

Eclética química

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ISSN (Versión impresa): 0100-4670

ISSN (Versión en línea): 1678-4618

BRASIL

2000

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Eclética química, año/vol. 25, número 001

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Araraquara, Brasil

CRYSTALLOGRAPHIC STUDIES OF FISH HEMOGLOBINS

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ABSTRACT: The present work reports our successful experience concerning crystallization of four fish hemoglobins from three Brazilian species of Teleosts: *Liposarcus anisitsi*, *Brycon cephalus* and *Piaractus mesopotamicus*. The data shown here is part of a systematic functional and structural study of fish hemoglobins with the aim of better understanding the outstanding range of functional and structural properties exhibited by these proteins. We also present a reduced sparse-matrix method for crystallization of fish hemoglobins, which can reduce the amount of hemoglobin initially used in the crystallization experiments.

KEYWORDS: Hemoglobin, biocrystallography, fish, X-ray, crystallization

Introduction

Fish hemoglobins have been extensively studied in the last few years. This is mainly due to the wide spectrum of functional properties identified in these proteins. When this variety occurs within a species, it is often associated with the assumption of being a selective advantage.⁹ This variety of functional behaviors presumably reflects evolutionary adaptation to different physiological and environmental needs.

The existence of iso-hemoglobins (iso-Hbs) prevails among fish species.⁶ Some researchers propose for them an adaptive role, since they would contribute by granting oxygen transport under a variety of physiological demands and environmental oxygen shortage.

Nevertheless, in order for this heterogeneity to have an adaptive function, it looks necessary that their functional properties should have meaningful differences. Accordingly, we would expect that when a change on oxygen availability occurs (such as changes on blood oxygenation, pH or temperature), these functional differences would increase the possibilities for an efficient response, contributing for a better species adaptation to the environment.²²

The study of the structures of other forms of hemoglobin could contribute to provide a better understanding of hemoglobin function. The animal kingdom is plenty of isomorphous hemoglobins working on a variety of physiological requirements and subject to environmental stress, being a natural source for studying structure-function relationships.²³ In order to improve our understanding of the structural basis for the variety of functional behaviors, present in fish hemoglobins, we have started a systematic functional and structural study of hemoglobins isolated from Brazilian fishes.^{5, 8, 10, 21, 23}

High-resolution determination of fish hemoglobins is essential for a detailed understanding of the wide spectrum of functional behaviors present by fish hemoglobins. The established technique to access the three-dimensional structure of hemoglobins is the biocrystallography^{3,7}, a technique which uses X-ray to obtain structural information of biological macromolecules. The first step to successfully solve a biological macromolecule structure is to obtain crystals of this molecule, and then expose them to a source of X-ray.

Here is described a general protocol to crystallize and solve three dimensional structures of fish hemoglobins and the preliminary results in the structural analysis of four fish hemoglobins. It is also described a reduced sparse-matrix method for crystallization of fish hemoglobins, which can reduce the amount of hemoglobin initially used in the crystallization trials.

Materials and Methods

Blood collection

Adult specimens of *Piaractus mesopotamicus* (Pacu), *Liposarcus anisitsi*, and *Brycon cephalus* were obtained at the Centro de Aqüicultura from the Universidade Estadual Paulista (CAUNESP) at Jaboticabal, State of São Paulo (Brazil).

The specimens were anesthetized by immersion in clean water containing benzocaine (1g/15 liters). Blood was collected from the caudal vein using disposable syringes containing buffer A: 0.1 ml of 1 % Saline buffered with 50 mM Tris-HCl pH 8.0, containing 1 mM EDTA and 0.2 % (w/v) D-glucose. The erythrocytes were washed three times by centrifugation against a large excess of the same solution. Hemolysis was carried out overnight inside a dialysis bag against buffer B: 50 mM Tris-HCl pH 9.5. All procedures were carried out keeping sample temperature around 4°C.^{21,23}

For stabilization the hemolysate was clarified by centrifugation and saturated with carbon monoxide under refrigeration and gentle stirring.

