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Crystallization and X-ray diffraction data analysis of human deoxyhaemoglobin A_0 fully stripped of any anions

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In this work, initial crystallographic studies of human haemoglobin (Hb) crystallized in isoionic and oxygen-free PEG solution are presented. Under these conditions, functional measurements of the O_2-linked binding of water molecules and release of protons have evidenced that Hb assumes an unforeseen new allosteric conformation. The determination of the high-resolution structure of the crystal of human deoxy-Hb fully stripped of anions may provide a structural explanation for the role of anions in the allosteric properties of Hb and, particularly, for the influence of chloride on the Bohr effect, the mechanism by which Hb oxygen affinity is regulated by pH. X-ray diffraction data were collected to 1.87 Å resolution using a synchrotron-radiation source. Crystals belong to the space group $P2_12_12$ and preliminary analysis revealed the presence of one tetramer in the asymmetric unit. The structure is currently being refined using maximum-likelihood protocols.

1. Introduction

Haemoglobin binds oxygen cooperatively, with an overall affinity modulated by heterotropic ligands, such as H^+, Cl^-, DPG (Baldwin, 1976) and water molecules (Colombo *et al.*, 1992, 1994). This complex network of cross-interactions, where the binding at one site alters the affinity of other ligands at distinct sites, guarantee efficient loading of O_2 at the lungs and delivery to the tissues. Crystallographic studies, pioneered by Perutz, demonstrated in the early 1970s that the fully oxygenated and the fully deoxygenated states of Hb assume two different quaternary structures, R and T, respectively (Perutz, 1970; Perutz *et al.*, 1987). Based on these two sets of coordinates, the classic two-state allosteric model of Monod *et al.* (1965) was put on a stereochemical basis. Thus, the major features of energy transduction among haem sites, bringing about cooperative binding, was explained in terms of an equilibrium between two alternative quaternary structures, the low-affinity T, constrained by salt bridges, and the high-affinity R; in the concerted transition between T and R, where it is not possible to explain the unequal O_2-linked release of Cl^- and of H^+ with oxygenation, the conjecture of sequential rupture of the salt bridges within the T structure with the earlier steps of O_2 binding was put forward (Perutz, 1970; Perutz *et al.*, 1987). Crystallographic studies of hybrids and partially ligated Hb in the T state, however, have not yet been able to confirm this conjecture (Paoli *et al.*, 1996).

One current structural explanation for the influence of chloride on the proton release linked with Hb oxygenation, the alkaline Bohr effect, is strictly coupled with the above conjecture. It has been formulated on the basis of the structure of the crystals of oxy- and deoxy-Hb grown at intermediate to high salt content and suggests that a few special Bohr groups, among which are those involved in salt bridges, are responsible for the change in O_2 affinity with pH (Perutz *et.al.*, 1998). In contrast to this interpretation, NMR measurements of the p*K*s of several surface histidyl groups on Hb molecules carried out in the absence and in the presence of anions have suggested that other residues than these few special residues contribute to the Bohr effect (Bush & Ho, 1990: Sun *et.al.*, 1997). It was observed that chloride changes the p*K* of several surface histidyl groups, suggesting global conformational changes in the Hb molecule upon anion binding. While NMR measurements have been carried out in the absence of anions, neither the crystallographic structure of oxy- nor of deoxy-Hb fully stripped of anions has been solved. This gap in information appears to contribute to the persistence of the controversy on the structural interpretation of the role of anions in the allosteric properties of Hb.

The present work is part of our efforts to better characterize the influence of anions on Hb structure and function. We have previously measured the O_2-linked binding of water molecules to Hb in solution *via* the dependence of $\log(p_{50})$ on $\log(a_w)$, where p_{50} is the

