Assembly of the Gigantic Hemoglobin of the Earthworm

*Lumbricus terrestris*

ROLES OF SUBUNIT EQUILIBRIA, NON-GLOBIN LINKER CHAINS, AND VALENCE OF THE HEME IRON*

(Received for publication, June 28, 1996, and in revised form, September 5, 1996)

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The extracellular hemoglobin of the earthworm *Lumbricus terrestris* has four major kinds of O₂-binding chains: a, b, and c (forming a disulfide-linked trimer), and chain d. Non-heme, non-globin structural chains, "linkers," are also present. Light-scattering techniques have been used to show that the ferrous CO-saturated abc trimer and chain d form an (abcd)₄ complex of 285 kDa at neutral pH. Formation of the full-sized 4-MDa molecule requires the addition of linker chains in the proportion of two linkers per (abcd)₄ and occurs much more rapidly in the presence of 10 mM calcium. This stoichiometry is supported not only by direct quantitative analysis of the intact hemoglobin but also by the fact that the addition of 50% of the proposed stoichiometric quantity of linkers results in the conversion of 50% of the (abcd)₄ to full-sized molecules. Isolated CO-saturated abc trimers self-associate to (abc)₂ and higher aggregates up to an apparent limit of (abc)₁₀—550 kDa. The CO-saturated chain d forms dimers, (d)₂, and tetramers, (d)₄. Oxidation of the (abcd)₄ complex with ferri cyanide causes complete dissociation of chain d from the abc trimer, but addition of CN⁻ maintains the (abcd)₄ complex. Valence hybrids have also been studied. The ferrous CO-saturated abc trimer and met (ferric) chain d also associate to form (abcd)₄, but the met abc trimer and ferrous CO-saturated chain d do not. Oxidation of the abc trimer and chain d to the ferric form causes the formation of a characteristic hemichrome spectrum with a maximum at 565 nm and a shoulder near 530 nm. These results show that interactions between the abc trimer and chain d are strongly dependent on the ligand and valence state of the heme iron. Light-scattering measurements reveal that oxidation of the intact Hb produces a significant drop in molecular mass from 4.1 to 3.6 MDa. The addition of CN⁻ prevents this drop. These experiments indicate that oxygenation causes the Hb to shed subunits. The observations provide an explanation for the wide variations in the molecular mass of *L. terrestris* Hb that have been observed previously.

The extracellular hemoglobins of annelids were first shown by Svedberg and Eriksson (1) to be gigantic molecules of at least 3 MDa in molecular mass. Although several subsequent studies gave molecular masses close to 4 MDa or higher for the Hb of *Lumbricus terrestris* and related species (2–4), recent studies by scanning transmission electron microscopy (5) have suggested masses of 3.5–3.6 MDa, and early light-scattering measurements suggested even lower values (6, 7). A possible reason for this great variation in reported molecular masses is oxidation, which has been shown to cause extensive dissociation of many annelid Hbs (8–10). Goss et al. (9) used light scattering to show that oxidation of *L. terrestris* Hb at neutral pH caused a large drop in molecular weight. Similarly, Ascoli et al. (10) found that oxidation of the similar Hb from *Octolasium complanatum* caused hemichrome formation and dissociation outside of a narrow range near pH 7.

The stoichiometry has also been uncertain. The Hb of *L. terrestris* has four major kinds of O₂-binding globin chains: a, b, and c (forming the disulfide-linked abc trimer) and chain d, together with non-heme structural chains, "linkers," designated L. Kapp et al. (11) and Vinogradov et al. (12) have reported that linkers comprise 33–36% of the total mass, but this conclusion was based largely on the staining of SDS-gels with Coomassie Blue. This dye, however, binds to different proteins to quite different extents (13, 14). Ownby et al. (15) redetermined the stoichiometry by reverse-phase high-performance liquid chromatography (HPLC). The weight proportions of linkers were found to be approximately 16.4% by two independent procedures: the integrated absorption of the HPLC peaks and amino acid analysis of these peaks. These results led to the conclusion that the overall stoichiometry is (abcd)₄ L. This stoichiometry is further supported by the results of SDS-capillary gel electrophoresis monitored at 214 nm (see our companion study (16)).

The goals of the present experiments are to redetermine the molecular mass by light scattering, to examine the equilibria between subunits, to investigate the effects of oxidation, and to examine the role of the linker chains in assembly. Vinogradov et al. (12) were the first to conclude that these chains are required for assembly. This conclusion was based on the finding that preparations of the abc trimer and chain d subunits

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* This work was supported in part by National Institutes of Health Grant GM 35847 and National Science Foundation Grants MCB 9205764 and 9511759 (to A. F. R.) and GM 48278 (to J. K. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 Reidentified as this species. See footnote 5 in Kapp et al. (11).

2 The abbreviations used are: HPLC, high performance liquid chromatography; bis-tris propane, 1,3-bis[(hydroxymethyl)methylamino]propane; DTT, dithiothreitol.
that were deficient in linkers failed to form full-sized molecules. This by itself does not exclude the possibility that the preparative procedure might have rendered the linker and chain d incompetent to assemble. A final goal of these studies is, then, to reassemble the full-sized hemoglobin by titrating an equimolar mixture of abc trimer and chain d with linkers. The titration itself should provide a further determination of the stoichiometry.

MATERIALS AND METHODS

Preparation—The CO-derivative of L. terrestris Hb was prepared as described previously (15). The HbCO was either (i) used directly, (ii) used for subunit preparation, (iii) frozen at −80 °C, or (iv) kept in 50% saturated (NH₄)₂SO₄ for a year at 0 °C under a CO atmosphere. We emphasize that saturation with CO is crucial because CO is itself a reducing agent (17) that helps to maintain the hemes in the ferrous state. All preparative steps were carried out at 0−4 °C.

The first subunits (preparation 1) were obtained by chromatography on an ACA44 Ultrogel column (LKB), 5 × 55 cm, with CO-saturated 0.1 M borate, 1 mM EDTA, pH 9.3, exactly as described previously (18). The linker, trimer, and chain d fractions were pooled and rechromatographed on the same column. The fractions were concentrated by an Amicon concentrator (YM-10 membrane) filled with CO prior to applying N₂ pressure. HPLC analysis performed as described elsewhere (15) showed that at least 90% of the fractions of linker, trimer, and chain d preparations were 0.045 and 0.005, respectively. These preparations were used to measure the pH and concentration dependence of self-association of abc (Fig. 7, a and b).

Preparation 2 of subunits was similar to the first except that Sephacryl S-200 HR (Pharmacia Biotech Inc.) was substituted for ACA44. This preparation yielded trimer and chain d fractions with weight proportions of linkers of 0.0115 and 0.0015, respectively, by HPLC analysis (15) (data not shown). This preparation was used in some of the titrations of abc + d with linkers (Fig. 12, a and b). The linkers used in these titrations were isolated on a column of DEAE-cellulose (DE52, Whatman), 2.5 × 8.0 cm, with a linear gradient between buffer A, CO-saturated 20 mM borate, pH 9.3, and buffer B, the same buffer with 350 mM NaCl and a flow rate of −1 ml/min. Fresh HbCO (271 mg in 4.5 ml) was dialyzed against buffer A overnight, and then was applied to the column equilibrated with buffer A. Approximately 25 ml of buffer A was passed through the column before starting the gradient. The linker chain fraction, eluting between 115 and 130 ml, was identified with a 280 nm/576 nm absorbance ratio of 6.37 ± 0.21 for the fraction. HPLC analyses (data not shown) gave the following polypeptide composition for the linker fraction: 62.6% linkers, 29.4% trimer, and 8.0% chain d. In the titration of (abc)d, with 100% of the stoichiometric quantity of linkers, the absorbance at 576 nm increased by 7.4%, and the linkers in the abc + d preparations increases the linkers by 4.8%. Application of these values to the data of Fig. 12b does not alter the total percent linkers. Residual linkers in abc and d were allowed for in the calculations for Fig. 12b.

