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Bracelet protein: A quaternary structure proposed for the giant extracellular hemoglobin of *Lumbricus terrestris*

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ABSTRACT **The complete dissociation of the hexagonal bilayer structure of *Lumbricus terrestris* hemoglobin (3900 kDa) at neutral pH, in the presence of urea, guanidine hydrochloride, sodium perchlorate, potassium thiocyanate, sodium phosphotungstate, and sodium phosphomolybdate, followed by gel filtration at neutral pH on Sephacryl S-200 or Superose 6, produced two fragments, II (65 kDa) and III (17 kDa); NaDodSO$_4$/polyacrylamide gel electrophoresis showed that peak II consisted of subunits D1 (31 kDa, chain V), D2 (37 kDa, chain VI), and T (50 kDa, disulfide-bonded trimer of chains II, III, and IV) and that peak II consisted of subunit M (16 kDa, chain I). When dissociation was incomplete, two additional peaks were present, peak Ia eluting at the same volume as the whole hemoglobin and peak Ib (200 kDa). Scanning transmission electron micrographs of peak Ia showed it to consist of whole molecules and of incomplete hexagonal bilayer structures, missing an apparent 1/12th. Peak Ib contained all four subunits but was usually deficient in subunits D1 and D2, was not always in equilibrium with the whole molecule, and could be dissociated further into II and III. The patterns of dissociation observed at neutral pH were very similar to those observed previously at alkaline pH and at acid pH and appear to be incompatible with the generally accepted multimeric model of *Lumbricus* hemoglobin subunit structure. A model is proposed in which it is postulated that the stoichiometries of some of the subunits need not be constant and that subunits D1 and D2 either form a "bracelet" decorated with complexes of T and M subunits or serve as "linkers" between the latter, to provide the appearance of a two-tiered hexagonal structure. Additional support for the proposed model comes from observations that the fragment II obtained subsequent to dissociation at pH 4, in sodium phosphotungstate, in sodium perchlorate, and in potassium thiocyanate was found to be in equilibrium with a hexagonal bilayer structure IaR(II), whose dimensions were ≈20% smaller than those of the native hemoglobin.**

The invertebrate extracellular Hbs can be classified into four separate groups based on their subunit structures (1): (*i*) monomeric single polypeptide chain proteins, (*ii*) aggregates of two or more chains each containing from 8 to 20 heme-binding domains, (*iii*) aggregates of two or more chains containing 2 heme-binding domains, and (*iv*) hexagonally symmetric arrays of small subunits, some of which are disulfide-bonded and not all of which carry heme. The extracellular Hbs and chlorocruorins of the annelids form the latter group. These 60S molecules have the characteristic two-tiered hexagonal appearance in electron micrographs (2, 3) and, unlike almost all other vertebrate and invertebrate Hbs and myoglobins, have a low iron content of 0.24% ± 0.03% (4–6). The Hb of *Lumbricus terrestris*, the common earthworm, is the most studied of the annelid Hbs: it has a mass of ≈3900 kDa, a diameter of 30 nm, a height of 20 nm and an iron content of 0.22% (7–10). NaDodSO$_4$/PAGE of the unreduced Hb shows that it consists of four subunits.‡ M (chain I, 16 kDa), D1 (chain V, 31 kDa), D2 (chain VI, 37 kDa), and T (50 kDa), a disulfide-bonded trimer of 16- to 19-kDa chains II, III, and IV (10).

The quaternary structures of most annelid extracellular Hbs and chlorocruorins are stable at neutral pH and dissociate only at pH above 8 or below 5 (4–6). The dissociation of *L. terrestris* Hb at both extremes of pH has been studied in detail in our laboratories (11, 12). In both cases, the Hb was found to dissociate into the same three types of fragments§: Ib (200 kDa), II (65 kDa), and III (17 kDa). NaDodSO$_4$/PAGE showed that although Ib contained all four subunits, it was deficient in subunits D1 and D2 and that fragments II and III consisted of subunits D1, D2, and T, and subunit M, respectively. In addition, Ib was not in equilibrium with the whole molecule and appeared to be a "pseudoprotomer"—i.e., it was not 1/12th of the whole molecule.

