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Crystallization, preliminary X-ray analysis and molecular-replacement solution of haemoglobin-II from the fish matrinxã (*Brycon cephalus*)

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Haemoglobins constitute a set of proteins with interesting structural and functional properties, especially when the two large animal groups reptiles and fishes are focused on. Here, the crystallization and preliminary X-ray analysis of haemoglobin-II from the South American fish matrinxã (*Brycon cephalus*) is reported. X-ray diffraction data have been collected to 3.0 Å resolution using synchrotron radiation (LNLS). Crystals were determined to belong to space group $P2_1$ and preliminary structural analysis revealed the presence of two tetramers in the asymmetric unit. The structure was determined using the standard molecular-replacement technique.

1. Introduction

Fish haemoglobins have been extensively studied in recent decades, mainly because of the presence of isoforms, the wide spectrum of functional properties identified in these proteins and the possible correlations with physiological requirements. When different functional properties are observed in the haemoglobins of a species, it is often assumed that the varying properties constitute a selective advantage (Smarra *et al.*, 1997, 1999, 2000) and accordingly reflect evolutionary adaptation to different physiological and environmental needs (De Young *et al.*, 1994; Fadel *et al.*, 2000).

Within the scope of the adaptive role, the isoforms would contribute by providing oxygen transport under a variety of physiological demands and environmental oxygen availability. Nevertheless, in order to associate an adaptive function with this heterogeneity, it is necessary to have the different haemoglobins present at significant relative concentrations, with isoforms that show functional differences. Furthermore, it would be expected that when a change in oxygen availability occurs (such as changes in blood oxygenation, pH or temperature), these functional differences would increase the possibilities for an efficient response, contributing to a better species adaptation to the environment (Smarra *et al.*, 1999; Honda *et al.*, 2000). However, comprehensive analysis has shown that no correlation can be established between the presence of isoforms and the animal's physiology and/or environment (Val & Almeida-Val, 1995).

Oxygen transport is adjusted according to physiological needs by allosteric control, exerted by heterotropic effectors, mainly phosphates, but also by chloride, protons, carbon dioxide, bicarbonate, urea and other chemical entities, depending on the species.

Usually, protons bind to haemoglobin (Hb) when the protein downloads oxygen at the tissue level (the Haldane effect) and they simultaneously produce a decrease in oxygen affinity (the alkaline Bohr effect) (Fago *et al.*, 1999; Seixas *et al.*, 1999; Delatorre *et al.*, 2000, 2001).

A functional study on the oxygen binding and allosteric control of two haemoglobins isolated from the fish *Brycon cephalus* revealed no Bohr effect for stripped BcHb-I (a minor component) and a high homology of its primary sequence with trout Hb-I, a canonical protein which possesses no allosteric control (Honda *et al.*, 2003). The present work was performed on the oxygenated form of BcHb-II from *B. cephalus*. This haemoglobin, comprising about 90% of the haemolysate, exhibited a large Bohr effect in the pH range 6.5–7.5 that was independent of the presence or absence of phosphates and is twice that found for human haemoglobin, although no proton-binding effects could be detected at higher pH values. It is also interesting that ATP was able to deoxygenate BcHb-II immediately at pH values below 7.0, decreasing saturation to 30–40%, behaviour that resembles the Root effect. Oxygen binding is cooperative for both haemoglobins (Honda *et al.*, 2000).

Since structural and functional studies on fish haemoglobins can provide further evidence to establish new conformational states that are possibly associated with allosteric control, we have performed an analysis of the haemoglobins from *B. cephalus*, a South American teleost freshwater fish from the Amazon Basin which is largely exploited for

aquaculture in Brazil (Centro de Pesquisa e Treinamento em Aqüicultura, 1994).

2. Materials and methods

2.1. Purification

Blood was collected from adult specimens at the Centro de Aqüicultura of the State University of São Paulo (CAUNESP) at Jaboticabal SP (Brazil). The animals were anesthetized by immersion in a solution of benzocaine (1 g per 15 l of water) and blood was collected by caudal vein puncture. 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–HCl pH 8.5 containing 0.2% D-glucose and 1 m*M* EDTA). Haemolysis was accomplished with buffer *A* (50 m*M* Tris–HCl pH 8.5 containing 1 m*M* EDTA) followed by centrifugation (1000*g* for 1 h) and filtration through Millipore membranes for removal of debris. For phosphate removal, haemolysate dialysis was performed using buffer *A*, followed by gel filtration on Sephacryl S-100 (Sigma) on a 2.6 × 30 cm column equilibrated with the same buffer containing 0.2 *M* NaCl.

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

Non-denaturing electrophoresis was performed in 10% polyacrylamide gel and cellulose acetate. The gel was digitalized without staining on a Genius EP scanner and analyzed using the shareware program *Bandleader* 3.00 (Magnitec, Israel).

