Ligand binding and stereochemical effects in hemocyanins

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The Bohr effect in the binding of O₂ and CO to *Helix pomatia* β -hemocyanin has been estimated by the potentiometric titration method. On the basis of the results, as well as other information available, we conclude that binding of O₂ to hemocyanin is associated with a quaternary allosteric transition (T $\rightarrow$ R), while binding of CO is not. This conclusion is in agreement with previous results, which indicate that homotropic interactions are observed only for the binding of O₂ to the active site of hemocyanins. This is in marked contrast to the behaviour of hemoglobins which, otherwise, share with hemocyanins a number of important features, in spite of basic chemical differences. The stereochemical requirements of O₂, which forms a bridge between the two copper atoms in a site, have to be considered essential for the onset of cooperativity in hemocyanins.

Key words: allosterism/hemocyanin/stereochemical effects

Introduction

Hemocyanins are the giant copper-containing proteins which act as oxygen carriers in a large number of arthropods and molluscs (Ghiretti, 1968; Van Holde and Van Bruggen, 1971; Lontie and Witters, 1981; Brunori *et al.*, 1982). Their structural and functional properties have been extensively investigated (see Lamy and Lamy, 1981), and the level of quantitative information available nowadays seems sufficient to present a stereochemical hypothesis for the cooperative effects observed in the binding of oxygen to these multisubunit proteins.

Deoxyhemocyanin binds reversibly O₂ and CO in the same molar ratio, i.e., 1 mol of ligand per binding site containing two copper atoms. The Bohr effect upon binding of both O₂ and CO to *Helix pomatia* β -hemocyanin measured by the differential titration method is reported here. As discussed elsewhere (Zolla *et al.*, 1978; Kuiper *et al.*, 1978), the Bohr effect in this protein is a reliable marker of the quaternary conformational change, which represents the structural basis of allosteric effects in functioning macromolecules (Monod *et al.*, 1965).

In this paper new and older results are correlated to known structural features of hemocyanins. The description of ligand binding and the structural basis of cooperative effects in hemoglobins (Wyman, 1964; Perutz, 1970; Antonini and Brunori, 1971; Perutz, 1980) are taken into consideration. This is justified, in our opinion, by the fact that, although the overall structure and molecular architecture of hemocyanins, their primary structure, their ligand binding site, and the stereochemistry of the binuclear copper center (where dioxy-

gen is reversibly bound) are indeed very different from the corresponding structural features of hemoglobins, there are outstanding analogies in the functional properties of these two families of oxygen carriers.

Results

A summary of the differential titration measurements carried out with both gaseous ligands (O₂ and CO) is presented in Table I. In agreement with equilibrium and kinetic data (Zolla *et al.*, 1978; Kuiper *et al.*, 1978), the combination of O₂ with deoxyhemocyanin is associated with a large negative Bohr effect, since protons are taken up upon O₂ combination (line 1 of Table I). On the other hand, binding of CO to deoxyhemocyanin is not associated with any significant Bohr effect (line 2 of Table I). Previous experiments on the same protein (Kuiper *et al.*, 1980), which suggested the existence of a small positive Bohr effect upon binding of CO, have not been confirmed by luminescence measurements (Brunori *et al.*, 1981), and may be due to inaccuracy of the results because of the small signal change and possible effects from scattering.

Lines 3 and 4 of Table I report the measured Bohr effect upon substitution of CO by O₂, or *vice versa*. It is clear that replacement is associated with a change in the number of protons bound by the protein which is the same as the value obtained upon binding of O₂ to deoxyhemocyanin.

These experiments provide independent information which substantiates the idea that at pH 7–7.5 binding of O₂ to deoxyhemocyanin is associated with a quaternary switch (indicated as T $\rightarrow$ R), while binding of CO occurs without change in quaternary structure.

