Cooperative Transition in the Conformation of 24-Mer Tarantula Hemocyanin upon Oxygen Binding*

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Hemocyanins are large respiratory proteins of arthropods and mollusks, which bind oxygen with very high cooperativity. Here, we investigated the relationship between oxygen binding and structural changes of the 24-mer tarantula hemocyanin. Oxygen binding of the hemocyanin was detected following the fluorescence intensity of the intrinsic tryptophans. Under the same conditions, structural changes were monitored by the non-covalently bound fluorescence probe Prodan (6-propionyl-2-(dimethylamino)-naphthalene), which is very sensitive to its surroundings. Upon oxygen binding of the hemocyanin a red shift of 5 nm in the emission maximum of the label was observed. A comparison of oxygen binding curves recorded with tryptophan and Prodan emission revealed that structural changes in tarantula hemocyanin lag behind oxygen binding at the beginning of oxygenation. Analyses based on the nested two-state model, which describes cooperative oxygen binding of hemocyanins, indicated that the transition monitored by Prodan emission is closely related to one of the four conformations (rR) predicted for the allosteric unit. Earlier, the allosteric unit of tarantula hemocyanin was found to be the 12-mer half-molecule. Here, fluorescence titration revealed that the number of Prodan binding sites/24-mer tarantula hemocyanin is ~2, matching the number of allosteric units/hemocyanin. Based on the agreement between oxygen binding curves and fluorescence titration we concluded that Prodan monitors a conformational transition of the allosteric unit.

Hemocyanins are respiratory proteins of numerous arthropods and mollusks occurring freely dissolved in the hemolymph (1–3). The hemocyanins of both phyla have almost identical active sites but completely different tertiary and quaternary structures. The basic structural element of arthropod hemocyanins is a hexamer. Simple hexamers as well as aggregates (2 × 6, 4 × 6, 6 × 6, and 8 × 6) are found (1). Each subunit has a molar mass of ~72 kg/mol and carries one oxygen-binding site (4, 5). The active site of hemocyanins consists of two copper ions ligated by three histidine residues of the polypeptide chain each. Oxygen is reversibly bound between the copper ions as a peroxide in a side on μ-η¹:η² coordination (6–9).

Because of their complex and hierarchic structure, arthropod hemocyanins reach a remarkable cooperativity in their oxygen binding behavior (10). Cooperative oxygen binding of hexameric hemocyanins could be described by the two-state MWC model (11, 12). This is not the case for arthropod hemocyanins larger than hexamers. Among various proposed models, the nested MWC-model seems to describe the cooperative and allosteric ligand binding behavior of arthropod hemocyanins best (13, 14). The nesting model assigns hierarchical allosteric equilibria to hierarchies in structure (15, 16). In the case of the 24-mer tarantula hemocyanin (Eurypeltma californicum), two hexameric units form dodecameric half-molecules, which represent the smallest structurally identical unit of the hemocyanin. The latter are thought to behave as allosteric units according to the MWC model, which occur as r or t-state (13, 14, 16). Two of these allosteric units dimerize isologously to form the native 24-mer tarantula hemocyanin, which can also adopt two states (R and T). Thus, each allosteric unit can adopt one of the four conformations (rR, tR, rT, tT) respecting the allosteric equilibria L = [T]/[R], lR = [tR]/[rR], and lT = [tT]/[rT]. This concept of the nested allostery was successfully applied to various 2 × 6-mer and 4 × 6-mer hemocyanins from crustacean and cheliceraes (13–18), as well as for extracellular hemoglobin (19) and GroEl (20). However, the conformational predictions have not been investigated so far.

