Molecular Shape, Dissociation, and Oxygen Binding of the Dodecamer Subunit of Lumbricus terrestris Hemoglobin*

(Received for publication, February 29, 1996, and in revised form, May 13, 1996)

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Small angle x-ray scattering of the 213-kDa dodecamer of Lumbricus terrestris Hb yielded radius of gyration of 3.74 ± 0.01 nm, maximum diameter = 10.59 ± 0.01 nm, and volume = 255 ± 10 nm³, with no difference between the oxy and deoxy states. Sedimentation velocity studies indicate the dodecamer to have a spherical shape and concentration- and Ca²⁺-dependent equilibria with its constituent subunits, the disulfide-bonded trimer of chains a-c and chain d. Equilibrium sedimentation data were fitted best with a trimer-dodecamer model, In K₄ = 7 (association K in liters/g) at 1°C and 4 at 25°C, providing ΔH = -20 kcal/mol and ΔS = 4.4 eu/mol. Oxydodecamer dissociation at pH 8.0, in urea, GdmCl, heteropolytungstate K₄[SiW₁₁O₃₉] and of metdodecamer at pH 7, was followed by gel filtration. Elution profiles were fitted with exponentially modified gaussians to represent the three peaks. Two exponentials were necessary to fit all the dissociations except in [SiW₄O₃₉]⁻⁻. Equilibrium oxygen binding measurements at pH 6.5–8.5, provided P₅₀ = 8.5, 11.5–11.9 and 11.9–13.5 torr, and n₅₀ = 5.2–9.5, 3.2–4.9, and 1.8–2.7 for blood, Hb, and dodecamer, respectively, at pH 7.5, 25°C. P₅₀ was decreased 3- and 2-fold in 100 mM Ca²⁺ and Mg²⁺, respectively, with concomitant but smaller increases in cooperativity.

The HBL Hb of the common North American earthworm Lumbricus terrestris is the most extensively studied of the annelid and vestimentiferan extracellular Hbs, giant (~35 MDa) heteromultimeric protein complexes of about 180 globin and nonglobin polypeptide chains that have a high cooperativity of oxygen binding and low iron and heme contents (1–3). The Lumbricus Hb is comprised of globin subunits T, a disulfide-bonded trimer of chains a-c (4, 5) and the monomer M (chain d) (6, 7), and four different types of linker chains of 24–32 kDa (8, 9). Dissociation of the oxyHb at neutral pH in the presence of urea, Gdm salts, and other chaotropes at concentrations that do not denature the subunits, provides a 213-kDa dodecamer complex, consisting of T and M subunits (10, 11). It has been shown recently that the dodecamer plays an important role in the dissociation and reassociation of the HBL structure (12). Furthermore, a preliminary x-ray diffraction study of oxydodecamer crystals has shown that it has an almost planar conformation (13). Here we report the results obtained regarding the molecular shape and physical homogeneity of the oxydodecamer in solution at neutral pH, its dissociation, and its equilibrium oxygen binding.

EXPERIMENTAL PROCEDURES

Materials—L. terrestris Hb was prepared as described previously in 0.1 M Tris buffer, pH 7.0, 1 mM EDTA, ~2 mM phenylmethylsulfonyl fluoride from live worms from around London, Ontario (Carolina Wholesale Bait Co., Canton, NC) (3). Blood was the supernatant from the first centrifugation to remove cellular debris. The dodecamer was isolated by preparative gel filtration at neutral pH, subsequent to exposure to 4 M urea (11) or 10–30 mM SiW or AsW at 7°C; the fractions were screened by SDS-polyacrylamide gel electrophoresis (14) and were pooled on the basis of their subunit content. The concentration was calculated from the absorbance of the cyano-met form at 540 nm using the extinction coefficient 0.656 ± 0.011 ml/mg·cm⁻¹ for the monomer subunit (6). GdmCl was purum grade from Fluka AG (9470 Buchs, Switzerland), and urea was from Sigma. The heteropolytungstates SiW and AsW were synthesized as described by Klemperer (15).

