Nested Allosteric Interactions in Extracellular Hemoglobin of the Leech *Macrobdella decora*

Received for publication, July 18, 2003, and in revised form, August 26, 2003 Published, JBC Papers in Press, August 27, 2003, DOI 10.1074/jbc.M307810200

Nadja Hellmann‡§, Roy E. Weber†, and Heinz Decker‡

From the ‡Institute for Molecular Biophysics, University of Mainz, Jakob-Welder-Weg 26, 55128 Mainz, Germany and the §Department of Zoophysiology, University of Aarhus, DK 8000 Aarhus, Denmark

Hemoglobin from the leech *Macrobdella decora* belongs to the class of giant extracellular hexagonal bilayer globin structures found in annelid and vestimentiferan worms. These complexes consist of 144 heme-bearing subunits, exhibit a characteristic quaternary structure (2 × (6 × (3 × 4))), and contain tetramers as basic substructures that express cooperative oxygen binding and thus provide a structural basis for a hierarchy in allosteric interactions. A thorough analysis of the isolated tetramer indicates that it functions as a trimer of cooperatively functioning subunits and a non-cooperative monomer rather than as four interacting subunits. A thermodynamic analysis of the whole molecule favors the application of a nested Monod-Wyman-Changeux; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxy-methyl)propane-1,3-diol.

In contrast to vertebrate animals that possess tetrameric hemoglobins in red blood cells and monomeric myoglobin in muscle cells, many invertebrates have extracellular hemoglobins with enormously high molecular weights. Based on electron microscopic and structural analyses, the extracellular hemoglobins of annelids exhibit unique structures that constitute the summit of complexity for oxygen-binding hemes. These proteins exhibit variable heterotropic and homotropic interactions: Bohr effects and cooperativity between the heme groups, and both the cooperativity coefficient and the oxygen affinity (Bohr factor (φ) of −0.4) are pH-sensitive (8, 9).

No conclusive information is available with regard to the influence of hierarchical structure on the expression of cooperative and allosteric interactions in annelid hemoglobins. This is in part because of differences in the functional criteria considered in different studies (O₂ affinity, cooperativity, Bohr effect, cation and temperature effects, etc.), the specific physicochemical conditions (pH, effector concentration, temperature, etc.), the species used for study, and the point of reference (whole blood or purified Hb) (5, 7, 10). Graphical analysis of precise O₂ binding data in terms of the Monod-Wyman-Changeux (MWC) model (11) indicates interactions between 5 and 12 oxygen-binding sites in *L. terrestris* Hb (7, 12).

In *M. decora* Hb that dissociates into tetrameric, dimeric, and monomeric units and few, if any, dodecamers (2, 8), the intact molecules and the tetrameric and monomeric subunits show increasing affinities (oxygen partial pressures at half-saturation (p₅₀) of 4.4, 1.9, and 0.3 torr, respectively, at pH 7.5 and 25 °C). Furthermore, decreasing Bohr factors (φ = −0.38, −0.30, and 0, respectively) and Hill coefficients at half-saturation (n₅₀ = 3.1, 1.4, and 1.0, respectively) were observed (8). Analysis of the data in terms of the MWC model (13) indicates 7–16 interacting heme groups in the intact Hb (8). For *L. terrestris* Hb, a similar value is indicated by the highest n₅₀ value observed (9.5 at pH −7.8) (13). In view of the complex quaternary structure of HBLs, we analyzed the oxygen binding curves of the whole 144-mer molecules and the isolated tetramers of *M. decora* in terms of both the two-state MWC model (11) and the nested MWC model, which takes into account the hierarchies within in the quaternary structure (14, 15).

MATERIALS AND METHODS

Hemoglobin Preparation and Oxygen Binding Curves

Solutions of native hemoglobin and its tetrameric dissociation product were prepared from pond leech *M. decora* as previously described (8). Oxygen binding curves were determined using a modified diffusion chamber technique (16, 17) in the presence of Tris/BisTris buffers at 25 °C and pH 7.26.

Analysis of Oxygen Binding Curves

MWC Model—The oxygen binding curves of leech hemoglobin were analyzed according to the MWC model using the following binding polynomial (Equation 1) (18).

\[ P_{MWC} = (1 + K_t x)^n + I_s (1 + K_s x)^s = P_t^n + I_s P_s^s \] (Eq. 1)
Thus, the molecule can adopt two different conformations (r and t) that are characterized by binding constants \( K_r \) and \( K_t \). The size of the allosteric unit is \( q \). The oxygen saturation curve \( \Theta \) is obtained as \( \Theta = \varphi \ln n \), where \( \varphi \) is the ligand activity.

