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Purification, crystallization and preliminary X-ray diffraction analysis of carboxyhaemoglobin-II from the fish *Piaractus mesopotamicus* (pacu)

V. Fadel,[a,c,d] R. T. Honda,[b] M. Dellamano,[a] A. L. S. Smarra,[a] P. Delatorre,[a] J. R. Olivieri,[a] G. O. Bonilla-Rodriguez[b] and W. F. de Azevedo Jr[a]*

[a]Departamento de Física, IBILCE-UNESP, CP 136, CEP 15054-000, São José do Rio Preto SP, Brazil, [b]Departamento de Química e Geociências-I, BILCE-UNESP, CP 136, CEP 15054-000, São José do Rio Preto SP, Brazil, [c]Departamento de Física, Faciba-FEB, Barretos, SP 14783-226, Brazil, and [d]Centro Universitário de Votuporanga, Votuporanga-SP, CEP 15500-030, Brazil

Correspondence e-mail: walterfa@df.ibilce.unesp.br

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Carboxyhaemoglobin-II isolated from the pacu (*Piaractus mesopotamicus*) has been crystallized and X-ray diffraction data were collected to 2.0 Å resolution using synchrotron radiation. Crystals were characterized as belonging to the space group *I*23; preliminary structural analysis reveals the presence of one dimer in the asymmetric unit.

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1. Introduction

The existence of iso-haemoglobins (iso-Hbs) prevails among fish species (De Young *et al.*, 1994). Some researchers have proposed an adaptive role for the isoforms, since they could contribute by allowing oxygen transport under a variety of physiological demands.

Nevertheless, in order for this heterogeneity to have an adaptive function, it seems necessary that the functional properties should have meaningful differences. Accordingly, we would expect 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.

The analysis of the functional behaviour and allosteric control of iso-Hbs has primarily contributed to improve our knowledge of the variety of operational mechanisms which govern these proteins. In addition, it has led to the finding of haemoglobins such as trout (*Salmo gairdneri*) Hb-I, which displays a very weak interaction with known allosteric effectors (Brunori, 1975; Tame *et al.*, 1996).

The purification, crystallization and preliminary X-ray diffraction analysis of the haemoglobin-II (PmHb-II) isolated from the pacu fish are reported in this paper. This fish presents aquatic surface respiration through extended lips when water oxygenation drops below critical levels (Saint-Paul & Bernardinho, 1988). This adaptation allows the fish to access the oxygen available at the upper layers of the water column, improving blood oxygenation.

2. Materials and methods

2.1. Blood collection

Adult specimens of *P. mesopotamicus* (pacu) were obtained at the Centro de Aqüicultura, Universidade Estadual Paulista (CAUNESP), Jaboticabal, State of São Paulo, Brazil.

Blood was collected from the caudal vein using disposable syringes containing buffer *A* [0.1 ml of 1% saline buffered with 50 m*M* Tris–HCl pH 8.0 containing 1 m*M* EDTA and 0.2%(*w*/*v*) D-glucose]. The erythrocytes were washed three times by centrifugation against a large excess of the same solution. Haemolysis was carried out overnight inside a dialysis bag against buffer *B* (50 m*M* Tris–HCl pH 9.5). All procedures were carried out maintaining the sample temperature at around 277 K.

For stabilization, the haemolysate was clarified by centrifugation and saturated with carbon monoxide (generated chemically by mixing formic and sulfuric acids) under refrigeration and gentle stirring.

2.2. Purification

Non-denaturating analytical electrophoresis was performed on 7% polyacrylamide slab gels for screening iso-haemoglobins in terms of their relative concentration and to estimate their probable isoelectric point in comparison with adult human hemoglobin.

Haemoglobin purification was performed by ion-exchange chromatography on DEAE-Sephadex (Sigma) using buffer *B* as the starting solution and buffer *C* (50 m*M* HEPES pH 6.5) to generate a pH gradient. Two fractions were obtained, named I and II according to their elution sequence from the column. Haemoglobin purity was checked by non-denaturating polyacrylamide electrophoresis (PAGE).

2.3. Crystallization

The haemoglobin used in the crystallization experiments was dissolved in water. Crystals of PmHb-II have been obtained under several different crystallization conditions using the hanging-drop vapour-diffusion and sparse-matrix methods (Jancarik & Kim, 1991). The

Table 1
Detailed X-ray diffraction statistics for synchrotron data from a PmHb-II crystal.