Preparation 3 was similar to preparation 2 in use of Sephacryl S200 but did not use DEAE cellulose for linker isolation. The trimer and chain d fractions contained weight fractions of linkers of <0.01 and 0.054, respectively. The linker chain fraction contained 17.6% linker and 24.4% chain d. Preparation 3 was used in the experiments of Figs. 6 and 8–10. The components in each fraction were considered in the reassembly experiments.

The following conditions gave poor separations: (a) either high or low ionic strength (2 M NaCl or 0.024 M borate, pH 9.3); (b) 4 M urea, pH 4.3, 4 °C (extensive oxidation); (c) application of sample to the column without prior dialysis; and (d) chromatography at room temperature because of extensive oxidation.

Part of the (NH₄)₂SO₄ preparation was processed further as follows. The HbCO, kept for 1 year at 0 °C under a CO atmosphere in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.0. The K₉Fe(CN)₆ was added in the same buffer. Light-scattering experiments were performed on these solutions with the size-exclusion column equilibrated with the same buffer. (ii) In the second set of experiments, HbO₂ (freshly prepared, unfrozen) with and without calcium was prepared with Ca²⁺, 25 mM bis-tris propane, 100 mM NaCl, 1 mM EDTA, pH 7.0. The latter preparation without calcium is the same solution used for the dry weight determinations (16). Aliquots of each of these solutions were oxidized in the dark by adding K₉Fe(CN)₆ as 10, 25, 50, 75, 90, or 100% of the molar quantity of heme-iron. The actual percent oxidation was estimated from the change in absorbance. The end point of the absorbance change was calculated approximately from the ratio between the extinction coefficients for human HbO₂ and metHb at 576 nm because slow transformation of the L. terrestris metHb to the hemichrome precluded direct determination of the end point.

Light Scattering—A DAWN model F multangle light-scattering photometer (Wyatt Technology, Santa Barbara, CA) with a 5-milliwatt He-Ne vertically polarized laser of wavelength 632.8 nm was used in all experiments. The construction and principles of operation of this instrument together with the associated interferometric refractometer hardware and software have been described in detail (22). The photometer is equipped with a 55 cm, 10 mm path length (2 cm internal diameter tubing). The flow rate for all experiments was 0.1 ml/min. A Beckman model 110A HPLC pump with a Waters model 440 absorbance photometer was used to deliver samples through a switching valve (catalog no. V240, Upchurch) either directly to the photometer or first through a Bio-Gel SEC 40Xl column (300 × 7.8 mm, Bio-Rad catalog no. 125-0604). Experiments shown in Fig. 13, a and b, used a Waters Protein-Pak 300 SW column (300 × 7.8 mm, catalog no. 800B). Injections loops of 20 μl, 180 μl, and 1 ml were used, depending on the experiment. Connections were made with Upchurch “PEEK” 0.1-inch internal diameter tubing. The flow rate for all experiments was 0.3 ml/min. A model LP-21 “Low-Pulse” pulse damper (Scientific Systems, Inc., State College, PA) was inserted after the pump. An Optilab-R903 interferometric refractometer (Wyatt Technology, Santa Barbara, CA) was connected in-line following the light-scattering photometer. The P2 sample cell used in the refractometer has a path length of 0.2 mm. A Hitachi model 100-10 uv-visible spectrophotometer with flow cell in the fluid line follows the refractometer.

Photometer Calibration—Calibration was performed as described in the Wyatt manual. The relationship between molecular weight and light-scattering is given (23, 24) by the equation:

\[ R_C = \frac{1}{R_L} = M_w \frac{F(\theta)}{2BC + \cdots} \]  

(Eq. 1)

°Ferricyanide is light-sensitive (20).
The Wyatt Optilab model 903 interferometric refractometer measures the difference between the refractive indices of sample and reference cells. The refractometer has the following features (22). Collimated light polarized at 45° is split by a Wollaston prism into vertically polarized sample and horizontally polarized reference beams. Recombination of these beams by a second Wollaston prism followed by a quarter-wave plate and analyzer filter gives a plane polarized beam rotated with respect to the initial 45°. The rotational phase difference is proportional to the refractive index difference between solvent and solution. An interference filter with maximal transmission at 630 nm is placed in front of the detector. A three-way valve makes possible fluid flow through both sample and reference cells (flush) or only through the sample cell. The cells are thoroughly flushed at a flow rate of 0.3 ml/min with deionized "nanapure" HPLC-grade water (resistance = 17.2 megohm) filtered through a 0.2-μm nylon filter (Schleicher & Schuell, catalog no. 00040). The valve is then turned so that fluid flows only through the sample cell. The temperature is maintained at 25°C for all experiments with water from an external bath. Calibration provides the difference in refractive index between sample and reference cells per volt from the signal detector. Both NaCl and sucrose were used as calibrants. NaCl (Aldrich, catalog no. 20–4439), 99.999% pure, was found to give satisfactory results with the value of the refractive index increment, dn/dc, taken to be 0.1741 ml/g at 25°C and 589 nm (25). This value was used in all calculations. Interpolation of the data of Krusi (26) yields a slightly higher value, 0.1744 ml/g at 632.8 nm and 25°C. The NaCl (Aldrich) was dried at 180°C overnight, weighed, and dissolved in a 1-liter volumetric flask with nanopure water passed through a 0.2-μm filter. All dilutions were made with great care using a single calibrated Pipetman. Calibration and dn/dc measurements were made either “off-line” directly into the refractometer by siphon or through the HPLC system but bypassing the column. For the latter procedure, a 1-ml injection loop was used with a flow rate of 0.3 ml/min. Sucrose (Baker, analytical reagent, catalog no. 4072–01) with a dn/dc value of 0.1422 ml/g (25) was used as a second standard. The calibration constants obtained from NaCl and from sucrose agreed to within 0.2%. The calibrating solutions were not filtered because filtration was found to give poor, non-reproducible results. Recalibration with sucrose one year later gave an identical result.

**Determination of the molecular mass by light scattering is critically dependent on the accurate determination of dn/dc. This was measured for L. terrestris Hb as follows. The last diastase used for the dry weight and hemoglobin determination (see our companion study (16)) was also used to obtain dn/dc. Dilutions were made by weight from the stock solution (0.1642 mg/mL heme). The dry weight determination (16) resulted in a dn/dc value of 0.185 ml/g. An identical value was obtained by assuming the (abcd)_L stoichiometry previously determined (15, 16) together with the measured heme concentration of 0.1642 mg/mL. The weight concentration c, in each slice in the elution pattern was calculated from the refractive index difference and the value of dn/dc, n_i - n_s = (dn/dc)_i.

The refraction per gram of amino acids has been determined by McMeekin et al. (27). These values, multiplied by the partial specific volumes (28) for each amino acid, should provide an estimate of the value of dn/dc for each amino acid. Values of dn/dc for the polypeptides of L. terrestris Hb, calculated on this basis, together with the weight-average value for the intact Hb, are given in Table I. We have assumed that the subunits of L. terrestris Hb have the same dn/dc value found for the whole molecule. Although partial specific volumes calculated from amino acid compositions have been widely used in ultracentrifugation, calculation of dn/dc for proteins from their amino acid composition remains to be tested for its possible general applicability.

**Interdetector Volume**—The volume, V_i, between the scattering volume of the photometer and the refractometer cell must be determined so that the light scattering and refractive index data can be properly matched. If the V_i value is too large, it will produce a positive slope in a plot of molecular mass versus elution volume for a monodisperse substance, and if too small, the slope will be negative (30). A plot of this slope against various values of V_i in one set of experiments with test proteins produced a straight line; the intercept corresponding to a zero slope gave a V_i volume of 91 μl at a flow rate of 0.3 ml/min. Alternatively, the data from the photometer and refractometer were matched with program “Align” (Wyatt Astra Program Version 2.11). Small changes in flow rate produce changes in the time interval required for a given element of fluid to reach the sample cell of the refractometer from the photometer. This time difference will appear as a change in apparent interdetector volume.

