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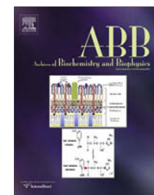

Original paper

Unusual oxygen binding behavior of a 24-meric crustacean hemocyanin

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Hemocyanins from Crustacea usually are found as 1×6 or 2×6-meric assemblies. An exception is the hemocyanin isolated from thalassinidean shrimps where the main component is a 24-meric structure. Our analysis of oxygen binding data of the thalassinidean shrimp *Upogebia pusilla* based on a three-state MWC-model revealed that despite the 24-meric structure the functional properties can be described very well based on the hexamer as allosteric unit. In contrast to the hemocyanins from other thalassinidean shrimps the oxygen affinity of hemocyanin from *U. pusilla* is increased upon addition of L-lactate. A particular feature of this hemocyanin seems to be that L-lactate already enhances oxygen affinity under resting conditions which possibly compensates the rather low intrinsic affinity observed in absence of L-lactate. The fast rate of oxygen dissociation might indicate that in this hemocyanin a higher cooperativity is less important than a fast response of saturation level to changes in oxygen concentration.
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Introduction

Mud shrimps spend most of their life in complex burrows, dug through littoral and sub-littoral soft sediments consisting of mud or muddy, fine sands. The oxygen circulation inside such a burrow is maintained exclusively through active water-pumping carried out by the crustacean. As shown for *Callianassa truncata* the oxygen concentration ranges from 5% to 40% of air-saturation inside the burrows, while oxygen is totally depleted in the surrounding sediments [1]. Mud shrimps exit these burrows for short periods in order to feed and reproduce [2,3]. Thus, in their daily life mud shrimps are exposed to water with different degrees of hypoxia. The lifestyle of these species suggests the evolution of a finely regulated oxygen delivering system in order to maintain an efficient oxygen supply [4].

In some arthropods, namely in Crustacea and Chelicerata, oxygen is transported in the hemolymph by large, copper containing proteins, the hemocyanins. The hemocyanins of arthropods are multiples of hexameric assemblies of ≈75 kDa subunits. Depending on the species hemocyanins are found as hexamers, dodecamers or multiples of dodecamers, displaying a rich set of quaternary structures of diverse levels of complexity and the propensity to exhibit highly cooperative oxygen binding behavior [5]. Short-time regulation of oxygen binding behavior is accomplished by the ability of the protein to adopt different conformations with different binding affinities for oxygen. The relative amount of these conformations and consequently the mean oxygen binding affinity is regulated by effectors [6–9]. The extent of cooperativity and the oxygen binding affinity found for a given condition (temperature, pH, salt concentration) is highly species dependent and is assumed to reflect the needs imposed by the specific living conditions.

In order to correlate the structural hierarchies with functional aspects, different models for cooperativity exist. Due to the large number of binding sites involved in case of hemocyanins mostly extensions of the concerted MWC model are used which is based on the assumption that a certain number of interacting binding sites are coupled (size of the allosteric unit) so that the whole structure can exist only in two conformations. These conformations determine the binding behavior at low and high saturation levels, respectively. For hemocyanins only the simple hexamer was found to exhibit binding behavior which is in agreement with this simplest model, but even for hexamers the model failed in some cases [10]. Dodecameric hemocyanins can sometimes be described by a linear extension of the MWC-model where a third conformation is postulated [11]. However, in most cases higher aggregation forms (2×6-, 4×6-, 8×6-meric) exhibit additional, hierarchical interactions. In this frame, it was suggested that structural hierarchies should be reflected in hierarchies of the equilibrium between different types of allosteric units, leading to the development of a theoretical model based on more than one level of cooperativity [12,13]. The nesting or nested MWC model is a hierarchical model where different allosteric units of the MWC-type are postulated and one level is embedded in the other [14,15].

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The hemolymph of thalassinidean mud shrimps generally contains a 24-meric aggregation state as major component. Besides this, two other aggregation states can be found: hexameric and dodecameric oligomers. The percentage of these low molecular weight oligomers is species dependent and was never found to exceed 15% of the total hemocyanin [16]. The occurrence of 24-meric hemocyanins is untypical for crustacean and seems to be specific for thalassinidean shrimps. Compared to the quasi-square planar 24-meric hemocyanin from Chelicerata, a different quaternary structure has been proposed for crustacean 24-meric hemocyanin [17].

For five thalassinidean shrimps the oxygen binding properties of the 24-meric fraction have been investigated, revealing a relatively high affinity (oxygen pressure at half-saturation p_{50} = 0.13–1.24 kPa), medium cooperativity (Hill coefficient n_{50} = 2.3–3.8), large Bohr effect (ΔLog p_{50}/ΔpH −1.06, −1.48) and no effect of L-lactate [18]).

In order to understand the structure–function relationship in this unusual hemocyanin we investigated in detail the interaction of oxygen with the 24-meric hemocyanin of another member of thalassinidean shrimps, *Upogebia pusilla*. To this end we performed studies on oxygen binding under equilibrium conditions under various conditions with respect to pH and L-lactate. These data indicate that despite the 4 × 6-meric structure the functional properties are in very good agreement with a model assuming the hexamer as allosteric unit without additional interactions between the hexamers. The three conformations which can be adopted by the hexamers are regulated by protons but also by L-lactate in contrast to the species investigated in a previous study [18]. Furthermore we determined the rates of oxygen dissociation from fully oxygenated hemocyanin in order to investigate the role of allosteric effectors on the kinetic properties of this hemocyanin.

