The Role of the Dodecamer Subunit in the Dissociation and Reassembly of the Hexagonal Bilayer Structure of Lumbricus terrestris Hemoglobin

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The dissociation of the ~3500-kDa hexagonal bilayer (HBL) hemoglobin (Hb) of Lumbricus terrestris upon exposure to Gdm salts, urea and the heteropolytungstates [SiW12O40]6− (SiW) and [Na26[BaAs4W40O140].60H2O] (AsW) appeared to be the key structural feature of HBL reassembly, which was minimal (10%) during HBL reassembly, which was minimal (10%)

without Group IIA cations. During reassembly, maximal (~60%) at 10 mM cation, D occurs at constant levels (~15%), implying the dodecamer to be an intermediate.

The giant, hexagonal bilayer (HBL)† extracellular Hbs and chlorocruorin of annelids and vestimentiferans are ~60 S proteins with an acidic isoelectric point, high cooperativity of oxygen binding, and a characteristically low iron and heme content, about two thirds of normal (1–4). They represent in many ways a summit of complexity for structures containing globins (5). The most extensively studied Hb is that of the common North American earthworm Lumbricus terrestris. Although it has been the subject of numerous studies since Svedberg determined its mass by centrifugation in 1933, the molecular architecture of this complex of ~180 polypeptide chains remains uncertain in the absence of a crystal structure. An early SDS-PAGE study showed that it consisted of at least six subunits (6), four of which were globins, comprising a monomer subunit M (7) and a disulfide-bonded trimer T (8), the remainder being linkers, chains of 24–32 kDa. The amino acid sequences of the T and M subunits have been determined (9, 10). Although only three linker chains were thought to exist (11), only one of which had been sequenced (12), a recent ESI-mass spectroscopy study provided a detailed inventory of all the constituent polypeptide chains and indicated the existence of four linker chains (13). Here we report the results of a study of the dissociation and reassembly of Lumbricus Hb, which support the role of the dodecamer of globin chains [3T + 3M] as a principal intermediate in both processes.

EXPERIMENTAL PROCEDURES

Materials—L. terrestris Hb was prepared as described previously in 0.1 M Tris/Cl buffer, pH 7.0, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, from live worms collected around London, Ontario (Carolina Wholesale Bait Co., Canton, NC) (4, 6). The concentration of the Hb was determined from the absorbance of the native form at 280 nm or of the cyanomet form at 540 nm (4), employing the respective extinction coefficients, 2.063 ± 0.032 mM−1 cm−1 and 0.442 ± 0.013 mM−1 cm−1 (13). The Gdm salts were purum grade from Fluka AG (9470 Buchs, Switzerland) and urea was from Sigma. The heteropolytungstate salts K5SiW12O40.14H2O, 3239.2 Da, Na26[Na26[NaSb9W21O86].24H2O], and Na26[BaAs4W40O140].60H2O, 11,731.7 Da were prepared according to Klemperer (14).

Analytical Gel Filtration—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 5/261 or 526 (Pharmacia). Flow rate was 0.14 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.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis in the presence of 0.1% SDS was carried out using the buffer system of Laemmli (15) and slab gels (1.5 mm × 10 cm × 8 cm) of 8–20% acrylamide. The gels were electrophoresed for 2–4 h, stained in 0.125% Coomassie Brilliant Blue R-250 in 45% methanol, 7.5% acetic acid, and destained in 25% methanol, 7.5% acetic acid.

Optical Spectrophotometry—The absorption spectra over the 200–650 nm range were obtained using an OLIS (Bogart, GA) spectrophotometer employing a Hewlett Packard diode array detector or a Hitachi model 2000 spectrophotometer.

