HEME PROTEINS: HEMOGLOBIN

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Progress in understanding structure and function of heme proteins has been so great during the past ten years that it is difficult to review it with any completeness*. The field has been and still is among the most active ones in Biochemistry not only for the physiological interest but also because heme proteins, particularly hemoglobin, have been taken as a prototype for studying (in general) function–structure relationships in proteins and the basic mechanisms underlying biological regulation at a molecular level.

The present review will be largely confined to hemoglobin and even so there will be many omissions and some important contributions may be neglected. Substantial progress has also been made in the elucidation of structure and reactivities of cytochromes, cytochrome oxidase and other heme proteins, but even a short mention of the main results in these areas would take up too much space.

Naturally, the development of the subject in the last decade has depended heavily on previous basic achievements, primarily on the initial determinations of the structures of myoglobin [5] and hemoglobin [6] by X-ray analysis. On these bases, many of the questions which have lately been answered, were clearly posed about ten years ago. With hemoglobin, some of the unanswered problems at the time were: The resolution of the structures of the ligand-bound and the ligand-free proteins at an atomic level; the structural details of the conformation change associated with ligand binding, the mechanism by which it was produced, and in turn, how this change could be quantitatively related to the characteristic functional properties of the protein; the exact relations between hemoglobin function, particularly interaction effects, and its subunit structure; the identification of the groups involved in the Bohr effect and in other oxygen linked interactions; the structural interpretation of many aspects of hemoglobin kinetics; the role of specific amino acid residues and the interpretation in structural terms of the functional changes brought out by chemical or physicochemical modifications of the protein.

As it will be shown below, most of these problems have now been solved, although some unclear aspects and uncertainties still remain and, as usual, new questions, open for future research, have arisen. On the whole, however, research on hemoglobin is now passing from a stage in which the basic aspects of the problem at a molecular level have been successfully attacked to one of refinement and sophistication of analysis.

In work on hemoglobin, the contributions from European laboratories have often been dominant; in many cases, important achievements have been the results of friendly and intense collaboration among scientists active in the field.

Three dimensional structure

From the initial model of horse oxyhemoglobin at 5.5 Å resolution, the work of the Cambridge group has admirably expanded in a continuous attempt to discover new details of the structure and to correlate the structural features to the function of the protein [7–11].

* For recent reviews on hemoglobin see refs. [1–4].

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The structures of oxy- and deoxyhemoglobin are now known at 2.8 Å resolution; this has allowed one to establish the residues in contact with the heme group and the interactions, on the α and β chain surfaces, responsible for stabilization of the $\alpha_2\beta_2$ tetramer.

The conformation change accompanying oxygenation (or ligand binding) has been accurately described in terms of changes in the quaternary and tertiary structures. The changes in quaternary structure consist of a rotation with a small translation of the subunits relative to each other; as a result of this there is a small change at the $\alpha_1\beta_1$ interface, but a rather larger one at the $\alpha_1\beta_2$ contact. The distance between the iron atoms changes: on oxygenation the distance between the iron atoms in the β chain decreases by 6.5 Å, that between the iron atoms in the α chains increases by 1 Å.

The changes in tertiary structure involve the iron atom, regions around the heme, and the C terminal ends of α and β chains. The iron atom is five coordinated in deoxyhemoglobin: as expected for a high spin compound the metal is out of plane in this derivative (by about 0.75 Å). On oxygenation the iron becomes low spin and falls into plane. The hemes, on ligand binding, slightly change position with respect to the polypeptide chains and, on approaching the iron of the β chains, the ligand displaces the methyl group of a valine.

The C terminal regions are free to rotate in oxyhemoglobin but immobilized in deoxyhemoglobin where they form six salt bridges at the contacts with neighbouring subunits.

The crystallographic analysis has also been extended to modified [10,12] and abnormal hemoglobins [13,14] with the aim of correlating functional alterations to changes in the structure.

The crystallographic studies on the monomeric hemoglobin from *Chironomus thummi-thummi,* have also led to important results of general significance [15]. The folding of the chain in this protein is similar to that in mammalian myoglobin and hemoglobin chains; 'the distal' residue facing the heme however is not an imidazole. Difference analysis of the CO versus the deoxy derivative has clearly shown the ligand bound to the heme and, especially relevant, that, as could be predicted theoretically, the iron is out of the porphyrin plane in the deoxy and almost in piane in the CO derivative. This movement of the iron was later also demonstrated in hemoglobin.

The structures of myoglobins from seal [6] and of the hemoglobin from Glycera [17] have also been investigated by X-ray analysis and again indicate a common structural pattern for oxygen binding hemoproteins.

