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Review

Hemoglobin, an "evergreen" red protein

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ABSTRACT

After more than a century of experimental, theoretical and computational studies, there is no general agreement yet on the mechanisms underlying the fine regulation of hemoglobin structural and functional properties. The experiments that we have carried out during the last two decades on hemoglobin immobilized in the crystal or in nanoporous silica gels have demonstrated that oxygen binding to a single quaternary structure is non-cooperative. This work finally settled the controversy among competing allosteric models. In addition, a vast amount of experimental evidence has been accumulated showing that tertiary conformational changes play a major role in the functional regulation of hemoglobin, even within a single quaternary state, an observation that is inconsistent with the classical MWC model. Experiments appear to be fully consistent with the Tertiary Two State allosteric model, recently proposed by Eaton and co-workers.

The theoretical and experimental approaches described in this review should help in providing a quantitative understanding of allosteric interactions in other multi-subunit protein complexes.

1. Introduction

On two occasions, the first in 1985 during a seminar at the NIH, in Bethesda, Maryland, and the second in 1986 in a lecture at a meeting on allosteric proteins in Paris, the Nobel laureate Max Perutz pointed out that no functional information was available for hemoglobin (Hb) in the crystal, in spite of the many structures already solved in different ligations and conformational states. These stimulating remarks, together with discussions with a leading expert in Hb investigation by spectroscopic methods, William A. Eaton, at NIH, and the expertise in single crystal microspectrophotometry [1,2] accumulated in the laboratory of Gian Luigi Rossi, University of Parma, Italy, with the collaborative support of Robert W. Noble, at Buffalo, prompted an extensive series of functional studies on single crystals of Hb and Hb mutants. These investigations, that have been going on for more than twenty years, were initially aimed at filling the gap of knowledge on the functional and regulatory properties of Hb in the crystal, in order to directly correlate Hb structure and function determined in the same physical state. The initial experimental results [3–7] were found to be consistent with the allosteric model of Monod, Wyman and Changeux (MWC) [8], and inconsistent with an alternative model proposed by Ackers and co-workers [9,10]. The key question was whether the quaternary **T** state binds oxygen cooperatively, as required by the Ackers' model, or non-cooperatively, as required by MWC. To further investigate this key issue, and to challenge the criticisms that crystal lattice constraints might hamper functionally relevant intersubunit communication, a novel approach was pursued starting from the mid 1990s. Hb was encapsulated in wet, nanoporous silica gels, a procedure known to enourmously slow down the rate of conformational transitions [11–17] without significantly affecting protein structure and function [18,19], thus allowing the separate investigation of the equilibrium and kinetic properties of the **R** and **T** quaternary structures. Measurements of oxygen binding to Hb gels confirmed the crystal results showing that the **T** quaternary structure binds oxygen non-cooperatively, even in the presence of allosteric effects by protons, chloride and organic phosphates [20]. This observation, together with the results of CO rebinding kinetics following laser flash photolysis in Hb gels, motivated the development of a new model for Hb allosteric regulation, the Tertiary Two State model (TTS) [6,16,21]. The TTS model, in which the tertiary rather than quaternary structure is the main determinant of subunit reactivity, could serve as a guide for unraveling the complexities found for other allosteric proteins.

This review summarizes the main results of our research on Hb. The applicability of the developed approaches to other, not necessarily allosteric proteins, is straightforward.

Abbreviations: CD, circular dichroism; Hb, hemoglobin; IHP, inositol hexaphosphate; MEM, maximum entropy method; MWC, Monod, Wyman and Changeux; PEG, polyethylene glycol; TTS, Tertiary Two State allosteric model

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2. The crystal "season", i.e. the determination of oxygen binding to Hb crystals

