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## A disulfide-bonded trimer of myoglobin-like chains is the principal subunit of the extracellular hemoglobin of *Lumbricus terrestris*

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The extracellular hemoglobin of *Lumbricus terrestris* (3900 kDa) consists of at least six different polypeptide chains: I through IV (16–19 kDa), V (31 kDa) and VI (37 kDa) (Vinogradov, S.N., Shlom, J.M., Hall, B.C., Kapp, O.H. and Mizukami, H. (1977) *Biochim. Biophys. Acta* **492**, 136–155). SDS-polyacrylamide gel electrophoresis of the unreduced hemoglobin shows that chains II, III and IV form a disulfide-bonded 50 kDa subunit. This subunit was isolated by gel filtration of the hemoglobin on Sephadex G-200 (a) at neutral pH in 0.1% SDS and (b) in 0.1 M sodium acetate buffer (pH 4.0); in the latter case it retains heme. The 50 kDa subunit obtained by method (b) was reduced and subjected to chromatofocusing on a PBE 94 column: the elution pattern obtained with Polybuffer 74 (pH 4.5) and monitored at 280 nm, consisted of three peaks A, B and C; peaks A and B but not C, had absorbance at 410 nm. SDS-polyacrylamide gel electrophoresis showed that peaks A, B and C corresponded to chains II, IV and III, respectively. Amino acid analyses and N-terminal sequence determinations identified chain II as the chain whose primary structure had been determined (Garlick, R. and Riggs, A. (1982) *J. Biol. Chem.* **257**, 9005–9015). Carbohydrate analysis of the native hemoglobin shows it to contain  $2.0 \pm 0.5\%$  carbohydrate consisting of mannose and *N*-acetylglucosamine in a mole ratio of about 9:1. The carbohydrate content of the 50 kDa subunit is  $1.8 \pm 0.5\%$ ; it consists of mannose and *N*-acetylglucosamine in the same ratio and it appears to be associated with chain IV. Rabbit polyclonal antisera to the 50 kDa subunit, prepared by method (a), and to the native hemoglobin were shown to cross-react with the hemoglobin and the 50 kDa subunit, respectively, by immunodiffusion. One of eight mouse monoclonal antibodies directed against the native hemoglobin reacted strongly with the 50 kDa subunit prepared by methods (a) and (b) in an enzyme-linked immunosorbent assay (ELISA). Another monoclonal antibody reacted strongly with the 50 kDa subunit obtained by method (b). Neither of the two hybridomas exhibited a strong reaction with any of the three constituent chains of the 50 kDa subunit. These results suggest that the unusual disulfide-bonded 50 kDa subunit, consisting of three myoglobin-like polypeptide chains of which only two have heme, is an integral part of the native *Lumbricus* hemoglobin molecule.

### Introduction

The extracellular hemoglobin of the common earthworm *Lumbricus terrestris* is the best studied

Abbreviations: HPLC, high-pressure liquid chromatography;  
ELISA, enzyme-linked immunosorption assay.

of the giant extracellular hemoglobins found in the annelid phylum [1–3]. It has a molecular mass of approx. 3900 kDa, contains  $156 \pm 5$  heme groups and exhibits the characteristic two-tiered hexagonal appearance in electron micrographs [1–6]. When the reduced hemoglobin is subjected to