Fitting of Elution Profiles—The elution curves were either digitized on a Summagraphics Summasketch MM18 tablet using a Sigma Scan

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The shape of chromatographic elution peaks (16–18), gaussian and a decreasing exponential and is known to represent well ion 2.0, Jandel Scientific). The EMG function is a convolution of a D, T
four EMGs, each representing the undissociated Hb (HBL) and peaks

\[ f(x) = a_0 \exp \left( \frac{a_2 - a_1}{a_3} x \right) \left[ 1 + \text{erf} \left( \frac{x - a_1}{a_2/a_3} \right) \right] \] (Eq. 1)

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; the falloff rate of the tail is controlled by the parameter \( a_3 \). The areas of the individual peaks were plotted as percent of total time and fitted to sums of exponentials,

\[ f(t) = a_0 \exp[-k_1 t] + a_1 \exp[-k_2 t] + a_2 \exp[-k_3 t] \] (Eq. 2)

using PSI-Plot software (Poly Software International, Salt Lake City, UT) employing the Marquardt-Levenburg method. The acceptability of fits was judged by the absence of systematic trends in the plot of residuals with time.

Dissociation of Lumbricus OxyHb—The dissociating agent was dissolved in 0.1 M TrisCl buffer, pH 7.0, 1 mM EDTA, and Hb stock solution added to obtain the desired concentration, \( \sim 3.6 \) mg/mL. The following dissociating agents were employed: urea, GdmCl, GdmSCN, SiW, SbW, and AsW. The dissociations of oxyHb at neutral pH and at pH 8.0 and 8.2 in 0.1 M TrisCl buffer, 1 mM EDTA, were followed by FPLC at neutral pH.

Oxidation of Lumbricus Hb—The oxidation of Lumbricus oxyHb was effected by the addition of potassium ferricyanide (Fisher) or of sodium nitrite (Aldrich) at molar ratios relative to heme, ranging from 1 to 1000, in 0.1 M TrisCl buffer, pH 7.0, 1 mM EDTA. The conversion of oxyHb to metHb was monitored using optical spectrophotometry over the 450–650 nm range and was complete in 5–20 min. The metHb solution was then immediately passed over a 1.5 \( \times \) 20-cm Sephadex G-25 column to remove the oxidizing agent, and the progress of the dissociation measured using FPLC at neutral pH.

Reassembly of HBL Structure from Completely Dissociated OxyHb—Lumbricus Hb (30–60 mg/ml) was dissociated in the presence of 4–8 M urea in 0.1 M TrisCl buffer, pH 7.0, 1 mM EDTA at room temperature. The completeness of the dissociation to \( T + L \) was checked by FPLC, urea was removed by dialysis against 2 \( \times \) 1.5 liters of TrisCl buffer for \( \sim 2 \) h and the reassociation followed by FPLC in the presence of Group IIA cations Mg\(^{2+}\), Ca\(^{2+}\), and Sr\(^{2+}\) over the 0–50 mM range; the solutions were kept at 7°C. Alternatively, the \( T + L \) fractions were obtained by preparative gel filtration of oxyHb exposed to 4 M urea, pooled, concentrated by pressure filtration using Centricon 10 concentrators (Amicon Division, W. R. Grace & Co., Danvers, MA), and its reassociation followed in the absence and presence of Ca\(^{2+}\).

STEM Imaging and Mass Measurement of Unstained Protein—The mass measurements were performed using the STEM at the Brookhaven National Laboratory (19). Preparation of the unstained specimens with TMV fibers as internal mass standards was carried out as described by Kapp et al. (20). Negative staining was with 0.5% (w/v) uranyl acetate. The STEM was operated at 40 kV, a dose level \(<10 \) e/\( \mu \)m\(^2\) and a resolution of \( \sim 0.25 \) nm. An interactive program (19) was used to select the electron micrographs on the basis of clean background and apparent quality of TMV fibers and protein particles; it computes the background and permits the operator to select the TMV segments for internal mass calibration and the particles for mass measurement. At least 3–5 good TMV segments are generally chosen to calculate the internal mass calibration. The individual particles are selected based on clean background around the particles and absence of visible flaws.

