X-ray crystallographic analysis of the evolution of vertebrate hemoglobins

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Molecular evolution of protein molecules have been studied mainly through the analysis of the sequences of aminoacids and nucleotides. If the evolution of the three dimensional structure of a protein along the phylogenetic tree were known, it would afford a basis to study the molecular evolution of protein functions. Vertebrate hemoglobin (Hb) is an appropriate protein to study it, because it is a best studied protein in both molecular evolution and X ray crystallography.

We have chosen blue fin tuna (bony fish), a sting ray and a shark (cartilaginous fish), a lamprey (an agnatha) and a sea cucumber as sources of Hbs, which constitutes animals around the branching point of vertebrate and invertebrate.

Lamprey belongs to agnatha (a jawless vertebrate) which is the most primitive vertebrate. Their Hbs appear to have an intermediate structure between vertebrate and invertebrate Hbs because $\alpha_2\beta_2$ type tetramer structure has not yet been established but still they show allostericity in oxygen binding.

Some of sea cucumbers express Hbs. They are nearest to vertebrate among the invertebrate animals expressing Hbs.

We collected X ray diffraction data from single crystals of lamprey COHb using this machine time at BL44B2.

The lampreys we are using is *Lampetra japonica*. They were caught by a fisherman and sent to us by air. The blood was collected from a caudal vein by syringe, washed by cold saline and lysed by cold distilled water. Main Hb component (more than 90%) was obtained by a column chromatography with DE cellulose. Crystals were grown by a batch method using 15% PEG 6000, 20mM phosphate pH7.5 under CO with Hb concentration of about 2.5%.

We have previously solved the crystal structure of deoxy form of the same lamprey Hb to a resolution of 2.5Å. It is in space group $P2_1$ with cell dimensions a=59.6 Å b=216.1 Å c=74.8 Å and β=96.26° . A unit cell has 24 globulin fold monomers showing a long b axis. The asymmetric unit is occupied by 12 monomers, which is composed of 2 hexamers and a hexamer is composed of 3 dimers. Their assembly to form dimers and hexamers was quite different from that of usual (jawed) vertebrate Hbs. The subunit-subunit interface of the dimer involves the beginning of E helix and the AB corner of both subunits. A molecular dyad axis which superimposes one subunit on the other by a 180° rotation passes through the interface. On the other hand the subunit-subunit interface of the hexamer involves the N terrminal loop and EF corner of one subunit and the end of F helix, the FG corner and the beginning of G helix of the other subunit. It is a directional contact.

The CO form of the lamprey Hb crystallized in the same space group with nearly the same cell dimensions as the deoxy form. We need higher resolution to solve the mechanism of the heme-heme interaction (at least the same resolution as the deoxy form) by the comparison of deoxy and CO forms. We tested more than twenty crystals, but could not find a good crystal in the machine time.