Small Angle X-ray Scattering—Scattering experiments were performed using a Kratky compact camera with slit collimation, a position-sensitive detector (M Braun PSD-50M), and a Philips PW2253/11 x-ray tube with a copper target, operated at 50 kV and 30 mA. Sample solutions were placed in a 1-mm diameter Mark capillary and kept at 4°C during the measurements. A 30-μm nickel filter was employed to eliminate polychromatic effects. Using a sample to detector distance of about 20.9 cm, the width per detector channel corresponded to 0.00964 nm⁻¹ on the h scale (h = 4π(λsinθ/2) is the scattering angle, λ = 0.154 nm is the wavelength of the CuKα line). Significant scattering data were collected in 300 channels corresponding to h values ranging from 0.056 to 2.0 nm⁻¹. The counting time was typically 1800 s, and each scattering curve was recorded several times in order to reduce the statistical errors. Two series of measurements for the oxy and deoxy forms of the dodecamer were performed using five different concentrations ranging from 5 to 53 mg/ml in 0.1 M TrisCl buffer, pH 7.0, 1 mM EDTA. Data evaluation, including smoothing, desmearing, and indirect Fourier transformation, was carried out with the computer program ITP. Details of the experimental technique and the evaluation procedure are described elsewhere (16, 17). A finite element method (small identical spheres) was used for model calculations to simulate the experimentally obtained scattering curves and distance distribution.

1 The abbreviations used are: HBL, hexagonal bilayer; Hb, hemoglobin; GdmCl, guanidinium chloride; K₄[SiW₁₁O₃₉] potassium undecatungstotetrasilicate; K₄[SiW₄O₃₉]₁₄H⁺O; Na₂AsW; sodium tetra-tatungstotetrasilicate; Na₂Ba₂SiW₂O₉H⁺O; BisTris, 2-bis(2-hydroxyethyl)-amino-2-(hydroxymethyl)-1,3-propanediol; FPLC, fast protein liquid chromatography; AU, absorbance unit; r.m.s., root mean square; EMG, exponentially modified gaussian; P₅₀, oxygen pressure at half-saturation; n₅₀, Hill's cooperativity coefficient; SAXS, small angle x-ray scattering; MWQ, Monod-Wyman-Changeux model.
functions (18). Deoxygenation of the oxydodecamer solution was performed directly in the Mark capillary by repeated evacuation and equilibration with purified nitrogen. The spectral alterations attendant upon the conversion of oxy to deoxy forms, namely the disappearance of the two peaks at 540 and 575 nm and the appearance of a broad peak at 550 nm, were followed via the measurement of visible absorption spectra over the 490–650-nm region using the Mark capillary as the sample cell in a specially constructed device equipped with two adjustable slits and an attenuation filter as reference, placed in a Zeiss PMQII spectrophotometer. The reversibility of the oxy to deoxy spectral alteration was checked by reoxygenation of the sample in the capillary in the presence of air.

Sedimentation Studies—For velocity sedimentation studies, 3- and 12-mm optical path double sector analytical ultracentrifuge cells were filled with oxydodecamer solutions (14 to 0.04 mg/ml) in 0.1 M Tris buffer, 1 mM EDTA, pH 7.0, using the dialysate for dilution of the stock solutions. The cell thickness (3 or 12 mm) and the wavelength of the light used to detect the gradient (280, 310, or 412 nm) were chosen so that the initial absorbance was ~2 for the high and ~0.1 for the low concentration samples. The appropriate dialysate was loaded in the reference side of each cell. The cells were centrifuged at 1.25 and 40 °C at 55,000 rpm in a Beckmann XL-A analytical ultracentrifuge, and the absorbance was measured radially for several hours until the boundaries had sedimented almost to the bottom of the cell.

For sedimentation equilibrium experiments, oxydodecamer solutions were diluted using the dialysate to provide concentrations of 2, 1, 0.5, 0.25, 0.125, and 0.04 mg/ml. Approximately 25 or 100 μl were loaded into the sample channels of 6-channel sedimentation equilibrium cells (19, 20) with 3- and 12-mm optical paths and 3 and 10 μl of high density fluorocarbon oil (M&MFC-43) added, respectively, in order to raise the bottom of the solution and provide column heights of 2 mm in all cases. The solvent channels were filled with about 30 and 110 μl of dialysate, respectively. The concentration gradients were measured from the absorbance at 280, 418, and 540 nm, depending on the protein concentration. The cells were centrifuged in a Beckman model XL-A analytical ultracentrifuge at 14,000 rpm at 1 °C for dodecamer from urea and 10,000 rpm at 1 and 25 °C for dodecamer from SiW dissociation, respectively. Absorbance data were taken radially at 0.001-cm intervals and scans made every 3 h. Sedimentation equilibrium was determined to have been reached when successive scans were not detectably different as determined by a program (MATCH developed by D. Yphantis). Data sets were also obtained upon reaching equilibrium at 19,000 and 24,000 rpm for dodecamer from urea dissociation and 20,000 rpm for dodecamer from SiW dissociation. This range of speeds was necessary to resolve species from monomer to dodecamer. A nonlinear least squares program (21) was used for data analysis.