The dissociation of annelid Hbs and chlorocruorins at neutral pH has not been investigated in detail. In studies using light scattering molecular mass measurements, Herskovits and Harrington had explored the effect of urea and its derivatives and of chaotropic salts such as $NaClO_4$ on the dissociation of *Lumbricus* Hb at neutral pH (13, 14). They interpreted their results in terms of equilibria between the duodecamer, the native hexagonal bilayer structure, hexamers, an assumed planar hexagonal half-molecule and monomers, the putative 1/12ths of the whole Hb, without providing any evidence for the existence in solution of the postulated species.

MATERIALS AND METHODS

All buffers were prepared with distilled deionized water, and all contained 1 mM EDTA. All experiments were carried out at 4°C. Live specimens of *L. terrestris* were obtained from Connecticut Valley Biological Supply Co. (Southampton, MA) and Forest City Bait Farm (London, ON). The Hb was prepared as described (7). The concentrations were determined using an absorptivity of 0.442 $ml \cdot mg^{-1} \cdot cm^{-1}$ for the *Lumbricus* cyanmetHb at 540 nm. The Hb used in our experiments was never more than 1 week old.

Gel filtration in 0.1 M Tris·HCl (pH 7), in 0.1 M sodium phosphate buffer (pH 7.0) (NPB), and in 0.1 M sodium acetate (pH 4.0), was carried out using 2 × 100 cm columns of Sephacryl S-200 or S-300 and a column of Superose 6 (1 ×

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Abbreviations: Gdn·HCl, guanidine hydrochloride; STEM, scanning transmission electron microscope.

‡The term subunit is used to denote the smallest building blocks of *Lumbricus* Hb obtained by NaDodSO$_4$/PAGE under nonreducing conditions.

§The term fragments is used to denote the dissociation products of *Lumbricus* Hb obtained by gel filtration either at an extreme of pH or in the presence of dissociating agents.

30 cm), using an FPLC system (Pharmacia). The molecular mass standards used in gel filtration were apoferritin (450 kDa), catalase (250 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), phosphorylase (96 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrate (29 kDa), sperm whale myoglobin (17.8 kDa), and cytochrome *c* (12.3 kDa).

$NaDodSO_4$/PAGE was carried out in slab gels of 15% polyacrylamide in the Laemmli (15) buffer system. All gels were stained with Coomassie brilliant blue R250. The gels were scanned on a soft laser scanning densitometer (Biomed).

Protein samples for electron microscopy were prepared as described (11, 12) and examined in the dark-field mode with the high-resolution field emission scanning transmission electron microscope (STEM) at the University of Chicago (16).

Lumbricus Hb in NPB (≈2.5 mg/ml) was exposed to a dissociating agent and subjected to centrifugation at 38,000 × *g* for 18 hr; the supernatant was subjected to gel filtration. *Lumbricus* Hb was dissociated at pH 4.0 by centrifugation in 0.1 M sodium acetate buffer; the supernatant was dialyzed against water and subjected to gel filtration on Sephacryl S-300 at pH 7 or to FPLC on a Superose 6 column as described above (12).

RESULTS

Dissociation of *Lumbricus* Hb. The supernatants obtained by centrifugation in the presence of 1 M, 2 M, 3 M, and 4 M urea were subjected to gel filtration on the Sephacryl S-200 column. There was no dissociation in 1 M urea. The supernatants obtained in 2 M and 3 M urea provided two peaks in their elution profiles, Ia and Ib. Peak Ia eluted at the same volume as the native Hb. The elution profile obtained with the supernatant in 4 M urea consisted of three peaks—Ib, II, and III. The percentage dissociation, calculated as $S/(S + P)$, where S and P are the quantity of Hb in the supernatant and pellet fractions, respectively, and corrected for pellet dissolution, was 12% in 2 M, 20% in 3 M, and 42% in 4 M urea.

The elution profiles of the supernatants obtained in 2 M and 4 M urea obtained by gel filtration on Sephacryl S-200 in NPB are shown in Fig. 1 *A* and *B*, respectively. The molecular masses of peaks Ib, II, and III obtained from FPLC on the Superose 6 column were 200 kDa, 65 kDa, and 17 kDa, respectively. It can be seen from the $NaDodSO_4$/PAGE patterns shown in Fig. 1 (*Inset*) that Ia (lane e) consisted of subunits M, D1, D2, and T in the same proportion as the native Hb fragment Ib (lanes b and f) consisted of the four subunits but was deficient in subunits D1 and D2; fragment II (lane c) consisted of subunits D1, D2, and T, and fragment III (lane d) consisted of subunit M only.

