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Influence of antibody binding on oxygen binding behavior of *Panulirus interruptus* hemocyanin

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Abstract Oxygen binding behavior of monomeric subunit *a* and the hexameric form of this subunit of hemocyanin of *Panulirus interruptus* is influenced by the binding of various monoclonal antibodies. These antibodies react with other surface parts of the subunit than its second domain in which the oxygen binding site is located. The influence of three monoclonal antibodies and their antigen binding fragments (F_{ab}) has been investigated. Two antibodies increase the oxygen affinity of monomeric hemocyanin from that observed in its low affinity T-state, while the third has little influence on this property. F_{ab} fragments abolish almost completely the cooperativity of oxygen binding by the hexameric hemocyanin molecule. The two antibodies which increase the oxygen affinity of the monomeric molecule stabilize high-affinity states of the hexameric molecule, while the third stabilizes the low-affinity state.

Key words: Cooperativity; Hemocyanin; Monoclonal antibodies; Oxygen binding; *Panulirus interruptus*

1. Introduction

Hemocyanin of the spiny lobster *Panulirus interruptus* is a well characterized oxygen binding hexameric protein built from subunits of about 75 kDa. Each subunit binds one oxygen molecule reversibly. The primary structure of its three subunit types *a*, *b* and *c* as well as its three-dimensional structure have been determined [1–5]. The latter structure is very similar to those of the oxygenated homohexamers of subunit II of the closely related hemocyanin of the horseshoe crab *Limulus polyphemus* [6,7]. Each subunit consists of three separate domains with different folding motifs. Domain 1 consists mainly of seven α-helices, designated α1.1 to α1.7. The second domain is also mainly α-helical and contains a four-α-helix bundle with two Cu atoms between which one molecule oxygen can reversibly be bound. Domain 3 is composed of several helices and twelve β-strands, the latter designated β3A to β3L. Its center consists of a seven-stranded β-barrel. A comparison of the oxy and deoxy forms of the homohexamer of subunit II of *Limulus polyphemus* hemocyanin indicates that the binding of oxygen is accompanied by a shortening of the Cu-Cu distance coupled by a rotation of the first domain relative to a core structure consisting of domains 2 and 3 [6,7]. These structural changes are found in all six subunits within the hexamer. This observation is supported by an analysis of CO/O_2 replacement experiments of *Panulirus* hemocyanin, which reveals a MWC-(Monod-Wyman-Changeux)-like [8,9] concerted transition of all six subunits between two conformations upon oxygenation designated as the low-affinity T-state and the high-affinity R-state. Allosteric effectors such as protons are thought to shift the equilibrium between the two conformations.

In order to identify structural elements of the subunits which are involved in the oxygen binding process, oxygen binding curves of monomeric subunit *a* and the reconstituted homohexamer of this subunit of *Panulirus interruptus* hemocyanin were recorded in the absence and presence of bound monoclonal antibodies. For these studies a panel of 14 monoclonal antibodies was available, indicated with letters A–M and T [10]. These antibodies react with the isolated subunit *a* of *Panulirus* hemocyanin, with dissociation constants ranging from 10^{-7} to 10^{-12} M and most of them recognize conformational isotopes. For the studies described here monoclonal antibodies D, E and J were used. The epitopes for antibodies E and J have been determined in detail by immuno-electron microscopy [11]. Antibody E binds to domain 3 at loops between strands β3D-β3E and between β3G-β3H, while antibody J binds to a region with the loop between helices α1.4 and α1.5 in domain 1 as the center of the epitope, but also includes the loops between helices α1.2 and α1.3 and between helix α1.6 and strand β1A. The epitope for antibody D is also localized on domain 1 [12], and does not overlap with that for antibody J [11], but has not yet been mapped into more detail.