Mindful of the finding that the dodecameric substructures of \( L. terrestris \) Hb consist of trimers (abc chains) plus monomers (d chains) that form \( abcd \) complexes and that this assembly has been confirmed by electron microscopic investigations (19) and the crystal structure at a resolution of 5.5 Å (6), the binding data were further analyzed using an alternative model. This model is based on a cooperative structure consisting of \( m \) trimers and loosely associated \( m \) monomers that do not participate in conformational transitions. For the analysis of the isolated tetramer, \( m = 1 \). The \( m \) trimers are described by the MWC polynomial \( P_{\text{MWCM}} \) analogous to Equation 1, and the \( m \) monomers by the corresponding binding polynomial \( P_m \) (Equation 2).

\[
P_M = \frac{1 + K_{dM}}{1 + K_{tM}}
\]

\[
P_{\text{MWCM},m} = 1 + P_{\text{MWCM}}^m
\]

\[
\Theta = \frac{1}{2} \frac{\varphi \ln n}{\varphi \ln n} + \frac{1}{2} \Theta_{\text{MWC},2}
\]

**Nested MWC Model—** For analysis of the whole 144-mer molecule, the nested MWC model (15) was additionally applied in the following form (Equation 4).

\[
P_{\text{neut}} = P_R + \Lambda P_T = \left( P_R + \frac{1}{2} P_T^{\text{b}} \right) + \left( \frac{1}{2} P_T^{\text{ab}} + \frac{1}{2} P_T^{\text{a}} \right)
\]

\[
\Lambda = \frac{1}{T} + \frac{1}{\bar{P}_T^{\text{a}}}
\]

\[
P_M = \frac{1 + K_{dM}}{1 + K_{tM}}
\]

\[
\alpha \beta = rR \cdot rR \cdot T \cdot T\cdot T\cdot T
\]

**Analysis of the Oxygen Binding of Isolated Tetramers—** A representative binding curve of the isolated tetramer is shown in Fig. 2A. The \( p_{50} \) is \(-3 \) torr. The tetramer obviously shows cooperative oxygen binding (Fig. 2B). The affinity constants for the first and last binding step can be estimated by extrapolation of the upper and lower asymptotes of the Hill plots, resulting in \( K_r \) and \( K_t \), values of \(<0.31 \) and \( 1 \) torr \(-1\), respectively. Fig. 2C shows the variation of the Hill coefficient with the degree of oxygenation. The maximum value of \(-2\) occurs near 80% saturation.

In a more detailed analysis, the two-state MWC model was applied. When no constraints were included, a \( q \) value of \(-13\) was obtained for the number of interacting oxygen-binding sites in the allosteric unit (Table I). This is obviously incorrect for isolated tetramers. The size of the allosteric unit for the tetramers was therefore fixed (\( q = 4 \)). The fit revealed a strong interdependence between the values for \( K_r \) and \( l_r \), which is reflected in the large errors for these two parameters.

An alternative approach was followed based on subunit analysis and information on the structure of \( Lumbricus \) Hb (1, 6, 7) indicating that each tetramer consists of a cooperative trimer and an ionically linked monomer that can readily be dissociated and does not contribute to cooperativity. A conformation-independent affinity is assigned to the monomer on the equilibrium constant between these conformations. Thus, the number \( q \) describes only how many subunits are coupled such that they always adopt the same conformations, whereas the Hill coefficient, as a measure of cooperativity, depends on both the size of the allosteric unit and all relevant equilibrium constants. The Hill coefficient can never exceed the number of interacting sites, which is the size of the allosteric unit in a concert model (\( \alpha_n \times q \)). Thus, the Hill coefficient represents a lower limit for the size of the allosteric unit (18).