No dissociation was observed when the Hb was centrifuged in 1 M sodium molybdate/1 M sodium tungstate. Dissociation was observed in 10–100 mM sodium phosphotungstate and sodium phosphomolybdate. When subjected to gel filtration at neutral pH, the Hb dissociated in sodium phosphotungstate or sodium phosphomolybdate gave four peaks (Ia, Ib, II, and III), whose elution volumes and $NaDodSO_4$/PAGE patterns were similar to those observed in the case of urea dissociation. When peak Ia was reexposed to 10 mM sodium phosphotungstate and subjected to gel filtration, four peaks (Ia′, Ib′, II′, and III′) were observed as shown in Fig. 2*A*. Fig. 2*A* (*Inset*) shows the $NaDodSO_4$/PAGE patterns of the first generation peaks Ia, Ib, II, and III (lanes b–e). Likewise, peak Ib reexposed to 10 mM sodium phosphotungstate also gave four peaks (Ia′, Ib′, II′, and III′), as shown in Fig. 2*B*. Fig. 2*B* (*Inset*) shows the $NaDodSO_4$/PAGE patterns of the fractions Ia′, Ib′, II′, and III′ obtained from peak Ia (lanes b–e) and from peak Ib (lanes f–i). It can be seen that, qualitatively, the $NaDodSO_4$/PAGE patterns of peaks Ia, Ib, II, and III (Fig. 2*A*) obtained in this case were similar to the patterns of the corresponding peaks obtained with the urea supernatants (Fig. 1).

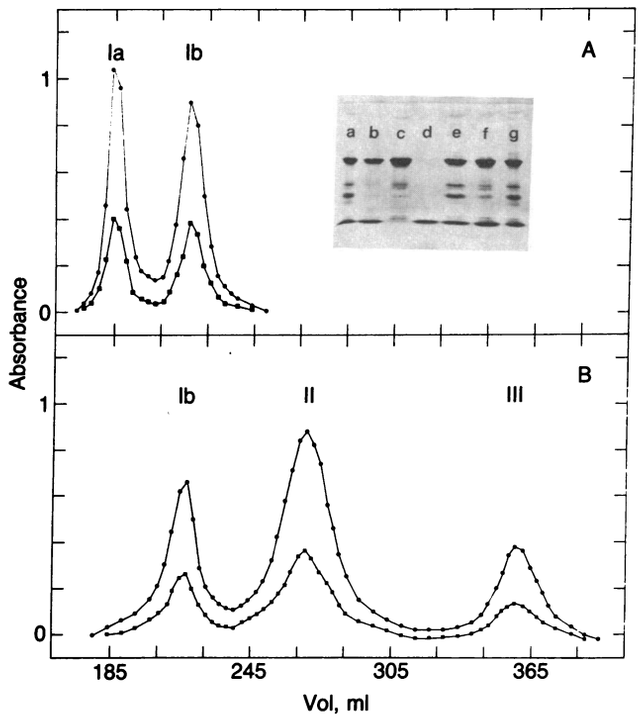

FIG. 1. Elution profiles at 280 nm (upper curve in each panel) and 415 nm (lower curve in each panel) of the supernatant of *Lumbricus* Hb in 2 M urea (*A*) and in 4 M urea (*B*). Arrow indicates elution volume of the undissociated Hb. (*Inset*) Laemmli $NaDodSO_4$/PAGE pattern of the unreduced Hb (lanes a and g); unreduced peaks Ia (lane e) and Ib (lane f) from *A*; and unreduced peaks Ib (lane b), II (lane c), and peak III (lane d) from *B*.

Lumbricus Hb dissociated in 1 M but not in 0.75 M $NaClO_4$ and KSCN. Gel filtration of the supernatants on Sephacryl S-200 at pH 7.0 gave three peaks (Ia, II, and III). Although no Ib was observed on Sephacryl S-200 gel filtration, a very small peak Ib was observable by FPLC on a Superose 6 column. The elution volumes and molecular masses of the three peaks were the same as before. Most of peak Ia was due to the dissolution of the pellet in the time period between the stopping of the centrifuge and the removal of the supernatant from the tube. The elution profile of the supernatant obtained in 1 M $NaClO_4$ is shown in Fig. 2*C*. The $NaDodSO_4$/PAGE patterns of the three peaks (*Inset*) are similar to the patterns of the peaks Ia, II, and III obtained in the presence of urea and sodium phosphotungstate (Figs. 1*B* and 2*A*).