2. Materials and methods

Antibodies, F_{ab} fragments and subunit *a* of *Panulirus* hemocyanin were purified as described earlier [10]. Continuous oxygen equilibrium binding curves were recorded with the fluorimetric-polarographic method as described by Loewe [13]. The hemocyanin concentration was about 0.25 mg/ml for experiments with hemocyanin, and 0.05 mg/ml for experiments in which hemocyanin was complexed with immunoreactants in order to avoid inner filter effects. This leads to an experimental error of about 10% in determining the fractional saturation, which makes a precise analysis of the O_2 binding curve as normally carried out less accurate. However, the observed differences in the oxygen binding curves are significant within the error ranges. Prior to oxygen binding measurements, the protein solutions were dialyzed overnight against the buffers in which the experiments were performed. Mixtures in which the effect of an immunoreactant was investigated were prepared at least 1 h before the performance of the oxygen binding experiment. Oxygen binding curves were performed in either 0.1 M Tris-HCl or 0.1 M glycine-NaOH at pH 9.0 at 20°C. Oxygen binding was measured in the presence of 10 mM $CaCl_2$ in order to stabilize the hexameric state and in the absence of divalent cations but in the presence of 10 mM EDTA to study the behavior of the monomerized hemocyanin [14–16]. In all samples 1 mM NaN_3 was present, which has no influence on the oxygen binding.

The subunits were incubated with IgG-D, IgG-E, and IgG-J in a molar ratio of antigen binding sites to subunit of 1.9, 1.4 and 1.3,

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Abbreviations: F_{ab}, antigen binding fragment obtained by digestion of immunoglobulin with papain

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respectively. Homohexamer *a* was incubated with IgG-D, F_{ab}-D, F_{ab}-E, and F_{ab}-J in a ratio of antigen binding sites to hemocyanin subunits in the hexamer of 2.3, 3.8, 1.8 and 3.5, respectively.

3. Results and discussion

3.1. Influence of antibodies on monomers

Incubation of monoclonal antibodies IgG-D, IgG-J and IgG-E shifts the oxygen binding of subunit *a* from $p_{50} = 23.9$ Torr (in the absence of any IgG) to higher affinities with $p_{50} = 17.1$ Torr, 8.5 Torr and 4.8 Torr after incubation with IgG-D, IgG-J and IgG-E, respectively (Fig. 1). Several conclusions can be drawn from these results: no negative cooperativity was observed, indicating that no heterogeneity was present, and that all subunits have bound antibodies. The observed shifts in the oxygen affinity were surprising, since none of the epitopes involves structural elements of the second domain where the active site is located. Nevertheless, binding of IgG-E on the top of the seven-stranded β-barrel in domain 3 at the loops between strands β3D-β3E and β3G-β3H strongly increases the affinity by a factor of 5. The β-strands, β3E and β3G, have contact with helix α2.2, which binds one of the two Cu atoms. Thus, any movement induced by binding of IgG-E may be transferred directly to the active site. A smaller but significant increase in affinity is also observed when antibody IgG-J is bound at the loops between α1.2/α1.3, α1.4/α1.5, and α1.6/β1A. The most intimate contact with the active site is that between α1.6 and α2.1, which binds the other Cu atom. Thus, any movement of helix α1.6 may have a direct influence on the oxygen binding. The almost negligible influence of IgG-D might indicate that no structural element of this epitope has direct contact with the active site. According to Perton et al. [12] the epitope of IgG-D is also located on domain 1, but does not overlap that of IgG-J. On the basis of our results one cannot deduce whether the binding of antibodies results in a closure or opening of the active site.

In order to understand the discrete shifts of the oxygen binding curves, one has to assume that monomeric subunit *a* can occur in various substates with different stabilities. The binding of antibodies may freeze one of these substates.