In this study we inspected structural changes of tarantula hemocyanin upon oxygen binding. We chose tarantula hemocyanin, because it develops strongly cooperative oxygen binding with Hill coefficients of ns ≈ 6–11, the highest observed for any biomolecule, and its ligand binding behavior is thoroughly analyzed (10, 13, 14, 16, 17). The fluorescence probe Prodan (6-propionyl-2-(dimethylamino)-naphthalene) was used to monitor the conformational change of the hemocyanin. Oxygen binding was measured by the quenching of the intrinsic tryptophan fluorescence (10, 21). The comparison of both signals obtained on the same hemocyanin sample revealed that the structural transition lags behind oxygen binding. The conformational transition follows the occurrence of the rR conformation when analyzed with the nesting model and, therefore, represents a transition of the allosteric unit.

EXPERIMENTAL PROCEDURES

Materials

Hemocyanin of tarantula E. californicum consists of four hexamers and has a molar mass of 1.72 × 10⁶ g/mol (23, 24). Seven different types of

1 The abbreviations used are: MWC, Monod-Wyman-Changeux; Prodan, 6-propionyl-2-(dimethylamino)-naphthalene.
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polypeptide chains (a–g) have been found and sequenced in *Eurypelta* hemocyanin (24). Each of the two hexamers within 1 half-molecule contains the subunits a, d–g, and either b or c (23). Tarantulas were purchased from Carolina Biological Supply. Hemocyanin extraction, purification, and deoxygenation has been described previously (21). The hemocyanin was stored in buffer (100 mM Tris, pH 7.8, 5 mM CaCl$_2$, 5 mM MgCl$_2$) at 4 °C. Protein concentration was calculated from the optical density; an absorption of 1.1 at 278 nm corresponds to a concentration of 1 mg/ml *Eurypelta* hemocyanin (10).

Prodan was purchased from Molecular Probes (Leiden, NL). Approximately 0.5 mg of the dye were dissolved in 600 µl of Me$_2$SO (dimethyl sulfoxide) and diluted 1:500 in water. Prodan concentration was determined by using the molar extinction coefficient of $\epsilon_{290} = 14,500$ M$^{-1}$ cm$^{-1}$ for watery solutions at 360 nm (25). To label the protein with the fluorescence probe, Prodan solution was added to hemocyanin in buffer and incubated for 1 h at 4 °C.

**Absorption and Fluorescence Spectroscopy**

Absorption was measured in a semimicrocuvette (fused silica) with a Cary 1E UV-visible spectrophotometer, and the fluorescence was measured with a Spex Fluorolog 2 spectrofluorometer. All fluorescence spectra were corrected both for excitation and emission. The sample temperature was adjusted to 20 °C. Static fluorescence anisotropy was recorded by using two polarizers in vertical (v) and horizontal (h) position, respectively. Prodan was excited at 370 nm, and its fluorescence emission ($F$) was measured at 490 nm. Anisotropy $A$ was calculated by

$$ A = \frac{F_{hv} - F_{vh}}{F_{hv} + 2F_{vh}} $$

(Eq. 1)

where $G = F_{vh}/F_{hv}$ is the grid factor (26). Prodan concentrations of 3 µM and hemocyanin concentrations of 2.3 µM (24-mer, corresponding to 4 mg/ml) have been used.

**Fluorescence Titration**

The binding of Prodan to hemocyanin was investigated by fluorescence titration and analyzed according to Mas and Colman (27). The ligand $L$ binds to the protein $P$ with a dissociation constant $K$. The binding of the dye is connected with an increase of its fluorescence intensity. The fluorescence intensity of the ligand in absence and presence of the protein is $F_0$ and $F$, respectively. The maximal increase in fluorescence intensity upon complete binding is represented by the enhancement factor $Q$. A linearized form describing the measured fluorescence intensities in dependence on the protein concentration is,

$$ \frac{1}{F} = \frac{1}{Q} \frac{[L]_\text{total}}{F_0} + \frac{1}{Q} $$

(Eq. 2)

and at high protein concentrations, $[L]_\text{free}$ can be replaced by $[L]_\text{total}$, which is justified in our case (30 nM dye and up to 10.5 µM protein, 24-mer). Based on the determined parameters K and Q, the concentration of bound ligand could be calculated according to

$$ [L]_\text{bound} = \frac{[L]_\text{total} - [L]_\text{free}}{Q} $$

(Eq. 3)

and the number of binding sites/protein, $n$, was computed with a converted form of the Scatchard equation

$$ n = \frac{[L]_\text{bound}}{[P]_\text{total}} (1 + \frac{K}{[L]_\text{free}}) $$