Fig. 1. FPLC elution profile at 280 nm of partially dissociated Lumbricus oxyHb in 0.1 M TrisCl buffer, 1 mM EDTA, pH 7.0. A, exposure to 1.5 M GdmCl; B, exposure to 12.4 mM SiW. The insets show the unreduced SDS-PAGE of native Hb (lane 1) and the indicated fractions. The undissociated peak is labeled HBL, and the three dissociated peaks are the dodecamer D, the trimer and linker subunits \( T + L \), and the monomer subunit M. The profiles were obtained with different columns. Note that in B peak M is overlapped by the SiW peak.

Fig. 2. EMG fits to the elution profiles at 280 nm obtained by FPLC. A, dissociation of oxyHb in 1.22 M GdmCl after 28 h; B, reassociation of completely dissociated oxyHb in 10 mM Ca\(^{2+}\) after 48 h. In A, the buffer also contained 1 mM EDTA. The differences in the elution volumes for the same subunits is due to the use of different columns.
RESULTS

Dissociation of OxyHb by Urea and Guanidinium Salts

Elution Profile of the Dissociation Products—Fig. 1A shows a typical FPLC elution profile of oxyHb dissociated at neutral pH in the presence of 1.5 M Gdm-Cl. In addition to the undissociated Hb (HBL), three peaks are observed, D, T+L, and M, at elution volumes corresponding to approximately 200, 60, and 25 kDa. Each species was determined by resolution of FPLC profiles using the EMG function and is expressed as percent of total area.

Fitting of Elution Profiles—Fig. 2A shows a representative fit of an FPLC elution curve with four EMG functions. The elution volumes of the four peaks remained unchanged throughout the course of the dissociation to ±3%, as did the fitted variables a1 (peak position). Fig. 2B shows a similar fit of an elution curve obtained following reassociation of T+L+M in 10 mM Ca2+.

Dissociation of OxyHb at Zero Time—In these experiments, the oxyHb was loaded on the column right after mixing with the dissociating agent and subjected to FPLC. The time elapsed between mixing and complete penetration of the sample into the column was 110–120 s. Plots of percent dissociation versus the concentration of the dissociating agent are shown in Fig. 3A.

Fig. 3. Zero time dissociation of Lumbricus oxyHb. A, percent undissociated oxyHb versus concentration of GdmSCN, GdmCl, urea, and GdmOAc. B, percent undissociated oxyHb (HBL) and peaks D, T+L, and M versus concentration of GdmSCN and GdmOAc. Each species was determined by resolution of FPLC profiles using the EMG function and is expressed as percent of total area.

Time Course of OxyHb Dissociation in 4 M Urea and the Effect of Ca2+—Fig. 4A shows the time course of oxyHb dissociation in 4 M urea; although it is almost complete within 2 h, peak D remains constant indicating its stability in 4 M urea. Fig. 4B shows the time course of oxyHb dissociation in 4 M urea in 2.5 mM Ca2+; it can be fitted as the sum of two exponentials. However, since substantial dissociation occurs within the dead time of the FPLC method (~2 min), there appear to be at least three separate dissociation processes with apparent t1/2 ~ 1 min, ~1 h, and ~50 h. Fig. 4C illustrates the effect of [Ca2+] on the dissociation of oxyHb in 4 M urea after 144 h.

Fig. 4. Time course of Lumbricus oxyHb dissociation in 0.1 M Tris-Cl buffer, pH 7.0. A, in 4 M urea and 1 mM EDTA; B, in 4 M urea and 2.5 mM Ca2+. The dotted lines show the two exponential functions fitted with the resulting residual below. C, in 4 M urea after 144 h as a function of [Ca2+].
**Fig. 5.** Time course of Lumbricus oxyHb dissociation in 1.75 mM urea in 0.1 M TrisCl buffer, pH 7, 1 mM EDTA. A, peak HBL; B, dodecamer; C, T + L subunits; D, M subunit, expressed as percent of total area. The insets show the dissociation over the first 200 h. The fits shown are to the sum of the three exponentials together with the resulting residuals. Note that the dodecamer reaches a maximum after ~250 h (B) and then decreases and the absence of any induction period in the formation of peaks T + L and M (C and D, respectively).