Lumbricus Hb dissociated in 2 M and 3 M but not in 1 M guanidine hydrochloride (Gdn·HCl). Gel filtration at neutral pH provided four peaks (Ia, Ib, II, and III), whose elution volumes and molecular masses determined by FPLC were similar to those found in the other cases. The $NaDodSO_4$/PAGE patterns of the four peaks were again similar to those of the corresponding peaks obtained previously.

Repeated Dissociation and Gel Filtration of Peaks Ia and Ib. Peaks Ia and Ib obtained by gel filtration subsequent to exposure to 2.5 M urea were subjected to two additional cycles of dissociation in 2.0 M urea followed by gel filtration on Sephacryl S-200 or FPLC on Superose 6 at neutral pH. Peak Ia gave peaks Ia′ and Ib′ and Ia″ and Ib″. The $NaDodSO_4$/PAGE of these fractions are shown in Fig. 3. The results of densitometric scans of the patterns for Ia, Ia′, and Ia″ (lanes b, d, and e) are given in Table 1. It can be seen that in the series Ia, Ia′, and Ia″ (lanes b, d, and e), there was a

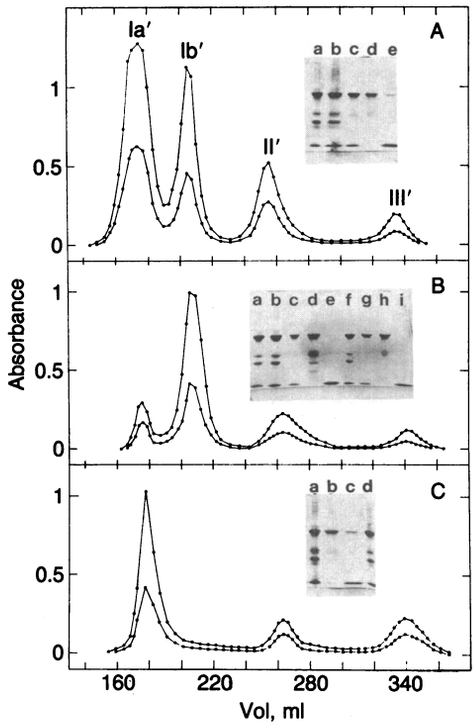

FIG. 2. Elution profiles at 280 nm (upper curve in each panel) and 415 nm (lower curve in each panel) of fractions Ia (*A*) and Ib (*B*) of *Lumbricus* Hb dissociated in 10 mM sodium phosphotungstate after a second dissociation in 10 mM sodium phosphotungstate and the supernatant fraction of *Lumbricus* Hb dissociated in 1 M $NaClO_4$ (*C*). (*A, Inset*) Laemmli $NaDodSO_4$/PAGE patterns of unreduced Hb (lane a) and of unreduced first generation peaks Ia (lane b), Ib (lane c), II (lane d), and III (lane e). (*B, Inset*) Laemmli $NaDodSO_4$/PAGE patterns of unreduced Hb (lane a); the unreduced second generation peaks Ia' (lane b), Ib' (lane c), II' (lane d), and III' (lane e) derived from the dissociation of peak Ia; and the unreduced second generation peaks Ia' (lane f), Ib' (lane g), II' (lane h), and III' (lane i) derived from the dissociation of peak Ib. (*C, Inset*) Laemmli $NaDodSO_4$/PAGE patterns of unreduced Hb (lane a), peak Ia (lane b), peak II (lane c), and peak III (lane d).

slight increase in the content of subunits D1 and D2. In the series Ib, Ib', and Ib" (lanes c, f, and g), there is no perceptible alteration in the patterns, provided one takes into account the differences in protein loads.

Peak Ib, when dissociated in 2 M urea and subjected to gel filtration at neutral pH, gave a very small peak Ia' and peaks Ib', II', and III'. When peak Ib' was dissociated in 2 M urea and subjected to gel filtration at neutral pH, it gave peaks Ib", II", and III", and no peak Ia". These results suggested that very little and no reassociation had occurred in the first and second dissociation and gel filtration cycles, respectively. The $NaDodSO_4$/PAGE patterns of peaks Ia, Ib, Ib', and Ib" are shown in Fig. 4 (lanes b–e). It can be seen that, although the pattern of the Ia peak was similar to that of the native Hb, the patterns of the Ib, Ib', and Ib" peaks were progressively more deficient in subunits D1 and D2. In fact, Ib" appeared to be almost completely devoid of the dimeric subunits. Table 1 gives the results of densitometric scans of the corresponding lanes: the proportions of subunits M and T in the series Ib, Ib', and Ib" were increased relative to those in the Hb at the expense of subunits D1 and D2.