(Eq. 4)

where $[L]_\text{free} = [L]_\text{total} - [L]_\text{bound}$ is the concentration of unbound ligand.

**Oxygen Binding Curves**

**Measurements**—The setup for detection of oxygen binding curves based on a modified SPEX Fluorolog II fluorometer (Jobin Yvon, France). It enabled simultaneous measurement of fluorescence and oxygen content of the sample. Via a flow meter, a humidifier, and a syringe three types of gas could be streamed into the measuring cuvette: 100% O$_2$, 5% O$_2$, and 100% N$_2$. A miniature paddle below the level of excitation light stirred the sample to guarantee optimal gas exchange. Cuvette and gas humidifier were held at a temperature of (20.0 ± 0.1) °C. A homemade Clark electrode measured the oxygen content of the sample and was calibrated with the values detected at 5% O$_2$ and 100% N$_2$. The sample volume was 700 µl, and the concentrations of hemocyanin and Prodan for these measurements were 3.5 µM (24-mer, corresponding to 6 mg/ml) and 1 µM, respectively. The emission was recorded in front face mode to avoid the inner filter effect (28).

Continuous movement of the Prodan emission maximum has been observed by successive recording of emission spectra between 450 and 500 nm excited at 390 nm from the beginning to the end of the deoxygenation. Oxygen binding of the hemocyanin was recorded by the fluorometric-polarographic method (10, 21); tryptophan was excited at a wavelength of 280 nm, and emission was measured at 340 nm.

**Analysis of Structural Changes**—For precise determination of the emission maxima of bound Prodan at different deoxygenation levels the spectra were fitted with a two-state model. First, the spectrum of free Prodan in watery solution was described in a fit with one gaussian curve plus a constant background. Second, spectra of completely oxygenated (A) and deoxygenated (B) hemocyanin were chosen. These spectra could be assumed to be composed solely of the emission of Prodan bound to one of the two states and of free Prodan. Thus, each of the two spectra (A and B) could be described as the sum of two gaussian curves (bound and free Prodan) and a variable background. For fitting these spectra, width and emission maximum of free Prodan were taken over from the previous fit. Finally, the rest of the spectra could be fitted as the superposition of various fractions of the two extreme spectra A and B. The recording time of each spectrum (~1 min) was small compared with the total recording time of the oxygen binding curve (range of 1 h). Therefore, each spectrum could be assigned to an oxygen content of the sample during the deoxygenation process.

**Analysis of Bound Oxygen According to the Nesting Model**—Oxygen binding curves based on the intrinsic tryptophan fluorescence were fitted according to the nesting model to deduce the relative fractions of the four predicted conformations $rR$, $rT$, $tR$, and $tT$ at every oxygen partial pressure. The binding polynomial $P$ of a nested molecule, describing the sum of the concentrations of all molecular states relative to a reference state, is given by (14, 16, 28)

$$ P = 1 + L \left( P_R^0 + LP_R^1 \right) $$

(Eq. 5)

where $N$ is the number of allosteric units, $P_R$ and $P_T$ are the binding polynomials of the two upper level states:

$$ P_R = 1 + \left( P_R^0 + 1P_R^1 \right) $$

(Eq. 6)

$$ P_T = 1 + \left( P_T^0 + 1P_T^1 \right) $$

(Eq. 7)