Discussion

As in the case of the hemoglobins (Perutz, 1970; Hopfield *et al.*, 1971), the classical two-state allosteric model (Monod *et al.*, 1965), based on the presence of only two functionally relevant quaternary states of the macromolecule in chemical equilibrium, is often adequate to describe cooperative effects in the binding of O₂ by hemocyanins. The features which differentiate the ligand-binding properties of hemoglobins and hemocyanins involve the absolute value of the overall free energy of interaction (ΔF_i) (Wyman, 1964), the maximum Hill coefficient (n_H) and the effects of different solution components on the slope and position of the O₂ binding curve. Some of the functional differences between the two classes of respiratory proteins (summarized in Table II) may be rationalized within a two-state model assuming that: (i) in hemoglobins the whole tetramer is the "basic functional unit" (Edelstein and Gibson, 1971), while (ii) in the case of hemocyanins, cooperative interactions do not necessarily extend over the whole molecule (which in some cases contains >100 binding sites), but involve only a limited number of sites, at least for the larger hemocyanins (Colosimo *et al.*, 1974). Thus, in the case of β -hemocyanin from *H. pomatia* the "basic functional unit" involves ~ 16 O₂ binding sites out of a total of 160–180 sites carried by the molecule (Zolla *et al.*, 1978). This is sufficient to account quantitatively for the

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Table I. Bohr effect in the binding of O₂ and CO to *H. pomatia* β -hemocyanin

Reaction	ΔH^+
1 Hc(T) $\rightarrow$ HcO ₂ (R)	+ 1.0; + 1.1
2 Hc(T) $\rightarrow$ HcCO(T)	0
3 HcCO(T) $\rightarrow$ HcO ₂ (R)	+ 1.0; + 1.2
4 HcO ₂ (R) $\rightarrow$ HcCO(T)	- 1.1

The shift in quaternary state (T and R) upon ligand binding or ligand substitution is indicated. Conditions: pH (deoxyhemocyanin) = 7.25 in 10 mM NaCl + 20 mM CaCl₂; 20°C; protein concentration = 17.7 mg/ml. Hc = deoxyhemocyanin; HcO₂ = oxyhemocyanin; HcCO = carbon monoxide hemocyanin; ΔH^+ is expressed in terms of protons taken up (+) or released (-) by the protein.

Table II. Comparison of some structural and functional properties of human hemoglobin and *H. pomatia* β -hemocyanin

	Hemoglobin	Hemocyanin
Ligand binding site	Proto heme-Fe(II)	Cu(I) ₂
Molecular weight	64 000	9 x 10 ⁶
Ligand binding sites/molecule	4	160–180
O ₂ binding ^a :		
n_H	2.9	7
ΔF_i^b	3 kcal/site	1.5 kcal/site
ΔH_{max}^+ ^c	- 0.5 H ⁺ /site	+ 1.1 H ⁺ /site
CO binding ^a :		
n_H	2.9	1
ΔF_i^b	3 kcal/site	0
ΔH_{max}^+ ^c	- 0.5 H ⁺ /site	0

^aConditions: hemoglobin pH 7 in phosphate buffer 0.1 M, 20°C; hemocyanin pH 7.2–7.3 in Tris-HCl 0.01 M + CaCl₂ 17 mM, 20°C (from Antonini and Brunori (1971) and Zolla *et al.* (1978)). ^b ΔF_i is the free energy of interaction per site (Wyman, 1964). ^c ΔH_{max}^+ is the maximum number of Bohr protons taken up (+) or released (-) per site upon binding of O₂ or CO. (See Materials and methods.)

fact that the O₂ binding isotherm is very steep in the middle saturation region, although the overall free energy of interaction per site is rather small as compared with hemoglobin (Table II).

The most striking functional difference, in our opinion, between hemoglobins and hemocyanins involves the binding of CO. Thus, in the case of hemoglobins the reaction with CO is associated with homotropic and heterotropic interaction phenomena which are indeed very similar, if not identical, to those observed in the binding of O₂ (Antonini and Brunori, 1971). On the other hand, binding of CO to hemocyanins is non-cooperative and the quaternary-linked Bohr effect is absent [although a small tertiary pH effect is reported for *Panulirus interruptus* (Brunori *et al.*, 1981) and *Callinectes sapidus* (Bonaventura *et al.*, 1974) hemocyanins]. As our results show, the measurement of the Bohr effect by the differential titration method proves that saturation of deoxyhemocyanin with CO is not associated with a quaternary conformational change, while replacement of CO by O₂ is accompanied by a full Bohr effect, identical to that observed in the transition from deoxy- to oxyhemocyanin.

