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Crystallization and preliminary crystallographic studies of bar-headed goose fluoromethaemoglobin with inositol hexaphosphate

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Bar-headed goose fluoromethaemoglobin (fluoromet-Hb) complexed with inositol hexaphosphate (IHP) has been crystallized using PEG 6000 as precipitant. The crystal belongs to space group $P2_1$, with unit-cell parameters a = 59.8, b = 72.0, c = 79.8 Å, β = 102.1°, and diffracts to 2.5 Å resolution. To prove the presence of IHP, the structure was determined by the molecular-replacement method. IHP was observed at the entrance to the central cavity between the N and C termini of two β subunits.

1. Introduction

Cooperativity exists in the binding to and release from haemoglobin (Hb) of oxygen, showing the presence of communication between polypeptide chains. Its structural basis is the allosteric effect, which is regulated by allosteric effectors (Perutz, 1970). Vertebrate Hbs have a variety of organic phosphates as allosteric effectors. These are 2,3-diphosphoglycerate (DPG) for mammal Hbs, ATP and GTP for fish Hbs and inositol pentaphosphate (IPP) for avian Hbs (Isaacks & Harkness, 1980) [*in vitro*, inositol hexaphosphate (IHP) was used in most of the experiments]. All of these effector molecules bind to the same site on the Hbs, *i.e.* at the entrance to the central cavity between the N and C termini of two β-subunits (Arnone, 1972; Perutz, 1983). Their binding to T-state Hb lowers the oxygen affinity of Hb and facilitates the unloading of oxygen from red cells to the tissues (Benesch & Benesch, 1969). The P_{50} values of bar-headed goose Hb and greylag goose Hb are 270 and 370 Pa in 100 mM Cl^-, respectively (at pH 7.2, 298 K), but these increase to 2720 and 4120 Pa in the presence of IPP, indicating that the oxygen affinity of both Hbs decrease about ten times (Rollema & Bauer, 1979). IPP is a more powerful allosteric effector than DPG and the binding of IPP to avian Hbs is tighter than to human Hbs. At pH 7.2 and 298 K, the IPP binding constants of bar-headed goose oxy- and deoxy-Hbs are 3×10^5 and 4×10^9 M^{-1}, respectively. Under similar conditions, the association constants of IPP binding to human oxy- and deoxy-Hbs are 2.5×10^3 and 1×10^6 M^{-1} (Rollema & Bauer, 1979), respectively, and for DPG binding they are 1.2×10^3 and 1.7×10^4 M^{-1}, respectively (de Bruin *et al.*, 1974). Several crystal structures of IHP or DPG bound to human Hbs have been determined. They are human deoxy-Hb bound to IHP at 3.5 Å resolution (Arnone & Perutz, 1974) and at 2.6 Å (Luisi *et al.*, 1990), human fluoromet-Hb bound to IHP at 3.5 Å (Fermi & Perutz, 1977), partially oxygenated T-state human Hb bound to IHP at 1.5 Å (Waller & Liddington, 1990) and human deoxy-Hb bound to DPG at 2.5 Å (Richard *et al.*, 1993). However, no crystal structure of an avian Hbs with IPP, IHP or DPG has yet been reported.

The bar-headed goose (*Anser indicus*), one of the most remarkable species in the goose family, lives at an altitude of 4000–6000 m in central Asia and Qinghai Lake in western China and migrates annually across the Himalayas at altitudes of about 9000 m (Swan, 1970). The bird has long been regarded as a favourable object for research on the mechanism of avian high-altitude hypoxia respiration. Its blood has a far higher oxygen affinity than that of closely related lowland species such as the greylag goose (*A. anser*) so that it can tolerate hypoxic conditions (Petschow *et al.*, 1977). There are only four amino-acid differences between greylag goose Hb and bar-headed goose Hb (Oberthur *et al.*, 1981). Biochemical and model-building experiments predicted that the mutation Proα119 (greylag goose) to Ala (bar-headed goose) eliminates a van der Waals interaction with Leuβ55 in the $\alpha_1\beta_1$ interface and is responsible for the high intrinsic oxygen affinity (Oberthur *et al.*, 1982; Perutz, 1983; Hiebl *et al.*, 1986). Genetic engineering experiments (Jessen *et al.*, 1991; Weber *et al.*, 1993) and our previously reported crystal structure of bar-headed goose oxy-Hb, the first crystal structure of an avian Hb (Zhang *et al.*, 1996), proved the prediction above. The present paper is the first report on the crystal of avian Hb complexed with its allosteric effector. A further crystal structure analysis will elucidate the mechanism of the avian Hb allosteric effect. An especially interesting structural question is how the

allosteric effector IPP can regulate the unloading of oxygen bound to bar-headed goose Hb possessing so high an oxygen affinity.