**TABLE I**

Kinetic parameters for Lumbricus oxyHb dissociation

| Exp. | $a_1$ | $k_1 \times 10^{-5}$ | $t_{1/2a}$ | $a_2$ | $k_2 \times 10^{-5}$ | $t_{1/2a}$ | $a_3$ | $k_3 \times 10^{-5}$ | $t_{1/2a}$ | $r^b$ |
|------|-------|----------------------|-----------|-------|----------------------|-----------|-------|----------------------|-----------|-------|
| 1.75 mM Urea | 16    | 49,000               | 1.4       | 25    | 2500                 | 28        | 54    | 140                  | 500       | 0.997 |
| 1.22 mM GdmCl | 25    | 33,000               | 2.1       | 35    | 1300                 | 53        | 36    | 180                  | 390       | 0.998 |
| pH 8.0 | 18    | 3100                 | 22        | 66    | 60                   | 1200      | 0.994 |
| pH 8.2 | 20    | 36,000               | 2         | 10    | 1400                 | 50        | 0.989 |
| 4 mM SiW | 45    | 1800                 | 39        | 56    | 53                   | 1300      | 0.989 |
| 12 mM SiW | 61    | 5600                 | 12        | 35    | 160                  | 430       | 0.993 |
| K$_3$Fe(CN)$_6$ | 11    | 32,000               | 2         | 89    | 5                    | 13,000    | 0.989 |
| NaN$_3$ | 10    | 37,000               | 2         | 90    | 2                    | 35,000    | 0.990 |

$^a$ $t_{1/2} = 0.693k$.

$^b$ The correlation coefficient $r = \frac{\sum XY}{(\sum X^2)(\sum Y^2)^{1/2}}$, where $X = x_i - x$ and $Y = y_i - y$.

$^c$ Dissociation subsequent to conversion of oxyHb to the met form and removal of oxidizing agent by gel filtration.

Kinetics of OxyHb Dissociation—Fig. 5A shows the time course of oxyHb dissociation in 1.75 mM urea and Fig. 5 (B–D), show the corresponding time courses of peaks D, T + L, and M, respectively. The insets show the initial phases. The points shown represent averages of values determined in three separate experiments. For all the processes shown in Fig. 5 and for oxyHb dissociation in 1.22 mM GdmCl (results not shown), at least three exponentials were necessary to obtain a good fit as
judged by the absence of any trends in the plots of residuals versus time provided at the bottom of each panel. Table I summarizes the amplitudes and kinetic constants determined from the fits.

**STEM Imaging and Mass Mapping of Undissociated OxyHb and Peak D**

Fig. 6 shows views of unstained, cryo-lyophilized undissociated oxyHb obtained at 11% (A and C) and 89% (B and D) dissociation, respectively. Fig. 7A shows a histogram of the STEM masses of the complete HBL structures observed at 89% dissociation. Although the mean mass, 3540 ± 260 kDa (n = 120), is similar to the value 3560 ± 130 kDa obtained previously for native Hb (13), the distribution of masses is more asymmetric at the lower end.

Fig. 6 (E and F) shows typical views of unstained, cryo-lyophilized peak D obtained by dissociation in SiW and at pH 8.3, respectively; the observed particles are ~10 nm in diameter and histograms of the STEM masses within the range 150–250 kDa (Fig. 7, B and C) had corresponding mean masses of 200 ± 26 kDa and 195 ± 21 kDa, respectively.