SDS-polyacrylamide gel electrophoresis, it dissociates into six polypeptide chains: I through IV, of 16–19 kDa and two chains, V and VI, of 33 and 37 kDa, respectively [4,6]. SDS-polyacrylamide gel electrophoresis of the unreduced hemoglobin shows it to consist of four subunits: a monomeric subunit of 16 kDa corresponding to chain I, two subunits in the 27–33 kDa range, which upon reduction correspond to polypeptide chains V and VI, and a subunit of approx. 50 kDa which consists of a disulfide-bonded trimer of chains II, III and IV. The 50 kDa subunit accounts for about 40% of the total molecule and chain I accounts for approx. 20% [1,2]. Although subunit I has been shown to be a heme-containing polypeptide chain of 16 kDa [6], the 50 kDa subunit has not been isolated under conditions which would allow it to retain heme. The distribution of heme among the subunits of the *Lumbricus* hemoglobin is of interest because it and all the known annelid extracellular hemoglobins are unique among vertebrate and invertebrate hemoglobins in having fewer heme groups than the number of constituent polypeptide chains of approx. 16 kDa. *Lumbricus* hemoglobin has an iron content of 0.22 wt.% [4]: hence, less than two-thirds of its constituent polypeptide chains contain heme. We have isolated the 50 kDa subunit of *Lumbricus* hemoglobin with the heme still present and are able to show that only two of the three polypeptide chains contain heme and that this subunit is an integral part of the native hemoglobin structure and not an artefact of SDS-polyacrylamide gel electrophoresis or of the isolation procedure.

## Experimental materials and methods

**Materials.** *L. terrestris* hemoglobin was prepared as described previously and stored in 0.1 M Tris-HCl buffer (pH 7.2)/1 mM in EDTA [4]. The trimer subunit was isolated by two methods: (a) gel filtration on 2.5 × 100 cm columns of Sephadex S-200 in 0.1 M sodium phosphate buffer (pH 7.0)/0.1% SDS and (b) gel filtration on 5 × 60 cm columns of Sephadex G-200 in 0.1 M sodium acetate buffer (pH 4.0). The concentration of *Lumbricus* hemoglobin was determined by using  $E_{540} = 0.492 \text{ cm}^{-1} \cdot \text{mg}^{-1} \cdot \text{ml}^{-1}$  for its cyanferri form [4]. The concentration of the 50 kDa subunit

was determined using  $E_{280} = 1.92 \text{ cm}^{-1} \cdot \text{mg}^{-1} \cdot \text{ml}^{-1}$  based on a dry weight determination (unpublished observations).

**Chromatofocusing.** The 50 kDa subunit of *Lumbricus* hemoglobin was converted to the cyanferri form by the addition of potassium ferri-cyanide and potassium cyanide [7] and reduced with 1 mM dithiothreitol/0.025 M imidazole buffer (pH 7.0) at 4°C [8] for 3–5 h. The reduced material was applied to a 0.9 × 50 cm column of PBE 94 (Pharmacia Fine Chemicals, Piscataway, NJ), previously equilibrated with the 0.025 M imidazole buffer (pH 7.0) and eluted with Polybuffer 74 (Pharmacia Fine Chemicals) diluted 10-fold and adjusted to pH 4.5 with HCl. The absorbance of the eluate was monitored at 280 nm and at 410 nm. The pooled fractions were subjected to chromatography on a 0.7 × 2.5 cm column of Phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals) in order to remove the Polybuffer. Ammonium sulfate was added to the pooled fractions to 80% of saturation and the samples applied to the column, they were washed with 80% saturated ammonium sulfate followed by distilled water and then eluted with 50% aqueous acetic acid. The eluted protein fractions were pooled and lyophilized.

**Polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis was carried out using either cylindrical gels or slab minigels at acrylamide concentrations ranging from 10 to 15% or using acrylamide concentration gradient between 5 and 20% and the buffer systems of Weber et al. [9] or of Laemmli [10]. The gels were stained with Coomassie brilliant blue R250 in methanol/acetic acid/water (4 : 1 : 10, v/v) solution and then destained in methanol/acetic acid/water (4 : 1 : 10, v/v).

**Amino acid analysis and sequence determination.** Protein samples were hydrolyzed in constant b.p. HCl at 110°C for 22 h, dried and derivatized to the phenylthiocarbamate form immediately before chromatographic analysis using a Waters Pico-Tag system [11]. Automated amino acid sequence determination [12] was performed using a Beckman 890M sequencer. 3 nmol of protein, previously denatured by performic acid oxidation [13], was degraded sequentially, and the resulting phenylthiohydantoin derivatives were separated by isocratic elution from a Beckman 2.0 × 250 mm ultra-

sphere C18 column using a 0.01 M sodium acetate buffer (pH 4.9)/acetonitrile mixture (62:38, v/v) [14] and a Waters HPLC system. Phenylthiohydantoin/norleucine was used as an internal standard.