Table 1. Results of densitometric scans of the $NaDodSO_4$/PAGE patterns of *Lumbricus* Hb, of peaks Ia, Ia', Ia'', Ib, Ib', and Ib'' obtained by repeated dissociation in urea followed by gel filtration and of the reassociated molecule IaR(II) (pH 4)

	Subunit			
	M	D1	D2	T
Hb	20 ± 3	22 ± 2	10 ± 2	48 ± 5
Hb − M*	—	27	13	60
Hb − (D1 + D2)†	30	—	—	70
Ia	18 ± 2	17 ± 3	12 ± 1	53 ± 4
Ia'	18 ± 3	19 ± 2	12 ± 2	51 ± 5
Ia''	16 ± 3	21 ± 2	14 ± 2	49 ± 3
Ib	24 ± 4	3 ± 1	6 ± 2	67 ± 5
Ib'	20 ± 2	3 ± 1	8 ± 1	69 ± 4
Ib''	24 ± 2	—	3 ± 1	73 ± 3
IaR(II) (pH 4)	—	26 ± 3	14 ± 2	60 ± 3

Values are expressed as mean (±SD).
*Calculated assuming no monomer present.
†Calculated assuming no D1 and D2 present.

Reassociation of Fragment II. Fragments II obtained by dissociation at pH 4, dialysis of the supernatant against water and then against 0.1 M imidazole chloride buffer (pH 7), followed by gel filtration at pH 7 (12), and the fragments II obtained by gel filtration at neutral pH subsequent to dissociation in 4 M urea/2 M Gdn·HCl/10 mM sodium phosphotungstate/1 M $NaClO_4$/1.5 M KSCN were checked for spontaneous reassociation by FPLC on the Superose 6 column at pH 7.0. Fragments II obtained from dissociation at pH 4 and in sodium phosphotungstate/$NaClO_4$/KSCN exhibited partial reassociation to a whole molecule as evidenced by the appearance of a small peak IaR(II), eluting at a slightly

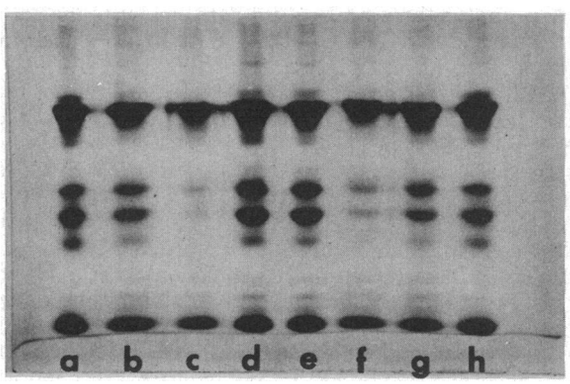

FIG. 3. $NaDodSO_4$/PAGE patterns of unreduced Hb (lanes a and h); unreduced peaks Ia (lane b) and Ib (lane c) of the Hb exposed to 2.5 M urea; and unreduced peaks Ia' (lane d), Ia" (lane e), Ib' (lane f), and Ib" (lane g) obtained from two additional cycles of dissociation of peak Ia in 2 M urea followed each time by gel filtration.

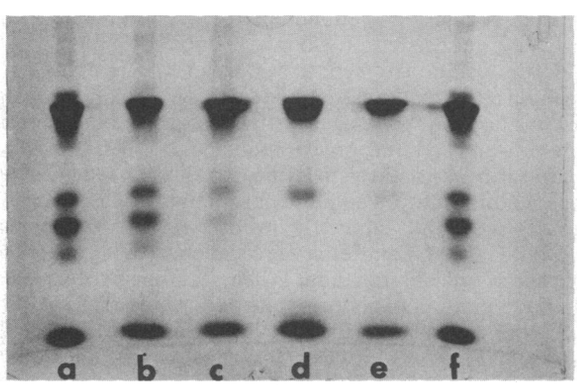

FIG. 4. $NaDodSO_4$/PAGE patterns of unreduced Hb (lanes a and f), unreduced peaks Ia (lane b) and Ib (lane c) of the Hb dissociated in 2.5 M urea, and unreduced peaks Ib' (lane d) and Ib" (lane e) obtained from two additional cycles of dissociation of peak Ib in 2 M urea, followed each time by gel filtration.