Analytical Gel Filtration and Fitting of Elution Profiles—Low pressure, isocratic gel filtration was carried out at room temperature (20 ± 2 °C) employing an FPLC system (Pharmacia Biotech Inc.) and 1 × 30-cm columns of Superose 512 or 56 (Pharmacia). Flow rate was 0.4 ml/min and the eluate was monitored at 280 nm. A constant amount of protein in a constant sample volume, ~800 μg/200 μl, was loaded each time. The FPLC elution curves were acquired using the Easiest System 8 (KettlMey Instruments, Inc. Rochester, NY) and an IBM PC386 computer and fitted with EMG’s representing the undissociated dodecamer D, trimer T, and monomer subunit M, employing least squares minimization (Peak Fit Version 2.0, Jandel Scientific). The EMG function is a convolution of gaussian and decreasing exponential functions, where $a_0$ is the amplitude, $a_1$ is the center, $a_2$ is the width of the gaussian, and $a_3$ is the width of the exponential. The EMG is asymmetric with an exponential tail on the right side, whose fall-off width is controlled by the parameter $a_3$. The EMG represents well the shape of chromatographic elution peaks (22, 23).

Oxygen Equilibrium Measurements—O2 binding equilibria were measured at 25 and 10 °C, using a modified gas diffusion chamber allowing stepwise increases in O2 pressure (24, 25). Variation in pH was obtained by adding Tris-Cl buffers of different pH. The chamber was coupled to cascaded Woesthoff types M301, M201 and M101a-f gas pumps for mixing pure N2 (99.998%), atmospheric air, and O2. The PaO2 and PNa+ were interpolated from linear plots of log[Si(1 – S)] versus log PNa+ for S (fractional saturation) between 0.3 and 0.7. The O2 equilibrium measurements at extremes of saturation were measured as described previously (26, 27). Errors resulting from possible incomplete saturation in the presence of pure O2 were minimized by upper end point extrapolation of log(A) versus 1/PNa+ plots to 1/PNa+ = 0. Lower end point correction was carried out applying an A versus PNa+ plot. The extrapolations were performed by fitting a cubic polynomial. The data were analyzed in terms of the two-state MWC equation (28):
TABLE I
SAXS molecular parameters for dodecamer and model

| Parameter                  | Dodecamer$^a$ | Model$^b$ |
|----------------------------|---------------|-----------|
| Radius of gyration (nm)    | 3.74 ± 0.01   | 3.73 ± 0.01 | 3.74 ± 0.05 |
| Maximum diameter (nm)      | 10.59 ± 0.01  | 10.59 ± 0.01 | 10.7 ± 0.1  |
| Volume (nm$^3$)            | 255 ± 10      | 250 ± 10   | 249 ± 2     |
| Molecular mass (kDa)$^c$   | 190 ± 19      | 191 ± 19   | 191 ± 19    |

$^a$ From Hb dissociation in urea.
$^b$ Consensus model (Fig. 2) based on averaging 33 well-fitting models using 265 overlapping spheres, 0.661 nm in diameter.
$^c$ Based on a calculated specific volume of 0.733.

**RESULTS**

SAXS of the Oxy and Deoxy Dodecamer—The final scattering curves $I(h)$ for the oxy and deoxy forms of dodecamer from Hb dissociation in urea, obtained after extrapolation to zero concentration, are shown in Fig. 1A and the corresponding pair distance distribution functions $p(r)$ in Fig. 1B. The molecular parameters derived from the two functions are provided in Table I; they appear to be identical within the error limits.