**Band Broadening**—Some spreading of the chromatographic peak occurs between the light-scattering volume in the flow cell and the refractometer (31). The effect of this dispersion for a sharp peak is to decrease the concentration at the peak maximum below that “seen” in the light-scattering cell so that the calculated molecular weight is
slightly too high. Similarly, down the sides of a sharp peak, the concentration increases so that the calculated molecular weight is too low. The overall effect is a small "frown" in the plot of the calculated molecular weight versus elution volume. However, if the weight-average molecular weight is calculated for an entire chromatographic peak for a monodisperse protein, these effects cancel out if the value of the second virial coefficient, $B$, is zero or if the concentration is sufficiently low so that the $B$ term can be neglected. This can be shown as follows. The light scattering Equation 1 gives the relation between the angle-dependent Rayleigh ratio and molecular weight.

$$
\frac{K_c}{K_v} = \frac{1}{M_i} + B_c
$$

(Eq. 4)

The function $P(\theta)$ in Equation 1 is omitted here because all data are extrapolated to zero angle. We assume $B = 0$. The actual concentration determined in the refractometer, $c_i'$, differs slightly from $c_i$ in the photometer, leading to the calculation of the wrong molecular weight, $M_i'$.

$$
\frac{M_i'}{M_i} = \frac{1}{\sum c_i' M_i'} = \frac{1}{\sum c_i M_i}
$$

(Eq. 6)

This shows that the band-broadening error cancels out if the weight-average molecular weight is calculated for the entire peak or half-peak for a monodisperse substance at sufficiently low concentrations. However, the light scattering data for most of the test proteins indicate some self-association. Use of the trailing half-peak appears to avoid complications from self-association in these proteins (see Table II). A P2 refractometer cell (Wyatt) was used in all of the experiments described in this report. The P10 cell (Wyatt) has been developed to eliminate or reduce secondary band broadening by ensuring complete mixing so that a uniform front enters the cell.

**Test Proteins**—Seven different proteins were examined by light scattering. Five of these were obtained from Sigma and were used without further purification: bovine carbonic anhydrase (catalog no. C7025), yeast alcohol dehydrogenase (catalog no. A8656), sweet potato $\beta$-amylase (catalog no. A8781), horse spleen apoferritin (catalog no. A3660), and bovine thyroglobulin (catalog no. T9145). Each of these was prepared DTT just before use. A dithiothreitol (DTT) solution in 100 mM phosphate, pH 7.0, 0.5 mM EDTA, was dialyzed overnight at 0°C against the same buffer made 1 mM in freshly prepared DTT just before use. A dithiothreitol (DTT) value of 0.19 cm$^3$/g was assumed for all of the above proteins. Hemocyanin from Octopus dolfinei, a gift from Dr. Karen Miller, was dialyzed at 0°C against 100 mM Tris-HCl, pH 7.6 (at room temperature), 50 mM MgCl$_2$ prior to use. The $dn/dc$ value was taken to be 0.194 cm$^3$/g, a value obtained for several hemocyanins from other species of octopus (34).

**Lactobacillus sp. strain 30a (32, 33), a gift from Dr. Marvin L. Hackert, was prepared similarly. The concentration of the decarboxylase was determined with an extinction coefficient, $\varepsilon_{280nm}$, 1.34 (32). A $\sim$1.6 mg/ml solution in 100 mM phosphate, pH 6.3, 0.5 mM EDTA, was dialyzed overnight at 0°C against the same buffer made 1 mM in freshly prepared DTT just before use. A $dn/dc$ value of 0.190 cm$^3$/g was assumed for all of the above proteins. Hemocyanin from Octopus dolfinei, a gift from Dr. Karen Miller, was dialyzed at 0°C against 100 mM Tris-HCl, pH 7.6 (at room temperature), 50 mM MgCl$_2$ prior to use. The $dn/dc$ value was taken to be 0.194 cm$^3$/g, a value obtained for several hemocyanins from other species of octopus (34).**

**Electron Microscopy and Analysis**—The protein was diluted to 100 mg/ml in 0.25% methylamine tungstate stain and applied by the spray method to the Butvar side of a carbon-coated Butvar film (35). The images were recorded with a JOEL JEM 1200 electron microscope at 50,000 and 100 kV using conventional irradiation procedures at an under focus of 0.5 mm. The micrographs were digitized as 4096 $\times$ 4096 $\times$ 12 bit arrays using an Eikonix 412 digitizer at a pixel size of 5.7 Å/pixel. The digitally generated power spectrum of the micrographs was utilized to check for drift and astigmatism.

The molecules were in random orientations, and those that were judged to represent 6- and 2-fold projections of the structure were selected for image analysis. Image analyses were performed as de-
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Table II
Accuracy of molecular masses of test proteins determined by light scattering

| Protein                          | Mass from Structurea | Light scatteringb | Apparent error | %  |
|----------------------------------|----------------------|-------------------|----------------|----|
| Carbonic anhydrase               | 29,023               | 29,800c (C = 0)   | +2.7           |    |
| Alcohol dehydrogenase            | 146,980c             | 149,000c (C = 0)c | +1.4           |    |
| β-Amylase                       | 224,349              | 228,000c          | +1.6           |    |
| Apoferritin                     | 476,316e             | 484,400c          | +1.7           |    |
| Thryoglobulin                   | 669,000c             | 670,000c          | +1.5           |    |
| Ornithine dehydroxylase         | 990,684c             | 978,000c          | -1.3           |    |
| Octopus hemocyanin              | 3,440,000c           | 3,450,000c        | +0.3           |    |

a All values are based on amino sequences except that for thyroglobulin.
b Symbols: R, right (trailing) half of chromatographic peak used; L, left (leading) half of chromatographic peak used; C = 0, data extrapolated to zero concentration. The value of ds/dc was taken to be 0.190 cm/g for all light-scattering measurements of test proteins except octopus hemocyanin for which the measured value of 0.194 cm/g was used (34).
c Masses of 29,021 and 47,922 Da in the mass spectra (38) indicate some dimerization.
d Mass spectrometry of sample used gave a monomeric value of 36,745 Da (×4 ≈ 146,980) which corresponds to yeast alcohol dehydrogenase type II (36,732 Da) (39) rather than type I (36,823 Da) (40).
e Pronounced concentration-dependence.
f Value based on monomer mass of 56,084.8 Da determined by mass spectrometry. The GenBank sequence (accession no. S35667) gives a molecular mass of 55,960.7 Da without an NH2-terminal Met so that a tetramer should have a mass of 223,843 Da. The discrepancy in molecular mass is unresolved. Although an NH2-terminal Met would largely account for this difference, this residue was not detected (41). The directly determined sequence (41) agrees completely with the cDNA-derived sequence (42) after the latter is corrected for translation errors, but both differ by two residues from those in GenBank™ (GenBank™ Refs. 41 and 42: Thr237 → Ala, Ile396 → Met).
g The molecular mass for a monomer calculated from the amino acid sequence is 19,846.5 (43). Apoferritin was found by x-ray crystallography to have 24 subunits (44) so that the total molecular mass is 476,316 Da.
h Value based on extensive sedimentation and diffusion data extrapolated to zero concentration (45). Light-scattering data (45), also extrapolated to C = 0, yielded 690,000 Da. Sedimentation equilibrium measurements of the reduced protein in 8 M urea gave 336,000 Da for the largest component (46), corresponding to 672,000 Da for the dimer. The mean value derived from all these measurements is 677 kDa [1/3(669 + 24 × 212)] ≈ 219,000 Da (47) to which N-linked carbohydrate adds about 17,900 Da (48). Phosphorylation, sulfation, iodination and O-linked glycosylation (referred to in Ref. 48) add a variable mass of 5–6%.
i Value calculated from DNA-derived sequence (33), 82,557 Da, and the x-ray crystallography (49) which shows that the molecule has 12 subunits.
j Value calculated from the amino acid sequence determined by K. I. Miller and K. E. van Holde (private communication) together with the carbohydrate content of 2.3% (w/w) (50).