Purification

Non-denaturing analytical electrophoresis was performed on 7 % polyacrylamide slab gels (PAGE) for screening iso-hemoglobins in terms of their relative concentration and to estimate their probable isoelectric point in comparison with adult human hemoglobin.^{21,23}

Hemoglobin purification was performed by ion-exchange chromatography on DEAE-Sephadex (Sigma) using buffer B as the starting solution and buffer C (50 mM Hepes pH 6.5) to generate a pH gradient. Two fractions were identified in hemolysate of *Piaractus mesopotamicus* (Pacu), named PmHb-I and PmHb-II, according to their elution sequence from the column. Four fractions were identified for hemolysate of *Liposarcus anisitsi* and named LaHb-I to IV, and two fractions were found in the hemolysate of *Brycon cephalus*, named BcHb-I and II. Purity was checked by non-denaturing polyacrylamide electrophoresis (PAGE).

Crystallization

The hemoglobin used in the crystallization experiments was dissolved in water. Crystals of the fish hemoglobins have been obtained in several different crystallization conditions, using the hanging drop vapor diffusion and sparse matrix methods.¹² The crystallization conditions for each fish hemoglobin are described on [table 1](#). Crystals were mounted in capillary tubes of borosilicate glass for X-ray data collection.

Table 1 - Crystallization conditions

Haemoglobin	Crystallization conditions
LcHb-I	0.1 M TRIS, 0.3 M Ammonium Sulfate, 0.1M HEPES pH 7.4, 1.6M Sodium Citrate, 0.2 M Magnesium Acetate, 0.1 M Chloroform, 30% PEG 4000, 0.2 M Ammonium Sulfate, 30% PEG 4000, 0.2 M Sodium Acetate, 0.1 M TRIS pH 8.0, 30% PEG 4000, 1.0 M Ammonium Sulfate, 30% PEG 1500.
LcHb-II	No Crystals
LcHb-III	1.1 M TRIS, 2.0 M Ammonium Sulfate, 1.2 2.0 M Ammonium Sulfate, 1.3 30% PEG 1500.
LcHb-IV	0.5 M Ammonium Sulfate, 30% PEG 4000, 0.2 M Sodium Acetate, 0.1 M TRIS pH 8.0, 30% PEG 4000, 2.0 M Ammonium Sulfate, 30% PEG 1500.
VcHb-I	No Crystals
VcHb-II	0.1 M TRIS, 0.3 M Ammonium Sulfate, 0.1 M Sodium Acetate pH 4.6, 2.0M Ammonium Sulfate, 0% Resorcinol, 0.2 M Sodium Acetate, 0.1 M TRIS pH 8.0, 30% PEG 4000, 2.0 M Ammonium Sulfate, 30% PEG 1500.
VcHb-III	0.1 M TRIS, 0.3 M Ammonium Sulfate, 1.1 M HEPES pH 7.4, 1.6M Sodium Citrate, 1.4 2.0 M Ammonium Sulfate, 1.5 30% PEG 1500.
VcHb-IV	0.1 M TRIS, 0.3 M Ammonium Sulfate, 0.2 M Ammonium Sulfate, 30% PEG 1500, 0.1 M Sodium Acetate pH 4.6, 2.0M Ammonium Sulfate, 0% Resorcinol, 0.2 M Sodium Acetate, 0.1 M TRIS pH 8.0, 30% PEG 4000.

Cryocrystallography

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-ray at room temperature. To overcome this difficulty we collected data from a flash-frozen crystal at 85K, using the procedures described earlier.^{1,2,10,18} In brief, prior to flash freezing, glycerol was

added, up to 25% by volume, to the crystallisation drops for cryoprotection.

X-ray data collection and processing

X-ray diffraction data were collected using the Synchrotron Radiation Source (Station PCr, Laboratório Nacional de Luz Síncrotron, LNLs, Campinas, Brazil) and a 34.5 cm MAR imaging plate detector (MAR Research).^{17,18} The programs DENZO and SCALEPACK¹⁶ were used in the X-ray data process. The overall statistics for the data collection for the four crystallized hemoglobins is described in [table 2](#).