For each of the possible models, the relevant equations for the dependence of oxygen saturation \( \Theta \) on the oxygen partial pressure were fitted to the data. For the analysis based on the MWC model, three parameters were determined: the two binding constants \( K_r \) and \( K_t \) and the allosteric equilibrium constant \( L_r \). In some cases, the size of the allosteric unit \( q \) was also optimized. The nested MWC model is described by seven parameters, i.e., four binding constants \( (K_r, K_t, K_{r0} \text{ and } K_{t0}) \) and three allosteric equilibrium constants \( L_r, l_r \) and \( L_t \). The fitting routine was based on a nonlinear regression analysis (Levenberg-Marquardt-routine) incorporated into the program Sigma Plot (SPSS Inc., Chicago, IL). The iterations stopped when the change in the square of residuals was \(<0.0001\).
The Hill coefficient was used to determine the affinity of the first and last binding steps, $K_1$ and $K_n$, for the monomer model (for details, see equations in the text). This model gives a better fit than the MWC model for oxygen binding of isolated tetramers from leech hemoglobin (at 25°C) as judged from the Hill plot. The maximum Hill coefficient ($n_H$) is $-5$ and occurs near the $p_{50}$ (Fig. 3). Thus, the allosteric unit within the intact 144-mer structure contains more than five interacting oxygen-binding sites. Based on the Hill plot, the affinity constant for binding of the last oxygen molecule is almost similar in the 144-mer molecule and tetrameric subunits: $K_1 = 1.2$ and 1 torr$^{-1}$, respectively. In contrast, the affinity of the first oxygen molecule is markedly lower in the 144-mer ($K_1 = 0.04$ torr$^{-1}$) than in the isolated tetramer ($K_1 = 0.31$ torr$^{-1}$).

How can these phenomena be explained on the basis of the structure, and which type of cooperative interactions might occur upon assembly of the tetramers? (a) In the simplest case, the two-state MWC model is applicable. One might expect that the association of tetramers to form the 144-mer leads to the formation of larger allosteric units. Furthermore, it is possible that, because of the formation of the larger structure, the uncoupled monomer, which is not part of the allosteric unit in the isolated tetramer, becomes functionally coupled. Indeed, the two-state MWC model is applicable to the binding data of the 144-mer. The best fit was obtained with a value of $q = 8.3 \pm 0.2$ for the size of the allosteric unit (Table I). Thus, in the framework of the MWC model, two tetramers are coupled to form an allosteric unit. This implies that the 144-mer is composed of $144/8 = 18$ non-interacting allosteric units, each containing eight interacting subunits. Bearing in mind the information about the structural hierarchy ($2 \times (6 \times (3 \times 4))$, this result can hardly be rationalized.

(b) Possibly the four subunits of the basic tetramer retain their allosteric properties also in the 144-mer. To take this possibility into account, the MWC model was extended to allow for a tetrameric structure, which consists of an interacting trimer and a non-interacting monomer (Equation 2). For the analysis of the 144-mer, $m$ of these $3 + 1$ tetramers might be coupled. In this case, the nonlinear regression yielded $m = 1.8 \pm 2$. Thus, the cooperative properties in this model again indicate an octameric structure, i.e. the functional coupling of two $3 + 1$ tetramers, which is unlikely (as case (a)).

(c) As a further alternative, we applied the nested MWC model for cooperativity (Equation 3), respecting the hierarchy in the structure of 144-mer leech hemoglobin. The simplest approach is to consider the tetramers as the basic allosteric unit ($s = 4$) and the dodecamer as the allosteric unit at the next level because the structural information indicates that three tetramers are coupled. The cooperative molecule in this model therefore consists of three cooperatively interacting tetramers, whose four subunits also interact cooperatively (Fig. 1). This model provided a better fit to the data than a nonhierarchical MWC model. However, we cannot exclude additional functional coupling at a higher structural level such as between the 12-mers within the 144-mer. In this scenario, the basic allosteric unit is assumed to be a 12-mer, six copies of which are coupled to form the 72-mer half-molecule (Table II). In this case, the tetramers seem to function as a cooperative trimer and a non-cooperative monomer instead of four cooperatively linked subunits.

FIG. 2. Oxygen binding of isolated tetramers. A, oxygen binding curve of purified tetramers from leech hemoglobin (at 25°C and pH 7.26), $p_{50} = 2.7$ torr. The curve drawn corresponds to the trimer + monomer model (for details, see “Results”). B, Hill plot. The maximum Hill coefficient was $-2$ at $p_{50} = 9$ torr. The straight lines correspond to slopes of unity; their intercepts with the $y$ axis at log $p_{50} = 0$ indicate values for the affinity of the first and last binding steps, $K_1 = 0.31$ and $K_n = 1$ torr$^{-1}$, respectively. The error bars were calculated based on an uncertainty in the saturation fraction of 0.01. C, saturation dependence of the Hill coefficient.
regression coefficient was slightly higher, which favors this variant of the nested MWC model. However, the difference is small, so the 3 × 4 model cannot be entirely excluded as a possible mechanism. When the data were analyzed based on a 12 × 12 model, the values for the parameters were similar to those found for the 6 × 12 model. The linear regression coefficient was slightly lower in the 12 × 12 model, which again favors the 6 × 12 model.