Abnormal hemoglobins

The number of abnormal human hemoglobins discovered up to now is more than one hundred. Many of them are not associated with evident clinical symptoms and their recognition is the result of systematic screening of blood samples [18]. The amino acid substitution has been identified in most cases; the study of their oxygen combining properties has often led to important conclusions on structure–function relationships in hemoglobin. The effects of the specific amino acid changes have been interpreted in the light of the three-dimensional atomic model according to the position of the substitution [19]. Thus, replacements in the external part of the molecule are generally without direct effects on function; replacements of residues near the heme, may be associated with loosening of the heme globin interaction or with increased methemoglobin formation in vivo and in vitro; replacements at the $\alpha_1\beta_2$ interface may produce large changes in functional behaviour.

Subunit structure of hemoglobin

Hemoglobin is made up by two pairs of α and β subunits corresponding to the tetramer $\alpha_2\beta_2$. The tetramer may reversibly dissociate, under a variety of conditions, into dimers and, under more extreme ones, into single chain molecules.

The dissociation into subunits and the properties of the dissociation products have been a central problem and have been the centre of

controversy for a long time. This can be easily understood since the functional interactions which dominate the reactions of hemoglobin with ligands are correlated with the subunit structure of the protein.

The reaction of the masked SH groups of human hemoglobin with *p*-mercuribenzoate promotes dissociation of human hemoglobin into single chains; based on this, native α and β chains can be easily prepared in large quantities [20], and their properties studied in great detail [21–23]. Isolated α and β chains have functional and structural properties widely different from those they show in the assembled molecule. Regardless of their state of polymerization (the α chains are mainly monomeric and the β chains tetrameric) the isolated chains show hyperbolic ligand equilibrium curves, high affinity, absence of Bohr effect and of other heterotropic interaction effects and very fast rates of combination with ligands. On mixing the two chains, hemoglobin is very quickly reformed [24], with identical properties to that of the original protein; the reassembly of hemoglobin from the chains is accompanied by conformational changes revealed by changes in spectral properties [25–27].

Reversible dissociation of ligand-bound hemoglobin into $\alpha\beta$ dimers [28] occurs under a variety of conditions and there is no great change in the functional properties of the protein [29]. This fact, in the absence of clear data on the dissociation of deoxyhemoglobin, led to the suggestion that free $\alpha\beta$ dimers had functional properties similar to those of the hemoglobin tetramer [30,31]. However, there was evidence against this view [32,33]; this controversial aspect of the hemoglobin problem has stimulated a large number of studies on the equilibrium and kinetic properties of hemoglobin under a great variety of conditions. The situation was finally clarified by the demonstration that, under conditions where ligand-bound hemoglobin was largely dissociated into dimers, deoxyhemoglobin was essentially undissociated, the dissociation constant between the two derivatives differing by several orders of magnitude [34]. With this new information previous results could be re-interpreted to indicate that free dimers have functional properties more similar to the isolated chains than to the tetramer [35].

Ligand binding equilibria

Studies on the oxygen equilibria of normal, abnormal and modified hemoglobins have been done to obtain quantitative data on the oxygen affinity, on the heme–heme interaction (cooperativity) and on the effect of third components. Very precise and automatic methods for the measurement of oxygen binding have been employed to determine numerical values of the individual equilibrium constants corresponding to the successive oxygenation of the various hemoglobin sites [36–38].

Equilibrium measurements have been extended to other ligands such as CO [39] and isocyanides [40,41] nitroso aromatic compounds [42], and to the oxidation–reduction process in the ferro–ferrihemoglobin system [43,44], arriving at the general conclusion that, apart from a scale factor, all the ligands behave similarly. However, there are significant differences in the degree of cooperativity of the binding curves for different ligands which may be ascribed, at least in part, to intrinsic differences in reactivity between the α and β chains. Studies on oxygen binding by cobalt-substituted hemoglobins have provided important results about structure–function relationships [45,46].

Ligand binding under equilibrium conditions has been followed by NMR measurements with the aim of establishing directly the distribution of intermediates present at the various stages of saturation [47–49].

Measurement of binding of CO to hemoglobin under conditions of photodissociation [50] have yielded valuable information in the framework of models for the reactions of hemoglobin with ligands.

Comparative studies on the ligand binding properties of hemoglobins from different animal classes [51–53], especially fishes, have shown relevant variations on a common functional theme.

Great advances have been made in clarifying

the effects of third components on the ligand equilibria of hemoglobin.