greater volume than the native Hb, 12.1 ml versus 11.5 ml. The results of densitometric scans of the $NaDodSO_4$/PAGE patterns of IaR(II) (pH 4) are given in Table 1. The proportions of subunits D1, D2, and T were the same for all the fragments and agreed closely with the proportions calculated on the basis of the relative proportions of the three subunits in the Hb. STEM views of IaR(II) (pH 4) are shown in Fig. 5 *C* and *D*. The dimensions and surface areas are given in Table 2 and compared with the dimensions of native *Lumbricus* Hb and of IaRs obtained after dissociation at alkaline pH (11) and to dissociation at acid pH (12).

Incomplete Hexagonal Bilayer Structures in Peak Ia. The peaks Ia, Ia′, and Ia″ obtained in urea dissociation when examined by STEM showed the presence of incomplete hexagonal bilayer structures—i.e., the presence of Hb molecules that appear to have lost an apparent 1/12th. A couple of typical images are shown in Fig. 5 *E* and *F*.

DISCUSSION

One of the two significant findings is that the hexagonal bilayer structure of *Lumbricus* Hb exposed to very different dissociating agents at neutral pH dissociated into the same three types of fragments—Ib, II, and III. The two smallest fragments (II and III) consisted of subunits D1, D2, and T, and subunit M, respectively. The size of ≈65 kDa for fragment II suggests that it is a complex of subunit T (50 kDa) and of an aggregate of subunits D1 and D2 (31 + 37 = 68 kDa). The molecular mass of 18 kDa for peak III is in agreement with the known size of subunit M (7). Fragment Ib has a mass of 200 kDa and, although it consists of all four subunits, it is invariably deficient in subunits D1 and D2. These results are very similar to those obtained in the dissociation of *Lumbricus* Hb at alkaline pH (11) and at acid pH (12). The Ib fragment is the counterpart of the 9S–11S fragments observed by ultracentrifugation of several annelid Hbs at alkaline pH (4–6). The Ib fragments obtained in this study and in the dissociations at alkaline and acid pH (11, 12) are unlikely to be the 1/12th protomers of *Lumbricus* Hb for the following reasons: (*i*) A 1/12th of an ≈3900-kDa molecule should have a mass >300 kDa; 200 kDa, obtained by FPLC, is too low. (*ii*) The subunit composition of Ib is always different from that of the native Hb; it is deficient in subunits D1 and D2. (*iii*) The Ib fragments are not always in equilibrium with whole Hb. (*iv*) Two cycles of dissociation and gel filtration of Ib obtained in urea dissociation leads to a fragment Ib″ which is almost totally devoid of subunits D1 and D2. These facts are not compatible with the generally accepted multimeric model of earthworm Hb subunit structure. In particular, it can be seen that the interpretations put forward by Herskovitz and Harrington to account for the dissociation of *Lumbricus* Hb at neutral pH: duodecamer–hexamer and hexamer–monomer equilibria in 2–3 M and 4 M urea, respectively; the simultaneous presence of duodecamers, hexamers, and monomers in 1 M $NaClO_4$; and a duodecamer–hexamer equilibrium in 1 M Gdn·HCl (13, 14), are not useful in explaining the experimental results obtained in this and in our previous studies.

Table 2. STEM dimensions of *Lumbricus* Hb and of the reassociated molecules

Molecule	Vertex to vertex diameter, nm	Surface area, nm²		
		Height	Top view	Side view
Hb*	30.8	20.1	790	480
P2 (pH 4)†	25.2	16.0	630	390
IaR(II)‡	24.8	16.2	610	395

*From ref. 11.
†From ref. 12.
‡Obtained by FPLC on Superose 6 of II produced by the dissociation of the Hb at pH 4 (12).

