inverse Bohr effect for the stripped Hb (in the absence of phosphates) and cooperative oxygen binding ($n_{50} \simeq 1.5$). In the presence of adenosine triphosphate (ATP), the response of oxygen affinity as a function of proton concentration disappears and cooperativity increases by about twofold (Honda *et al.*, 1999). This functional characteristic is unusual and deserves further study.

2. Materials and methods

2.1. Purification

Blood was collected by caudal vein puncture from adult specimens at the Centro de Aqüicultura of the State University of São Paulo (Caunesp-Unesp) at Jaboticabal SP (Brazil). The animals were anaesthetized using benzocaine (1 g per 15 l of water). Subsequent procedures were carried out at low temperature (around 277 K). Red blood cells were washed by centrifugation four times with buffered saline (50 m*M* Tris pH 8.5 containing 0.2% D-glucose and 1 m*M* EDTA). Haemolysis was accomplished with buffer *A* (50 m*M* Tris pH 8.5 containing 1 m*M* EDTA), followed by centrifugation (1000*g* for 1 h) and filtration through Millipore membranes for debris removal. For phosphate removal, haemolysate dialysis was performed against buffer *A*, followed by gel filtration on Sephacryl S-100 (Sigma) on a 2.6 × 30 cm column equilibrated with the same buffer.

Haemoglobin purification was performed on DEAE–Sephadex A-50 using a pH gradient between 50 m*M* Tris buffer pH 9.2 and 50 m*M* bis-Tris pH 6.5. The isolated components were further deionized by several passages through mixed-bed Amberlite MB-1 (Sigma), were concentrated by centrifugation on Amicon microconcentrators and were stored in liquid nitrogen until use.

Table 1
Haemoglobins used as search models for molecular replacement.

PDB code	Protein
1hho	Oxy Hb from *Homo sapiens*
1t1n	Carboxy Hb from the fish *Trematomus akajei*
1ouu	Carboxy Hb from the fish *Oncorhyncus mykiss*
1cg8	Carboxy Hb from the fish *Dasyatis akajei*
1hbh	Deoxy Hb from the fish *Pagothenia bernacchii*
1out	Deoxy Hb from the fish *O. mykiss*
1pbx	Carboxy Hb from the fish *P. bernacchii*
1spg	Carboxy Hb from the fish *Leiostomus xanthurus*
1cg5	Deoxy Hb from the fish *D. akajei*

Non-denaturing electrophoresis was performed in 10% polyacrylamide gel and

Table 2
Data-processing statistics for BcHb-I.

Values for the highest resolution shell are given in parentheses.

Resolution range (Å)	20.0–2.5 (2.56–2.50)
Total No. of observations ($I > 1\sigma$)	61431
No. of unique reflections	13667
Completeness (%)	98.3 (98.7)
Redundancy	4.5 (4.52)
Overall $I/\sigma(I)$	15.64 (5.16)
$R_{\rm merge}$† (%)	7.5 (24.9)

† $R_{\rm merge} = 100\sum_{hkl}[\sum_i(|I_{hkl,i} - \langle I_{hkl}\rangle|)]/\sum_{hkl,i}(I_{hkl})$, 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.

Table 3
Eulerian angles and fractional coordinates after translation-function computation.

Protein	α (°)	β (°)	γ (°)	x	y	z	CC (%)	R factor† (%)
1hho	45.18	44.43	187.50	0.0561	0.4834	0.3196	22.3 (21.6)	57.7 (56.9)
1t1n	58.70	136.87	1.62	0.1178	0.7214	0.0387	21.1 (20.8)	57.0 (57.1)
1ouu	29.81	77.16	41.97	0.2342	0.1704	0.3413	55.3 (37.1)	43.4 (50.9)
1cg8	5.56	135.70	190.71	0.3635	0.6433	0.2703	20.6 (19.7)	55.8 (56.3)
1hbh	1.06	44.44	91.62	0.4833	0.9100	0.2573	21.7 (20.8)	56.6 (56.0)
1out	−0.63	44.93	92.55	0.9143	0.5610	0.3638	20.2 (19.5)	57.0 (57.2)
1pbx	55.20	136.64	0.16	0.2380	0.0386	0.4509	23.1 (21.3)	55.4 (56.7)
1spg	28.35	79.70	42.75	0.2338	0.1718	0.3425	51.6 (36.6)	45.4 (51.3)
1cg5	11.85	95.18	101.82	0.0598	0.6828	0.2637	19.6 (19.5)	56.5 (56.9)

† $R = 100\sum|F_{\rm obs} - F_{\rm calc}|/\sum(F_{\rm obs})$, the sums being taken over all reflections with $F/\sigma(F)^2$ cutoff. The values in brackets are those found for the next peaks.

cellulose acetate. The gel was digitized without staining on a Genius EP scanner and analysed using a freeware program: *Bandleader* 3.00 (Magnitec, Israel).