The scattering curves and $p(r)$ functions of a large number of models were calculated using small overlapping identical spheres to represent the protein mass (17) and compared with the experimental results. Only a few models were found to exhibit scattering equivalent to the dodecamer, i.e. fulfilling the following requirements within the mean square deviation, 1) same radius of gyration, 2) same scattering curve up to $h = 1.9$ nm$^{-1}$ (corresponding to about 3 orders of magnitude in intensity), 3) same maximum diameter and shape of $p(r)$ function, and 4) similar volume. As a result of the averaging, the spheres are no longer of equal weight; the filled spheres occur in 32 or all models and the empty ones occur in only one or two models and the gray spheres have an intermediate weight. B, planar model for the dodecamer using spheres of equal weight (side view and tilted at 40° to the x axis).

where $S$ denotes saturation, $P$ the partial pressure of $O_2$, $L$ the allosteric constant, $K_T$ and $K_R$ the association equilibrium constants for the low affinity (T, tense) and high affinity (R, relaxed) forms, respectively, and $q$ the number of interacting binding sites. Nonlinear least squares fitting of the data in the form $\log[S/(1 - S)]$ versus $\log(P)$ was performed using the software package Mathematica (Wolfram Research Inc., Champaign, IL 61820) employing the Marquardt-Levenberg method.

Attempts to fit the experimental data with more oblate models were unsuccessful. Fig. 2B shows a planar model (223 spheres, radius 0.661 nm$^{-1}$) which had the following molecular parameters, radius of gyration 3.75 nm, maximum dimension 10.8 nm, volume 270 nm$^3$, dimensions $x = y = 9.5$ nm, and dimension $z = 4.2$ nm. The scattering curve of this model
deviates from the experimental one (Fig. 3A) in the angular range $h_{1nm}$ to $h_{2nm}$, whereas the model shown in Fig. 2A fits the experimental curve up to $h_{1.9nm}$. Comparison of the pair distance distribution functions in Fig. 3B also shows considerable discrepancies in the case of the planar model. As a consequence of planarity, the shape of the $p(r)$ function is changed in a way that the frequency of distances representing the "thickness" of the model is increased, leading to a shift of the $p(r)$ function maximum to the left.

Sedimentation Velocity Studies of the Oxydodecamer—The scans were analyzed by the dc/dt method (29) to provide weight distributions of sedimentation coefficients ($s$), called $g^*$'s. Fig. 4 shows the results obtained at 1°C for the oxydodecamer from Hb dissociation in urea, in the absence and presence of 10 mM Ca$^{2+}$, analyzed by the dc/dt method (29). B, 0.0390 mg/ml in the absence (circles) and 0.0406 mg/ml in the presence (squares) of 10 mM Ca$^{2+}$.

Sedimentation Velocity Studies of the Oxydodecamer—The experiments with oxydodecamer from urea dissociation produced 1818 data points in the absence and 1568 points in the presence of Ca$^{2+}$ at 1°C. Global fits using the NONLIN program (21) were obtained by assuming various models of self-association. The program calculates the value of the reduced molecular weight ($M' = M(1 - v_p/v)$), where $v$ is the specific volume, $M$ is the molecular weight of the molecule, and $p$ is the density of the solution), the values of other constants related to the model, i.e. second virial coefficient, equilibrium constants, etc., and the value of the r.m.s. error. $M'$ was converted to molecular weight using $v = 0.733$, calculated from the amino acid compositions (30) of the globin subunits of Lumbricus Hb (5, 7) and solvent densities estimated from density tables. The monomer-trimer and monomer-tetramer models gave the best fits; the results are provided in Table I. The experiments with oxydodecamer from SiW dissociation produced 12 sets of data consisting of about 1300 data points at both 1 and 25°C; the results of global fits with the NONLIN program are shown in Fig. 5.
Table III and the residuals of the fits in Fig. 6. Although the figure shows little systematic error for the two best fits, the monomer-tetramer model provided the best fit based on r.m.s. error, with $\ln K_4$ (association $K_4$ in liters$^3$/g$^3$) being 7 at 1°C and 4 at 25°C.

Dissociation of Oxydodecamer from Hb Dissociated in Urea—Fig. 7A shows a typical FPLC elution profile at 280 nm of partially dissociated dodecamer and illustrates the fitting of the three peaks with EMG functions. The elution volumes of the peaks were unchanged during the course of the dissociation, the standard deviation of constant $a_1$ in the EMG fit being <2%.

The time course of oxydodecamer dissociation in 1.75 molal urea is shown in Fig. 7, B–D. The points represent averages of two separate experiments. The dissociation of the dodecamer was followed to >80% completion, ~1400 h. The insets show the initial 10% of the processes. In all cases, the data could be properly fitted only with two exponentials. The resulting residuals are shown at the bottom of the plots. Table IV provides the amplitudes and kinetic constants obtained from the fits. The presence of 10 mM Ca$^{2+}$ did not have much effect on the dissociation of the oxydodecamer in urea.