Resolution range (Å)	No. of independent reflections	Overall completeness (%)	$I/\sigma(I)$	Redundancy	Overall R_{merge}† (%)
20.00–5.39	1050	92.8	13.32	2.59	8.8
5.39–4.30	1084	98.7	13.87	2.58	8.4
4.30–3.76	1088	99.4	13.84	2.58	8.3
3.76–3.42	1065	98.5	13.74	2.57	8.9
3.42–3.17	1050	98.8	13.47	2.58	9.8
3.17–2.99	1082	98.6	13.12	2.59	9.4
2.99–2.84	1039	98.9	12.64	2.59	9.9
2.84–2.71	1079	99.0	11.70	2.60	10.3
2.71–2.61	1068	99.2	11.00	2.58	10.2
2.61–2.52	1055	99.2	10.56	2.57	10.8
2.52–2.44	1053	99.5	10.12	2.53	11.1
2.44–2.37	1075	99.7	9.63	2.53	11.2
2.37–2.31	1040	99.8	8.89	2.55	11.7
2.31–2.25	1056	99.6	8.27	2.54	12.1
2.25–2.20	1067	99.4	7.78	2.53	12.6
2.20–2.15	1080	99.5	7.45	2.54	12.8
2.15–2.11	1045	99.6	7.03	2.52	14.3
2.11–2.07	1067	99.4	6.50	2.54	15.6
2.07–2.03	1045	99.3	5.62	2.54	16.2
2.03–2.00	1063	99.4	5.06	2.54	17.4
All reflections	21251	98.9	12.07	2.56	9.0

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 reservoir solution. 2.0 *M* ammonium sulfate, 0.1 *M* sodium acetate (pH 4.6) was used as the reservoir solution. Crystals were mounted in borosilicate glass capillary tubes for X-ray data collection.

2.4. X-ray data collection and processing

X-ray diffraction data were collected at a wavelength of 1.378 Å using the 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), using a crystal-to-detector distance of 150.0 mm at temperature of 280 K. Using an oscillation range of 0.85°, 24 images were collected and integrated to 2.0 Å resolution using the program *DENZO* and 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.

2.5. Molecular replacement

The crystal structure of PmHb-II was determined by standard molecular-replacement methods using the program *AMoRe* (Navaza, 1994). The atomic coordinates of the haemoglobin isolated from the sea cucumber (Mitchell *et al.*, 1995; PDB code 1hlb) were used as search model. All solvent molecules were removed from the search model, the temperature factors for all atoms were set to 20.0 Å^2 and the haem groups were kept in the model. The atomic coordinates for the search model were translated in order to position the centre of gravity at the origin and rotated in order to orient the principal axes of inertia of the search model along the orthogonal cell axes.

Cross-rotation functions were calculated in the following resolution ranges: 10–4.5, 8–3 and 6–3 Å, with a sampling step of 2.5° using the program *AMoRe* (Navaza, 1994). These calculations were carried out with an integration radius of 20 Å. The rotation which generated the highest correlation coefficient (CC) was applied to the search model and was used in the subsequent translation-function computations based on data in the same resolution range.

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

3. Results and discussion

Two iso-haemoglobins present in the specimens were purified by ion-exchange chromatography and were named I and II according to their elution order.

Crystals of PmHb-II suitable for X-ray diffraction experiments have average dimensions of about 1.5 × 1.0 × 1.0 mm. The crystal has the body-centered cubic Bravais lattice, with unit-cell parameters $a = b = c = 123.9$ (1) Å. Assuming the asymmetric unit content to be one dimer of molecular weight 32.5 kDa, the V_m value is 2.44 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 1.19 g cm^{-3}. Detailed data-collection statistics for synchrotron data are given in Table 1.

The correlation coefficient after translation-function computation was 51.6% and the *R* factor was 50.7% in the resolution range 10–4.5 Å. Further refinement using the slow-cooling protocol will be performed using the program *X-PLOR* (Brünger, 1992). The amino-acid sequence will be determined by automated Edman degradation. The refined model of PmHb-II will be used for detailed comparison with other haemoglobins.

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