**DISCUSSION**

The 144-mer Hb from the leech *M. decora* has been extensively investigated structurally and functionally (3, 8, 20). However, cooperative oxygen binding is not well defined by a cooperative model especially when the hierarchical structure is respected. When oxygen binding curves of 144-mer HBL from *M. decora* were analyzed on the basis of the MWC model, the size of the allosteric unit was found to be 7–16 depending on the experimental conditions and weighting of the data points in the fitting procedure (8). Furthermore, the binding constant for the high affinity R conformation changes with pH, which does not agree with a concerted model like the MWC model (21). Accordingly, the simple MWC model needs to be extended by including further conformations.

Similar observations were made for other extracellular respiratory proteins, which often show hierarchically arranged oligomeric structures (4, 5, 22, 23). In a number of cases, the simplest concerted model of cooperativity, the MWC model, cannot adequately describe the oxygen binding properties of these molecules (8, 21). An extension of the MWC model that takes into account the structural hierarchy is the nested MWC model (14, 15), which was applied here to describe the cooperative binding properties of leech HBL.

The fact that leech hemoglobin molecules do not dissociate into the 72- or 12-mer substructures (halves and one twentieths of the intact HBL molecules, respectively), but form tetramers upon removal of Ca²⁺ ions, suggests that the tetramers may play an important role in the cooperative interactions. Therefore, the oxygen binding properties of the isolated tetramer were analyzed and compared with the properties of tetramers embedded in the 144-mer structure.

**Isolated Tetramers**—The MWC model provides a good description of the binding curve of the isolated tetramers. However, the cooperative “trimer + monomer” model used for the analysis provides the better fit of the MWC model to the data among those parameter sets, which yield reasonable values for the size of the allosteric unit (q ≤ 4). This result also explains why the Hill plot is not symmetrical. The non-symmetric Hill plot appears to be attributable to the superimposition of non-cooperative oxygen binding to the monomer, which dominates at low oxygen pressure, and cooperative binding to the trimer, which dominates at higher oxygen pressure. Thus, the maximum Hill coefficient is found at 80% saturation, and *n₅₀* < *nₙ₅ₐ₅*When the Hill coefficient is calculated for the cooperative trimer and plotted against the saturation of the trimer, a symmetric distribution is found as expected (data not shown). In the framework of this model, the value for *Kₚ* as determined from the Hill plot largely corresponds to that for oxygen binding to the monomers (*Kₚ* = *Kₚ*/4).

It should be noted that the 3 + 1 tetrameric structure seems to be common in oligochetes and polychetes, whereas achete (leech) hemoglobins seem to dissociate into monomers and disulfide-bonded dimers (4, 8). However, the observation that the Hill coefficient for oxygen binding of isolated tetramers of leech hemoglobin has a value of −2 indicates that the allosteric unit is larger than a dimer. Indeed, when an MWC model foreseeing a dimeric allosteric unit plus two uncoupled monomers (Equation 3) is fitted to the data, the regression coefficient is lower for the 2 + 2 model than for the 3 + 1 model (r² = 0.9993 and 0.99966, respectively). Furthermore, the values for *lₚ* and *Kₚ* are very large, indicating that the model has to be stretched to its physical limit (q = *nₙ₅ₐ₅*) to describe the data. Thus, the oxygen binding data are consistent with structural evidence for the 3 + 1 tetramers (6), indicating that the hierarchy in functional coupling in the oligomeric structure does not necessarily reflect the dissociation products of HBLs.

**Intact 144-mer Molecules**—The MWC model also provides a good description of oxygen binding of the whole 144-mer molecules. However, the number of interacting subunits (q = 8.3) seems to be inconsistent with three-dimensional reconstructions for *Lumbricus* Hb, which show three tightly coupled tetramers (6). One might be tempted to interpret q = 8.3 as an indication that the size of the allosteric unit is q = 9 with three interacting trimers and the three remaining monomers being loosely attached, as found for the isolated tetramer. However, when such a model is applied (Equation 2), the model is compatible with the data only when the number of interacting subunits is about six (q = 3m ~ 3 × 2). The only way to restore three trimers as an allosteric unit is to assume that the loosely associated monomers do not bind oxygen at all. However, because no indications for a loss of function were observed in the analysis of the isolated tetramer, this hypothesis can be discarded.