Here, $n$ is the number of binding sites within one allosteric unit and $P_{xy}$ are the binding polynomials for one binding site of the four lower level states,

$$ P_{xy} = 1 + k_{xy}[O_2] $$

with the oxygen binding constants $k_{xy}$. The equilibrium constants, one for the upper level and two for the lower level, are

$$ L = \frac{[T]\} + \frac{[R]}{[R]}}{[L]} $$

(Eq. 8)

Based on the binding polynomials one obtains the fractional saturation $\Theta$ according to Ref. 28,

$$ \Theta = \frac{\frac{\partial \ln P}{\partial [O_2]} Nn}{\frac{\partial \ln P}{\partial [O_2]} Nn P \frac{\partial [O_2]}{\partial [O_2]}} $$

(Eq. 9)

For tarantula hemocyanin, the values of the oxygen binding constants $k_{xy}$ were 2 Torr$^{-1}$, $k_{xy} = 0.05$ Torr$^{-1}$, $k_{xy} = 3.5$ Torr$^{-1}$, $k_{xy} = 0.012$ Torr$^{-1}$, as well as number and size of the allosteric units, $N = 2$ and $n = 12$, were taken from literature (16). The fit of $\Theta$ to the measured oxygen binding curves yielded the three equilibrium constants $L$, $l_R$, and $l_T$. The relative fractions $f_{xy}$ of the four conformations $xy$ were calculated by considering the terms in the binding polynomial $P$, which contain the binding polynomial of the certain configuration $P_{xy}$ normalized by $P$, as follows,
Hemocyanin concentration was varied in the range from 0 up to 10.5 μM (24-mer, corresponding to 5 mg/ml), and the Prodan concentration was 8 μM.

\[
    f_{\text{rel}} = \frac{1}{1 + L (1 + k_0 \alpha)} \left( l_0 P_{\text{rel}} + l_0 P_{\text{rel}} P_{\text{bound}} \right) \frac{1}{P}
\]

\[
    f_{\text{em}} = \frac{1}{1 + L (1 + k_0 \alpha)} \left( l_0 P_{\text{rel}} + l_0 P_{\text{rel}} P_{\text{bound}} \right) \frac{1}{P}
\]

\[
    f_{\text{ex}} = \frac{L}{1 + L (1 + k_0 \alpha)} \left( l_0 P_{\text{rel}} + l_0 P_{\text{rel}} P_{\text{bound}} \right) \frac{1}{P}
\]

\[
    f_{\text{ex}} = \frac{L}{1 + L (1 + k_0 \alpha)} \left( l_0 P_{\text{rel}} + l_0 P_{\text{rel}} P_{\text{bound}} \right) \frac{1}{P}
\]

where the sum of the four relative fractions is 1.

RESULTS

Fluorescence-spectroscopic Characterization

Fluorescence spectra of Prodan freely dissolved in watery solution and bound to tarantula hemocyanin have been detected, and the normalized emission spectra are shown in Fig. 1. Fluorescence of the free dye showed peak intensities for excitation and emission at wavelengths of 365 and 520 nm, respectively. The binding of the probe to oxygenated hemocyanin resulted in very strong shifts of the excitation and emission maxima to 391 and 476 nm, respectively. The binding was accompanied by an increase in the fluorescence quantum yield by a factor of 6. A further blue shift in the fluorescence maxima occurred when the Prodan-labeled hemocyanin became completely deoxygenated. The excitation and emission maxima moved to 385 and 471 nm, respectively, and a further increase of the fluorescence quantum yield by a factor of 1.4 could be observed.

Static fluorescence anisotropy measured with Prodan freely dissolved in solution showed a low anisotropy of A = 0.04, which did not change upon deoxygenation. The binding of Prodan to oxygenated hemocyanin results in a strong increase of anisotropy (A = 0.33), but again no difference was found between oxygenated and deoxygenated hemocyanin.