Fig. 8 shows the time courses of oxydodecamer dissociation in 1.22 molal GdmCl at pH 7 (A), at pH 8.0 (B), in 10 mM SiW (C), and upon conversion to the met-form (D) at pH 7, together with the exponential fits and the resulting residuals. Although two exponentials are necessary to provide a reasonable fit to the dissociations in GdmCl and at pH 8, the dissociations in SiW and upon oxidation can be fitted with only a single exponential. The parameters obtained from the fit are given in Table IV.

Equilibrium O$_2$ Binding—The parameters for Lumbricus blood Hb, and the dodecamer from Hb dissociation in urea, in the absence and presence of Mg$^{2+}$ and Ca$^{2+}$, are shown in Figs. 9 and 10 and Table V; the extended Hill plots are presented in Fig. 11 and the calculated MWC parameters in Table VI. The parameters for dodecamer from SiW dissociation are also provided in Tables V and VI.

**DISCUSSION**

The Molecular Shape of Dodecamer from SAXS—The data shown in Fig. 1 and Table I demonstrate that the molecular shapes of the oxy and deoxy forms of dodecamer from Hb dissociation in urea are the same within the experimental error of the SAXS method. Similar experiments carried out with...
Lumbricus Hb Dodecamer Subunit

native Lumbricus Hb also lead to a similar conclusion,\(^2\) in agreement with the results of a Raman spectroscopic study (31).

Although oxydodecamer from urea dissociation appears to be fairly planar in the crystal (13), our consensus model (Fig. 2) indicates it to be domed-up rather than planar in solution, in agreement with the requirement that the superposition of two dodecamers equal the \(200 \text{ nm height observed in electron micrographs of native HBL structures (32).}\)

Molecular Shape of Oxydodecamer from Sedimentation Velocity—The oxydodecamer from urea dissociation (Fig. 4, Table II) appears to consist of three species, \(8.5-9.4 \text{ S}, 3.6-4.4 \text{ S},\) and \(1.9 \text{ S},\) present in concentration-dependent equilibria with each other. The net effect of Ca\(^{2+}\) appears to be stabilization of the \(3.6-4.4 \text{ S peak at the expense of the other two peaks. Fig. 5 shows the results obtained for the oxydodecamer from SiW dissociation. There is a concentration-dependent equilibrium between an 8.6–10 S species, presumably the dodecamer and a 3.5–4.0 S species (Table II). Since there is no 1.9 S species in this case, the 3.5–4.0 S species is either the complex T + M or a mixture of T and M\(_3\). Comparing the results obtained at 1°C (Figs. 4 and 5), it is obvious that the oxydodecamer from SiW dissociation is substantially more stable than from urea dissociation.

Since \(s_{20,w} = 1.9 \text{ S for the slowest component observed in Fig. 4 is a likely value for the monomer (chain d, 17 kDa), one can estimate, using the Kirkwood-Riseman approach (33), the } s_{20,w}\)

\(a \beta_0 = 0.693 K.\)

\(b\) The correlation coefficient \(r = \Sigma XY/[(\Sigma X^2)(\Sigma Y^2)]^{1/2},\) where \(X = x_i - \bar{x}\) and \(Y = y_i - \bar{y}.\)
values to be expected for the trimer, tetramer, and dodecamer. For spherical, planar, and linear models, they are 3.93 S, 3.78 S, and 3.46 S, respectively, for the trimer and 4.95 S, 4.65 S, and 4.09 S, respectively, for the tetramer. All the experimental $s_{20,w}$ values for the intermediate component (Table II) except one (4.41 S) are in the range 3.5–4.0 S (mean 3.7 ± 0.2 S, n = 8), suggesting it is a trimer with a planar conformation. Using 3.7 S for the trimer, one calculates for the dodecamer, 9.1 S for a spherical, and 8.5 S for a planar model. Since the mean $s_{20,w} = 9.1 ± 0.6 S$ (S.D., n = 7) (Table II), the sedimentation velocity results favor the spherical model for the dodecamer, in agreement with the SAXS results. Although it is tempting to add that the oxydodecamer obtained by dissociation in urea appears to be spherical in the absence of Ca$^{2+}$ and planar in its absence (Table II), the scatter in the $s_{20,w}$ values (Table II) suggests caution. Metal analysis indicates that there is a difference in calcium content between the urea and SiW preparations of dodecamer, <1Ca/mol versus ~3Ca/mol, respectively. ³ Quantitative studies of calcium binding to Hb, dodecamer, trimer, and monomer are under way.