Table 2 - Overall X-ray Collection Statistics

Hemoglobin	Wavelength (Å)	Resolution (Å)	Number of independent reflections	R _{int}
LactHb-I	1.0 - 1.07	2.00	10816	0.0
LactHb-II	1.0 - 1.0	2.00	10816	0.0
FishHb-II	1.0 - 1.0	2.00	10816	0.0
FishHb-I	1.0 - 1.0	2.00	10816	0.0

R_{int} = $\frac{1}{N} \sum_{hkl} \frac{\sum_{i=1}^N |I_{hkl}(i) - \bar{I}_{hkl}|}{\sum_{i=1}^N I_{hkl}(i)}$, where \bar{I}_{hkl} is the intensity of an individual measurement of the reflection with indices h, k and l, and $\sum_{i=1}^N I_{hkl}(i)$ is the mean intensity of that reflection.

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.

Molecular replacement

The crystal structure of the fish hemoglobins were determined by standard molecular replacement methods using the program AMoRe.¹⁵ The atomic coordinates of the hemoglobin isolated from fish hemoglobins deposited in the PDB were used as search model. [Table 3](#) describes all search models used for molecular replacement. All solvent molecules were removed from the search model and the temperature factors for all atoms were set to 20.00 Å², the heme groups were kept in the model. The atomic coordinates for the search model were translated so that their center of gravity is at the origin, they were also rotated so that the principal axes of inertia of the search model is parallel to the orthogonal axes.

Table 2 - Search models used for molecular replacement.

PDB Accession number	Protein
1T16	Carbonaceous Nanographite from fish (Hemichromis sp.) ¹⁴
1G00	Carbonaceous Nanographite from fish (Hemichromis sp.) ¹⁴
1C98	Carbonaceous Nanographite from fish (Hemichromis sp.) ¹⁴
1HLE	Graphite from sea cucumber (Cassida exilis) ¹⁴
1Q97	Layer Graphite from fish (Hemichromis sp.) ¹⁴
1P3X	Carbonaceous Nanographite from fish (Pogonias cromis) ¹⁴
1C95	Layer Graphite from fish (Hemichromis sp.) ¹⁴
1SFC	Carbonaceous Nanographite from fish (Latesoma senhousii) ¹⁴

Cross-rotation functions were calculated in the following resolution ranges, 10-4.5 Å, 8-3 Å, and 6-3 Å with a sampling step of 2.5° using the program AMoRe.¹⁵ These calculations were carried out with integration radius of 20 Å. The rotation which generated the highest correlation coefficient (CC) (Equation 1) was applied to the search model and 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} (Equation 2) and correlation coefficient.

$$CC = \frac{\sum_{hkl} (|F_{obs}| - |F_{calc}|) \cdot (|F_{obs}| - |F_{calc}|)}{\left[\sum_{hkl} (|F_{obs}| - |F_{calc}|)^2 \sum_{hkl} (|F_{obs}| - |F_{calc}|)^2 \right]^{1/2}} \quad \text{Equation 1}$$

$$R = \frac{\sum_{hkl} |F_{obs} - kF_{calc}|}{\sum_{hkl} |F_{obs}|} \quad \text{Equation 2}$$

where F_{obs} and F_{calc} are observed and calculated structure factors, respectively, and k is a scale factor.⁶ Sums are made over all available hkl reflections.

Partial refinement

The best models identified in the molecular replacement were submitted to a crystallographic refinement using the program X-PLOR⁴. These models were initially submitted to 40 cycles of rigid-body refinement using the tetramer as rigid-body, for LaHb-I, LaHb-IV and BcHb-I, and

the dimer for PmHb-II, in order to optimize the overall positions. The resolution range used was 8-3 Å. Further refinement was then performed using 80 cycles of conjugate gradient minimization.⁴ This partially refined model was submitted to simulated annealing refinement using initial and final temperatures of 3000 K and 300 K respectively, and time step of 0.5 fsec. A set of reflections comprising approximately 10 % of the data were randomly selected to compute the "free R_{factor} ", as means of cross-validating the model. The simulated annealing refinement was carried out against data with $F_{\text{obs}} > 2\sigma(F_{\text{obs}})$. The computer graphics program XtalView¹³ implemented on an O2 silicon graphics workstation (R10000) was used for all model visualization.