**Carbohydrate analysis.** Lyophilized protein samples were analyzed by gas chromatography of their trimethylsilylmethyl glycosides following the procedure of Bhatti et al. [15]. Hydrolysis was performed in methanol/0.5 M HCl at 80°C for 7 h followed by neutralization with silver carbonate and re-N-acetylation with acetic anhydride. Samples were silylated (22°C, 45 min) with Sylon HTP (Supelco, Bellefonte, PA). The trimethylsilylmethyl glycosides were separated and quantitated by gas chromatography on a DB-5 bonded-phase fused silica capillary column (30 m × 0.32 mm) using a temperature program and a Varian Model 6000 gas chromatograph. Perseitol, 5 nmol, was used as an internal standard.

**Production of rabbit antisera.** Rabbits were immunized with antigen dissolved in 0.15 M NaCl to a final concentration of 1.2 mg/ml mixed with an equal volume of Freund's adjuvant containing Pertussis vaccine and emulsified. The animals were injected intradermally at 15–20 sites over their backs [16]. Booster injections were administered 3 months later using the antigen dissolved in incomplete Freund's adjuvant. 1 week after boosting, 50 ml of whole blood was collected from the proximal ear vein, the blood clotted for 4 h at 37°C and the antisera harvested by centrifugation at 2000 × g.

**Immunodiffusion.** A 1% agar solution in 0.015 M Tris-HCl buffer (pH 7.6)/0.1 M sodium chloride/2 mM calcium chloride/0.02% sodium azide was used to prepare 75 × 25 mm plates. A five-hole rosette pattern of wells, each with 10 µl capacity, was punched out. The antiserum was placed in the central well and the antigens were placed in the peripheral wells. Diffusion was allowed to proceed for 24 h at 4°C in a humidified chamber.

**Production of hybridoma monoclonal antibodies.** The hybridomas were obtained by using a modification of the procedure of Kohler and Milstein [17]. BALB/c mice were immunized with 0.1 ml of hemoglobin (2 mg/ml) in complete Freund's adjuvant in both rear-foot pads. 1 month later, the same concentration of hemoglobin in incomplete Freund's adjuvant was injected into both rear-foot

pads and intradermally into each flank. 1 month later the spleen cells were removed and fused to azaguanine-resistant NS1 cells [18] obtained from Dr. M.D. Poulik (Beaumont Hospital, Royal Oak, MI) in the presence of 50% poly(ethylene glycol) 4000 (Merck and Co.). After 15 days the supernatants of all wells containing clones were tested for anti-hemoglobin activity using the ELISA described below. All positive clones were then recloned twice using the method of limiting dilution and spleen cell feeder layers. To obtain ascites, hybridoma cells were injected into BALB/c mice which had been primed with pristane (2,6,10,14-tetramethylpentadecane) 2 weeks previously. After 2 weeks, the ascites fluid was harvested and the cells were removed by centrifugation at 3000 × g for 20 min.

**Monoclonal antibody isotyping.** The monoclonal antibodies were isotyped using rabbit anti-mouse immunoglobulins and goat anti-rabbit IgG-peroxidase conjugate in a double antibody binding assay (Kit No. 55050-K, HyClone Labs.).

**Enzyme-linked immunosorbent assay. (ELISA).** A double antibody binding assay was used for the screening of culture supernatants. Round-bottom 96-well microtiter plates (Linbro, New Haven, CT) were coated overnight at 4°C with 50 µl of the hemoglobin solution or of one of its subunits (approx. 30 µg/ml) in 0.015 M sodium phosphate buffer (pH 7.2)/0.15 M NaCl (phosphate-buffered saline), washed with phosphate-buffered saline and incubated for 1 h with 50 µl of 3% bovine serum albumin in phosphate-buffered saline. 50-µl aliquots of tissue culture supernatant or ascites fluid were added to the wells and incubated for 4 h at room temperature. The plates were washed with phosphate-buffered saline and 50 µl of alkaline phosphatase-coupled rabbit anti-mouse IgG (heavy and light chains) (Jackson Immuno Research, Avondale, PA) were added and incubated for 2 h at room temperature. After washing three times with phosphate-buffered saline, 200 µl of substrate (*p*-nitrophenylacetate in glycine buffer (pH 9.8)) was added and incubated for 20 min at room temperature. The antibody present was quantitatively measured by reading the absorbance at 405 nm in an EIA reader model EL 307 (Bio-Tek Instruments).