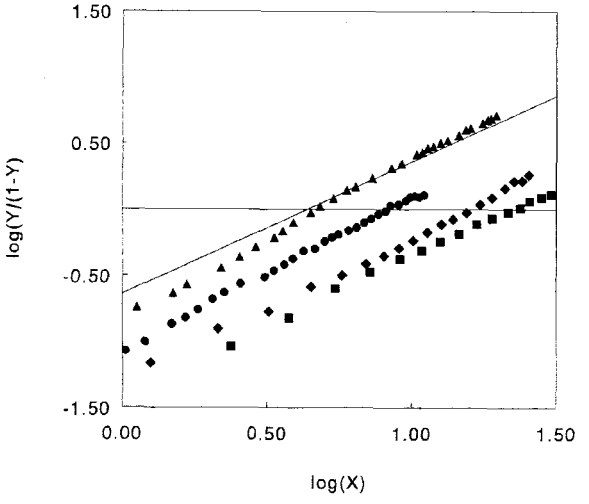

Fig. 1. Influence of monoclonal antibodies on the oxygen binding of monomeric subunit *a* of *Panulirus interruptus* hemocyanin: ■, no antibody present; ♦, with IgG-D; ▲, with IgG-E; ●, with IgG-J. The slope of the drawn line indicates non-cooperativity.

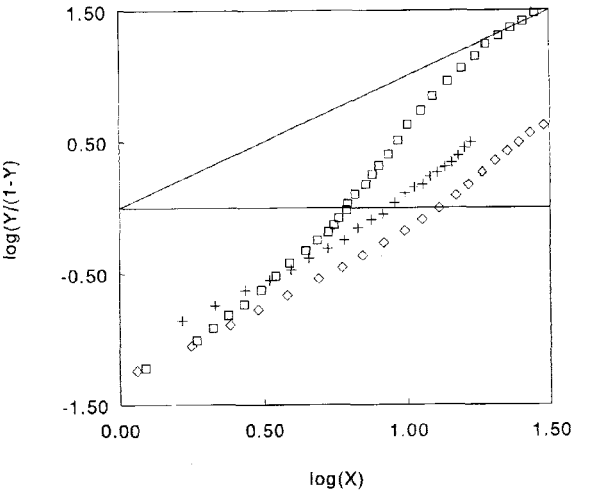

Fig. 2. Oxygen binding curves of homohexamer *a*: □, no antibody present, +, with IgG-D; ◇, with F_{ab}-D. The slope of the drawn line indicates non-cooperativity.

Monomeric subunits occur predominantly in a low-affinity substate. IgG-D binds to a such a low-affinity substate, while IgG-E binds a high-affinity substate and IgG-J an intermediate one. In addition, our results reveal another important observation: binding of antibodies influences the oxygen affinity although the epitopes of the antibodies are located on domains different from where the oxygen binding site is located, which indicates a rather large flexibility of interdomain contacts. This contrasts with the observation based on a comparison of crystal structures of the oxy and deoxy forms of the homohexamer of the related hemocyanin of subunit II from *Limulus polyphemus* [6,7], that there is a rigid connection between domains 2 and 3 which rotates as a unit by 8° with respect to domain 1 upon oxygenation.

3.2. Influence of antibody binding on the homohexamer (subunit a)

The influence of monoclonal antibodies on the reassembled homohexamer built up by subunit *a* was studied at pH 9.0, in the presence of 10 mM $CaCl_2$ in order to guarantee the hexameric state.

Firstly, we compared the influence of IgG and F_{ab} fragments of clone D on the oxygen binding curve of homohexamer *a* (Fig. 2). The curves are very similar and indicate that IgG-D and F_{ab}-D freeze the homohexamer in the low-affinity state and abolish almost completely the cooperativity. As binding of IgG to one subunit may partially cover the other epitopes on the connected subunits, due to the size of the IgG molecules, we have continued binding studies to the hexamer only with F_{ab} fragments. Although F_{ab} fragments bind more weakly than intact IgG molecules, the used molar excesses and the affinity constants of the monoclonal antibodies [10] support our assumption that all six subunits in the hexameric hemocyanin molecules had bound a F_{ab} fragment under the applied conditions.