Sedimentation Equilibrium Studies of Dodecamer Dissociation—The program NONLIN analyzes the sedimentation equilibrium data only in terms of a simple monomer $\leftrightarrow$ n-mer equilibrium. The monomer-trimer and monomer-tetramer models produce the best fits for both preparations of oxydodecamer (Table III and Fig. 6). If the four globin chains can form a putative tetramer, then the monomer-trimer equilibrium is,

$$3(a + b + c + d) \leftrightarrow \text{dodecamer} \quad \text{(Eq. 4)}$$

³ R. Klockenkämper, unpublished observations.
Based on the lower r.m.s. error and better correspondence between the fitted and known masses of the dodecamer (213 kDa), the monomer-tetramer model is clearly superior to the monomer-trimer one, suggesting that what is occurring is a "pseudo monomer-tetramer equilibrium."

\[ 3(a + b + c) + (d) \leftrightarrow \text{dodecamer} \]  \hspace{1cm} (Eq. 5)

This is a reasonable interpretation in view of the known self-association of chain d (6). Using 52 and 210 kDa as the putative masses of trimmer and dodecamer (Table III), we calculate from the known \( K_d, \Delta H = -20 \text{ kcal/mol} \) and \( \Delta S = -4.4 \text{ e.u./mol} \) for the tetramerization. We are presently investigating the self-association of chain d.

Kinetics of Oxydodecamer Dissociation—The time courses of oxydodecamer (from urea dissociation) dissociation at neutral pH in the presence of 1.75 molal urea and 1.22 molal GdmCl and at pH 8 (Figs. 7 and 8, Table IV) can be fitted only with two exponentials, suggesting that the underlying mechanism is more complicated than a simple one-step unimolecular decomposition,

\[ D \rightarrow 3T + 3M \]  \hspace{1cm} (Eq. 6)

or a two-step decomposition through an unstable tetramer intermediate,

\[ D \rightarrow 3[T + M] \rightarrow 3T + 3M \]  \hspace{1cm} (Eq. 7)

since a single overall exponential would then be sufficient to fit the data (34). The slower of the two phases of oxydodecamer dissociation in 1.75 molal urea and 1.22 molal GdmCl, \( t_{1/2} \sim 100-200 \text{ h} \) and \( t_{1/2} \sim 4100 - 4600 \text{ h} \) could be due to dissociation of metdodecamer formed by autoxidation, since the metdodecamer was found to dissociate rather rapidly (Fig. 8D, Table IV), requiring only a single exponential fit, with \( t_{1/2} \sim 14 \text{ h} \). It is worth noting that the monophasic dissociation is over 3 orders of magnitude faster than that of the metHb, \( t_{1/2} \sim 14 \text{ h} \) versus 13,000-35,000 h (12). The presence of superoxide dismutase activity in the native Hb but not the dodecamer may be responsible for this disparity in behavior of metHb and metdodecamer.

The heteropolytungstate anion SiW is known to form 1:1 complexes with myoglobin at neutral pH, with association constants in the \( 10^5-10^6 \text{ M}^{-1} \) range and concomitant formation of hemichrome type-visible absorption spectra (35). Since the time course of dissociation of oxydodecamer from urea dissociation in 10 mM SiW (Fig. 8C) can be fitted by a single exponential, its mechanism is likely to be the simple decomposition (1). It is worth noting that the monophasic dissociation in SiW versus the biphasic dissociation in urea, GdmCl, and pH 8 may be related to the much larger size of SiW (3,239 Da) than urea, GdmCl, and OH\(^-\) ion and its consequent inability to penetrate into the dodecamer interior. Likewise, although three exponentials were required to fit the kinetic data obtained for Hb dissociation in urea, GdmCl, and at pH 8, only two were needed for Hb dissociation in SiW (12). Another interesting point was a delay of \( \sim 50 \text{ h} \) before the onset of the dissociation (Fig. 8C).

**Fig. 10.** Effect of Ca\(^{2+}\) and Mg\(^{2+}\) cations on \( P_{50} \) and \( n_{50} \) values of dodecamer from urea dissociation, Hb, and blood at 25 °C. Empty column, at pH 7.86 in the absence of cations; solid column, at pH 7.18 in the absence of cations; hatched column, at pH 7.18 in the presence of 100 mM Mg\(^{2+}\); cross-hatched column, at pH 7.18 in the presence of 100 mM Ca\(^{2+}\).