We would like to consider a radically different model of the quaternary structure of *Lumbricus* Hb, in which the relative stoichiometries of the four subunits need not be constant. In this "bracelet" model we assume that subunits D1 and D2 form a closed circular collar or bracelet decorated with 12 complexes of several T and M subunits each, providing the electron microscopic appearance of a symmetrical hexagonal bilayer. Fig. 6 shows a diagrammatic representation of this model. Such a structure may fragment in a variety of ways, ranging from the formation of a Ib fragment capable of reassociation to the whole molecule (case A) to another Ib fragment where the contents of subunits D1 and D2 are insufficient for the regain of the hexagonal bilayer structure (case B). Although *Lumbricus* Hb appears to produce only type B fragments, the Hb of *Eisenia foetida*, whose

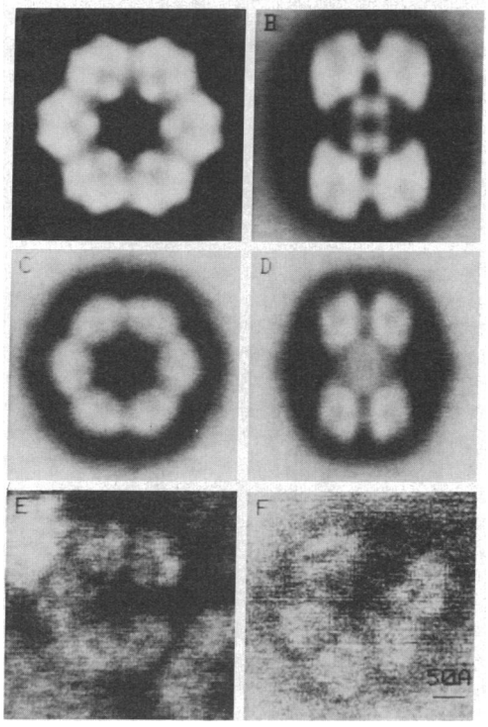

FIG. 5. Top and side views of *Lumbricus* Hb (*A* and *B*), of the reassociated fractions IaR(II) (pH 4) (*C* and *D*), and of incomplete hexagonal bilayer structures found in fraction Ia″ prepared by repeated dissociation in 2 M urea (*E* and *F*), obtained by summing and averaging the digitized STEM images, 57, 60, 19, and 20 in cases *A–F*. The images were grey level expanded after processing.

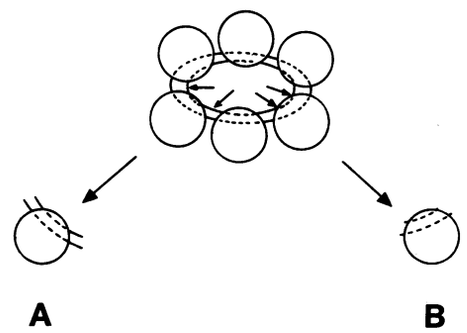

FIG. 6. Qualitative model of the quaternary structure of *Lumbricus* Hb consisting of a bracelet of D1 and D2 subunits decorated with complexes of T and M subunits. Arrows indicate the points at which scission of the bracelet structure will lead to the formation of two types of Ib fragments.

unreduced $NaDodSO_4$/PAGE pattern is almost identical to that of *Lumbricus* Hb (17), appears to produce both types. The type B fragment is produced by exposure to alkaline pH or through aging, and the type A fragment is produced by freezing and thawing (18). The $NaDodSO_4$/PAGE patterns of the two type of fragments are very convincing: relative to the type A fragment, the type B fragments are markedly deficient in subunits D1 and D2 (18).

The results of our experiments on repeated dissociation in urea of peaks Ia and Ib are completely consistent with our model and the view that the dissociation of *Lumbricus* Hb occurs by the shearing off of the "pseudoprotomers" together with some subunits D1 and D2. The evidence for it can be summarized briefly as follows. (*i*) Incomplete hexagonal bilayer structures missing one or more such pseudoprotomers are observed by STEM (Fig. 5 *E* and *F*). (*ii*) Densitometry of the $NaDodSO_4$/PAGE patterns of Ia, Ia′, and Ia″ reveals a slight progressive increase in the contents of subunits D1 and D2. (*iii*) Dissociation and gel filtration of Ib produced a small peak Ia′ and Ib′, II′ and III′; dissociation and gel filtration of Ib′ produced no peak Ia″ and peaks Ib″, II″, and III″; thus, the regaining of a hexagonal bilayer structure was possible provided there was some D1 and D2 present. (*iv*) $NaDodSO_4$/PAGE of Ib, Ib′, and Ib″ showed a progressive disappearance of subunits D1 and D2 (Fig. 4 and Table 1): Ib″ consisted of subunits T and M and was unable to reassociate to anything larger than itself. Its presence demonstrated that complexes of T and M subunits of ≈200 kDa do exist.