O_2 pressure at half saturation and a_w is the activity of water, using the osmotic stress method (Colombo *et al.*, 1992, 1994; Colombo & Bonilla-Rodrigues, 1996). These studies demonstrated that about 70 water molecules bind to Hb upon the conformation change from the fully deoxygenated to the fully oxygenated Hb in a 100 m*M* NaCl solution. This hydration change, Δn_w, was found to agree with the change in accessible surface area computed by Chothia *et al.* (1976) arising from the re-orientation of the $\alpha_1\beta_1/\alpha_2\beta_2$ dimers, which buries more interface area in the T state, and owing to the exposure of the T-state's salt bridges which are ruptured in the R state. Recent studies from our laboratory have demonstrated an unanticipated dependence of Δn_w on chloride and DPG concentration; in salt-free HEPES buffer only ~25 H_2O are linked with oxygen saturation of Hb, while with the gradual increase in anion concentration Δn_w smoothly increases from 25 to 70 H_2O within an anion-concentration range which corresponds to that where Cl^- (or DPG) binds specifically to the deoxy-Hb (Seixas, 1998). These results were interpreted suggesting that in salt- and oxygen-free solutions Hb is fully stabilized in a new allosteric conformational state. Further experiments have also shown that the influence of Cl^- on the Bohr effect is parallel to that on the water effect (Castro *et al.*, 1998). These findings appear to be in accord with the previously mentioned NMR results (Bush & Ho, 1990) and give further evidence that the conformation of the deoxy-Hb in solution depends on anion binding. The determination of the high-resolution structure of the salt-free crystal of deoxy-Hb may reveal the structural changes the deoxy-T Hb structure undergoes upon chloride release in solution and how these changes are coupled with (i) the larger hydration of the T-state, (ii) the change in the Bohr effect with anions and (iii) the change in the intrinsic O_2 affinity of the T state with anion release.

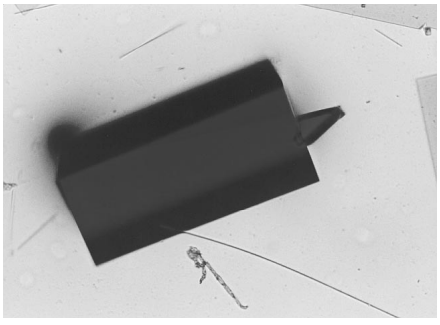

Figure 1
Crystal of deionized human haemoglobin.

2. Materials and methods

2.1. Haemoglobin purification

Haemoglobin A_0 was purified from the blood of a healthy non-smoking adult as described by Colombo & Bonilla-Rodrigues (1996). After purification, haemoglobin solution was deionized in a ion-exchange mixed-bed column of Amberlite MB-1 (Sigma) and its purity was checked by non-denaturing PAGE.

2.2. Crystallization

Crystals of isoionic fully deoxygenated haemoglobin have been obtained using the method of crystallization in batches. The crystallization process occurred in a deoxygenated environment. The best crystals were obtained after one week of growth in a deionized solution of 12% polyethylene glycol 3350 (Sigma) pH 7.02 using a solution of approximately 10 mg ml^{-1} protein in an N_2 atmosphere. The crystal was mounted in borosilicate glass capillary tubes for X-ray data collection.

2.3. X-ray data collection and processing

X-ray diffraction data were firstly collected from a single haemoglobin crystal (crystal I) at room temperature (300 K) using an R-AXIS IV imaging-plate system and graphite-monochromated Cu $K\alpha$ X-ray radiation generated by a Rigaku RU300 rotating-anode generator operated at 50 kV and 100 mA at a crystal-to-detector distance of 150 mm. 40 frames were collected using a oscillation range of 2.5°. The exposure time per frame was 20 min. The X-ray diffraction data were processed to 2.8 Å resolution and scaled using the program *PROCESS* (Higashi, 1990). A second X-ray diffraction data set (crystal II) was collected at a wavelength of 1.38 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 30 cm MAR Research imaging-plate detector at a crystal-to-detector distance of 160 mm at a temperature of 299 K. Using an oscillation range of 1.0°, 95 images were collected and the raw X-ray diffraction data were processed to 1.87 Å resolution using the program *DENZO* and scaled by 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.

Two weeks after X-ray data collection, the crystal was dissolved in an oxygenated HEPES buffer solution and showed the presence of only 2.3% of methaemoglobin.

2.4. Partial refinement

It was not necessary to use the molecular-replacement technique to solve the haemoglobin structure, as it crystallized in the same space group as low-salt haemoglobin with only very small differences in the unit-cell parameters, less than 1.0 Å. The low-salt haemoglobin atomic coordinates (PDB code 1hbb; Kavanaugh *et al.*, 1992), without water molecules and haem groups, were used for rigid-body refinement against the haemoglobin synchrotron data using the program *REFMAC* (Murshudov *et al.*, 1997). Each monomer was used as a single rigid body. The *R* factor after 40 cycles of refinement decreased to 24.5% with data in the resolution range 10–1.87 Å. Further refinement in *REFMAC* (Murshudov *et al.*, 1997) continued with 50 cycles of the maximum-likelihood method, which reduced the *R* factor to 23.0%.