**TABLE V**

Oxygenation parameters of Lumbricus blood, Hb, and dodecamer

| Parameters | \( P_{50} \) (torr) | \( d^a \) | \( n_{50} \) | \( \Delta H \) (kJ/mol) |
|------------|-------------------|--------|--------|----------------|
| pH         | 7.5               | 7.2-7.8| 7.2    | 7.8            | 7.5 |
| Temp °C    | 10                | 25     | 25     | 25             | 25  |
| Blood      | 3.2               | 8.5    | -0.42  | 5.2            | 9.5 | -45 |
| + 100 mM Mg\(^{2+}\) | 3.7         | -0.62  | 7.0    |                |     |
| + 100 mM Ca\(^{2+}\) | 2.9         | -0.53  | 6.0    |                |     |
| Hb         | 4.0               | 11.5   | -0.24  | 4.5            | 4.9 | -50 |
| + 100 mM Mg\(^{2+}\) | 4.3         | -0.56  | 5.8    |                |     |
| + 100 mM Ca\(^{2+}\) | 3.4         | -0.49  | 5.7    |                |     |
| Dodecamer\(^a\) | 4.1         | 13.5   | -0.19  | 1.8            | 2.2 | -56 |
| + 100 mM Mg\(^{2+}\) | 5.2         | -0.61  | 2.6    |                |     |
| + 100 mM Ca\(^{2+}\) | 4.3         | -0.46  | 2.0    |                |     |
| Dodecamer\(^b\) | 11.7         | 11.9   | -0.17  | 3.2            |     |
| Dodecamer\(^c\) | 11.9         | -0.16  | 2.7    |                |     |
| Dodecamer\(^d\) | 12.3         | -0.15  | 2.1    |                |     |

\(^a\) Bohr coefficient = \( \Delta \log K_o / \Delta \text{pH} \).

\(^b\) From Hb dissociation in 4 M urea.

\(^c\) From Hb dissociation in 0.1 M NaCl, 1 mM EDTA (11).

\(^d\) From Hb dissociation in SiW.

\(^\ast\) From Hb dissociation in AsW.
Clearly, the interaction of SiW with Hb and dodecamer requires further study.

Equilibrium O2 Binding of Dodecamer, Hb, and Blood—The dodecamer preparations and the Hb appear to have similar O2 affinities (P50 = 10–12 torr at pH 7.5, 25 °C) and Bohr coefficients in contrast to blood which exhibits higher affinity (P50 = 8.5 torr) and a more pronounced Bohr effect (Fig. 9, Table V). The cation sensitivities are similar, and the effects of Ca2+ and Mg2+ are comparable, the P50 values are decreased 2.9–3.4-fold in 100 mM Ca2+ and 2.3–2.7-fold in 100 mM Mg2+. The increase in the Bohr effect in the presence of cations results from greater cation-induced increases in O2 affinity at high than at low pH, which suggests ionic interactions. It is appropriate to note that group IIA cations stabilize the quaternary structure of Hb to apparently similar extents, inhibiting dissociation, whether at alkaline pH or at neutral pH and promote reassembly to the HBL structure (12). The oxygenation parameters obtained for Hb in the absence and presence of Ca2+ are in general agreement with earlier findings (36) and results obtained with other earthworm Hbs (37, 38). The differences between P50 and Pm (Table VI) are greater for both dodecamer preparations than for blood or Hb and are due to asymmetry in the O2 binding curves (Fig. 11).

Although there is some variation in the n50 values for blood, Hb and dodecamers (Tables V and VI), the differences are small at pH 6.5 and become important only at pH > 7 (Fig. 9). At pH 7.8, where blood cooperativity is maximal (Fig. 10), the n50 of the dodecamers is ~50% of the Hb and ~25% of the blood values (Table V). The smaller n50 are associated with corresponding increases in log KT (reflected in leftward displacement of the lower asymptotes in Fig. 11), whereas log K50 remains relatively constant. This behavior is consistent with decreasing free energies of heme-heme interaction for blood, Hb, and dodecamer (ΔG = 9.0, 7.6, and 6.2 kJ/mol, respectively, Table VI). The differences in bond energies between the T states of dodecamer and Hb (calculated as ΔGT = RT ln(KT, Dodecamer/KT, Hb)) and between Hb and blood are small, ~1 kJ/mol heme and ~2.0 kJ/mol heme, respectively, for fits where q is free (Table VI). The effect of 100 mM Ca2+ or Mg2+ is to increase the n50 of blood, Hb, and dodecamer by 20–40%. Since the concentration of these ions in earthworm plasma is ~10 mM (38), it is unlikely that the difference in the n50 for blood and Hb could be due to cation effects.