A protein crystal is an ordered array of molecules, that has properties closer to an oriented solution than a crystal of organic material. The composition of a protein crystal is typically 50% protein and 50% solvent, leading to an environment that allows free diffusion of ligands in and out the crystal lattice and a significant degree of conformational flexibility [1]. Meaningful spectroscopic measurements can be carried out on protein crystals provided that i) linearly polarized light is used and ii) crystals are oriented in such a way that the electric vector of the polarized light is parallel to one of the crystal optical axes. For deoxy-Hb crystals, grown from polyethylene glycol (PEG) solutions, the crystal space group is orthorhombic $P2_12_12_1$, with the *a* and *c* crystallographic axes parallel to the major and minor edges, respectively, and *b* perpendicular to the crystal plane (Fig. 1, inset). These directions are also the crystal optical axes along which polarized light travels without depolarization, thus the Beer–Lambert law is obeyed and absorbance depends on the concentration of the absorbing chromophore, the path-length and the extinction coefficient ε [22]. To rigorously compare crystal and solution spectra, it is necessary to calculate the average of the polarized absorption spectra taken in three mutually perpendicular directions. For a crystal belonging to an orthorhombic space group, the so-called isotropic extinction coefficient at each wavelength can be obtained from $1/3(\varepsilon_a + \varepsilon_b + \varepsilon_c)$ where *a*, *b* and *c* are both the axes of the unit cell of the crystal and the optical axes for polarized absorption measurements.

Polarized absorption spectra were recorded as a function of oxygen pressure for **T** state Hb crystals (Fig. 1), grown from PEG solutions, in the absence and presence of the allosteric effectors inositol hexaphosphate (IHP) and bezafibrate [4,7,23]. The fraction of deoxygenated, oxygenated and oxidized hemes was estimated by fitting the observed spectra to a linear combination of the pure species, Hb, oxy-Hb and met-Hb, respectively, and a baseline [4]. The average p50, i.e. the oxygen pressure at 50% saturation, measured along different crystal axes, was of the order of 130–150 Torr at 15 °C, independent of pH and the presence of allosteric effectors. This value is the same as K_T, the dissociation constant for oxygen binding to **T** state Hb (assuming the MWC model), or K_1, the dissociation constant for binding of the first oxygen molecule to the Hb tetramer (in a model-independent analysis) [24,25], determined in solution in the presence of strong allosteric effectors. This value has been proposed to be associated with the extreme low affinity **T** state Hb, a protein conformation essentially devoid of cooperativity [26]. Thus, **T** state Hb in the crystal behaves as one of the conformations present in solution. Furthermore, the oxygen binding curve for Hb crystals was characterized by a Hill coefficient very close to unity, indicating no cooperativity in oxygen binding within the **T** state, in agreement with the prediction of the MWC model for a molecule remaining in a single quaternary state. Rivetti et al. explained their crystal results by postulating the presence in solution of high and low affinity subunit conformations of **T**, associated with broken and unbroken intersubunit salt bridges [4]. Only the latter is populated in **T** state crystals grown under deoxy conditions. Accordingly, no effect of pH was observed indicating that ligand binding in the crystal does not lead to breakage of salt bridges, in agreement with crystallographic evidence [27–32] and the stereochemical mechanism proposed by Perutz [33].

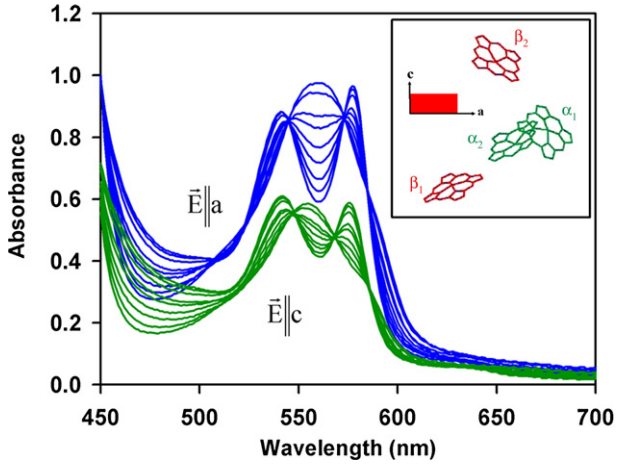

Fig. 1. Polarized absorption spectra recorded for **T** state Hb crystals, grown from PEG solutions, at different oxygen pressures between 0 and 760 Torr. Spectra were recorded with the electric vector of incident light parallel to the *a* (blue) or *c* (green) crystal axes, at 15 °C, pH 7. Inset: projections of the four hemes of the asymmetric unit of crystals onto the *ac* crystal face of the optical measurements.