2.2. Crystallization

The haemoglobin used in the crystallization experiments was dissolved in water. Crystals of BcHb-II have been obtained under several different crystallization conditions using hanging-drop vapour-diffusion and sparse-matrix methods (Jancarik & Kim, 1991). The best crystals were obtained after 3 d of growth in drops in which 2 µl haemoglobin solution (25 mg ml^{-1}) was mixed with an equal volume of reservoir solution (25% polyethylene glycol 4000, 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* sodium chloride). A crystal was mounted in a borosilicate glass capillary tube for X-ray data collection.

Table 1
List of search models and the results of molecular replacement using these search models.

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

PDB code	Protein	CC (%)
1t1n	Carbonmonoxy Hb from *Trematomus akajei* (Mazzarella *et al.*, 1999)	29.8 (28.7)
1ouu	Carbonmonoxy Hb from *Oncorhyncus mykiss* (Tame *et al.*, 1996)	58.5 (37.9)
1cg8	Carbonmonoxy Hb from *Dasyatis akajei* (Chong *et al.*, 1999)	26.5 (26.0)
1hbh	Deoxy Hb from *Pagothenia bernacchii* (Ito *et al.*, 1995)	32.8 (30.0)
1out	Deoxy Hb from *O. mykiss* (Tame *et al.*, 1996)	30.8 (30.0)
1pbx	Carbonmonoxy Hb from *Pagothenia bernacchii* (Camardella *et al.*, 1992)	29.7 (29.5)
1spg	Carbonmonoxy Hb from *Leiostomus xanthurus* (Mylvaganam *et al.*, 1996)	29.3 (28.4)
1cg5	Deoxy Hb from *D. akajei* (Chong *et al.*, 1999)	23.2 (22.8)

2.3. X-ray data collection

X-ray diffraction data were collected at a wavelength of 1.544 Å using the Brazilian National Synchrotron Laboratory (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) using a crystal-to-detector distance of 200.0 mm at temperature of 281 K. Using an oscillation range of 1.2°, 135 images were collected and integrated to 3 Å resolution using *DENZO* and scaled with *SCALEPACK* (Otwinowski, 1993).

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

2.4. Molecular replacement

The crystal structure of BcHb-II was determined by molecular-replacement methods using the program *AMoRe* (Navaza, 1994). The atomic coordinates of eight different fish haemoglobins deposited in the Protein Data Bank (PDB; Berman *et al.*, 2000) were used as search models. The names and PDB accession numbers 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 28.0 Å. The rotation which generated the highest correlation coefficient (CC) was applied to the models and was 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 space group *P*2 has also been computed using the same resolution range and the best model, in order to confirm the correct choice of the space group.

Table 2
Data processing statistics for BcHb-II.

Values in parentheses are for the highest resolution shell.

Resolution range (Å)	30.0–3.0 (3.1–3.0)
Total No. of observations ($I > 1\sigma$)	48397
No. of unique reflections	19510
Completeness (%)	82.6 (85.8)
Redundancy	2.48 (2.56)
$I/\sigma(I)$	10.60 (2.63)
$R_{\rm merge}$† (%)	8.1 (21.3)

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

3. Results and discussion

Two haemoglobins were identified in the haemolysate by electrophoresis and named BcHb-I and 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 crystal of BcHb-II belongs to the monoclinic space group $P2_1$, with unit-cell parameters a = 64.4 (7), b = 89.0 (7), c = 106.1 (3) Å, β = 102.8 (3)°. The volume of the unit cell is 5.93 × 10^5 Å^3, which is compatible with two tetramers in the asymmetric unit with a $V_{\rm M}$ value of 2.28 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 of the crystal is 46% and the calculated crystal density is 1.19 g cm^{-3}. The X-ray diffraction statistics are summarized in Table 2.

The results of molecular replacement using the eight different search models are listed in Table 1. The correlation coefficients after translation-function computation range from 23.3 to 58.5% and the *R* factors range from 42.3 to 55.1%. The search model which presented the best correlation coeffi-

cient and R factor was the trout haemoglobin (PDB code 1ouu; Tame *et al.*, 1996).

The translation function for the space group ($P2$) was computed using the coordinates of the model 1ouu as a search model. The correlation coefficient after translation-function computation was 42.1% and the $R_{\rm factor}$ was 49.1%, which strongly indicating the correct space group to be $P2_1$. Repeated cycles of refinement were carried out using the simulated-annealing method as implemented in *X-PLOR* (Brünger, 1992). The present values of the R factor and $R_{\rm free}$ are 26.2 and 34.0%, respectively, using the sequence of trout haemoglobin (PDB code 1ouu).

Amino-acid sequencing of BcHb-II 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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