The second striking result obtained in this study is the partial reassociation of fragment II even though it consists only of subunits D1, D2, and T. The elution volume of IaR(II) (pH 4) on Superose 6 was consistent with its dimensions determined from STEM (Table 2). The smaller size was expected because it is missing subunit M, which accounts for ≈20% of the whole protein. This result is fully consistent with our model, since according to it, the regaining of a hexagonal bilayer structure by a dissociated fragment is predicated on the latter having a sufficient number of D1 and D2 subunits to form the bracelet.

The bracelet model of *Lumbricus* Hb explains a number of contradictory observations from different laboratories concerning the fragments produced by the dissociation of several earthworm Hbs at alkaline pH and their respective ability or inability to be in equilibrium with whole molecules. Heretofore, this morass of contradictory experimental observations has been explained as the manifestation of a "molecular heterogeneity": ". . . the native Hb consists of molecules differing slightly in the pH of their dissociation; under dissociating conditions, namely alkaline pH, a fraction of the molecules would dissociate irreversibly, whereas the remaining molecules would remain undissociated" (4). It appears to us that our model provides a much simpler explanation.

Although the results obtained in this study and in our studies of the dissociation of *Lumbricus* Hb at both extremes of pH (11, 12) are consistent with the proposed model, they do not provide direct proof for the existence of a bracelet structure. It is possible that subunits D1 and D2 do not form a continuous bracelet structure but may act as linkers between complexes of T and M subunits.

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1. Vinogradov, S. N. (1985) *Comp. Biochem. Physiol.* **82B**, 1–15.
2. Kapp, O. H., Vinogradov, S. N., Ohtsuki, M. & Crewe, A. V. (1982) *Biochim. Biophys. Acta* **704**, 546–548.
3. Vinogradov, S. N., Kapp, O. H. & Ohtsuki, M. (1982) in *Electron Microscopy of Proteins*, ed. Harris, J. (Academic, New York), Vol. 3, pp. 135–163.
4. Antonini, E. & Chiancone, E. (1977) *Annu. Rev. Biophys. Bioeng.* **6**, 239–271.
5. Chung, M. C. M. & Ellerton, H. D. (1979) *Prog. Biophys. Mol. Biol.* **35**, 53–102.
6. Vinogradov, S. N., Shlom, J. M., Kapp, O. H. & Frossard, P. (1980) *Comp. Biochem. Physiol.* **67B**, 1–16.
7. Vinogradov, S. N., Shlom, J. M., Hall, B. C., Kapp, O. H. & Mizukami, H. (1977) *Biochim. Biophys. Acta* **492**, 136–155.
8. Vinogradov, S. N., Shlom, J. M., Kapp, O. H. & Frossard, P. (1981) in *Invertebrate Oxygen Binding of Proteins*, eds. Lamy, J. & Lamy, J. (Dekker, New York), pp. 193–200.
9. Pilz, I., Schwartz, E. & Vinogradov, S. N. (1980) *Int. J. Biol. Macromol.* **2**, 279–283.
10. Shlom, J. & Vinogradov, S. N. (1973) *J. Biol. Chem.* **248**, 7904–7912.
11. Kapp, O. H., Polidori, G., Mainwaring, M. G., Crewe, A. V. & Vinogradov, S. N. (1984) *J. Biol. Chem.* **259**, 628–639.
12. Mainwaring, M. G., Lugo, S. D., Fingal, R. A., Kapp, O. H., Crewe, A. V. & Vinogradov, S. N. (1986) *J. Biol. Chem.* **261**, 10899–10908.
13. Herskovits, T. T. & Harrington, J. P. (1975) *Biochemistry* **14**, 4964–4971.
14. Harrington, J. P. & Herskovits, T. T. (1975) *Biochemistry* **14**, 4972–4976.
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
16. Crewe, A. V. (1983) *Science* **221**, 325–330.
17. Frossard, P. (1982) *Biochim. Biophys. Acta* **704**, 524–534.
18. Frossard, P. (1982) *Biochim. Biophys. Acta* **704**, 535–541.