The binding of Prodan to oxygenated tarantula hemocyanin was further characterized by titration experiments. The final Prodan concentration was kept constant at 30 nM, and the final hemocyanin concentration was varied in the range from 0 up to 10.5 μM (24-mer, 0–18 mg/ml). The increase of Prodan fluorescence intensity was used to monitor the binding of the dye. The analysis performed with Equation 2 delivered a fluorescence enhancement factor of Q = 22.2 and a dissociation constant of K = 2.4 μM (Fig. 2a). The number of binding sites (n) could not be determined by using plots according to Scatchard, Eadie-Hofstee, or Lineweaver-Burk. This is most likely because of the effect that no complete ligand binding could be achieved under the conditions described above (see below). However, a direct calculation of n for each data point with Equation 4 and subsequent averaging yielded a value of n = 1.85 ± 0.12 binding sites/24-mer (Fig. 2b).

Oxygen Binding Curves

Prodan emission spectra and oxygen partial pressure were simultaneously measured. The precise position of the emission maxima in each of the successive spectra was analyzed by a fitting procedure based on a two-state model. The model took into account the presence of unbound Prodan ensuring that the calculated emission maxima represent exclusively the shift of the bound dye. The graph of the normalized Prodan emission maxima in dependence on the oxygen partial pressure (Fig. 3, dots) exhibits a sigmoid form. Around 8 Torr the emission maxima increased but slowed down after the turning point at ~13.3 Torr. Around 20 Torr and above no significant shift could be observed. The graph clearly displays a transition between two distinct states. An alternative analysis of the emission spectra was tested, assuming a stepless shift in the Prodan emission maxima. The resulting oxygen binding curves were similar (turning point also at 13.3 Torr) but showed more noise. Therefore, analysis based on the two-step model was used for further investigations.

The amount of bound oxygen was measured under the same conditions with the same sample via the quenching of the intrinsic tryptophan fluorescence. Fig. 3 (straight line) shows the normalized fractional oxygen saturation versus the oxygen partial pressure. The graph has a sigmoid form with typical values for the oxygen partial pressure at half-saturation of p_50 = 12 Torr and the Hill coefficient of n_Hill = 5.7. No changes of the oxygen binding behavior using the tryptophan signal were found when Prodan was present or absent.

Oxygen binding curves (tryptophan signal) like those plotted in Fig. 3 (straight line) were analyzed according to the MWC and nesting model. Although lower systematic deviations occurred with the MWC model, the experimental curves could be fitted neatly with Equations 5–9 yielding typical values for the equilibrium constants of log(L) = 2.67, log(l_P) = 14.91, and

FIG. 1. Fluorescence emission spectra of Prodan. Fluorescence emission (F) of Prodan freely dissolved (dashed line), bound to oxygenated hemocyanin (solid line), and bound to deoxygenated hemocyanin (dotted line) was excited at λ_ex = 390 nm. The spectra were normalized with respect to their peak intensity. Measurements were performed in 100 mM Tris buffer, pH 7.8, containing 5 mM CaCl_2, 5 mM MgCl_2, at 20 °C. Hemocyanin concentration was 2.9 μM (24-mer, corresponding to 5 mg/ml), and the Prodan concentration was 8 μM.

FIG. 2. Fluorescence titration of hemocyanin and Prodan. The Prodan concentration was kept constant at c = 30 nM in Tris buffer (pH 7.8). Total hemocyanin concentration (24-mer) was stepwise reduced from 10.5 down to 0.3 μM. Prodan fluorescence was excited at a wavelength of λ_ex = 390 nm and detected at λ_em = 500 nm. a, double-reciprocal plot of the titration data according to Equation 2. The linear fit yields a fluorescence enhancement factor of Q = 22.2 and a binding constant of K = 2.4 μM. b, number of binding sites, n, computed with Equation 4. The average of all values is n = 1.85 ± 0.12 and drawn as a dotted horizontal line.
DISCUSSION

The hemocyanin of tarantula *E. californicum* shows highly cooperative oxygen binding, possess 24 oxygen-binding sites, and is able to adopt different conformations (10, 30, 31). However, transitions between these conformations are scarcely investigated so far. There is one study showing proton release and uptake upon oxygen binding (32). In our study, the fluorescence probe Prodan served as a monitor for structural changes of the 24-mer tarantula hemocyanin upon oxygen binding. Prodan possesses a large dipole moment making it highly sensitive to the polarity of its surroundings (25). Prodan was shown to bind non-covalently to hydrophobic sites in proteins and has been used as a monitor in structural investigations of many proteins (33–36).