We have evaluated the accuracy of the light-scattering methodology with seven test proteins. Fig. 2 shows the 90° light-scattering and refractive index patterns for these proteins. The results of the light-scattering measurements are summarized in Table II.

Carbonic Anhydrase—The molecular weights calculated for the left and right half-peaks (Fig. 2a) are 33,900 and 32,300 (only the segment to 10.3 ml was used). These values suggest some self-association. Extrapolation of the concentration dependence to C = 0 yielded a molecular weight close to the structural value. The perturbation at the end of the right (trailing) peak suggests a small difference between column buffer (diisylate) and the buffer of the sample. Prolonged (48 h) dialysis against the elution buffer did not eliminate the irregularity in the baseline. However, the perturbation disappeared in a later experiment in which the sample was centrifuged and not filtered.

Yeast Alcohol Dehydrogenase (Fig. 2b)—Substantial nonideality is present in the light-scattering results for alcohol dehydrogenase. A plot of IMapp versus total quantity in peak was linear and extrapolated to a mass within 1.4% of the structurally determined value. It is curious that the alcohol dehydrogenase migrates in SDS gel electrophoresis much closer to egg albumin (45 kDa) than to glyceraldehyde-3-phosphate dehydrogenase (36 kDa) (data not shown). Nevertheless, the expected molecular mass of the alcohol dehydrogenase in this sample was determined by mass spectrometry (see footnote d of Table II).

Sweet Potato β-Amylase—The right-half peak (Fig. 2c) yielded a value of 228 kDa, which is within 1.6% of the structurally derived value.

Horse Spleen Apoferritin—The calculated weight-average molecular weight for the right-half of the major peak (Fig. 2d), 484,400, is only 1.7% higher than the value expected for a homopolymer of the L-chain of this protein with 24 subunits (44) of molecular weight 19,846.5 (GenBank™) (19,846.5 × 24 = 476,316). This result, obtained at a weight-average concentration of only 0.06 mg/ml, indicates that the protein is relatively stable to dissociation, in agreement with prior studies by sedimentation equilibrium (51) and by light scattering (52). The chromatogram (Fig. 2d) shows a minor component of apparent mass 1.15 MDA that is probably a dimer. Horse spleen apoferritin has two types of chain, H (10%, ~21 kDa), and L (~90%, ~19 kDa), that form heteropolymers (53). The weight-average molecular mass increases across the major peak (Fig. 2d, right to left) from 484 kDa (right, trailing half) to ~540 kDa (left, leading half), as expected from the composition. The amino acid sequence of the H-chain of horse spleen apoferritin has not been determined, but its mass is about 10.5% greater than that of the L-chain. On this basis, the expected molecular mass of the H-chain homopolymer would be 526 kDa (24 × 1.105 × 19847), close to the 540 kDa observed for the left, leading half-peak.

Bovine Thyroglobulin—A shoulder on the leading edge of the peak (Fig. 2e, arrow) suggests some self association. The weight-average molecular weight for the right-half peak, 679,000, is within 1.5% of the 669,000 value obtained by sedimentation (45).

Ornithine Decarboxylase—This dodecameric enzyme has a subunit molecular weight of 82,557 (33) and a total molecular weight of 990,685 (Fig. 2f). Guirard and Snell (32) obtained a molecular mass of 1.04 MDa by sedimentation equilibrium and observed that omission of DTT from the buffer caused variable aggregation to 1.5–3.5 MDa. The initial light-scattering data

scribed previously (36) using Silicon Graphics 4D25 and Indigo workstations and our SUPRIM image-processing software (37).

RESULTS

Molecular Weight

Test Proteins
gave 1.16 MDa for the right half-peak in the presence of 1 mM DTT (prepared from an old stock solution), suggesting some aggregation. When the experiment was repeated with freshly prepared DTT, the molecular weight for the right-half peak was 978,000, within 1.3% of the structurally determined value.

Octopus Hemocyanin—This O₂-carrying copper protein is a decamer with a molecular mass of 3.48 MDa measured by sedimentation equilibrium (50) and a mass of 3.44 MDa from amino acid sequence analysis4 and determination of a carbohydrate content (50). The light-scattering measurements (Fig. 2) are in close agreement with these values.

Measurements made with these diverse proteins, summarized in Table II, demonstrate that light-scattering is capable of yielding molecular weights within 2% of those obtained by structural analysis. These results were obtained by assigning the same $dn/dc$ value, 0.190 cm³/g, to six of the proteins. An accuracy better than 1% should be achievable with a careful $dn/dc$ determination for each protein combined with measurement of the concentration dependence of the light-scattering and an analysis of the pH-dependent deviations from ideality.

Hemoglobin of L. terrestris

Molecular Weight

Fig. 3a shows the 90° light-scattering and refractive index difference patterns for a dilute solution of freshly prepared L. terrestris HbCO. The calculated weight-average molecular mass from these data is shown as a function of elution volume in Fig. 3b. The calculated weight-average molecular mass for the entire peak is 4.10 ± 0.1 MDa on the basis of the measured $dn/dc$ value of 0.185 ml/g. These measurements (Fig. 3) were made at sufficiently low concentrations (≤84 ng/ml) so that correction for the second virial coefficient could be ignored. The value of $B$ (Equation 1) has been estimated to be only $5 \times 10^{-9}$ liter-mol/g² (7). This would change our value of the molecular mass by less than 0.3%. Similarly, light-scattering measurements of Hb from the related earthworm (54), O. complanatum1, in the range 25–250 ng/ml showed no concentration dependence.

The angular dependence of scattering (see Fig. 1) has been used to estimate the radius of gyration, $R_G$, by use of equations (1) and (2) for the same experimental data used for Fig. 3. This yielded a value of $12.7 \pm 2.8$ nm ($n = 22$) for $R_G$ which is 13% higher than the more precise value of $11.2 \pm 0.1$ nm determined by low angle x-ray scattering (55). Somewhat higher accuracy may be possible with careful redetermination of the normalization factors for each detector, particularly at low angles (see “Materials and Methods”) performed at the same time as experiments such as those shown in Fig. 3. However, the $R_G$ value is only 1.8% of the wavelength of the scattering light, and the slope of the line in Fig. 1 is very small so that we are near the experimental limit.

We have compared L. terrestris HbCO samples freshly prepared and frozen with those quickly frozen in liquid N₂ or slowly frozen to −80 °C. The weight-average molecular masses obtained after each of these treatments were indistinguishable: 4.06 MDa (unfrozen), 4.13 MDa (liquid N₂ frozen), and 4.10 MDa.

4 K. I. Miller and K. E. van Holde, private communication.
MDa (−80 °C frozen). It may well be that freezing causes damage induced by ice crystals that is not reflected in the total molecular mass. We suspect, however, that previously observed dissociation induced by freezing may be due primarily to oxidation of HbO$_2$ that readily occurs at high freezing temperatures of −10 °C to −20 °C.

Results very similar to those shown in Fig. 3 for HbCO were obtained for HbO$_2$ (data not shown). The HbCO was converted to HbO$_2$ with light and an atmosphere of O$_2$ at 0 °C. metHb was prepared from HbO$_2$ by oxidation with an excess of K$_3$Fe(CN)$_6$. Spectral changes that accompany partial oxidation with K$_3$Fe(CN)$_6$ are shown in Fig. 4. Table III summarizes the light-scattering results following storage of the HbO$_2$ and metHb solutions at 4 °C for varying lengths of time. These results show that oxidation causes a decrease of 10–30% in molecular mass. The lowest mass for metHb, 3.0 MDa, was obtained after prior storage for 9 days at 4 °C as HbO$_2$. However, no decrease in molecular mass was observed in CN-metHb.