Fragments F_{ab}-D, F_{ab}-J and F_{ab}-E have a strong but different influence on the oxygen binding curves (Fig. 3). While F_{ab}-E freezes a high-affinity state, F_{ab}-J freezes a conformation between the high- and low-affinity state, and F_{ab}-D freezes the T-state. The values for p_{50} were determined to 3.8 Torr, 5.5 Torr, and 13 Torr, respectively. In all cases,

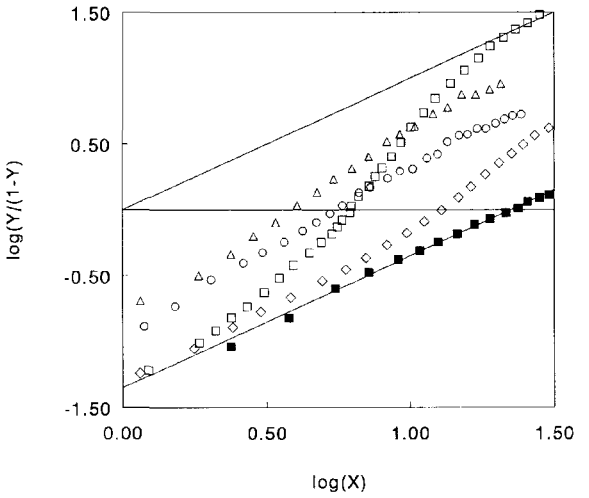

Fig. 3. Influence of F_{ab} fragments on the oxygen binding of reassociated hexamers of subunit *a* of *Panulirus interruptus* hemocyanin. □, no antibody present; ◇, with F_{ab}-D; △, with F_{ab}-E; ○, with F_{ab}-J; ■, monomeric subunit *a* (no antibody present). The slope of the drawn line indicates non-cooperativity.

F_{ab} fragments abolish the cooperativity almost completely. In no case was negative cooperativity detected. This indicates that not only all the hexamers but also all the subunits within the homohexamers bind oxygen in the same way.

The results obtained for homohexamers were consistent with those obtained for the subunits. While IgG-D binds to a low-affinity T-state, IgG-E binds to a high-affinity R-state, and IgG-J to one in between. The antibodies had been elicited in mice by injection with hexameric hemocyanin. Evidently antibodies against both the R-state and the T-state, and states in between had been selected for. As the hexameric form is required for the high affinity R-state, we assume that antibodies E and J had been elicited by the hexameric protein. But antibody D may also have been formed against the low-affinity T-state.

These data can be interpreted in the following way. Each subunit has the potential to adapt several conformations, characterized by their particular binding affinity [17,18]. In the case of complete cooperativity there are at least two conformations, the low-affinity and high-affinity states, designed as T- and R-states. These conformations are stabilized by association with other subunits, allosteric effectors, but also by other proteins such as monoclonal antibodies. The binding of antibodies or F_{ab} fragments stabilizes the subunits in the hexamer in one of these states.

Binding of antibodies to oligomeric hemocyanins seems to stabilize conformations which are already present at the level of subunits rather than inducing new conformations. The oxygen binding behavior of monomeric subunit *a* is very similar to that of the T-state. This has also been observed earlier by others [14–16]. The oxygen binding curves of subunits and hexamers after incubation with antibodies or F_{ab} fragments show comparable shifts to higher affinity independent of the aggregation state, but dependent on the antibody clone. The influence of the association of subunits on the oxygen binding behavior of assembled hexamers may be understood in the following way: each subunit has the potential to adapt several conformations, characterized by particular oxygen binding affinities [17,18]. These conformations are stabilized either by association with other subunits to establish the oxygenated R-state or by other proteins such as antibodies to establish substates between the T- and R-states. The association of the subunits still allows the subunits themselves within the hexamers to adopt conformations between the low-affinity T- and high-affinity R-states. However, due to the association and the tight contact between the subunits, all subunits are forced to adopt the same substate; the association hinders the formation of a mixture of different substates on the level of subunits. Thus the binding of antibodies still stabilizes the subunits in the hexamer in one of these states rather than inducing new conformations. This obviously occurs when homohexamers *a* are incubated with various F_{ab} fragments. The results obtained for homohexamers are consistent with those obtained for the subunits. While IgG-D binds to a low-affinity T-like state, the IgG-E binds to a high-affinity R-like state and IgG-J binds to a state in between.

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