## Results

Fig. 1 shows the result of chromatofocusing of the *Lumbricus* hemoglobin 50 kDa subunit obtained by gel filtration at pH 4.0. The three peaks A, B and C eluted at pH  $6.30 \pm 0.09$ ,  $5.77 \pm 0.08$  and  $5.58 \pm 0.07$ , respectively; they were identified by SDS-polyacrylamide gel electrophoresis, shown as the inset in Fig. 1, to be subunits II, IV and III, respectively. Because of the overloading of the gels, slight contamination with a neighboring peak can be observed. It can also be seen that only peaks A and B have absorbance at 410 nm associated with the 280 nm absorbance, i.e., only subunits II and IV contain heme. The N-terminal sequence of the polypeptide chain II isolated by chromatofocusing was found to be Lys-Lys-Gln-Cys-Gly-Val-Leu-Glu-Gly-Leu-Lys-Val. This sequence identifies our chain II as the chain A-III isolated and sequenced by Garlick and Riggs [19]. The N-terminal residues of chains III and IV together with their amino acid compositions (not shown here), identify chains III and IV with the polypeptide chains A-II and A-I, respectively, isolated previously by Garlick and Riggs using ion-exchange chromatography in 8 M urea [19,20].

Table I presents the results of carbohydrate

analysis of native *Lumbricus* hemoglobin, the 50 kDa subunit isolated by SDS gel filtration and of each of the three chains isolated by chromatofocusing. The results show that the carbohydrate portion of *L. terrestris* hemoglobin consists exclusively of mannose together with a small amount of *N*-acetylglucosamine (mole ratio Man : GlcNAc = 9 : 1). Within the 50 kDa subunit, the carbohydrate side-chains which also contain approximately the same ratio of mannose to *N*-acetylglucosamine, appear to reside exclusively on chain IV. If the carbohydrate portion of the latter is taken to consist only of one GlcNAc and eight mannose the percentages by weight of carbohydrate in chain IV (19 kDa) and in the 50 kDa subunit should be approx. 8.5 and 3.4%, respectively. Thus, the difference between the experimental carbohydrate contents and the minimum content assuming only one carbohydrate side-chain, suggests that the glycosylation of the 50 kDa subunit is heterogeneous.

Fig. 2 shows the results of double-diffusion experiments using the rabbit polyclonal antisera to native hemoglobin in well A and to the 50 kDa subunit isolated by SDS gel filtration, in well B. It can be clearly seen that there are precipitin lines between the antiserum to the hemoglobin (well A) and the 50 kDa subunit isolated by gel filtration at