Results and discussion

We were unable to obtain crystals for PmHb-I and LaHb-II. Microcrystals were obtained for LaHb-III and BcHb-II. The failure in obtaining X-ray quality crystals for these hemoglobins indicate that further purification steps may be necessary to improve crystal quality. Most of the diffracting crystals presented dimensions larger than 1 mm, which facilitates the crystal mounting (Photos of the hemoglobin crystals are available on www.biocristalografia.df.ibilce.unesp.br). Especially interesting is the concentration of positive results in few crystallization conditions. We have used the standard sparse-matrix method for crystallization of the hemoglobins.¹² In this method 50 crystallization conditions are initially tried, varying pH, salts, and precipitant agents. In favorable cases X-ray diffracting crystals are obtained, or even when only microcrystals are obtained, improvements can be reached, using one or more of the 50 initial conditions as a start point. A comparison of the crystallization results, using the 50 different crystallization conditions, strongly indicates that a reduction in the number of the crystallization conditions, may reduce the amount of protein initially used in the crystallization trials, and the time expended in the crystallization experiments, since the positive crystallization results systematically appear in the same conditions. These conditions are shown on [Table 4](#).

Table 4 - Reduced sparse-matrix method for crystallization of fish hemoglobins

Number of the condition in the sparse-matrix method (Jancarik & Kim, 1991) ¹⁵	Crystallization conditions
38	0.1M HEPES pH 7.4, 1.4M Sodium Citrate.
18	0.2 M Magnesium Acetate, 0.1 M Cacodylate, 25% PEG 8000.
4	0.1 M TRIS, 2.0 M Ammonium Sulfate.
30	0.2 M Ammonium Sulfate, 30% PEG 8000.
22	0.2 M Sodium Acetate, 0.1 M TRIS pH 8.5, 30% PEG 4000.
47	0.1 M Sodium Acetate pH 4.6, 2.0M Ammonium Sulfate, 5% Isopropanol.
32	2.0 M Ammonium Sulfate.
43	30% PEG 1500.

Table 5 shows the calculated values of V_m^{14} , solvent content, crystal density, cell parameters and space group for the four hemoglobins which had X-ray diffraction data collected. The content of the asymmetric unit the V_m values range from 2.41 to 2.78 $\text{\AA}^3 \text{Da}^{-1}$. Assuming a value of 0.74 $\text{cm}^3 \text{g}^{-1}$ for the protein partial specific volume, the calculated solvent content in the crystal range from 49.0 to 56 % and the calculated crystal density from 1.16 to 1.19 g cm^{-3} .

Table 5 - Crystallographic information for the fish hemoglobins studied in this paper

Hemoglobin	Cell parameters	Space Group	V_m ($\text{\AA}^3 \text{Da}^{-1}$) ¹⁴	Solvent content (%)	Calculated crystal density (g/cm^3)
LcHb-1	a=48.8, b=61.0, and c=35.7 \AA $\beta=92.3^\circ$	P6 ₃	2.68	51.5	1.17
LcHb-1B	a= 52.6, b=103.8 and c=113.8 \AA	P6 ₃ /2 ₁	2.41	49.0	1.16
FaHb-3I	a=b=c=125.0 \AA	I23	2.46	50.0	1.18
BcHb-1	a=b=45.0, c=615.4 \AA , $\beta=120^\circ$	P6 ₃ /22	2.78	52.0	1.18

¹⁴ $V_m = V/Z \cdot MW$, where V is the volume of the unit cell, Z is the number of asymmetric units in the unit cell, and MW is the protein's molecular weight. The reported values for V_m for protein molecules ranges from 1.7 to 3.0 $\text{\AA}^3 \text{Da}^{-1}$.