**Fluorescence-spectroscopic Characterization**

As has been observed for other proteins, the binding of Prodan to oxygenated tarantula hemocyanin was connected with a blue shift in the emission maximum of the dye, a red shift in the excitation maximum, a strong increase in the fluorescence quantum yield, and a strong increase in the fluorescence quantum yield. The fluorescence titration revealed that Prodan binds to tarantula hemocyanin with a dissociation constant of $K = 2.4 \, \mu M$ and a stoichiometry of $n = 1.85 \pm 0.12$ molecules/24-mer. The number of binding sites $n$ is close to 2, thus, each 24-mer tarantula hemocyanin seems to possess two Prodan-binding sites. Therefore, it is very likely that Prodan interacts with the two structurally identical 12-mer half-molecules. The binding of Prodan to tarantula hemocyanin is connected with an increase in its fluorescence intensity. The fluorescence enhancement factor obtained by fluorescence titration ($Q = 22.2$) is obviously much larger than that reported in connection with the spectra shown in Fig. 1 (factor 6). Although the analysis of fluorescence titration extrapolates for infinite protein concentrations, in practice, complete dye binding could not be achieved even at higher protein concentrations. Therefore, free Prodan is always present and has to be taken into account during further analysis, in particular for the oxygen binding curves.

When hemocyanin becomes deoxygenated, the fluorescence emission of bound Prodan revealed further changes. A 5-nm blue shift of the emission maximum, a 6-nm blue shift of the excitation maximum, and an increase in fluorescence quantum yield by a factor of 1.4 could be observed. The emission blue shift indicates that the surrounding Prodan becomes even more hydrophobic upon deoxygenation. The increase in fluorescence quantum yield is most likely because of the absence of a quenching process in deoxygenated hemocyanin, which recently has been discussed in detail (37). One may think that the emission blue shift and the increase of the fluorescence quantum yield could also be caused by a heightened binding of free Prodan to the hemocyanin, because the trend is the same as for Prodan binding to oxygenated hemocyanin. However, the blue shift of the excitation maximum is in the opposite direction and disproves this possibility.

Prodan fluorescence emission does not show any changes upon deoxygenation in the absence of hemocyanin. Therefore, all changes of the dye properties in presence of hemocyanin are because of changes of the protein.

**Conformational Transition**

To monitor a transition between different hemocyanin conformations we measured two different types of oxygen binding curves, structural changes based on Prodan emission and the amount of bound oxygen based on tryptophan emission (Fig. 3). The oxygen partial pressure of 12 Torr at half-saturation of bound oxygen (tryptophan signal) is in full agreement with published data (16). Both curves in Fig. 3 display similar sigmoidal shapes. However, the partial pressure of the half-maximal signal is higher for the Prodan curve, and the slope at the turning point is steeper than that of the tryptophan signal. Binding curves reflecting the amount of bound oxygen of tarantula hemocyanin (tryptophan signal) measured in the presence and absence of Prodan showed no deviations and were superimposable. Therefore, the dye can be assumed not to interfere as an effector in the oxygen binding behavior of tarantula hemocyanin, and its emission maximum can serve as a structural monitor of the protein. The differences between the tryptophan and Prodan fluorescence curves can solely be ascribed to asynchronous changes of oxygen load and conformational transition in the protein.