Table 1
Equilibrium oxygen binding parameters for hemoglobin crystals and silica gels, at 15 °C.

Hb	Conditions	p50[a] (Torr)	Hill *n*[a]	Reference
HbA	Gel, no allosteric effectors	26	0.95	[16]
"	Gel, + IHP, BZF, Cl^-	134	0.93	[16]
"	Crystal, no allosteric effectors	136/133	1.00/1.01	[23]
"	Crystal, + IHP	139/132	0.94/0.95	[23]
"	Crystal, + BZF	138/127	0.94/0.97	[23]
Des(Arg)	Crystal	12.7/9.6	0.97/0.99	[39]
Des(His)	Crystal	81/76	0.98/1.01	[40]
Cowtown	Crystal	44/45	0.99/0.98	[41]
$\alpha(Fe^{2+})_2\beta(Ni^{2+})_2$	Crystal	95/87	0.96/0.90	[37]
$\alpha(Ni^{2+})_2\beta(Fe^{2+})_2$	Crystal	123/102	0.90/0.90	[5]
$\alpha(Fe^{2+})_2\beta(Zn^{2+})_2$	Crystal	81/81	1.08/1.10	[34]
$\alpha(Zn^{2+})_2\beta(Fe^{2+})_2$	Crystal	155/152	1.13/1.08	[34]

[a] For crystal experiments, two p50 and Hill coefficient *n* values are reported, referring to oxygen binding curves recorded along two orthogonal crystal axes.

The oxygen binding curves of Hb crystals, measured with light polarized along the *a* and *c* crystal axes, do not exhibit the same p50 (Table 1), with a lower value for the binding curve recorded along the *c* axis. The α and β hemes make different projections along the *a* and *c* axes (Fig. 1, inset), and, in particular, the α hemes contribute more to the absorbance along the *c* axis. When binding curves are analyzed taking into account the different α and β heme orientations, oxygen binding curves for α and β hemes can be separately determined, information that is unique to polarized absorption measurements on oriented single Hb crystals. It was found that the α hemes bind oxygen with an affinity two-fold higher than β hemes [23], in agreement with data obtained using metal hybrids Hb in solution [34–36]. This conclusion was further confirmed by oxygen binding curves measured on $\alpha(Fe^{2+})_2\beta(Ni^{2+})_2$, $\alpha(Ni^{2+})_2\beta(Fe^{2+})_2$, $\alpha(Fe^{2+})_2\beta(Zn^{2+})_2$, $\alpha(Zn^{2+})_2\beta(Fe^{2+})_2$ crystals [5,34,37] (Table 1). This ruled out the possibility that a large decrease in the Hill n from α–β inequivalence masks positive cooperativity to yield a Hill coefficient close to unity.

Additional information that can be obtained from polarized absorption spectra of Hb crystals concerns differences in the heme orientation in different ligation or oxidation states. These are reflected in variations of the polarization ratio, i.e. the ratio of the optical densities in two orthogonal directions at wavelengths where the electronic transitions are almost perfectly *x*, *y* polarized [22,38]. The observed changes in the polarization ratios indicate that tertiary conformational transitions take place in **T** state Hb crystals upon oxygen binding [4].

According to Perutz's stereochemical mechanism the salt bridges are key elements in the stabilization of the **T** quaternary state and the control of oxygen affinity [33]. However, measurements of oxygen affinity in solution upon removal of salt bridges cannot distinguish between a shift in the quaternary equilibrium and a change in the intrinsic affinity of **T** state subunits. Oxygen binding curves in the crystal allowed separation of the contribution of individual salt bridges to the stabilization of the **T** state from their direct effect on oxygen affinity. Oxygen binding curves were recorded for desArgHb