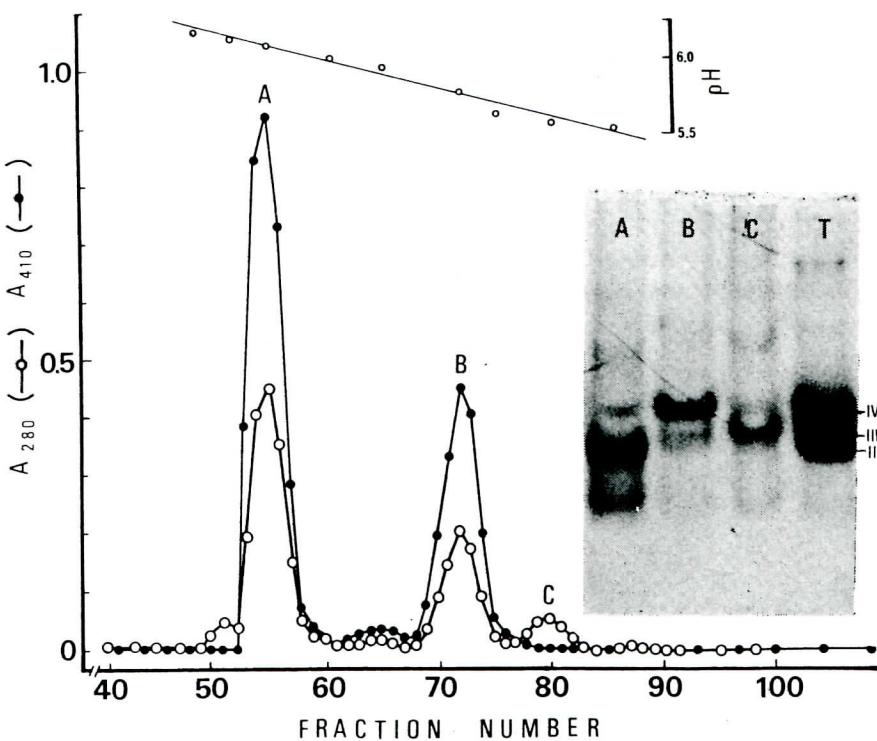


Fig. 1. Chromatofocusing elution profile of the reduced 50 kDa subunit of *L. terrestris* hemoglobin isolated by gel filtration at acid pH. The inset shows a Laemmli SDS slab gel electrophoresis of peaks A, B and C obtained by chromatofocusing: (A) peak A (chain II), (B) peak B (chain IV), (C) peak C (chain III) and (T) 50 kDa subunit of *Lumbricus* hemoglobin.

TABLE I

CARBOHYDRATE ANALYSIS OF *L. TERRESTRIS* HEMOGLOBIN, THE 50 kDa SUBUNIT AND ITS THREE CONSTITUENT POLYPEPTIDE CHAINS

Material	Carbohydrate content (wt.%)	Mannose <sup>a</sup>	<i>N</i> -Acetyl glucosamine <sup>a</sup>	Mole ratio Man : GlcNAc
Hemoglobin	2.0 ± 0.5 <sup>b</sup>	113 ± 29 <sup>b</sup>	12 ± 3 <sup>b</sup>	9.4 ± 0.5
50 kDa subunit	1.8 ± 0.5 <sup>b</sup>	92 ± 28 <sup>b</sup>	11 ± 4 <sup>b</sup>	8.4 ± 1.0
Chain II	n.d. <sup>c</sup>	n.d.	n.d.	—
Chain III	n.d.	n.d.	n.d.	—
Chain IV	4.8 <sup>d</sup>	17.4 <sup>d</sup>	2.4 <sup>d</sup>	7.4

<sup>a</sup> values in ng/mg protein.

<sup>b</sup> Average of four determinations (± S.D.).

<sup>c</sup> Not detected.

<sup>d</sup> Average of two determinations.

acid pH (well 6). Furthermore, there is a reaction between the antiserum to the 50 kDa subunit isolated by SDS gel filtration (well B) and the native hemoglobin (well 2) as well as the 50 kDa subunit isolated by gel filtration at acid pH (well 4). In addition, although the antiserum to the hemoglobin (well A) gave weak precipitin lines with the three isolated chains, the antiserum to the 50 kDa subunit isolated by SDS gel filtration (well B) gave a definite precipitin line with chain II only (chains III and IV not shown here). The dark halos around wells 1 on the left and wells 1, 3 and 5 on the right, are due to absorption by heme because of the high hemoglobin concentration. Reaction with the hemoglobin in both cases occurs only at a dilution of 1:100. In separate experiments, not shown here, it was determined that the antiserum to the 50 kDa subunit isolated by SDS gel filtration did react with the 50 kDa subunit isolated by gel filtration.