3. Results and discussion

Fig. 1 shows a photomicrograph of a crystal of deoxy-Hb, fully free of anions and suitable for X-ray diffraction experiments. The crystals, with average dimensions of about 2.5 × 1.0 × 1.0 mm, show a prismatic morphology. Ward *et al.* (1975) have previously reported the crystallization of human deoxyhaemoglobin from a solution of 100 mg ml^{-1} haemoglobin, 17% PEG 6000, 10 m*M* phosphate buffer pH 7.1. Under these conditions, they determined the space group to be $P2_12_12$, with unit-cell dimensions a = 97.1, b = 99.4, c = 66.0 Å. In addition, Kavanaugh *et al.* (1992) crystallized human deoxyhaemoglobin from a solution of 10 mg ml^{-1} haemoglobin, 1–10.5% PEG 6000, 100 m*M* potassium chloride, 10 m*M* potassium phosphate (pH 7.0), 4 m*M* sodim dithionite and determined the same space group with unit-cell dimensions a = 97.1, b = 99.3, c = 65.8 Å. The crystal of deoxyhaemoglobin stripped of anions also has the orthorhombic space group $P2_12_12$, with unit-cell dimensions a = 97.1, b = 99.4, c = 66.0 Å for crystal II (LNLS); the unit-cell volume is 639 438.2 $Å^3$, compatible with one tetramer in the asymmetric unit with a V_m value of 2.48 $Å^3$ 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 50.4% and the calculated crystal

Table 1
Summary of X-ray diffraction data-collection statistics of the two crystals of deionized and deoxygenated human Hb.

Source	R-AXIS IV	LNLS
Unit-cell dimensions (Å)	a = 97.3 (3), b = 100.2 (3), c = 66.3 (3)	a = 97.3 (1), b = 99.4 (1), c = 66.1 (1)
No. of unique reflections $[I/\sigma(I) > 2.0]$	14776	51413
Maximum resolution (Å)	2.8	1.87
Completeness of data to maximum resolution (%)	92.7	1.87
R_{sym}† (%)	6.17	6.20

† $R_{sym} = 100\sum|I(h) - \langle I(h)\rangle|\sum I(h)$, where $I(h)$ is the observed intensity and $\langle I(h)\rangle$ is the mean intensity of reflection h over all measurements of $I(h)$.

Table 2
Detailed X-ray diffraction statistics for synchrotron data of deionized deoxygenated human Hb crystal.

Resolution range (Å)	Number of independent reflections	Completeness (%)	R_{sym} in range (%)
999.00–4.61	3690	95.9	5.8
4.61–3.66	3602	98.4	5.9
3.66–3.20	3543	97.8	7.2
3.20–2.91	3500	97.3	7.3
2.91–2.70	3467	96.8	8.2
2.70–2.54	3470	96.7	9.2
2.54–2.41	3422	95.9	9.5
2.41–2.31	3392	95.7	10.8
2.31–2.22	3394	95.4	12.1
2.22–2.14	3362	95.0	14.6
2.14–2.07	3343	94.5	15.9
2.07–2.01	3332	93.7	19.0
2.01–1.96	3325	94.1	23.2
1.96–1.91	3328	93.5	29.3
1.91–1.87	3240	92.6	34.8
All reflections	51413	95.6	6.2

density is 1.18 g cm^{-3}. The X-ray diffraction statistics for the two data sets are summarized in Table 1. Detailed data-collection statistics for the synchrotron data are given in Table 2.

Crystal I (R-AXIS IV) and crystal II (LNLS) presented a difference of 0.8 Å in the b dimension. Differences in unit-cell parameters have been observed previously in some isomorphous crystals. Unit-cell parameters with a difference of greater than 1.2% have been reported for isomorphous crystals of cyclin-dependent kinases (Kim *et al.*, 1996; de Azevedo *et al.*, 1996; de Azevedo *et al.*, 1997). The reasons for such differences can be a consequence of different indexing algorithms or of X-ray diffraction data being collected under different experimental conditions, such as temperature and X-ray source. Furthermore, for the crystals I (R-AXIS IV) and II (LNLS) of deoxyhaemoglobin stripped of anions with an observed difference of 0.85% in the b dimension, each unit-cell parameter has an error of 0.3 Å for crystal I and of 0.1 Å for crystal II. In addition, crystal II (LNLS) was selected from a different crystallization batch to crystal I (R-AXIS IV). The above differences combined may be the reason for the difference of 0.85% in the b dimension observed for the two isomorphous crystals of deoxyhaemoglobin stripped of anions.

Difference maps will be used for localization of the haem groups. The water-molecule search protocol implemented in the program *XtalView* (McRee, 1992) will be used for localization of the water molecules. Further refinement in the complete atomic model using maximum-likelihood protocols will be performed using the program *REFMAC* (Murshudov *et al.*, 1997).

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