Recent spectroscopic information on hemocyanin provides a structural basis for the rationalization of some of these effects. The structure of the binuclear copper center in oxy- and deoxyhemocyanin shows dramatic differences, indicating O₂-linked conformational changes. The Cu(II) oxidation state of

the two metal atoms in oxyhemocyanin is related to the formation of a symmetrical peroxo-Cu(II)₂ complex; and the stereochemistry of the site involves coupling of the two metals through an endogenous protein ligand (possibly oxygen from a phenolate) and a copper–copper distance of 3.6–3.7 Å. This model is supported by chemical and spectroscopic data (Freedman *et al.*, 1976; Himmelwright *et al.*, 1980; Brown *et al.*, 1980; Jori *et al.*, 1981; Co Man Sung *et al.*, 1981a, 1981b). In deoxyhemocyanin, the binuclear center has very different stereochemistry and coordination and, in spite of some controversy, breakage of the endogenous bridge, changes in the copper–copper distance (3.4 Å or 5–6 Å), and decrease in the number of coordinated ligands are accepted facts (Brown *et al.*, 1980; Co Man Sung *et al.*, 1981a, 1981b). Given the species chosen for these studies and the experimental conditions used, these structural features may be taken to reflect the stereochemistry of an R-state site in oxyhemocyanin and of a T-state site in deoxyhemocyanin. It is therefore expected that the bridging nature of the oxygen bound to the binuclear center may result in a considerable rearrangement of the site, and this effect may possibly be correlated with the biphasic nature of the O₂-binding kinetics displayed by hemocyanin in the T state (as described several years ago by Brunori, 1971).

It is, therefore, proposed that the structural changes necessary to fit an oxygen molecule symmetrically bound between the two copper atoms may well be the key event in the onset of cooperativity observed in the binding of this ligand. Somewhat in line with Perutz's (1970) stereochemical model for ligand binding to hemoglobin, it may be postulated that the low O₂ affinity of hemocyanin in the T-state is related to the energy necessary to optimize the structure of the binuclear copper center to fit an O₂ molecule. Thus, the bridging nature of dioxygen, which imposes a stereochemical constraint on the metal-protein complex, would be an essential step in the trigger of the conformational events which help the free energy balance in favor of the R-state.

In line with this hypothesis, the non-cooperative binding of CO to hemocyanins and the absence of a Bohr effect may be correlated to the stereochemistry of the CO bound to the binuclear copper center. Thus, although the stoichiometric ratio corresponds to one CO per two metals, i.e. spectroscopy (Fagen and Alben, 1972; Van der Deen and Hoving, 1979) has shown that CO is bound to only one of the two copper atoms in a site, and interactions with the other metal in the same site are not apparent. Therefore, the non-bridging nature of CO may explain why binding of this ligand is not associated with a T to R quaternary switch.

In conclusion, cooperative effects in hemocyanins are limited to the binding of O₂ and are to be related to the bridging properties unique to this ligand, which operates as a "clamp" between the two copper atoms. The stereochemical constraint imposed by dioxygen produces local perturbations which set into motion structural changes leading to the quaternary switch and, therefore, to cooperative behaviour. Binding of CO, which coordinates with only one of the two metals in a site, exerts no trigger on the quaternary structural change. Spectroscopic studies of hemocyanins frozen in either the T or the R state, at the same degree of ligation, will provide interesting information in relation to this hypothesis.

Materials and methods

H. pomatia β -hemocyanin was purified from freshly drawn hemolymph according to Heirwegh *et al.* (1961). Its functional properties were checked by

reference to the pH-dependent O₂-binding data reported by Zolla *et al.* (1978).

Protein concentration was determined by absorbance at 280 nm, taking the extinction of 1.4 for a 1 mg/ml solution of β -hemocyanin in 2% borate pH 9.15. Pure gases (O₂ and CO) were obtained from Caracciolo and S.I.O. (Rome, Italy) and were bubbled through aqueous solutions of NaOH and HCl, and through water before use.

Differential titration experiments were carried out in 10 mM NaCl containing 20 mM CaCl₂ at 20°C. The procedure used was essentially according to Antonini *et al.* (1963) and the number of protons taken up (or released) was determined by back titration with 0.1 N HCl or NaOH.

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