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OXYGEN BINDING PROPERTIES OF *COLUS GRACILIS* HAEMOCYANIN AND ITS SUBUNITS

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1. Introduction

The respiratory pigments of most gastropods are copper-containing haemocyanins which are capable of binding oxygen co-operatively. These multisubunit proteins typically have a mol. wt. of 9×10^6, and contain about 180 oxygen binding sites per molecule [1]. A considerable amount of information on the subunit structure of the molecules is available from electronmicroscopic and analytical ultracentrifuge studies and it is therefore of interest to relate this structure to the functional properties of the molecules.

Gastropod haemocyanins undergo a pH-induced dissociation which is prevented by the presence of calcium ions. It is well established that whereas whole or native haemocyanin molecules bind oxygen co-operatively, one-tenth molecules obtained by subjecting whole molecules to a small pH change in the absence of Ca^{2+}(e.g. from pH 7.5 to 8.5), do not bind oxygen co-operatively [2,3]. This is in spite of the fact that one-tenth molecules contain about 18 functional oxygen-binding sites. The fact that the wholes–tenths dissociation is reversible upon lowering the pH and adding calcium ions [1], suggests that the one-tenth molecules have not suffered any major destruction of conformation in the process of dissociation. One may therefore ask what is the smallest fraction of a haemocyanin molecule which can exhibit co-operative binding.

With most gastropod haemocyanins the pH-induced dissociation results in the formation of a mixture of whole, one-half, and one-tenth molecules [1]: at higher pH values only one-tenths exist. In the present work the oxygen-binding properties of the haemocyanin from the whelk, *Colus gracilis* (da Costa) were investigated. Within a certain narrow pH range this haemocyanin, unlike other gastropod haemocyanins that have been studied, dissociates completely to one-half molecules, no one-tenths being formed [1]. One-tenth molecules are produced at higher pH values. Upon lowering the pH to 7.0–8.0 these do not re-associate to form whole molecules unless calcium ions are present. This made it possible to determine the oxygen equilibrium curves for whole, one-half, and one-tenth molecules independently at the same pH value and under almost identical conditions. It is shown that the one-half molecule is the smallest structure capable of exhibiting co-operative oxygen binding.

2. Experimental

2.1 *Material*

Haemocyanin was obtained from specimens of *C. gracilis* as described previously [1]. By careful adjustment of both the pH and the Ca^{2+} concentration, solutions containing entirely whole, entirely one-half, or entirely one-tenth molecules could be obtained. For example, to obtain solutions of these at the same pH value, portions of a stock solution of haemocyanin were dialyzed against (a) 0.1 M Tris-HCl, pH 7.82, containing 0.05 M $CaCl_2$ (wholes), (b) 0.1 M Tris–HC1, pH 7.82, containing 0.01 M EDTA (halves), or (c) 0.05 M borate, pH 9.2, followed by 0.1 M Tris–HCl, pH 7.82, containing 0.01 M EDTA (tenths). The dissociation state was checked by sedimentation velocity experiments in a Beckman Model E analytical ultracentrifuge equipped with a schlieren optical system.

2.2 *Oxygen binding curves*

Oxygen binding curves were determined by means of a modification of the spectrophotometric and oxygen electrode method described by Imai et al. [4], taking advantage of the fact that oxy- but not deoxyhaemocyanin absorbs at 346 nm. The cuvette containing the haemocyanin solution was placed in the thermostatted cell holder of a Unicam SP 1800 spectrophotometer. The pO_2 and the absorbance at 346 nm were then simultaneously and continuously recorded as pure nitrogen was gently bubbled through the stirred haemocyanin solution.

3. Results

The oxygen binding data for whole, one-half, and one-tenth *C. gracilis* haemocyanin molecules are shown in fig. 1 in the form of Hill plots. It will be recalled that for multisubunit proteins which bind oxygen co-operatively, the binding curve is sigmoidal and the Hill plot has a high intermediate slope (n), but a slope approaching unity at high and low oxygen tensions [5]. For non-co-operative binding the curve is hyperbolic and the Hill plot has a slope of 1.0 throughout. It is clear from fig. 1 that both whole and one-half molecules, but not one-tenth molecules, bind oxygen co-operatively. Table 1 lists further data in the form of p_{50}, and the slope of the Hill plot at p_{50}, n_H. It will be seen that n_H for one-tenth molecules is always about 1.0, whereas for whole and one-half molecules it is in the region of 2.0.

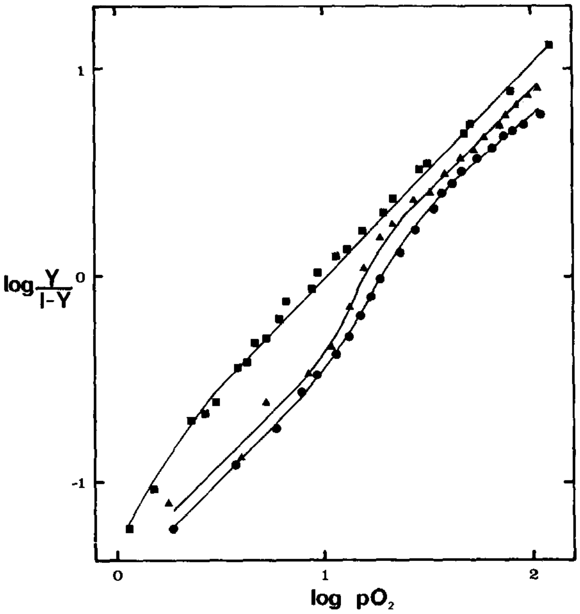

Fig. 1. Hill plots of oxygen equilibrium of *C. gracilis* heamocyanin at 20°C. Protein concentration approx. 1.4 mg/ml, pH 7.82, buffer, 0.1M Tris–HCl (see text for details). (●—●—●) whole molecules; (▲—▲—▲) one-half molecules; (■—■—■) one-tenth molecules.