A large number (over 50) of monoclonal mouse

antibodies to *Lumbricus* hemoglobin were obtained. Ten of the strongest reacting antibodies were kept. Of these two (EHb 1 and EHb 9) were

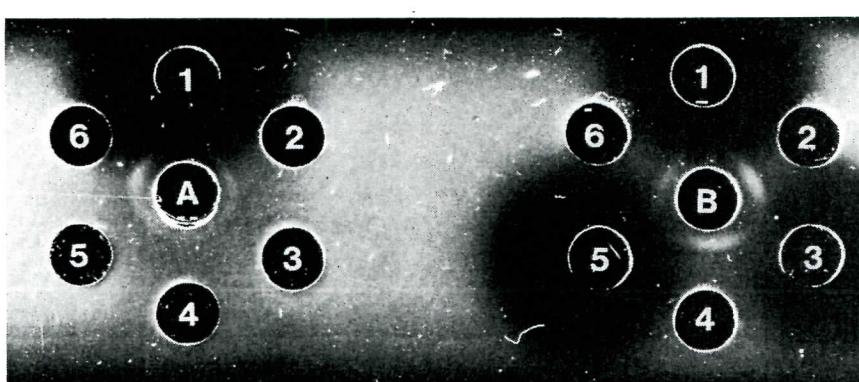
TABLE II

ELISA OF *L. TERRESTRIS* HEMOGLOBIN, THE 50 kDa SUBUNIT AND ITS CONSTITUENT POLYPEPTIDE CHAINS WITH MURINE MONOCLONAL ANTIBODIES TO THE NATIVE HEMOGLOBIN

Absorbance at 405 nm after 20 min reaction time using undiluted tissue culture supernatant. NS, tissue culture supernatant from the lymphoma to which the immunized spleen cells were fused.

	Monoclonal antibody			
	EHb 1	EHb 6	EHb 9	NS
Hemoglobin	1.7	1.7	1.7	0
50 kDa subunit (a)	1.6	0	1.6	0
50 kDa subunit (b)	0	0	1.6	0
Chain II	0.16	0	0.15	0
Chain III	0.16	0	0.16	0
Chain IV	0.16	0	0.15	0

Fig. 2. Immunodiffusion in agar gel using rabbit polyclonal antisera to *L. terrestris* hemoglobin (A) and to its 50 kDa subunit isolated by SDS gel filtration (B). (A) Wells: 1, *Lumbricus* whole blood; 2, *Lumbricus* hemoglobin, 1:100 dilution; 3, chain II; 4, chain III; 5, chain IV and 6, 50 kDa subunit isolated by gel filtration at acid pH. (B) Wells: 1, *Lumbricus* whole blood; 2, *Lumbricus* hemoglobin, 1:100 dilution; 3, *Lumbricus* hemoglobin, 1:10 dilution; 4, 50 kDa subunit isolated by gel filtration at acid pH; 5, *Lumbricus* hemoglobin, 1:5 dilution and 6, chain II.



found to react with the 50 kDa subunit obtained by gel filtration at acid pH. Table II shows the results of a typical ELISA; hybridoma EHb 6 was used as a control. All three antibodies were found to be IgG<sub>1</sub>. Although EHb 9 reacted with the 50 kDa subunit isolated by both methods, hybridoma EHb 1 did not react with the 50 kDa subunit isolated by SDS gel filtration. Both of these monoclonals gave very weak ( $A < 0.16$ ) reactions against the three polypeptide chains isolated by chromatofocusing from the 50 kDa subunit obtained by gel filtration at acid pH.

## Discussion

The giant, annelid extracellular hemoglobins are unique among all known hemoglobins, intra- or extracellular, in having an iron content that ranges between 0.20 and 0.27 wt.% considerably below the normal iron content of about 0.33 wt.% of other invertebrate and vertebrate hemoglobins and myoglobins. Our results on the separation of the three polypeptide chains of the 50 kDa subunit of *L. terrestris* hemoglobin represent the first evidence for the existence of a non-heme-binding constituent polypeptide chain of an extracellular hemoglobin. It is possible that the 50 kDa subunit isolated by gel filtration at acid pH may have lost some heme; however, we would expect that if all three chains II, III and IV bind heme in the intact hemoglobin, that they would also bind it at least partially when isolated by chromatofocusing. If it proves correct that polypeptide chain III of *Lumbricus* hemoglobin does not contain heme, it might be due to alteration(s) in the primary structure of its heme-binding domain. This may be similar to the case of *Ascaris lumbricoides* hemoglobin, the only non-annelid hemoglobin to also have a low iron content [21,22]: this hemoglobin is an octamer of approx. 38 kDa subunits each containing only one heme group. Chromatographic separation of the heme-containing polypeptide chains of several annelid hemoglobins has been achieved: *Travisia japonica* hemoglobin [23] and *Perinereis brevicirris*, *Tylorrhynchus heterochaetus* and *Neanthes diversicolor* hemoglobins [8]. However, at the same time no heme-free polypeptide chains were isolated, thus presenting the conundrum of reconciling the low iron content of the