2. Methods and results

Bar-headed goose aquomet-Hb was prepared and purified by the previously reported method (Lu *et al.*, 1989) and its fluoromet-Hb form was prepared according to the method of Fermi & Perutz (1977). Aquomet Hb was prepared by adding solid $K_3Fe(CN)_6$ to Hb solution, running through Sephadex G-100 followed by concentration. By addition of KF solution to 0.1 *M*, aquomet-Hb was converted to fluoromet-Hb; excess IHP was added in a 4:1 molar ratio. Crystallization was carried out by the hanging-drop vapour-diffusion method at 293 K. A 10 µl droplet containing 25 mg ml^{-1} Hb, 5%(*w*/*v*) PEG 6000 and 0.05 *M* phosphate buffer pH 6.8 was equilibrated against 1 ml reservoir solution containing 28%(*w*/*v*) PEG 6000. Crystals were obtained with maximum dimensions of 0.5 × 0.5 × 0.2 mm after one week.

X-ray diffraction data were collected on a MAR 345 image-plate system using Cu $K\alpha$ radiation (λ = 1.5418 Å) from a Rigaku R-AXIS II X-ray generator with a crystal-to-detector distance of 200 mm. The data were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The crystal diffracts to 2.5 Å. A total of 126 567 observations of 15 868 unique reflections within the 50–2.5 Å resolution range were collected with an $R_{\rm merge}$ of 6.9% (23.4% in the resolution range 2.66–2.50 Å) and a multiplicity of 2.65 (2.19 in the resolution range 2.66–2.50 Å). The effective resolution is 2.8 Å. The completeness of the overall data set is 81.1% and the completeness of the highest resolution shell (2.8–2.9 Å) is 60.5%, calculated with the program *X-PLOR*. The percentage of the overall data set with $I > 3\sigma(I)$ is 59.4% and with $I > 2\sigma(I)$ is 72.3%. The crystal space group is $P2_1$, with unit-cell parameters a = 59.8, b = 72.0, c = 79.8 Å, β = 102.1°. If there is one Hb tetramer molecule per asymmetric unit cell, the calculated V_m is 2.497 Å^3 Da^{-1}, which is within the normal range for protein crystals (Matthews, 1968).

To prove the binding of IHP to the Hb, the crystal structure was determined using the molecular-replacement method with the bar-headed goose oxy-Hb as the model molecule (PDB code 1a4f; Zhang *et al.*, 1996), followed by rigid-body refinement and simulated annealing using *X-PLOR* (Brünger, 1992). Manual adjustment of the model was carried out using *FRODO-TURBO* and refinements of the coordinates were performed. The $2F_o - F_c$ and $F_o - F_c$ electron-density maps were calculated. Additional electron densities can be seen at the entrance to the central cavity between the N and C termini of two β-subunits. There is a main central density with several branches extending outward, which we propose to be IHP. The two β-chains were superimposed with those of human deoxy-Hb with bound IHP (crystal structure at 2.6 Å resolution; PDB code 1nih; Luisi *et al.*, 1990) and the coordinates of IHP from 1nih were then fitted to the additional electron densities and followed by refinements. In the resolution range 8–2.8 Å, R = 0.2104 and $R_{\rm free}$ = 0.2522. The r.m.s.d.s of the bond lengths and bond angles from standard values are 0.011 Å and 1.5°, respectively. The structure is reasonable and IHP exists at the entrance (Fig. 1). Further structure refinement and analysis are in progress.

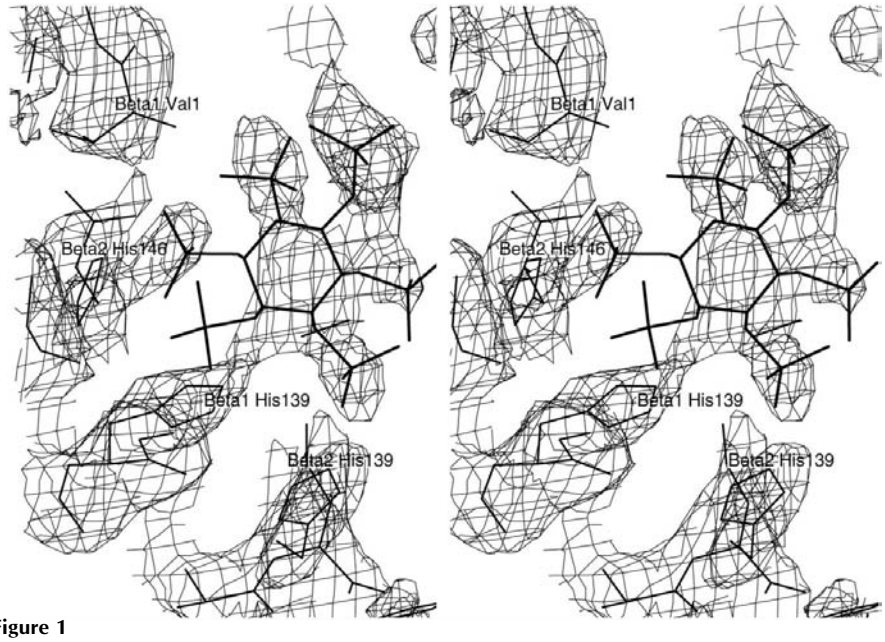

Figure 1
Stereo electron-density map around the IHP binding site displayed at a level of 0.8σ. The thick line represents the IHP molecule and the thin line the Hb molecule.

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