Oxygen Binding and Subunit Interactions in *Helix pomatia* Hemocyanin†

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ABSTRACT: Hemocyanin of *Helix pomatia* contains approximately 180 oxygen binding sites, and can be dissociated into ten equal subunits. We have compared the oxygen binding properties of (a) undissociated molecules, containing only native subunits; (b) undissociated, hybrid molecules, which contain native subunits as well as inactivated subunits, which were unable to bind oxygen; and (c) native protein, dissociated into subunits. The results indicate that the interactions between native subunits are essential for cooperative oxygen binding. It is concluded that the cooperative oxygen binding unit is larger than one subunit. Possibly hemocyanin is a model for other extended, highly organized, biological systems.

Hemocyanin of the Roman snail *Helix pomatia* is a very large respiratory protein, with a mol wt of 9×10^6 at neutral pH. Hemocyanin can be dissociated into subunits. From the copper content (Konings *et al.*, 1969b) it can be calculated that hemocyanin contains approximately 180 oxygen binding sites. Under certain conditions these binding sites interact with each other, resulting in cooperative oxygen binding.

It is conceivable that oxygen binding sites in the same subunit interact with each other, whereas sites in different subunits do not interact. In that case both the undissociated and the dissociated protein should bind oxygen cooperatively. It is also possible that all 180 oxygen binding sites interact with each other, so that dissociation will result in loss of cooperativity of oxygen binding. Wyman (1969) described possible allosteric effects in such highly extended biological systems.

Hemocyanin can be dissociated into subunits of a tenth of the molecular weight of the undissociated protein (Konings *et al.*, 1969a). Each of these one-tenth subunits contains approximately 18 oxygen binding sites. This study compares the oxygen binding properties of free one-tenth subunits and one-tenth subunits incorporated in the undissociated molecule. It was found that the cooperative oxygen binding unit is larger than a one-tenth subunit and is probably the half or whole molecule, containing 90 and 180 oxygen binding sites, respectively. Interactions between one-tenth molecule subunits appear to be essential for cooperative oxygen binding.

Materials and Methods

α-Hemocyanin from the snail *Helix pomatia* was isolated and stored as described previously (Siezen and Van Driel, 1973). Immediately before use the lyophilized protein was dissolved in Tris-HCl buffer, pH 7.0, and dialyzed for 24 hr against several changes of the same buffer. Subsequently, the protein was incubated at 20°, overnight, in the presence of a tenfold molar excess of hydroxylammonium chloride, relative to protein–copper, to possibly regenerate aged protein (Konings *et al.*, 1969b). Hydroxylammonium chloride was removed by dialysis against the appropriate buffer. Buffers were prepared as described previously (Siezen and Van Driel, 1973) and were made up to ionic strength 0.1 with NaCl. The Tris concentration was 50 mM. Dialysis was carried out at 20°.

Determination of hemocyanin concentration, analytical ultracentrifugation, and determination of oxygen binding curves were performed as previously described (Konings *et al.*, 1969b). Separation of whole and one-tenth subunit molecules was carried out by differential centrifugation as described before (Siezen and Van Driel, 1973). Apohemocyanin was prepared by dialysis of a protein solution against Tris-HCl buffer, pH 8.2, containing 10 mM $CaCl_2$ and 10 mM KCN, for 48 hr at room temperature, to remove copper from the protein. Subsequently the apoprotein solution was extensively dialyzed against Tris-HCl buffer, pH 7.0.

Hybrid molecules were prepared by mixing undissociated native protein and apoprotein in the desired ratio, in Tris-HCl buffer, pH 7.0, at 20°. The total protein concentration was 4 mg/ml. With a small amount of 2 N NaOH the pH was brought to 8.3, resulting in a rapid dissociation into one-tenth subunits of the native and apoproteins. Within 1 min the pH was readjusted to 7.0 with 2 N HCl. After 48 hr at 20°, approximately 60% of the protein was reassociated. The reassociated molecules were separated from the remaining one-tenth subunit molecules by differential centrifugation. The unreassociated protein was discarded. After dialysis of the reassociated protein against Tris-HCl buffer, pH 8.2, containing 10 mM $CaCl_2$, oxygen binding curves were determined. This $CaCl_2$ concentration was used to prevent dissociation of the reassociated molecules.