**Dissociation of OxyHb by Heteropolytungstates, at Alkaline pH and upon Conversion to MetHb**

The complex heteropolytungstate anions SiW$^{8-}$, SbW$^{18-}$, and AsW$^{27-}$ are known to form 1:1 complexes with metMb at neutral pH with association constants in the $10^3$ to $10^6$ M$^{-1}$ range and concomitant formation of hemichrome type visible absorption spectra (21). All three dissociate oxyHb; a typical elution curve is shown in Fig. 1B. Fig. 8 (A and B) shows the time courses of dissociation in 4.12 mM and 12.4 mM SiW, together with the fits to sums of two exponentials. It is well known that HBL Hbs dissociate at $\text{pH } 8$ (7, 22). Fig. 8 (C and D) shows the time courses of dissociation at pH 8.0 and 8.2. Again, it is evident that a third, rapid phase occurs within the dead time of the FPLC (~2 min). Thus, there appear to be three dissociation processes with $t_1/2$ ~ 1 min, 2–22 h, and 50–1200 h.

An early observation by Ascoli et al. (23) suggested that oxidation of earthworm Hb led to the dissociation of its quaternary structure. We reinvestigated this phenomenon because Lumbricus oxyHb was slowly altered to the met form during the dissociations in urea and GdmSCN. Fig. 8 (E and F) shows the time courses of dissociation following the conversion of oxyHb to metHb and the removal of oxidant by gel filtration. The fitted parameters for all the dissociations are provided in Table I.
Reassembly of HBL Structure

Fig. 9 shows some representative results obtained with the reassembly of HBL structures from completely dissociated oxyHb. In the absence of Group II A cations, reassembly was limited, generally much less than 10%. However, in the first 24 h, there is a spontaneous formation of dodecamers illustrated in Fig. 9A. The same result is also observed in Fig. 9B, which depicts the time course of reassembly to HBL in 5 mM Mg²⁺. Fig. 9C illustrates the effect of cation concentration on the extent of HBL reassembly, and Fig. 9D shows that although there may be differences in the extent of reassembly achieved initially, the final [HBL] is remarkably similar for all three cations after 200 h.

Reassociation, starting with peaks T+L and M isolated by gel filtration of oxyHb dissociated in 4 M urea, shows that a spontaneous reassociation of T and M to about 20% D had occurred within 6 h prior to the first FPLC (Fig. 10), even though reassembly to the HBL was almost nonexistent (1%). Fig. 10 also shows the reassembly time courses in 2.5 mM and 10 mM Ca²⁺; although the relative contents of T and M declined steadily, the level of peak D remained fairly constant at 10–15%. STEM images of unstained HBL[T+L+M] are indistinguishable from those of native Hb, and the mass distributions are similar to those determined for native Hb (13). The time courses of HBL reassembly could be fitted reasonably well with a single asymptotic exponential.

DISCUSSION

A Dodecamer [3T+3M] Is Observed in All Dissociations of Lumbricus OxyHb—The dissociation of the HBL structure at neutral pH by Gdm salts and heteropolytungstate anions and at mildly alkaline pH (Figs. 1 and 3) provide remarkably similar pictures; a 200-kDa dodecamer D ([3T+3M]), deficient in linker subunits, is always formed in addition to the M, T, and L subunits. In particular, dissociation in the weakest dissociating agent, namely Gdm-OAc (Fig. 3), shows that D accounts for about half of the initial dissociation products. The time course of dissociation in 4 M urea (Fig. 4A) also shows that D accounts for 40–50% of the dissociation products. In addition, it appears that D is fairly stable in the presence of 4 M urea, in agreement with earlier findings (24).

Fig. 3 summarizes the effect of urea and several Gdm salts on the dissociation of Lumbricus oxyHb determined by FPLC at zero time. The order of decreasing effectiveness is Gdm-SCN > Gdm-Cl > urea > Gdm-OAc, with the order of the anions in line with the well known Hofmeister series (25, 26). The order of increasing effectiveness of the three heteropolytungstates, SiW < SbW < AsW, appears to be correlated with
their total charge and mass, −8 (3239 Da), −18 (7178 Da), and −27 (11,732 Da), respectively, and not with the surface charge density. Although SiW is spherical, SbW is a trigonal pyramid, and AsW is a parallelepiped, the charge per unit area is approximately the same: −1.8, −2.0, and −2.1/100 Å², respectively (27).

