X-ray crystallographic studies on structure and fucntion of hemocyanins.

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Summary

Hemocyanin is the oxygen transport pigment in many arthropods and molluscs. In this respect, hemocyanin has the same function as hemoglobin has in many other organisms. This functional homology has resulted in the development of a number of special properties in both protein classes, e.g. cooperative oxygen binding, regulation by several allosteric effectors and the aggregation into muli-meric complexes. However, the molecular basis by which hemocyanins and hemoglobins achieve these properties is completely different.

In hemocyanin, molecular oxygen is bound to a dinuclear copper site. The copper ions are directly coordinated by the protein through 6 histidine side-chain atoms. Upon oxygenation, both copper ions are oxidised from Cu(I) to Cu(II). Accordingly, the oxygen binds as a peroxide (O_2^{2-}). In the oxygenated state, hemocyanins display several characteristic spectroscopic features, e.g. strong absorption at 340 and 580 nm (the 580 band gives the protein its characteristic blue colour), an unusual low resonance Raman frequently for the peroxide bond and the absence of the usual Cu(II) EPR-signal.

The cooperative oxygen-binding in hemocyanins indicates that the multi-meric protein complex exists in (at least) two distinct conformations that differ in oxygen affinity. The equilibrium between these conformations depends on the ligational state of the subunits and on the presence of heterotropic allosteric effectors. The linkage between changes in the protein structure and changes in the dinuclear copper site forms the core of this thesis.

In chapter 2, the crystal structure of deoxygenated *Limulus polyphemus* subunit type II (*Limulus* II) is presented. The oxygenated structure of this protein was determined in the group of Dr. Magnus (Cleveland, Ohio). A third structure, that of deoxygenated *Panulirus interruptus* hemocyanin, had previously been solved in our laboratory. Comparison of the two *Limulus* II structures has allowed to determine the oxygenation linked changes in the dinuclear copper site. The most striking observation in this respect is that, upon oxygenation, the Cu-Cu distance is reduced from 4.6 to 3.6 Å. The comparison between the *Limulus* II structures and the *Panulirus* hemocyanin structure revealed considerable changes in the tertiary and quaternary structures. On the basis of these observations, a regulation mechanism for arthropodan hemocyanins has been proposed. In this model it is postulated that the *Panulirus* and *Limulus* structures represent the high and low affinity conformations respectively. The main difference between these two conformations is a 7.5° rotation of the amino terminal domain with respect to the remainder of the protein. This rotation is structurally coupled to the dinuclear copper site by the interaction between Phenylalanine 49 in domain 1, the 'allosteric sensor', and the copper ligand Histidine 328 in domain 2. The rotation of domain 1 also gives an elegant explanation for the observed regulation by chloride ions.

In chapter 4, it is shown that nitrate can bind at the same position as chloride ions. Considering the structural homology between nitrate and HCO3-, it may be expected that HCO3- can also bind at this position. In hemoglobins, HCO3- is known to be an important allosteric effector. Oxygen-binding studies have to confirm whether or not a similar regulatory mechanism also exists in *Limulus* II hemocyanin.

Chapter 4 also proposes an extension to the two-state concerted model for allosteric regulation. In this model, two tertiary conformations are allowed within the low affinity

quaternary conformation of the hemocyanin hexamer. The new model can explain the observed regulation properties of chloride ions and protons, which deviate from a two-state concerted model. In addition, the model explains the observed relation between regulation by chloride ions and protons.

In chapter 5, the initial crystallisation and X-ray diffraction results with the hemocyanin of *Helix pomatia*, a mollusc, are described. Electron microscopy and amino acid sequence determinations have shown that the molluscan and arthropodan hemocyanins have very different structures. The hemocyanin in *Helix pomatia* is a 20-meric complex with a molecular mass of ~9 MDa. This makes it one of the largest protein complexes that have been investigated by X-ray crystallography. As a consequence, the data collection and processing turned out to be non-trivial tasks. The problems encountered and the first results will be presented.

As a side-study, the folding of Greek key β-barrel proteins was investigated based on the structural homology between the Greek key β-barrels observed in: the C-terminal domain of arthropodan hemocyanins, immuno globulins, Cu,Zn-superoxide dismutase, and a class of anti-tumour proteins. These 4 protein classes do not have a known evolutionary of functional relationship. Accordingly, it was assumed that conserved features in their amino acid sequences would be related to their structural homology. Analysis of the amino acid sequences, after alignment based on the structural homology, showed that the sequences of these proteins are very dissimilar. However, we observed to conserved patterns of large hydrophobic residues in alternating positions, separated by a short peptide. This patterns represent the two central stands in the b-barrel. The conservation of side-chain size and loop length are remarkable, since other studies have shown that these features are normally not well conserved. The conservation of these features can also not be explained satisfactorily from thermodynamic considerations. In contrast, the conserved side-chain size and loop length fits well within a model where the two central b-strands play an important role in the nucleation of protein folding.