Based on the three-dimensional reconstructions, which show a structural hierarchy (2 × (6 × (3 × 4))), one would expect multiples of tetramers, 12-mers, 72-mers, or the 144-mers as allosteric units. Furthermore, the hierarchy in the quaternary structure favors the nested MWC model over the simple MWC model. The nested MWC model moreover provides better agreement between the model and data.

The structure of 144-mer leech hemoglobin might be interpreted in terms of two possible functional hierarchies. In one model, the basic allosteric units are tetramers that are further functionally coupled (s × 4) into larger allosteric units. Alter-
natively, the dodecamers form the smallest basic allosteric unit, 6 or 12 of which are functionally coupled to form the half-molecule and the native molecule, respectively (6 × 12 or 12 × 12). The results for these two models slightly favor the 6 × 12 model due to the higher regression coefficient compared with the 3 × 4 and 12 × 12 models.

![Graph A](image)

**Fig. 3. Oxygen binding of the whole 144-mer.** A, oxygen binding curve of whole leech hemoglobin molecules (at 25 °C and pH 7.26). $p_{O_2} = 4.8$ torr. The curve drawn corresponds to a fit based on the nested MWC model with tetramers as the basic allosteric units that are coupled to intercepts with the $K_m$ of the first and last binding steps, curve of whole leech hemoglobin molecules (at 25°C and pH 7.26).

![Graph B](image)

**Fig. 3. Oxygen binding of the whole 144-mer.** B, Hill plot. The maximum Hill coefficient was 5 at $p_{O_2} = 5.4$ torr. The straight lines correspond to slopes of unity; their intercepts with the y axis at log $p_{O_2} = 0$ indicate values for the affinity of the first and last binding steps, $h_1 = 0.048$ and $h_n = 1.1$ torr$^{-1}$, respectively. For other details, see the legend to Fig. 2B. C, saturation dependence of the Hill coefficient.

Are there any additional similarities in the hierarchy of the functional properties when respiratory proteins from arthropods and annelids are compared? The oxygen binding data of the 12-mer lobster hemocyanin could be described in terms of a $2 × 6$ model, and those of the 24-mer spider and scorpion hemocyanins in terms of a $2 × 12$ model (21, 24). Thus, in each case, the half-molecules correspond to the basic allosteric unit. When the oxygen binding of the isolated half-molecules was investigated, it turned out that these could well be described by the simple MWC model. Furthermore, the binding affinities of the two conformations in the MWC model correspond to the binding affinities of the R-state of the nested MWC model, which describes the oxygen binding behavior of the whole complex (21, 24–26).

Can such a pattern also be found when the binding properties of the tetrameric leech globins are compared with those of the intact 144-mer hemoglobin? Because the binding constant for the R-state in the tetramer is not very well defined, the following strategy was performed. The binding data for the tetramer were reanalyzed with values for $K_r$ kept equal to the values $K_{R,T}$ and $K_{R,R}$ found for the nested MWC model describing the 144-mer. Binding constants are in torr$^{-1}$. For details, see "Discussion."

**Table II**

Allosteric parameters for intact Hb analyzed using the nested MWC model

| $w = 4$, $s = 3$ | $w = 12$, $s = 6$ | $w = 12$, $s = 12$ |
|------------------|-------------------|-------------------|
| $\log I_R$      | 1.9 ± 15          | 7.1 ± 0.2         | 7.0 ± 0.2         |
| $\log I_T$      | 5.8 ± 11          | 2.0 ± 0.2         | 3.0 ± 0.3         |
| $\log I_T$      | 1.0 ± 2.2         | 10.0 ± 0.5        | 8.8 ± 0.7         |
| $K_{R,T}$       | 0.008 ± 0.006     | 0.035 ± 0.002     | 0.041 ± 0.0022    |
| $K_{R,T}$       | 0.19 ± 0.35       | 1.41 ± 0.2        | 1.11 ± 0.2        |
| $K_{R,R}$       | 0.38 ± 4.6        | 0.065 ± 0.002     | 0.063 ± 0.002     |
| $K_{R,R}$       | 1.9 ± 21          | 0.83 ± 0.04       | 0.81 ± 0.05       |
| $r^2$           | 0.999964          | 0.999967          | 0.999957          |