The difference in cooperativity between the dodecamer and Hb is probably due to additional interactions in the native HBL structure, globin-linker interactions between dodecamer and linker chain(s) or globin-linker-globin interactions between neighboring dodecamers mediated by linker chain(s), or both. Furthermore, globin-linker-globin interactions could be either equatorial or axial, within the plane of a hexagonal ring or perpendicular to it. Recent three-dimensional reconstructions by cryoelectron microscopy, of the chlorocruorin from Eud-

![Fig. 11. Extended Hill plots of Lumbricus blood (triangles) at pH 7.29, 25 °C, and at pH 7.43, 10 °C (filled circles) and Hb (squares) and dodecamer from Hb dissociated in urea (diamonds) at pH 7.25 and 7.18, respectively. Dotted lines with slopes of unity correspond to fitted values of K50 and K9 for q = 9 obtained using the MWC model (see Table VI).](image)

**Table VI**

| T | pH  | q  | P50  | n50  | nmax | K50  | S.E.  | K9   | S.E.  | L   | r.m.s. | ΔG  |
|---|-----|----|------|------|------|------|-------|------|-------|-----|--------|-----|
|   | torr| 104 | torr |      |      |      | torr−1|      | torr−1|     |        | kJ/mol |
| Dodecamer | 25  | 7.18 | 4.4  | 15.5 | 1.83 | 1.96 | 0.0360 | 0.0019 | 0.903 | 1.00 | 5.7 × 10^4 | 0.057 | 6.17 |
| Hb  | 25  | 7.29 | 13.2 | 12.3 | 7.24 | 7.31 | 0.0104 | 0.0007 | 0.485 | 0.014 | 3.0 × 10^6 | 0.035 | 7.72 |
| Blood | 25  | 7.29 | 13.2 | 12.3 | 7.24 | 7.31 | 0.0104 | 0.0007 | 0.485 | 0.014 | 3.0 × 10^6 | 0.035 | 7.72 |

*Standard error estimates from the diagonal elements of the curvature matrix associated with the fit.

b Root mean square error, Σ[(x – x) (yi – y)]^2/n, where n = degrees of freedom = no. of data points – number of parameters fitted.

c Dodecamer prepared by Hb dissociation in SiW.*
haustial and Hbs from Macrobdella and Lumbriacus (39, 40), have demonstrated a close similarity of their quaternary structures at ~30 Å resolution. Furthermore, since they all exhibit a relative angular displacement of ~14° between the two hexagonal layers, Lamy has proposed that the relative movement of the latter may be involved in the globin-linker-globin interactions responsible for the full cooperativity in the native Hbs and chlorocruorins. This suggestion also explains the lack of measurable shape alterations in dodecamer and Hb upon change from oxygen to deoxy states observed by SAXS.

The MWC model fails to account for the asymmetry in the O2 binding curves. It is also difficult to find any physical basis for the number of interacting sites determined from the MWC fits when q is free, given in Table VI, q = ~5 for the two dodecamers, ~10 for Hb, and ~12–13 for blood. Since the overall cooperativity in HBL Hbs would be due to two or more different types of concomitant interactions, an adequate description of O2 binding would require a new allosteric model.

Conclusions—The SAXS results show that the molecular shape of oxydodecamer remains unaltered upon deoxygenation and, together with sedimentation velocity results, suggest it to be spherical in solution. Although the O2 affinities of dodecamer and Hb are similar, the dodecamer cooperativity is about half that of the Hb. The oxygenation parameters of dodecamer, Hb, and blood are equally affected by Ca2+ and Mg2+. Thus, it is likely that the dodecamer is the principal functional subunit of the Hb, in addition to being the principal structural subunit (12). Furthermore, our results make it quite clear that the overall cooperativity of an HBL structure is likely to consist of globin-globin interactions within the dodecamer and of linker-mediated globin interactions, whose mechanisms are almost sure of being quite different from the ones known presently.

Acknowledgment—We thank Dr. H. Malte for developing the MWC fitting program and assistance in its use.

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