Effect of Ca²⁺ on Urea Dissociation of OxyHb—Ca²⁺ exerts a markedly protective effect on the quaternary structure of oxyHb in the presence of 4 M urea (Fig. 4). Although dissociation is almost complete (−95%) after 2 h in 4 M urea (Fig. 4A), even 2.5 mM Ca²⁺ reduces dissociation to −75% after 144 h in 4 M urea (Fig. 4B). The maximum protective effect is reached at [Ca²⁺] ≈ 10 mM (Fig. 4C). Alkaline earth (Group IIA) cations are known to stabilize the HBL structure of annelid Hbs with respect to dissociation at alkaline pH (22, 28, 29), at acid pH (30, 31), as well as thermal unfolding and autoxidation (32). In some cases, such as Amphitrite Hb (33) and Myxococar chlorocruorin (34), Ca²⁺ is necessary for maintaining the HBL structure even at neutral pH.

The Kinetic Heterogeneity of Lumbricus OxyHb Dissociation—Our results show that dissociation of oxyHb followed by FPLC over several weeks is not accompanied by alteration in the properties of either the starting material or the products. 1) The elution volumes of the undissociated Hb (HBL) and of the products of its dissociation (peaks D, T+L, and M) remain unaltered. 2) The subunit compositions of all the peaks as judged by SDS-PAGE remain unchanged. 3) The STEM images of the HBL peak at an early (10%) and a late stage of dissociation (89%) indicate no major alterations in dimensions (Fig. 6, A–D). Furthermore, the STEM mass distribution at 89% dissociation (Fig. 7A) compared to that of the native Hb (13) exhibits only a slight asymmetry at the low end, probably due to the presence of a relatively small number of "deficient" HBLs, missing 1/6 and 2/6 of the HBL structure that can be observed in Fig. 6 (A–D).

The time courses of oxyHb dissociation in 1.75 M urea (Fig. 5A) and 1.22 M GdmCl at neutral pH can be satisfactorily represented as the sum of three first-order processes with $t_{1/2} \approx 1–2$ h, 30–50 h, and 400–500 h (Table I). Fig. 4 also shows that there are at least three processes occurring in the dissociation of oxyHb in 4 M urea in the absence and presence of Ca²⁺.

Three first-order processes are also observed in oxyHb dissociation at alkaline pH, $t_{1/2} \approx 1$ min, 2–20 h, and 50–1200 h (Table I). OxyHb dissociation in the presence of SiW (Fig. 8, A and B) can be fitted with two first-order processes, $t_{1/2} \approx 10–40$ h and 400–1300 h (Table I). The latter values correspond roughly to the $t_{1/2}$ for the two slower dissociation processes in urea and GdmCl and at alkaline pH.

Two points must be considered before discussing possible mechanisms for the dissociation of Lumbricus oxyHb. 1) Whether slow oxidation of oxyHb to metHb could be responsible for one of the dissociation processes observed. MetHb dissociation (Fig. 8, E and F, and Table I) consists of two phases: a small (−10%) initial dissociation ($t_{1/2} \approx 2$ h), followed by a dissociation that is slower by more than 1 order of magnitude than the slowest phase of the oxyHb dissociations ($t_{1/2} \approx$...
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3.4% dissociation (Fig. 6, 1) of D from (1) and (3) to T simultaneous. does the appearance of the final product C in the foregoing

tations A-L and M. STEM images of Hb at 11% Cl requires two exponentials for a satisfactory fit with

The dissociation of the metHb. It is likely that oxydodecamer dissociation (t1/2 ~ 100–200 h) occurs mostly in the later stages of oxyHb dissociation, following the accretion of peak D observed in the first 50–300 h (Fig. 5B).