**Table III**

Allosteric parameters for the isolated tetramer with fixed values for $K_r$

Data for oxygen binding of the isolated tetramer were reanalyzed with values for $K_r$ kept equal to the values $K_{R,T}$ and $K_{R,R}$ found for the nested MWC model describing the 144-mer. Binding constants are in torr$^{-1}$. For details, see "Discussion."

| $K_r$ | $K_{R,T}$ (6 × 12) | $K_{R,R}$ (6 × 12) | $K_{R,R}$ (12 × 12) |
|-------|--------------------|--------------------|--------------------|
| $K_{R,T}$ | 1.7 ± 0.2 | 1.6 ± 0.2 | 1.6 ± 0.2 |
| $I_R$ | 1.53 ± 0.02 | 2.15 ± 0.02 | 1.86 ± 0.02 |
| $K_{R,T}$ | 0.013 ± 0.007 | 0.049 ± 0.006 | 0.035 ± 0.007 |
| $K_{R,R}$ | 0.83 (fixed) | 1.41 (fixed) | 1.11 (fixed) |
| $r^2$ | 0.99939 | 0.99956 | 0.99950 |
values is slightly better with the 6 × 12 model than with the 12 × 12 model (Table III).

In the analysis of the ATPase activity of GroEL based on the nested MWC model (27) and in the analysis of the oxygen binding data of 12-mer lobster hemocyanin and 24-mer spider and scorpion hemocyanins (21, 24), a typical pattern for the parameter values describing the nested MWC mode was found: viz. \( K_{R} < K_{R} < K_{R} < K_{R} < K_{R} \) and \( L_{1} < L_{2} < L_{3} \). The same pattern was found when the 6 × 12 and 12 × 12 models were applied to leech HBL. This represents an additional similarity in functional properties for different hierarchical proteins.

Data on the pH dependence of the oxygen binding behavior of leech HBL further support the validity of the nested MWC model for this respiratory protein. The conformational distribution for the 144-mer depicted in Fig. 4 shows that, at pH 7.26 and low oxygen saturation, the apparent oxygen binding constant corresponds to binding to conformation \( t^{+} \). Indeed, \( K_{t} \) (the value determined from extrapolation of the Hill plot) is similar to \( K_{t} \) (0.04 and 0.035 torr \(^{-1} \)), respectively. In contrast, at high oxygen saturation, the apparent binding constant as given by \( K_{t} \) is an average of conformations \( K_{R} \) and \( K_{R} \) (\( K_{R} = 1.2, K_{R} = 0.83, \) and \( K_{R} = 1.41 \) torr \(^{-1} \) at pH 7.26). The change in \( K_{R} \) with pH as observed for leech HBL (\( K_{R} = 0.7 \) torr \(^{-1} \) at pH 7.08) (8) indicates that protons favor the low affinity conformations. At low oxygen saturation, the conformation with the lowest affinity already dominates at pH 7.26, so no significant effect of pH on \( K_{t} \) can be observed (\( K_{t} = 0.06 \) torr \(^{-1} \) at pH 7.08) (8). In contrast, at high oxygen saturation, the fraction of conformation \( t^{+} \) probably dominates at pH 7.08, so \( K_{t} \) is similar to \( K_{R} \). Thus, the hierarchical nested MWC model can explain the pH dependence of oxygen binding reported for leech HBL. As shown in Fig. 4, all conformations postulated by the nested MWC model are adopted by leech HBL within the physiologically relevant \( pO_{2} \) range (1–15 torr). Thus, the different conformations available to this hemoglobin in the framework of the nested MWC model might be important in adapting its oxygen-transporting functions in the blood to changes in environmental and physicochemical properties.

**Conclusion**—Analyses of the oxygen binding data of isolated tetramers and the whole 144-mer molecules of leech HBL indicate that the association of basic tetramers in the native structures creates a hierarchy of cooperativity. The nested MWC model neatly describes the cooperative binding properties. Furthermore, typical values for the oxygen binding parameters and the allosteric equilibrium constants are obtained for the 6 × 12 and 12 × 12 models. The Hb from the leech provides an example of a large multisubunit protein whose quaternary structure shows clear hierarchies to which allosteric equilibria could be assigned. Other examples are the respiratory hemocyanins and the chaperonin GroEL (16, 24, 27). This hierarchy in allosteric equilibria allows different effectors to modulate the binding behavior at different structural levels. In addition, the range of cooperative interaction is broadened. Thus, proteins exploiting the nesting principle gain functional plasticity.

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