A dependence of oxygen affinity of mammalian hemoglobin on concentration and type of ions had been known for a long time; however, it has only recently been discovered that certain polyanions, such as 2,3-diphosphoglycerate and ATP, which are present in red blood cells, have a very large effect on oxygen binding, correlated with the higher affinity they have for deoxy rather than for ligand-bound hemoglobin [54,55]. Thus, it was established that the organic polyphosphates have an important physiological role in regulating the oxygen affinity of hemoglobin in red cells [56–59]. A great amount of work has since been directed to the study of the interaction of polyphosphates with hemoglobin. The stoichiometry of binding is one polyanion per hemoglobin tetramer, the site of attachment, inferred from chemical studies, has been shown by X-ray analysis [60,61], to be in the central cavity where the polyanion interacts with positively charged groups on the β chains which are in a stereochemical favorable situation in the deoxy derivative.

The Bohr effect, the effect of hydrogen ions on the oxygen affinity of hemoglobin, is due to changes in pK of ionizable groups on oxygen binding. Chemical [62,63] and crystallographic studies [64] have led to the identification of most, if not all, of the groups, and to the explanation of their change in pK as a result of the conformation changes associated with ligand binding. The groups responsible for the alkaline Bohr effect have been identified with the imidazole of the C-terminal histidine on the β chain (His 146), the α amino of the N-terminal valine on the α chain and possibly the imidazole of histidine 122 also on the α chain. The effect of CO_2 on the oxygen equilibrium of hemoglobin has been clarified. It has been established that CO_2 lowers the O_2 affinity of hemoglobin independently of the Bohr effect, by preferential formation of carbamino compounds with the terminal α and β chain amino groups of deoxyhemoglobin [65].

A number of studies have been devoted to the interaction of hemoglobin with haptoglobin and to the effect of haptoglobin binding on the functional properties of hemoglobin [66,67].

Ligand binding kinetics

The kinetics of ligand binding to hemoglobin represent a formidable problem in view of the complexity of the system and of the very large number of individual reaction steps reflecting binding of the ligand itself, conformation changes, reaction with third components, and dissociation into subunits [68]. However, outstanding progress has been made during the past decade in understanding hemoglobin kinetics with the use of rapid mixing [69], flash photolysis [70] and relaxation (temperature jump) techniques [71,72] combined with sophisticated analysis of the data. Apart from attempts to give a detailed description of hemoglobin kinetics in the framework of reaction schemes corresponding to different models [73], basic achievements have been obtained at a phenomenological level. The main results may be summarized as follows: Cooperativity of ligand binding is reflected kinetically in changes in the combination and dissociation velocity constants as saturation proceeds [69,74]; intramolecular conformation changes and the linked reactions with protons or organic phosphates are rapid in respect to ligand binding [75–77]; quaternary changes occur late in the sequence of reactions leading to saturation [78]. Ligand linked dissociations into subunits (mainly dimers) appear as 'slow', protein concentration-dependent, processes [79]. In some association or dissociation reactions the α and β chains are not equivalent, the differences depending on the nature of the ligand [80,81].

Conformation changes and ligand binding: models for interaction effects in hemoglobin

In the last ten years, ligand-linked conformational transitions in proteins have acquired a key significance in understanding biological regulation at a molecular level. Hemoglobin has played a central role in the development of the subject

and has been taken as a model system for the study of 'allosteric' phenomena. Thus, both experimentally and theoretically, the bases on which 'allosteric theory' now relies have been largely founded on the knowledge of the structural and functional properties of hemoglobin [82,83].

This is not the place to discuss the general or restricted allosteric models which have been applied to hemoglobin and within which mechanistic descriptions of the hemoglobin reactions have been given [84–86]; all of them have the merit of relating, quantitatively, structural changes to changes in reactivity and (within the limits of their degeneracy) to be subjected to experimental verification.

Experimentally, the conformation changes associated with oxygenation have been revealed in great detail by X-ray analysis and a stereochemical interpretation of the interaction effects based on a two-state allosteric model has been proposed [11].

High resolution NMR spectroscopy and ESR measurements of spin labels have been successfully used to follow conformational transitions at various stages of ligand binding both at the tertiary and quaternary levels [87–91]. A great advantage of these studies has been the preparation and analysis of 'artificial intermediates' or 'valency hybrids' in which one or the other of the two kinds of chains, α or β, are frozen in the ligand-bound state (mainly ferric-cyanide) and only the partner is in the deoxy state and able to react with the ligand. Thus, structural events and reactivities associated with only partial and specific ligations of the hemes could be directly followed [92–96]. On the theoretical side, general and comprehensive treatments of cooperative effects associated with conformation transitions have developed from the original linked function theory [97–99].

This brief summary of the progress made during the last ten years in the hemoglobin field may be concluded on a note of satisfaction. In few other instances as in this one, confluence of theoretical and experimental work has led to a very rapid advancement of knowledge toward the solution of the problems of understanding of biological phenomena in terms of exact molecular events.

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