Isoelectric focusing was performed in a Rotofor Prep Cell (BioRad) using an EPS 3501 (Pharmacia) source power and 3-10 ampholytes. The fractions were read on a Scan 100 (Varian) spectrophotometer and the pH was measured using a 350 Orion pH meter.

2.2. Crystallization and X-ray data collection

The carboxy form of haemoglobin I (BcHb-I) was concentrated to 10 mg ml^{-1} in 50 m*M* Tris buffer pH 9.5.

Crystallization trials were performed using the hanging-drop vapour-diffusion method (Jancarik & Kim, 1991).

Hexagonal-shaped crystals were obtained after 2 d growth from drops in which 5 µl of haemoglobin solution (10 mg ml^{-1}) was mixed with an equal volume of reservoir solution. 0.1 *M* HEPES pH 7.5 and 1.4 *M* trisodium citrate dihydrate was used as the reservoir solution.

Preliminary X-ray studies on BcHb-I showed that these crystals diffracted to 2.5 Å resolution, although they decayed quickly when exposed to X-rays at room temperature. To overcome this difficulty, we collected data from a flash-frozen crystal at 85 K, using procedures described previously (de Azevedo *et al.*, 1996, 1997; Kim *et al.*, 1996). In brief, prior to flash-freezing, glycerol was added to 25%(*v*/*v*) to the crystallization drops for cryoprotection. X-ray diffraction data were collected from a BcHb-I crystal at a wavelength of 1.547 Å using a synchrotron-radiation source (Station PCr, Laboratório Nacional de Luz Síncrotron, LNLS, Campinas, Brazil; Polikarpov, Oliva *et al.*, 1998; Polikarpov, Perles *et al.*, 1998) and a 34.5 cm MAR imaging-plate detector (MAR Research) with an exposure time of 4 min per image. The crystal-to-detector distance was 150 mm and 130 images were collected using an oscillation of 0.35°. The raw X-ray diffraction data were processed to 2.5 Å resolution using the program *DENZO* (Otwinowski, 1993) and were scaled with the program *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 BcHb-I was determined by standard molecular-replacement methods using the program *AMoRe* (Navaza, 1994). The atomic coordinates of eight different fish haemoglobins and one human haemoglobin deposited in the PDB (Protein Data Bank; Abola *et al.,* 1997) were used as search models. The atomic coordinates for all models were translated so that their centre of gravity is at the origin; they were also rotated so that the principal axes of inertia of the search models are parallel to the orthogonal axes. The PDB accession numbers and identifications of the search models are listed in Table 1. Cross-rotation functions were calculated in the resolution range 10–4.5 Å using a sampling step of 2.5°. These calculations were carried out with an integration radius of 23.0 Å. The rotation which generated the highest correlation coefficient (CC) was applied to the models and used in the subsequent translation-function computations based on data in the same resolution range.

The best solutions for each model were selected based on the magnitude of the *R* factor and correlation coefficient. The translation function for the enantiomorphic space group $P6_522$ was also computed using the same resolution range and the best model in order to confirm the space group.

3. Results and discussion

Two haemoglobins were identified in the haemolysate by electrophoresis and named BcHb-I and BcHb-II (20 and 80% of the haemoglobin content, respectively). After isoelectric focusing, the pH values for the peaks were 8.3 for BcHb-I and 7.1 for BcHb-II.

The BcHb-I crystal suitable for X-ray diffraction experiments has average dimensions of about 0.5 × 0.5 × 0.1 mm. The crystal belongs to the hexagonal space group $P6_122$, with unit-cell parameters a = 63.03 (2), b = 63.03 (2), c = 315.4 (1) Å. The volume of the unit cell is 1.085×10^6 Å^3, compatible with one dimer ($\alpha\beta$) in the asymmetric unit with a $V_{\rm M}$ value of 2.78 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 56% and the calculated crystal density is 1.16 g cm^{-3}. The X-ray diffraction statistics are summarized in Table 2.

The results of molecular replacement using the nine different search models are listed in Table 3. The correlation coefficients after translation-function computation range from 19.5 to 55.3% and the *R* factors range from 43.4 to 57.2%. The search model which presented the best correlation coefficient and *R* factor was that of trout haemoglobin (PDB code 1ouu; Tame *et al.*, 1996).

Translation functions for the enantiomorphic space group ($P6_522$) were computed using the coordinates of the model 1ouu as search model. The correlation coefficient after translation-function computation was 31.8% and the *R* factor was 56.2%, which strongly indicates that the correct space group is $P6_122$.

Amino-acid sequencing of BcHb-I using the automated Edman technique is in progress. Further refinement will be carried out as soon as the sequence becomes available.

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