Results

Oxygen Binding Properties of Whole and One-Tenth Subunit Molecules. In Tris-HCl buffer of pH 8.2, containing 2 mM $CaCl_2$, ~20% of the protein was dissociated into one-tenth subunits. After separation, we obtained solutions, at the same pH and $CaCl_2$ concentration, of only whole and only one-tenth subunit molecules. These preparations were stable (Siezen and Van Driel, 1973). Figure 1 shows the oxygen binding curves of whole and one-tenth subunit molecules. The binding curves are presented as Hill plots: $\log (Y/1 - Y)$ *vs.* $\log p(O_2)$; Y is the fraction of the binding sites occupied by

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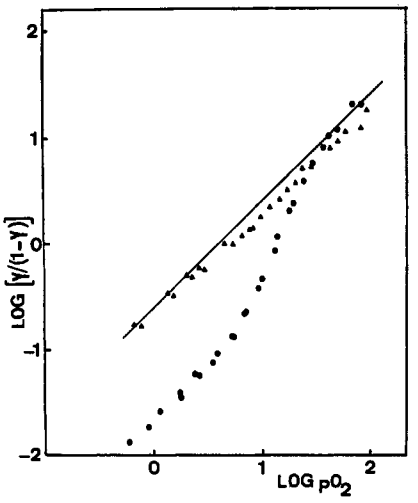

FIGURE 1: Hill plot of the oxygen binding curve in Tris-HCl buffer, pH 8.2, containing 2 mM $CaCl_2$, at 20°: (●) undissociated molecules; (▲) one-tenth subunit molecules. The line represents the oxygen binding curve expected for the oxy state of the protein; $p(O_2)$, oxygen pressure (mm); Y, fractional saturation of hemocyanin with oxygen.

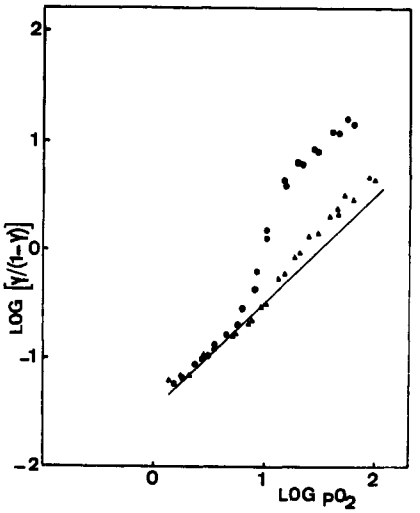

FIGURE 2: Hill plot of the oxygen binding curve in Tris-HCl buffer, pH 8.2, containing 10 mM $CaCl_2$, at 20°: (●) reassociated, 100% native protein; (▲) hybrid of 10% native and 90% apoprotein. The line represents the oxygen binding curve expected for the deoxy state of the protein; $p(O_2)$, oxygen pressure (mm); Y, fractional saturation of hemocyanin with oxygen.

oxygen; $p(O_2)$ is the partial oxygen pressure. The whole molecules bound oxygen cooperatively, whereas the one-tenth subunit molecules bound oxygen noncooperatively. In fact, the Hill coefficient of the one-tenth subunits was 0.9, suggesting some heterogeneity in the binding sites. When separation was carried out after dissociation of 80% of the protein into one-tenth subunits, the same binding curves were obtained. This indicates that the microheterogeneity in association–dissociation behavior, as described by Siezen and Van Driel (1973), is not reflected in the oxygen binding properties. As is theoretically expected (Wyman, 1968) and was found for *Levantina* hemocyanin (Er-el *et al.*, 1972), the slope of the Hill plot of the cooperative whole molecules approaches unity asymptotically, at values of Y close to 0 and 1, respectively. The asymptotes represent the hypothetical oxygen binding curves for the deoxy and oxy states of the protein, respectively (Er-el *et al.*, 1972).

Oxygen Binding Properties of Hybrid Molecules. Oxygen binding properties of mixtures of undissociated native and apoproteins did not differ from those of native protein alone. However, if the mixture was dissociated into one-tenth subunit molecules and subsequently reassociated, the oxygen binding properties differed considerably. As the ratio of native to apoproteins decreased, the oxygen binding properties of the reassociated protein differed increasingly from the oxygen binding properties of the native protein, indicating that hybrid molecules were formed. The results are shown in Figures 2 and 3. If the relative amount of native protein was low, the Hill coefficient approached unity, indicating that the cooperativety of oxygen binding vanishes (Figure 3A). The oxygen pressure at which 50% of the binding sites are occupied, p_{50}, increases (Figure 3B). The oxygen binding properties of hybrid molecules, containing an excess of apo one-tenth subunit molecules, approach those of the deoxy state of the native protein. Figure 3C shows the oxygen binding capacity (ΔE_{346} per milligram of protein) (Konings *et al.*, 1969b) of reassociated, hybrid molecules, after separating them from the nonreassociated one-tenth subunit molecules, as a function of the percentage of native protein in the mixture prior to hybridization. This yields an approximate straight line, indicating that the incorporation velocity of native and apo one-tenth subunit molecules is the same. It is important to note that the oxygen binding properties of the fully native, reassociated protein, as shown in Figures 2 and 3, are very similar to those of the native protein that had not undergone this treatment. There is no difference in shape between native and hybrid whole molecules as judged by analytical ultracentrifugation and electron microscopy.