The results of the molecular replacement using the 8 different search models are listed in **Table 6**. The correlation coefficients after translation

function computation range from 51.6 to 67.1% and the R_{factor} s range from 36.6 to 50.7 %.

Table 6 - Eulerian angles and fractional coordinates after translation function computation.

Hemoglobin	Search model	Donor	α (°)	β (°)	γ (°)	T_x	T_y	T_z	R_{factor} (%)	CC (%)
LaHb-I	1OUU	$\alpha1\beta1$	258.0	121.8	127.0	0.2888	0.0910	0.1707	38.8	67.1
		$\alpha2\beta2$	78.5	84.2	139.5	0.3480	0.3548	0.3191	38.6	67.1
LaHb-IV	1OUU	$\alpha1\beta1$	87.5	58.8	63.0	0.3072	0.0129	0.4158	39.0	62.7
		$\alpha2\beta2$	78.3	55.3	24.7	0.8661	0.8949	0.7090	39.0	62.7
PmHb-II	1HLE	$\alpha1\beta1$	22.1	81.2	70.7	0.4908	0.5751	0.4909	50.7	51.6
		$\alpha2\beta2$	78.6	57.1	275.0	0.0610	0.9778	0.0140	50.7	51.6
BcHb-I	1OUU	$\alpha\beta$	28.8	77.2	42.0	0.2842	0.1704	0.3403	38.9	66.2

The initial refinement was performed using the slow-cooling protocols implemented in the program X-PLOR⁴ for LaHb-I, LaHb-IV and BcHb-I. The present values of R_{free} range from 34.2 to 39.2 % and the values of R_{factor} range from 25.4 to 32.1 %. The amino acid sequencing for LaHb-I, LaHb-IV, PmHb-II, and BcHb-I using automated Edman technique is under progress. The refined model of the fish hemoglobins will be used for detailed comparison with other hemoglobins.

Conclusion

In the present paper 8 fish hemoglobins were studied, 6 were successfully crystallized and 4 had high resolution X-ray diffraction data collected, using the crystallization conditions described in [Table 1](#). The positive crystallization results are limited to 8 conditions. Using the positive crystallization results we propose a reduced sparse-matrix method for crystallization of hemoglobins, presented in [Table 4](#), which can reduce the amount of hemoglobin used the crystallization trials. Furthermore, the time expended in the crystallization trials can also be reduced, using the reduced sparse-matrix method.

Acknowledgments

We thank Dr. P. Kuser, José Brandão and Dr. I. Polikarpov (LNLS) for their help in the synchrotron data collection. The authors also thank Dr. Elisabeth Urbinati who kindly supplied specimens from the Centro de Aqüicultura (Caunesp-Unesp). This work was supported by grants from FAPESP (96/8279-7, 98/04452-1, 97/11135-0), CNPq, CAPES, Fundo Bunka, and FUNDUNESP (Brazil).

DELATORRE, P. et al. Estudos cristalográficos de hemoglobinas de peixes. *Ecl. Quím. (São Paulo)*, v.25, p. , 2000

RESUMO: O presente trabalho relata nossa experiência relacionada à cristalização de quatro hemoglobinas de peixe de três espécies brasileiras de Teleostes: *Liposarcus anisitsi*, *Brycon cephalus* e *Piaractus mesopotamicus*. Os dados, aqui apresentados, são parte de um estudo funcional e estrutural sistemático de hemoglobinas de peixe com o objetivo de melhor entender a ampla faixa de propriedades funcionais e estruturais exibidas por estas proteínas. Nós também apresentamos um método otimizado para cristalização de hemoglobinas de peixes, que pode reduzir a quantidade de hemoglobina inicialmente usada nos experimentos de cristalização.

PALAVRA-CHAVES: Hemoglobina, biocristalografia, peixe, Raios X, cristalização.

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Recebido em 4.3.2000
Aceito em 7.4.2000

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