There seem to be two simple explanations for the kinetic heterogeneity of oxyHb dissociation. 1) Since peak Hb, whose area is a measure of undissociated Hb structures, contains "complete" HbLs as well as the deficient HbLs lacking 1/6 and 2/6 of the structure, one explanation is that the observed three first-order processes reflect the dissociation of the complete and deficient HbLs. However, the STEM appearance and STEM mass distributions at a late stage of dissociation (Figs. 6, B and C, and 7A) indicate the presence of limited numbers of deficient HbLs. 2) Another possibility is that the native Hb consists of four unequal populations of Hb structures differing in their stabilities toward dissociation, each population of HbLs exhibiting its own rate of dissociation in the presence of a given concentration of the dissociating agent. In this view, the deficient HbLs are likely intermediates in the overall dissociation processes observed in urea and GdmCl and at pH 8.0 (t1/2 ~ 22–53 h and 400-1200 h, Table I).

Role of the Dodecamer in Hb Structure Reassembly—Our results (Figs. 9 and 10) demonstrate that dodecamers are formed both in the presence and absence of the linker subunits L and in the absence and presence of Group IIA cations. Likewise, in the presence of Mg2+ and Sr2+ (Fig. 9B), but not Ca2+, there is an increase in D which occurs in the first 24 h, prior to the formation of any significant amount of HbL. These facts suggest that formation of the dodecamer precedes that of HbL and that the dodecamer is an obligatory intermediate in the reassembly of the HbL structure (Fig. 11C). In the presence of Ca2+ (Fig. 10), the formation of HbL is accompanied by only a small decrease in D, the latter remaining at a fairly constant level between 10 and 15%. At optimum concentrations of Mg2+,
Ca\(^2+\) and Sr\(^2+\) ~10 mM in all three cases (Fig. 9C), the extent of HBL reassembly reaches 50–60%, again indicating a dominant role for Group IIA cations in stabilizing the HBL structure.

In contrast to the kinetic heterogeneity of HBL dissociation (Figs. 4, 5, and 8), the time course of HBL reassembly (Figs. 9 and 10) is readily fitted with a single asymptotic exponential. The first step of dodecamer formation (Fig. 11) appears to be relatively fast; hence, the observed process is likely to be the second step of dodecamer combination with linker subunits to form HBL structures (Fig. 11). Peaks intermediate between relatively fast and D occur in the elution profiles of reassociating mixtures (peaks 11 and 12 in Figs. 2B and 4B). At present, we do not know whether they are intermediates or reassembly-incompetent side-products.

Is Subunit Stoichiometry Constant in HBL Structures?—Native Lumbricus Hb examined by STEM mass mapping and sedimentation equilibrium, exhibits a fairly broad range of masses from 3200 to 3900 kDa (13). HBL structures can be reassembled from T+L subunits (38), and we have recently shown them to have STEM masses ranging from 2500 to 3600 kDa.\(^3\) Although the distribution is asymmetrical with a tail at lower masses than 3000 kDa, the surprising observation is that a considerable fraction of the masses are higher than 3000 kDa, the mass of native Hb, 3560 kDa, minus the contribution of the M subunit, 575 kDa (13). A possible explanation is that there may occur extensive formation of “pseudo-dodecamers” consisting of 4T subunits instead of [3T + 3M], which preserves the local 3-fold symmetry found in the dodecamer crystal (39).

Furthermore, the recent three-dimensional reconstructions from cryoelectron microscopic images of Eudistylia chloro-cruorin, Macrobdella Hb, Lumbricus Hb, and reassembled HBL missing one of the linker subunits of Lumbricus Hb by Lamy and collaborators (40, 41)\(^4\) demonstrate that all the HBL structures are very similar. An obvious explanation is that HBL structures may not require a fixed stoichiometry of globin and linker subunits. Hence, structural heterogeneity of Lumbricus Hb may lie at the heart of the kinetic heterogeneity of its dissociation.

Conclusion—The results presented here extend our earlier findings (24, 42) and provide conclusive evidence for the dodecamer [3T + 3M] being the principal structural intermediate in the dissociation and reassembly of Lumbricus Hb.

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