To examine this asynchronous behavior of the protein, the fluorescence emission maximum of bound Prodan was plotted as a function of bound oxygen (Fig. 5). A direct correlation of both quantities should result in a linear graph with a slope of...
1. In contrast, the graph is bent to the right. Between an amount of bound oxygen of 0 and ~80%, the structural signal lags remarkably behind the oxygen signal. A similar observation of non-linearity was reported for the proton binding/release of tarantula hemocyanin (32). Measured at different pH values, the proton release lagged behind oxygen binding. The course of the curve at pH 7.95, a pH value close to this study, is remarkably similar to that of our result indicating that the conformational transition observed by Prodan emission may also be connected with the binding/release of protons.

Similar investigations have previously been performed with other hemocyanins. In two different studies, Makino (38, 39) used chromophores that show different binding affinities for the oxygenated and deoxygenated forms of hemocyanins. He found structural changes in the protein matrix that are connected to oxygen binding. In contrast to our study, in both cases the structural signals preceded the functional signal. However, the Makino investigations (38, 39) had a major drawback, the oxygen binding behavior of the hemocyanins was affected by the label. In both studies, the oxygen affinity of the labeled proteins was increased compared with the unlabeled ones. This means that the dye induced a conformational change of the hemocyanins toward the high affinity (oxy) state.

**Comparison with the Nesting Model**

Next we wanted to address the question of whether the Prodan signal is a sensor for a certain conformation of tarantula hemocyanin. The analysis of the oxygen binding behavior according to the nesting model predicts four conformations, rR, tR, tT, and tT, for tarantula hemocyanin (13, 14, 16). Their fractions vary with the changing oxygen load of the active sites as shown in our study (Fig. 4). A direct comparison of the Prodan signal with the distributions of the four conformations is shown in Fig. 6. The four distributions were normalized in a way that each is spanning the full range between 0 and 1. A phenomenological comparison of the curves reveals that the course of the Prodan signal obviously follows the fraction of the hemocyanin in the rR conformation. At oxygen partial pressures above the p50 value the two curves match completely. Comparison of the Prodan signal with the other three conformation curves displays large deviations. There is also no match when the Prodan signal is compared with the distributions of R, T, r, or t (not shown). Thus, the emission maximum of Prodan bound to tarantula hemocyanin is sensitive to only one conformation (rR) and monitors the transition from the three other conformations into rR. These findings are plausible because of the following reason: rR is a state of the allosteric unit described by the nesting model. The 12-mer half-molecule has been recognized as allosteric unit in nested oxygen binding (13, 14, 16). Consequently, each 24-mer hemocyanin molecule should be able to bind at least 1 Prodan/half-molecule, and this is exactly what was found in the titration experiments. Thus, our results reveal a conformational transition of the allosteric unit and support the theory of the nesting model.

In a former study Sterner and Decker (22) reported a similar observation for the 12-mer hemocyanin from Carcinus maenas. There, the absorbance of the bound dye neutral red also correlates with the occurrence of the rR state upon oxygenation. However, as in the works of Makino (38, 39), binding of the dye increased the oxygen affinity of the protein thereby inducing a conformational change.

**Fig. 5. Prodan versus tryptophan signal.** A plot of the Prodan signal (hemocyanin conformation) at a certain oxygen partial pressure versus the tryptophan signal (bound oxygen) at the same oxygen partial pressure is shown. Before plotting, both signals were normalized as in Fig. 3. A straight dotted line with a slope of 1 is drawn as a guide for the eyes, indicating the case of linear relation between the two signals. The two extremes, oxy and deoxy state, are indicated by arrows.

**Fig. 6. Comparison of the nesting model with the Prodan signal.** The four fractional conformations (solid lines) predicted by the nesting model (in Fig. 4) were normalized in a way that each is spanning the full range between 0 and 1. In addition, the curve describing conformation tT was inverted. The curves are compared with the structural transition (dote) found by means of the Prodan probe (in Fig. 3).
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