Subjecting Hb to prolonged storage as HbO$_2$ and then oxidizing it (see Table III) causes a change in the pattern of components seen in reverse phase HPLC: a new peak designated “pseudotrimer” (PT) can be seen that elutes earlier than the abc trimer (Fig. 5). This peak has been identified as trimer-derived by reduction with DTT and isolation of chains b, c, and a, together with numerous additional peaks absent from the HPLC profile of the DTT-treated trimer peak (data not shown). The pseudotrimer is markedly heterogeneous and has a variable mass of 40–45 kDa by SDS-gel electrophoresis and by mass spectrometry (15). No pseudotrimer was detected in any HbCO preparation even after prolonged storage, and none was detected in metHb stored for one day. The lowered molecular mass of the pseudotrimer indicates proteolysis. Proteolytic attack by proteases. Molecular masses of 3.7 MDa or less were often associated with proteolysis when the storage of the metHb was prolonged.

The apparent rough correlation between time “as HbO$_2$” and quantity of pseudotrimer (Table III) is puzzling. The HbO$_2$ stored 10 days (Table III) is the same sample from which aliquots were taken for oxidation. Absorption spectra of the HbO$_2$ stored for various times did not suggest any oxidation. One interpretation is that storage as HbO$_2$ does result in the cleavage of a few susceptible bonds but that this is insufficient by itself to promote dissociation. However, upon oxidation to metHb, additional proteolysis would be rapid. In any event, fully ligated ferrous Hb is resistant to dissociation.

These observations help to explain the wide variation in molecular mass observed in different laboratories. We have been able to reproduce this variation by oxidizing the Hb. These data support the hypothesis that the variation in molecular mass results from the oxidation-dependent shedding of subunits.

**Table III**

| Derivative | Days at 4°C | Weight-average molecular mass (MDa) | Presence of pseudotrimer$^a$ |
|------------|-------------|------------------------------------|-----------------------------|
| HbCO       | 14          | 4.1 ± 0.1                          | 0                           |
| HbO$_2$    | 10          | 4.2                                | 0                           |

| As HbO$_2$ | As met Hb |
|------------|-----------|
| metHb      | 0         | 1         | 3.6  | 0   |
| 3          | 1         | 3.7      | ++  |
| 9          | 1         | 3.0      | +++ |
| CN-metHb   | 0         | 1         | 4.1  | 0   |
| 0          | 7         | 4.1      | ND$^d$|

$^a$ “Pseudotrimer” detected by HPLC (Fig. 5); see text.

$^b$ Length of prior storage at 4°C as HbO$_2$ at pH 7.0 before oxidation.

$^c$ After 18 days of storage of this sample, some pseudotrimer has formed.

$^d$ Not determined.

**Subunits**

**abc Trimer**—Fig. 6a shows the voltage signals from the detectors for 90° light-scattering and the refractometer as a function of elution volume. The calculated weight-average molecu-
lar mass (weight) versus elution volume is given in Fig. 6b. These data show that the ferrous CO-derivative of the \( \text{abc} \) trimer self-associates extensively at pH 6.8. Although the \( \text{abc} \) trimer elutes in what appears to be a single peak with a shoulder, the peak is far from monodisperse. The apparent weight-average molecular mass decreases smoothly across the peak from left to right, from \( \sim 250 \text{kDa} \) to \( \sim 100 \text{kDa} \). More than 75% of the \( \text{abc} \) trimer associates to \( (\text{abc})_2 (\sim 109 \text{kDa}) \) and larger products. The apparent weight-average molecular mass for the peak between 7 and 9 ml is 206 kDa. A distinct component which elutes at 6–7 ml can be accounted for by traces of linkers and chain d in the preparation. The data suggest the following self-association equilibrium.

\[
\text{abc} \rightleftharpoons (\text{abc})_2 \rightleftharpoons (\text{abc})_3 \rightleftharpoons (\text{abc})_{10} \\
\text{abc association}
\]

The trailing edge of the \( \text{abc} \) peak in Fig. 6b suggests that substantial dissociation of \( (\text{abc})_2 \) occurs only at concentrations below 0.1 mg/ml. This is confirmed in Fig. 7a which shows the overall weight-average molecular mass of the \( \text{abc} \) trimer as a function of concentration and pH. The \( \text{abc} \) trimer associates reversibly to \( (\text{abc})_2 \) or larger products at all pH values at concentrations above 0.1–0.2 mg/ml. The trimer associates only to \( (\text{abc})_2 \) at pH 9 even at a concentration of 1.8 mg/ml. These results indicate that the \( \text{abc-abc} \) interface in \( (\text{abc})_2 \) is not pH-dependent. The pH dependence of \( \text{abc} \) trimer association (Fig. 7b) shows an apparent maximal mass of \( (\text{abc})_{10} \) near \( \sim \text{pH 6} \) with half-dissociation at about pH 6.8. However, the data for pH 6.0 (Fig. 7a) extend only to 0.6 mg/ml because higher concentrations saturate the detectors so that the maximal value for \( \text{abc} \) association cannot be accurately assessed. Nevertheless, the 570 kDa shoulder in Fig. 6b is consistent with a maximum of \( (\text{abc})_{10} \) in Fig. 7b.

Oxidation of the \( \text{abc} \) trimer (Fig. 8) results in the dissociation of all species larger than \( (\text{abc})_2 \). The only significant association of the met trimer is \( 2\text{abc} \rightleftharpoons (\text{abc})_2 \). However, ligation with CN causes reassociation to larger products. The resulting elution pattern (7.5–8.5 ml in Fig. 8) is similar to that for the oxy \( \text{abc} \) trimer shown in Fig. 6b. These results indicate that oxidation
of the heme destabilizes the \( (abc)_2 \) interfaces but has relatively little or no effect on the \( (abc) \) interface. The association properties of the CN-Met and CO derivatives of the \( abc \) trimer are very similar.

**Chain \( d \)—** The CO-derivative of chain \( d \), like the CO-\( abc \) trimer, self-associates. The puzzling elution pattern (Fig. 9) indicates that the predominant species must be larger than \( (d)_2 \). The two \( d \) components (56), \( d_1 \) and \( d_2 \), are treated here together as \( (d) \). The same pattern is observed with CN- \( d \) trimer (data not shown). The apparent weight-average molecular mass for the entire double peak between 8.5 and 10.2 ml is \(-66 \) kDa, suggesting that \( (d)_4 \) is a major species. Our interpretation of the pattern is based on the finding that the weight-average molecular mass is about 7% lower than that obtained at pH 6.8, indicating some dissociation (data not shown). This result explains why the chromatographic separation of trimer, linkers and chain \( d \) at pH 9 requires two passes through a size-exclusion column. A single pass never completely separates the components. The results of experiments with oxidized subunits are given in Table IV. These data show that oxidized \( abc \) trimer and chain \( d \) do not combine with each other at all: no \( (abcd)_4 \) is formed and the oxidized \( (abc) \) and \( (d) \) subunits self-associate only to \( (abc)_2 \) and \( (d)_2 \). However, when the ferric hemes of an equimolar mixture of \( (abc)_2 \) and \( (d)_2 \) are ligated with CN\(^-\), the \( abcd \) complex does form. A small quantity of linkers is always present in preparations of the \( abcd \) complex. It is important to note that HPLC analysis of the fractions depicted in Fig. 10 shows that 100% of these contaminating linkers are sequestered into 4-MDa molecules, and none is left in the fractions of lower molecular mass. This means, as described below, that all linkers are competent to participate in reassembly.