Materials and methods

Hemocyanin sampling and purification of the 4 × 6-meric oligomers was carried out as described in [19] by a combination of ultracentrifugation and size exclusion chromatography. Protein concentration was determined based on an extinction coefficient of 1.11 ± 0.05 ml/mg at 278 nm [19]. Oxygen binding curves were measured in 100 mM Tris/HCl buffer containing 20 mM $CaCl_2$ at a pH ranging from 7.2 to 8.0. The effect of L-lactate (0–20.0 mM) was determined in the same buffer as above at pH 7.6.

The aggregation state of hemocyanin at low and high pH-values was checked based on a combination of analytical size exclusion chromatography and Multi-Angle-Light-Scattering (MALS)[2]. A DAWN DSP from Wyatt Technology, Erkrat, Germany, was employed equipped with a 6CL-B column from Pharmacia (GE Healthcare, Chicago, USA).

Oxygen binding curves

The fluorimetric polarographic method [20] was employed to investigate the oxygen binding behavior of *U. pusilla* hemocyanin. This method is based on the quenching of the tryptophan fluorescence when oxygen is bound to the active site, forming a $[\mathrm{Cu(II)} - \mathrm{O}_2^{2-} - \mathrm{Cu(II)}]$ – complex with a characteristic absorption at 338 nm [21,22]. Therefore, the fluorescence intensity increases with decreasing saturation level of the protein. Fluorescence was measured with a standard fluorimeter (Hitachi F4500, Binninger Analytic, Germany) at 338 nm while the oxygen concentration was determined simultaneously with an oxygen electrode (Microelectrodes. Inc., Bedford, USA) equipped with a home-built amplifier. This electrode allows determination of oxygen partial pressure to a precision of 0.1 kPa. All experiments were performed at 20 ± 0.05 °C at a protein concentration of 0.25 mg/ml. Thus, distortions due to the inner filter effect can be neglected.

The fractional saturation Y was calculated from the fluorescence at a given oxygen concentration (x) by normalization with respect to the fluorescence in absence of oxygen (F_o) and under fully saturated conditions (F_S)

$$Y(x) = \frac{F_o - F(x)}{F_o - F_S} \quad (1)$$

In order to obtain a measure for cooperativity (n_{50}) and the oxygen partial pressure necessary to yield half-saturation (p_{50}), oxygen binding data were plotted according to the Hill equation

$$g = \log\frac{Y}{1-Y} = n_h \log x - n_h \log\ p_{50} \quad (2)$$

Here, the slope around $g = 0$ (when $x = p_{50}$) corresponds to n_{50}, the hill-coefficient around p_{50}.

Allosteric models

Binding data were analyzed based on different models. The three-state model [6,23] is a linear extension of the simple MWC-model [24]. In the three-state model three different conformations exist, which are characterized by different oxygen binding constants (K_R, K_T, K_S). As in the MWC-model the size of the allosteric unit (n) describes how many subunits are strongly coupled so that they are always in the same conformation (T, S or R). The equilibrium between the conformations is described by the corresponding allosteric equilibrium constants (L_T and L_S) which are modulated by the presence of allosteric effectors. The binding polynomial for the three-state model is given by the following equation:

$$P = (1+K_R x)^n + L_T(1+K_T x)^n + L_S(1+K_S x)^n$$
$$L_T = \frac{[T_o]}{[R_o]} \qquad L = \frac{[S_o]}{[R_o]} \quad (3a)$$

By convention, the allosteric equilibrium constants denote the ratio of the corresponding conformation in the unliganded state ($[T_o]$,$[S_o]$,$[R_o]$). The simple MWC-model can be regarded as special case where $L_S = 0$ which reflects that the conformation S is not present.

The hybrid model is another possible extension of the simple MWC-model which has been applied to the functional properties of hemocyanins [25] and is characterized by the following binding polynomial

$$P = (1+K_R x)^n + 2qL_T(1+K_R x)^{n/2}(1+K_T x)^{n/2} + L_T^2(1+K_T x)^n$$
$$L_T^2 = \frac{[T_o^{n/2}T_o^{n/2}]}{[R_o^{n/2}R_o^{n/2}]} \qquad 2qL_T = \frac{[T_o^{n/2}R_o^{n/2}[}{[R_o^{n/2}R_o^{n/2}]} \quad (3b)$$

If $q = 1$ the model describes a simple MWC-model with an allosteric unit of size $n/2$.

The Nested MWC model assumes that two levels of allosteric units exist (n and m) which are embedded into each other, yielding the following binding polynomial [14,15]:

$$P = \left((1+K_{rR}x)^n + L_R(1+K_{tR}x)^n\right)^m + \Lambda\left((1+K_{rT}x)^n + L_T(1+K_{tT}x)^n\right)^m$$
$$L_T = \frac{[tT_o]}{[rT_o]} \qquad L_R = \frac{[tR_o]}{[rR_o]} \qquad L = \frac{[T_o]}{[R_o]} \qquad \Lambda = L\frac{(1+L_R)^m}{(1+L_T)^m} \quad (3c)$$

The saturation degree Y for a given model can be expressed as a function of oxygen partial pressure (x) based on the binding polynomial and its derivative [26]:

[2] *Abbreviation used:* MALS, Multi-Angle-Light-Scattering.

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