Discussion

Figure 1 demonstrates that under identical conditions undissociated molecules can bind oxygen cooperatively, whereas

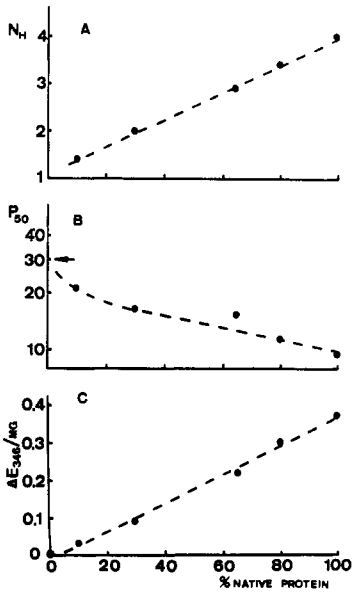

FIGURE 3: Relation between the relative amount of native protein in the hybridization mixture, and (A) the Hill coefficient, n_H; (B) the oxygen pressure at 50% saturation, p_{50} (mm) on a logarithmic scale; (C) the oxygen binding capacity, ΔE_{346}, per mg of protein. The arrow in part B indicates the p_{50}, expected for the deoxy state of the native protein. Experimental conditions are as in Figure 2.

one-tenth subunit molecules bind noncooperatively. The oxygen binding properties of the one-tenth subunits, free in solution, are similar to those of the oxy state of the undissociated molecule. The same was found for undissociated and dissociated *Levantina* hemocyanin in the presence of 20 mM $CaCl_2$ and in the absence of $CaCl_2$, respectively.

This result does not prove that the cooperative oxygen binding unit is larger than a one-tenth subunit. It is quite possible that, upon dissociation, the structure of the subunit changes, resulting in a loss of cooperative interaction between the 18 oxygen binding sites, within the subunit. To test this hypothesis, the oxygen binding properties of a one-tenth subunit, incorporated into an undissociated, whole molecule, have been studied.

Upon reassociation of a mixture of native one-tenth and inactive, apo one-tenth subunits, hybrid whole molecules were formed, containing both native and apo one-tenth subunits. This can be inferred from the observation that the oxygen binding properties depend on the composition of the mixture, *i.e.*, the proportion of native and apo subunits in the reassociated molecule. In contrast to this, mixtures of whole native and whole apomolecules give the same oxygen binding curves as native protein alone. The conclusion from Figure 3C, that the incorporation velocity of native and apo one-tenth subunit molecules is the same, suggests that the incorporation of native and apo one-tenth subunits is random. At a low relative amount of native protein, most hybrid molecules will contain a minimum number of native subunits. Most native subunits will then be surrounded by apo subunits. Figures 3A and 3B show that for a large excess of apo one-tenth subunits, the Hill coefficient approaches unity and the p_{50} value increases. The oxygen binding properties approach those of the deoxy state of the native protein, as indicated in Figure 2.

These results indicate that the cooperative oxygen binding unit, in the native, undissociated molecule, is larger than a one-tenth subunit. This is striking, since a one-tenth subunit contains not less than 18 oxygen binding sites. The symmetry of the hemocyanin molecule, determined from electron microscopic observations (Van Bruggen *et al.*, 1962; Mellema and Klug, 1972), suggests that the cooperative entity is as large as a half-molecule or comprises the whole molecule, with 90 and 180 oxygen binding sites, respectively.

It can be suggested that the low oxygen affinity of the native, undissociated, deoxy protein is induced by tension between the one-tenth subunits. Upon binding of oxygen or dissociation into one-tenth subunit molecules, this tension is relaxed, resulting in a high oxygen affinity. This is similar to the hemoglobin system, although the interacting structures are much larger in hemocyanin. It is remarkable that the oxygen binding sites in a hybrid molecule, containing an excess of apo one-tenth subunits, are fixed in the low-affinity, deoxy-like state. Obviously the transition to the high-affinity oxy state is closely linked to a structural change of the one-tenth subunit molecules in the undissociated, native protein. This structural change is inhibited by the interactions with several apo one-tenth subunit molecules. It would be very interesting to know to what extent the transition from deoxy to oxy structure of one subunit induces changes in the structure of neighboring one-tenth subunits, in the native protein.

DePhillips *et al.* (1970) found that subunit interactions in *Busycon* hemocyanin depend on the binding of oxygen: upon deoxygenation this hemocyanin reversibly dissociates into half-molecules. The oxygen binding properties of *Helix pomatia* hemocyanin agree quite well with those of *Levantina* hemocyanin, as determined by Er-el *et al.* (1972). This suggests that the conclusions reached in this paper are valid for other hemocyanins too.

In a very recent paper on another large respiratory protein, erythrocruorin, Wiechelman and Parkhurst (1972) concluded that the undissociated protein can bind oxygen cooperatively, whereas the one-twelfth molecule, containing 16 heme groups, binds noncooperatively. This makes it plausible that the conclusions drawn for these large hemocyanins can be extended to other large, highly organized biological systems.

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