Valence Hybrids—We have also prepared a series of valence hybrids. Addition of ferric \( (d) \) to the CO-\( (abc) \) trimer causes the immediate formation of an \( (abcd) \) complex. Thus, oxidation of chain \( d \), by itself, does not inhibit the formation of the \( (abcd) \) complex. These results are summarized in Table IV. In contrast, no \( (abcd)_4 \) forms in the reverse experiment: \( (abcd) \) mixed with CO-\( d \). One caveat of these experiments is that the light-scattering measurements must be performed immediately after oxidation and mixing. If the mixed samples are stored 2.5–3.5 days, then a small amount of \( (abcd)_4 \) is observed from the mixture of \( (abc) \) and CO-\( d \). We believe that this effect may result from redistribution of oxidation states and CO.

\[
\text{Fe}^{3+} \text{trimer} + \text{Fe}^{2+} \text{CO-d} \rightarrow \text{Fe}^{2+} \text{CO trimer} + \text{Fe}^{3+} \cdot \text{d}
\]

**Redistribution of Oxidation States**
Assembly of Earthworm Hemoglobin

Such a redistribution would be aided by the action of CO as a reducing agent (17).

Absorption Spectra of Oxidized Subunits—Oxidation of the abc trimer and chain d with ferricyanide causes the appearance of a characteristic hemichrome spectrum (A in Fig. 11) with maxima at 412 and 532–534 nm and a shoulder at ~565 nm. Reduction with dithionite (B in Fig. 11) results in a spectrum with maxima at 428 and 559 nm and a shoulder at 530 nm. Similar results were obtained with chain d (data not shown). The spectra are very similar to those described for hemichrome and hemochrome formation in human Hb (61) and in that of the related annelid, O. complanatum (10). Hemichrome formation in the subunits of L. terrestris Hb appears to be characteristic of the dissociated subunits.

Similar observations have been made in several unrelated invertebrate Hbs in three different phyla: the unligated metHbs of Phoronopsis viridis (62), Scapharca inaequivalvis (63), and Caudina arenicola (64) are monomeric but become dimeric as CN-metHb. This suggests that a common mechanism may exist in L. terrestris Hb and all these Hbs that links dimers with the CN-met form and the monomers in the presence of CN\textsuperscript{−}. The common mechanism might involve a similar relationship between a critical subunit interface and the heme iron in each of these Hbs.

Recombination of L. terrestris Hb from Trimer, Chain d, and Linkers—We seek to answer the following questions: (i) How does the yield of recombined Hb vary with the amount of linker chains added? (ii) How does the yield vary with time of incubation? (iii) What is the effect of Ca\textsuperscript{2+} on the recombination process? Three sets of experiments have been performed to address these questions:

Set no. 1: The first experiments were carried out by (a) mixing equimolar quantities of CO-abc trimer and CO-chain d to form (abcd)\textsubscript{4}. (b) adding various amounts of linkers at pH 6.5, then (c) raising the pH to 9.0, followed by (d) dialysis back to pH 7.0 overnight; all steps were performed at 4°C. The CO-saturated buffer at each pH was 50 mM bis tris propane, 100 mM NaCl and 1 mM EGTA. The rationale for the last two steps was that the linkers are known to self-associate, and it was believed that this association might retard formation of the 4-MDa Hb if dissociation of the linkers were a rate-limiting step. Initial dissociation at pH 9 might therefore remove this complication.

The results of adding 50% of the stoichiometric quantity of linkers to the (abcd)\textsubscript{4} complex are shown in Fig. 12a. The measurements were made approximately 18 h after the start of the dialysis. Two features of the weight-average molecular mass distribution are noteworthy: 1) 100% of the linkers added are incorporated into large structures of which more than 90% exceed 3.5 MDa in mass. The material between 5.9 and 8.1 ml was collected and subjected to HPLC analysis. The results were consistent with the values expected on the basis of the stoichiometry already determined (16) (data not shown). This demonstrates that all linkers are competent for reassembly. 2) The weight-average molecular mass decreases in two phases; a gradual decrease from 4 to 2 MDa between 6.0 and 8.2 ml, and a precipitous drop from 2 MDa to ~280 kDa between 8.3 and 9.0 ml. 3) A small but definite component comprising about 4% of the total protein, corresponding to half-molecules of ~2 MDa occurs at 8.0–8.2 ml. The concentration of material between 280 kDa and 2 MDa is extremely low, indicating a highly cooperative process of reassembly. We have assumed here that the rate at which equilibrium is reached is slow compared with the time for the chromatography.

Although the addition of 50% of the stoichiometric quantity of linkers to CO-saturated (abcd)\textsubscript{4} yielded approximately 50% of the reassembled molecules, an increase of linkers to 100% increased the yield only to 75–80% of the molecules with mass greater than 3 MDa (see Fig. 12b). The data, however, reveal that all linkers added to (abcd)\textsubscript{4} up to 50% of the stoichiometric

![Image: The elution pattern of the (abcd)\textsubscript{4} complex formed by mixing equimolar amounts of CO-abc and CO-d.](image)

**Fig. 10.** The elution pattern of the (abcd)\textsubscript{4} complex formed by mixing equimolar amounts of CO-abc and CO-d. The peaks at 6.5 and 7.6 ml correspond to 4-MDAs and 550-kDa molecules that result, respectively, from the presence of a small quantity of linker chains and a slight excess of the abc trimer. Buffer as in Fig. 6. Protein concentration at maximum refractive index difference (at 8.05 ml), 0.17 mg/ml.

**TABLE IV**

| Components | Time of incubation | Weight-average molecular mass of products |
|------------|--------------------|----------------------------------------|
| CO-abc + CO-d | Overnight, 0°C | 278 kDa |
| CO-abc + d\textsuperscript{+} | 1 min, 25°C | 265 kDa |
| CN-abc\textsuperscript{+} + d\textsuperscript{+} | Overnight, 0°C | 293 kDa |
| CN-abc\textsuperscript{+} + d\textsuperscript{+} | 1 min, 25°C | 258 kDa |
| CN-abc\textsuperscript{+} + CN-d\textsuperscript{+} | Overnight, 0°C | 289 kDa |
| abc\textsuperscript{+} + CO-d | 1 min, 25°C | No (abcd)\textsubscript{4} |
| abc\textsuperscript{+} + d\textsuperscript{+} | Overnight, 0°C | No (abcd)\textsubscript{4} |
| abc\textsuperscript{+} + CN-d\textsuperscript{+} | 1 min, 25°C | No (abcd)\textsubscript{4} |
| abc\textsuperscript{+} + CN-d\textsuperscript{+} | Overnight, 0°C | No (abcd)\textsubscript{4} |

\*The symbols abc\textsuperscript{+} and d\textsuperscript{+} mean the completely oxidized ferric forms.

\*b 1 min, 25°C refers to the time after mixing and before injection onto the column. The (abcd)\textsubscript{4} complex elutes in about 27 min.

\*c The variation in the mass of the (abcd) complex is not significant. The mean of the three values obtained after incubation overnight at 0°C is 287 kDa, essentially identical with the mass of (abcd)\textsubscript{4}, 286 kDa.
amount are competent to participate in assembly. We conclude that the partial inability to reassemble rests solely with the abc trimer and/or chain d. If the time of exposure of dissociated subunits (trimer, chain d and linkers) to high pH is extended to a week, no reassembly can be detected within 24 h.

Set no. 2: The second set of experiments was similar to the first except that a pH of 6.5 was maintained throughout. Various quantities of linkers were added to the trimer and chain d mixture (all in the CO-form), and the final mixture was incubated at 0 °C overnight and up to a month or longer. HPLC analysis was used to determine the subunit composition of each component of the mixture. No calcium was added in these experiments. The results, summarized in Fig. 13, a and b, shows that two distinct kinetic phases are present. When 200% of the stoichiometric quantity of linkers are added, the yield of 4-MDa Hb reaches 50% within 16.5 h, but 71 days are required to reach 78%. These observations reveal that a primary limitation on recombination is kinetic.

