G. Fermi and I tried to determine the chloride binding sites in bovine deoxy hemoglobin by collecting X-ray data of crystals in polyethylene glycol with 50 mM $Na_2HPO_4 \pm 0.1$ M NaCl or with HEPES ± 0.1 M NaBr. We found no halide binding sites in either medium. When told of this result, A. Arnone collected X-ray data from human deoxy hemoglobin crystals in polyethylene glycol ± 0.3 M NaF, NaCl or NaBr. He found a weak Br^- binding site near Val 1β, but no chloride binding site.

On the basis of earlier experiments by J. Manning, the Bonaventuras, J. Kilmartin and myself it was clear that chloride binds in the central, water filled cavity of hemoglobin which widens on loss and narrows on uptake of oxygen. The cavity contains 8 pairs of cationic and 3 pairs of anionic amino acids. The Bonaventuras proposed some years ago that electrostatic repulsion by the excess positive charges in the cavity destabilises the T-structure and therefore raises the oxygen affinity.

On the basis of all these results and ideas, I argued that widening of the cavity on deoxygenation allows additional chloride ions to diffuse in the cavity and to stabilise the T-structure by neutralising the excess positive charges. Earlier experiments had shown that neutralisation of only two pairs of charges, Val 1α and Lys 82β was sufficient to inhibit the chloride effect. I now argued that neutralisation of **any** one pair of positive charges in the cavity should halve the chloride effect, neutralisation of **any** two pairs should inhibit it and introduction of additional ones should enhance it.

D. Shih, D. Williamson and I found a series of abnormal human hemoglobins which confirmed that this is indeed the case. On the other hand, neutralisation of any pair of **external** positive charges did not diminish the chloride effect.

These findings suggested that abnormal human hemoglobins with fewer positive charges in the central cavity should have an intrinsically lower oxygen affinity than hemoglobin A and vice versa. A survey of all substitutions reported in the literature shows this to be true, with a few exceptions due to special stereochemical effects. In human hemoglobin half of the Bohr effect is inhibited in the absence of chloride. This chloride dependent Bohr effect is absent when two pairs of positive charges have been removed from the central cavity, which shows that the effect must be due to the raising of the pK_a of cationic groups in the cavity by chloride.

Regulation of the oxygen affinity by electrostatic interactions with diffusible anions not bound to any specific site represents a new kind of allosteric mechanism.

Scapharca dimeric hemoglobin: A new mechanism of information transfer between globin chains

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Abstract

The homodimeric hemoglobin component present in the red cells of the bivalve mollusc *Scapharca inaequivalvis*, HbI, is endowed with high cooperativity in ligand binding[1]. This behaviour is in contrast with that of vertebrate hemoglobins in which cooperativity is associated with a tetrameric assembly and the presence of two types of chain. Analysis of the aminoacid sequence[2] and immunological data[3] suggested that the assembly of HbI differed from that characteristic of vertebrate hemoglobins and hence that cooperativity had an unusual structural basis. Indeed the X-ray structures of the carbonmonoxy and deoxy derivatives at 2.4Å resolution showed that in HbI the heme carrying E and F helices are not exposed to solvent as in the vertebrate hemoglobin tetramer, but form the subunit interface and bring the two heme groups practically in direct contact through a network of hydrogen bonds[4]. Ligand binding brings about marked structural changes that are limited to the heme environment, whereas quaternary changes are only minor. The structural changes in the heme environment result in alterations in the network of interactions between the heme groups which lead to changes in ligand affinity[5,6]. In HbI therefore cooperativity in ligand binding is achieved through direct heme-heme communication as opposed to the long range information transfer operative in the vertebrate hemoglobin tetramer.

The direct communication between hemes is reflected in a number of properties with distinctive characteristics relative to vertebrate hemoglobins: information transfer between the hemes occurs in times no longer than a few nanoseconds, about 1000 times faster than in vertebrate hemoglobins[7]; the ligand-linked conformational changes take place with a half-time around 1 μs, a time regime characteristic of tertiary structural changes in mammalian hemoglobins[8,9]. In

Scapharca HbI the direct structural linkage between the hemes therefore results in a tight functional coupling which does not permit regulation of oxygen affinity. Regulation can be achieved only by separating the heme groups via the globin moiety thereby taking advantage of its flexibility. It is no surprise that the mechanism underlying cooperativity in vertebrate tetrameric hemoglobins operates in this way.

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The dimeric and co-operative myoglobin of *Nassa mutabilis*. A peculiar case

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Abstract

The myoglobin present in the radular muscle of the Prosobranchia sea snail *Nassa mutabilis* is a peculiar case among myoglobins. It is a dimer showing co-operative oxygen binding equilibrium curves with $pO_{2_{1/2}} =$ 4.7 mmHg, invariant with pH, and $n = 1.6$. Although the globin is composed of 147 amino acid residues, corresponding to a molecular mass of 15760 D, gel filtration chromatography of the native myoglobin indicate $Mr = 26000 \pm 2000$ D. Similarly, acrylamide electrophoretic analyses in SDS and velocity sedimentation indicate a molecular mass of about 13000 D for the denatured globin. The molecule is highly unstable and forms slowly a chromogen when aged or immediately upon oxidation to the ferric state. The visible region of the absorption spectrum of the O_2 or CO liganded myoglobin derivatives indicate an altered heme environment. Circular dichroism analyses confirm this indication showing negative Cotton effects in all regions of the heme absorption spectra of the MbO_2 and MbCO derivatives. Interestingly, the CD spectrum of the oxidised met-form shows a positive band almost symmetrical with respect to that of the MbO_2 derivative. This is similar to what reported for the monomeric hemoglobin of *Glycera dibranchiata* for which a reversed heme orientation was proposed. Detailed resonance Raman spectroscopic studies have permitted a more direct investigation of the interactions between the heme and the protein. The proximal Fe-Im bond shows a stretching mode frequency down shifted by 5 cm^{-1} with respect to the corresponding band of horse heart myoglobin, in good correlation with the much higher instability of *Nassa m.* myoglobin and its much lower oxygen affinity. The unusual bond instability finds additional support in a kinetic study in which the myoglobin is mixed with CO in buffered solutions at different pH values. This approach gives evidence that the Fe-Im bond is broken upon lowering the pH, with a pK of 4.0 ± 0.2, the highest among those of deoxy hemoproteins. The rupture of the proximal bond appears to occur with a proton-linked transition showing $n = 1.8 \pm 0.1$, again indicating cooperativity between the two subunits. The vinyl and propionate heme substituents show resonance Raman spectroscopic bands indicating different modes of interaction with their environment with respect to other myoglobins. Most interestingly, the vinyl stretching mode frequency, typically a single band, appears split in two bands in *Nassa m.* myoglobin. This splitting is evident in all the investigated derivatives of the myoglobin, indicating that vinyl 2 and 4 are not equivalent in this molecule. A similar splitting has been found so far only in *Chironomus t.t.* hemoglobin.

The appearance of a chromogen electronic spectrum upon myoglobin oxidation finds a counterpart in the resonance Raman spectrum of the met-derivative of *Nassa m.* myoglobin that shows the properties of a six coordinated low spin heme, indicating the formation of a bond between the hem-iron and an endogenous distal ligand upon oxidation of the metal. The heme environment of *Nassa m.* myoglobin has been studied also by EPR spectroscopy of the NO derivative in phosphate buffer at different pH values. The results show a spectrum that is similar to that of monomeric mammalian myoglobins at pH 8.7, indicating an axial symmetry of the heme pocket. Upon lowering the pH, the g_x component of the spectrum changes and at pH 7 the pattern is similar to that