Table 1
Oxygen equilibrium data for *C. gracilis* haemocyanin. In each case the haemocyanin concentration was approx. 1.4 mg/ml, and the temperature was 20°C

Dissociation State	Buffer Composition	pH	p_{50} (mm Hg)	n_H
Wholes	0.1M Tris–HCl, $0.05M\ CaCl_2$	7.82	19.0	1.90
Halves	0.1M Tris–HCl, 0.01M EDTA	7.82	15.5	2.10
Tenths	0.1M Tris–HCl 0.01M EDTA	7.82	10.2	1.03
Tenths	0.05M borate	9.20	4.1	0.96
Wholes	0.1M Tris–HCl, $0.05M\ CaCl_2$ + 0.5M NaCl.	7.84	20.1	1.98

The conditions used in these experiments are somewhat artificial as the ionic composition of the haemolymph of these animals probably approximates that of sea water. However, as table 1 shows, the addition of 0.5 M NaCl to the haemocyanin solution, giving a final total molarity of over 0.6M, makes little difference either to p_{50} or to n_H, indicating that the curves obtained are not significantly different to those for conditions closely approximating those in vivo.

Like many other haemocyanins from marine gastropods, that from *C. gracilis* has a reverse or negative Bohr effect (fig. 2). Over the pH range 7.4 to about 8.0, whole molecules in the presence of Ca^{2+} showed a Bohr shift, ϕ ($\Delta \log p_{50}/\Delta$ pH), of +0.52. Haemocyanins from terrestrial snails also have a reverse Bohr effect [6,7], but that from a freshwater snail, *Lynm-*

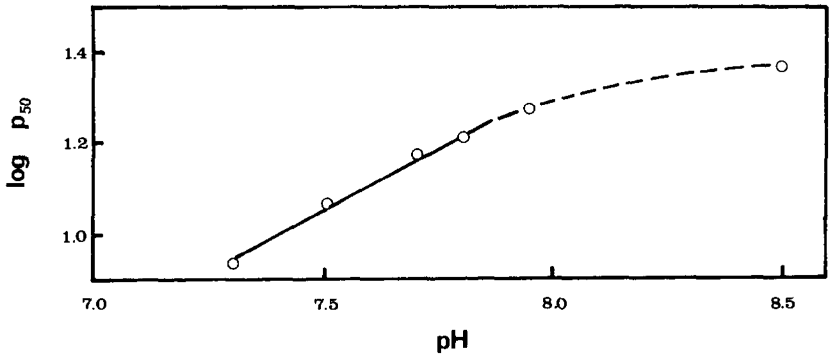

Fig. 2. Bohr effect for *C. gracilis* haemocyanin whole molecules at 20°C, protein concentration approx. 1.4 mg/ml. Buffer: 0.1M Tris–HCl containing 0.05M $CaCl_2$.

naea, [8], and also that from a slug, *Agrolimax,* [9], have normal or positive Bohr effects. An explanation for the reverse Bohr shift is lacking, although Redmond [9] has suggested a way in which it might be beneficial to a relatively inactive animal having a poorly developed circulatory system. It should be noted that the Bohr effect, in contrast to the co-operativity effect, is not dependent upon the integrity of one-half molecules, as one-tenth molecules of *C. gracilis* haemocyanin clearly show a Bohr effect (table 1).

4. Discussion

Some years ago Gruber [10] pointed out that for *Helix pomatia* haemocyanin there was a strong correlation between the presence of one-half molecules (in a mixture of wholes, halves, and tenths) and of a sigmoidal oxygen binding curve. The present work with *C. gracilis* haemocyanin confirms that one-half molecules, are required for co-operative binding to be shown and that one-tenth-molecules, even though they possess a large number of oxygen binding sites, do bind oxygen co-operatively. Recently van Driel [11], working with *H. pomatia* α-haemocyanin, used the photoelectric scanner of an analytic ultracentrifuge at 346 nm to follow the state of oxygenation of whole, one-half, and one-tenth molecules in a mixture, and reached essentially the same conclusion. Electron microscopy [12] shows that in both whole and one-half molecules the subunits are arranged in rings. When dissociation to one-tenths takes place the ring structures are lost [13]. This suggests that for co-operativity to be exhibited a closed ring of five one-tenth molecules must exist. One can envisage that a conformational change resulting from the binding of oxygen at one point on the ring can induce the rest of the subunits in the ring to change their conformation too, the protein thus being constrained in a low affinity state. It appears that this mechanism is independent of the one which mediates the Bohr effect.

It is interesting to recall that the giant multisubunit haemoglobins (erythrocruorins and chlorocruorins) of invertebrates which bind oxygen co-operatively [14,15] also show ring structures in the electron microscope. In fact the molecules appear to be made up of a stack of two rings, each consisting of six smaller rings [16]. The preliminary evidence of Weber [17] was that these smaller rings (=one-twelfth molecules) were capable of co-operative oxygen binding and this was confirmed by the work of David and Daniel [18]. It seems therefore that there may be a fundamental difference between vertebrate haemoglobins and invertebrate respiratory pigments in the way that co-operative interactions are mediated. Vertebrate haemoglobins are tetrametric, the subunits being arranged in a tetrahedron, whereas invertebrate pigments have very many subunits arranged in ring structures.

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