Comparison of Figs. 12 and 13 shows that the subunits used in the Fig. 13 experiments reassociated to a much lesser extent than in the experiments shown in Fig. 12. This difference presumably resulted from the use of freshly prepared Hb and subunits in the Fig. 12 experiments and the use of Hb long stored in (NH₄)₂SO₄ in the experiments shown in Fig. 13.

Set no. 3: This set of experiments examines the effect of calcium on reassembly. A dramatic increase in the rate of formation of 4-MDa Hb occurs in the presence of added Ca²⁺. In one experiment in 25 mM bistris propane, 100 mM NaCl, pH 6.8, the apparent half-time for the recombination was 30 days. The addition of 10 mM Ca²⁺ reduced the time to only 5.3 days, corresponding to a 6-fold increase in rate (data not shown). These experiments were done at 0 °C to avoid oxidation known to occur at higher temperatures where the rates of recombination would presumably be much faster.

Electron Microscopy—The shape and design of the average images of the 6- and 2-fold projections of the native and reconstituted complex appear identical (Fig. 14). Correspondence analysis followed by hierarchical ascendent classification was applied to the data for each average image. The analysis revealed no significant differences in the cluster averages so that we reasonably conclude that the grand averages are representative of the data sets. These average images are similar to the corresponding average images of the hemoglobin derived from frozen, hydrated material (65). However, it was apparent that the reconstituted preparation consisted of numerous particles that appeared to represent incompletely-formed structures (data not shown). These particles were not chosen for image analysis. The excellent concordance between the two orthogonal projections of the native and reconstituted structures indicates that the subunits have self-assembled to form the native complex. The preparation used is similar to that for the point in Fig. 12b with 100% of the stoichiometric quantity of linkers added and was not fractionated.

DISCUSSION

Our understanding of the assembly of L. terrestris Hb rests on four sets of observations: 1) the stoichiometry of globin and linker polypeptides: eight globin chains per linker; 2) the determination of the molecular mass of CO-saturated native Hb, close to 4.1 MDa; 3) the finding that the ferrous CO-saturated abc trimer and chain d combine to form (abcd)₄; and 4) the stoichiometric titration of CO-saturated (abcd)₄ with linkers to form 4.1-MDa molecules.

Stoichiometric Considerations

Our analysis (16) shows that the weight proportion of linkers is 17% in contrast to the prior reports by Vinogradov and associates (see Ref. 16 and references therein) that the linkers comprise ~33% of the total mass. They also propose (66) that the primary structural unit is (abcd)₄ which has a mass of ~216 kDa. The consequences of these different stoichiometries are summarized in Table V which shows four possible molecular masses. Stoichiometries 2 and 3 can be eliminated because
5-MDa molecules have never been observed and our results show that a 3-MDa value is only found after oxidation, subunit dissociation and proteolysis. Stoichiometry 1, favored by Vinogradov and colleagues (66), is inconsistent with our determination of the mass of the complex and our measurement of the globin/linker proportions. We conclude that only stoichiometry 4 (17% linkers) and a principal subunit of \((abcd)_4\) is consistent with the observed molecular mass of 4.1 MDa.

**Molecular Mass**

The accuracy of the molecular mass determined by light scattering is critically dependent on both the determination of \(dn/dc\) and the concentration dependence of the apparent molecular mass. The close correspondence between dry weight and amino acid analysis (16) provides strong support for our value of \(dn/dc = 0.185\) cm\(^3\)/g. Herskovits and Harrington (7) reported a value of 0.193 cm\(^3\)/g but their preparations were not protected from oxidation by saturation with CO. They estimated the protein concentration with a previously determined extinction coefficient \((E^{1\%}_{1\%} = 5.95)\) at 540 nm for HbO\(_2\) (54). The presence of only 8% metHb would be sufficient to reduce the calculated protein content enough to raise the value of \(dn/dc\) to 0.193 cm\(^3\)/g because the extinction coefficient of metHb at 540 nm is only half that of HbO\(_2\) (67). Their value of \(-3.1\) MDa for the molecular mass suggests that oxidation has occurred. We can reproduce this value, but only under conditions of oxidation and often proteolysis. The recently reported lower molecular masses of \(-3.5-3.6\) MDa for L. terrestris Hb (5) can be reproduced by oxidation alone without proteolysis. Although oxidation of the \((abcd)_4\) complex is accompanied by complete dissociation to \(\text{Hb}d_2^+\) and \(\text{Hb}c_2^+\) oxidation of all chains is not required for this dissociation because combination of oxidized chain \(d_2^+\) with CO-saturated \(\text{abc}\) trimer still produces \((abcd)_4\) molecules, whereas the combination of oxidized \(\text{abc}\) trimer with CO-saturated chain \(d\) does not occur (see Table IV).

We suggest that oxidation of only a small fraction of the total...
number of hemes is sufficient to cause dissociation. If one chain, for example \(a\) or \(b\), forms crucial intersubunit contacts, oxidation of this critical chain would weaken the contacts and cause dissociation. If so, then oxidation of 25% of the total number of hemes might be enough to cause 100% dissociation. Oxidation of 3.8% of the hemes would then suffice to give 15% dissociation and thereby reduce the molecular mass from 4.1 to \(\sim 3.5\) MDa. Furthermore, the effect of oxidation on dissociation of subunits is likely to be cooperative. Just as \(O_2\) binding by the 192 hemes in the Hb is highly cooperative (68), we expect that oxidation has a similar cooperative effect just as it does in human Hb. Preliminary measurements\(^5\) support this suggestion: partial oxidation of only a few percent results in a disproportionately greater dissociation of the Hb. We assume that those interfaces critical for \(O_2\) binding are the same as those involved in oxidation-dependent dissociation. If the cooperative mechanism is also similar, it would mean that the number of oxidized hemes necessary for 15% dissociation may well be much less than 3.8%. Such small levels of oxidation would be hard to detect and could easily be overlooked.

We do not know which chain(s) might be critical for oxidation-induced dissociation. However, isolated chains \(d\) and \(c\) are relatively resistant to autoxidation whereas chains \(a\) and \(b\) autoxidize rapidly (16, 18). If, as seems likely, similar differences in autoxidation also exist in the intact Hb, chains \(a\) and/or \(b\) would be prime candidates for the critical chain.

**Extent of Recombination**—The experiments summarized in Fig. 13, \(a\) and \(b\), reveal that a fraction of dissociated subunits reassociates only very slowly. We have shown that the subunits responsible for the slow reassociation are the \(abc\) trimer and/or chain \(d\) (see “Recombination of \(L. terrestris\) Hb from Trimer, Chain \(d\), and Linkers,” “Set no. 1”). A possible explanation for the slow rate of reassociation is provided by the concept of conformational drift whereby dissociated subunits of oligomeric proteins, freed of conformational constraints, often appear to change to new conformations and return to the native state very slowly (69–71). This idea has been fruitful in the analysis of pressure-induced dissociation of subunits of a similar Hb from the worm, *Glossoscolex paulistus* (72).

Recent measurements\(^6\) of the dissociation resulting from oxidation of the Hb and its reassociation upon the addition of \(CN^-\), \(N_2O_3\), or \(NO_2\) show that the reassembly of subunits occurs within the time required to add the ligand, mix and perform a light scattering experiment (<25 min). These experiments indicate that slow reassociation of \(L. terrestris\) Hb is characteristic only of subunits that have been dissociated for long periods of time (>48 h). Our observations are reminiscent of the report by David and Daniel (2) that dissociated subunits of the Hb of a related annelid lost their ability to reassemble after 24 h.

The close correspondence between the images of native and reconstituted hemoglobin (Fig. 14) is consistent with the molecular weight values for reassembled Hb and with the analytical results that indicate that the reconstituted Hb has the same stoichiometry of subunits.

**Model of Assembly**

A striking feature of the present experiments is that the subunit association processes are strongly dependent on both the ligand and valence state of the iron. The results show that processes at the heme are strongly linked to conformational changes at the interfaces responsible for association of the subunits. This conclusion applies to all globin subunits and their major association products.

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\(^5\) H. Zhu and A. F. Riggs, unpublished observations.

\(^6\) E. Karpova, C. K. Riggs, and A. F. Riggs, unpublished observations.
The final assembly to 4-MDa molecules from (abcd) and linkers is demonstrated to be highly cooperative by the extremely low concentrations of any intermediates evident in the elution pattern of Fig. 12a. The distribution of molecular weights in Fig. 12a suggests that the final self-assembly occurs in two steps. The first entails the highly-cooperative formation of a ring structure corresponding to the half-molecule, (abcd)2, of molecular mass ~2 MDa. This half-molecule corresponds to one layer of the hexagonal structure (see Fig. 14). The second step would be a non-cooperative but tight combination of the two half-molecules.

Calcium greatly accelerates the formation of 4-MDa molecules, but this observation does not explain how this effect is mediated. It may be either that Ca2+ accelerates the formation of intermediate species or that the primary effect of Ca2+ is to stabilize the Hb molecule once it is formed. The latter possibility is suggested by the observation that calcium stabilizes the Hb against dissociation at high pH. Thus it remains to be determined whether Ca2+ accelerates any step in assembly or slows the dissociation process. It is possible that Ca2+ affects both processes.

An essential feature of this model is the (abcd)2 complex. We assume that this structure retains its integrity within the intact Hb. However, it is likely that the conformation of the isolated (abcd)2 is perturbed to some extent upon binding linkers and incorporation into the larger structure. This may account for the fact that the (abcd)2 complex has an oxygen equilibrium identical with that of the intact Hb at pH 6.8 but that this match gradually disappears as the pH is raised (18). One reason this may occur is that high pH destabilizes the (abcd) complex more than the intact Hb.

The Dodecamer Model—An alternative model for the Hb of *L. terrestris* has been advanced by Vinogradov and colleagues (see Refs. 5, 66, and 73, and references therein). They propose that the molecule has 12 (abcd)2 dodecamer subunits and 36 linkers. This model is based on (a) the lower heme content observed by them, (b) a lower molecular mass of 3.5 ± 0.1 MDa, and (c) the isolation of an apparent dodecamer of 216 kDa from a size-exclusion column of products dissociated by 4 M urea. We evaluate their measurement of heme content in our companion study (16). Here we make the following observations on their conclusions concerning the lower molecular mass and the dodecamer.

First, comparison of the absorption spectrum7 of the HbO2 in Fig. 5A of Martin et al. (5) with our data for HbO2 freshly prepared from HbCO (Fig. 4, this report) reveals that approximately 30% of their “HbO2” is oxidized. This means that their preparation cannot be completely homogeneous. Our finding that oxidation causes a decrease in molecular mass means that the partially oxidized Hb studied by Martin et al. must have a lower weight-average molecular mass than it would have with all its iron atoms ferrous.

Second, Fig. 5B of Martin et al. (5) shows that treatment with ferricyanide causes dissociation. However, the authors attribute the dissociation not to oxidation but to the assumed binding of ferro- or ferricyanide to the Hb. This conclusion was reached because treatment with a 330-fold excess of nitrite oxidizes the Hb without a drop in molecular mass. However, oxidation with nitrite gives aquo-metHb only when Hb is treated with an equimolar amount of nitrite (74). Nitrite is a weak ligand (74, 75), and a 330-fold excess should saturate the Hb. We have found that the effects of the ligands, CN−, N3, and NO2, are all very similar.8 Oxidation with ferricyanide or nitrite will produce low molecular weights, and this can be reversed and prevented with any of these reagents. These experiments show that the binding of ferro- or ferricyanide is not responsible for the observed decrease in molecular weight.

Third, sedimentation equilibrium measurements made on three preparations of HbO2 by Martin et al. (5) yielded values for the molecular mass of 3.03, 3.25,9 and 3.95 MDa. Such a variation of 30% indicates an uncontrolled variable which we believe to be oxidation-induced subunit dissociation. These values were obtained with the use of a partial specific volume (\(\bar{\nu}\)) value of 0.714 cm3/g determined with the highly accurate Paar DMA-60 density meter. However, the value of \(\bar{\nu}\) depends critically on the determination of the concentration of protein. Oxidation and resultant heme-loss will change any weight-based extinction coefficients at 540 nm for CN-metHb used for these calculations. The net effect will be to increase the apparent concentration so that \(\bar{\nu}\) is reduced. They also calculated \(\bar{\nu} = 0.734 \text{cm}^3/\text{g}\) from the amino acid compositions of the constituent chains. This value yielded masses of 3.26, 3.50, and 4.25 MDa, the last value being close to that which we have obtained for the mass of completely ferrous HbCO by light scattering.

Fourth, the dodecamer subunit was prepared by “mild” dissociation of HbO2 with 4 M urea (66, 73). However, as discussed above, the starting material is clearly partially oxidized. Our observations show that all subunits (except d) are much more sensitive to oxidation than the original Hb, so the dodecamer is likely to be oxidized to a greater extent. We have found no evidence for a ferrous (abcd)2 complex of ~200 kDa. Our experiments show that oxidation of the abcd complex causes complete separation of the abc trimer from chain d at pH 6.8. Furthermore, the data of Herskovits and Harrington (7) on the effects of 4 M urea on *L. terrestris* Hb suggest that 5–9% of the Hb may become denatured by this treatment. Because some of the “dodecamer” is oxidized and therefore dissociated, the preparation cannot be completely homogeneous and must contain both free abc trimer and chain d. This conclusion is consistent with the rather broad distribution of molecular masses observed for the dodecamer by scanning transmission electron microscopy (66, 76).

Fifth, the composition of the crystals of the putative dodecamer was examined by SDS-polyacrylamide gel electrophoresis and found to contain both abc trimer and chain d (73). However, this preparation must be non-homogeneous because of the dissociation of abc and d as a result of oxidation. The extent to which the crystals were washed with the protein-free crystallizing solution is unclear. The observation would be uninterpretable without meticulous exhaustive washing.

Sixth, Sharma et al. (76) have assumed that high concentrations of guanidinium salts, urea, or heteropolyltungstates are required to dissociate the Hb at neutral pH to produce the presumed dodecamer and other products. The dissociation and subsequent reassociation upon removal of the agent were followed by HPLC chromatography with the fitting of the resulting patterns to a series of modified Gaussian distributions. Some of the experiments involved treatment with 8 M urea in

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7 The top tracing between 530 and 580 nm in Fig. 5 of Martin et al. (5) is not identified, but is the HbO2 before reaction with ferricyanide (S. N. Vinogradov, personal communication).

8 Calculated from the mean and the highest and lowest values provided.
which oxidation, denaturation and heme loss occur rapidly. We believe that the heterogeneous kinetics observed for dissociation under these and similar conditions may have resulted from the combined effects of dissociation caused by oxidation and heme loss as well as partial denaturation by treatment with the dissociating agent. Although they concluded that oxidation-induced dissociation is far too slow to be significant, our data clearly show that it can occur rapidly.

We conclude from this analysis that the presence of an (abcd) dodecamer as a principal subunit of L. terrestris Hb is not well supported.

Acknowledgments—We are indebted to the following individuals for valuable discussions and help in many ways: Wen-Yen Kao, Elizabeth Karpova, Kazuhito Matsumura, Lena Nilsson, David Shortt, and Qiang Xie. We are grateful for the assistance of Steven J. Kolodziej in performing image analyses and to Richard M. Caprioli and Terry B. Farmer of the Analytical Chemistry Center of the Health Science Center of the University of Texas at Houston for mass spectrometric measurements.

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