foregoing hemoglobins with the presence only of heme-containing polypeptide chains. It must be realized that since the iron content of annelid extracellular hemoglobins is about two-thirds that of the vertebrate and other invertebrate hemoglobins, at least a third of all the constituent monomeric chains must have no heme [24]. It is therefore necessary to admit that at least one of the dimeric subunits, chains V and VI, also contain little or no heme. The 50 kDa subunit which is ubiquitous in all oligochaete and polychaete hemoglobins examined so far by SDS-polyacrylamide gel electrophoresis [24–26] but which does not appear to be present, at least not in disulfide-bonded form, in leech hemoglobins [27–29], represents approx. 40% of the total protein. Since the monomeric, heme-containing subunit (chain I in *Lumbricus* hemoglobin) is also ubiquitous in annelid extracellular hemoglobins, including the leeches [27–29], it is evident that the presence of only one non-heme-binding chain in the 50 kDa subunit is insufficient to explain the low iron content of *Lumbricus* hemoglobin. Recent work in our laboratories suggests that chain VI has heme but that chain V does not have any heme associated with it (unpublished observations). It is possible that exposure to acid pH results in a partial loss of heme in the 50 kDa subunit. However, the experimental fact that we are trying to account for in *Lumbricus* hemoglobin in particular, and in annelid hemoglobins in general, is the presence of too little iron, i.e., too little heme, not of normal or elevated iron or heme contents.

The results of carbohydrate analysis (Table I) show that *Lumbricus* is a high-mannose glycoprotein. Besides the 50 kDa subunit, carbohydrate is known to be present in subunits V and VI but not I (Yurewicz, E.C. and Vinogradov, S.N., unpublished observations). In the 50 kDa subunit, it appears that most of the carbohydrate side-chain groups reside on polypeptide chain IV. The discrepancy between the percent carbohydrate by weight of the 50 kDa subunit and that calculated assuming that the latter contains at least one GlcNAc and eight mannose suggests that not all 50 kDa subunits are equally glycosylated, due perhaps to unequal availability during post-translational modification.

The immunochemical results show that poly-

clonal antisera as well as monoclonal antibodies directed against the whole *Lumbricus* hemoglobin molecule recognize the 50 kDa subunit isolated by SDS gel filtration, a denaturing method and by gel filtration at acid pH, assumed to be a non-denaturing method. Furthermore, the polyclonal anti-serum to the 50 kDa subunit isolated by SDS gel filtration at acid pH, assumed to be a non-denaturing method, recognizes the subunit isolated by acid pH gel filtration as well as the whole *Lumbricus* hemoglobin. Of the monoclonal antibodies, hybridoma EHb 9 reacts with the 50 kDa subunit obtained by both methods and hybridoma EHb 1 reacts only with the subunit obtained by the non-denaturing method; thus, it is clear that SDS alters the conformation of a portion of the 50 kDa subunit while leaving other areas structurally intact. Together with the fact that the two antibodies react very weakly with the three constituent polypeptide chains of the 50 kDa subunit, our findings indicate that the two epitopes towards which the hybridomas EHb 1 and EHb 9 are directed, are conformation-dependent, i.e., they depend on the presence of the native conformation of the disulfide-bonded trimer of chains II, III and IV and not on the presence of any one chain or portion thereof. The conclusion that can be drawn is that the 50 kDa subunit is a structural building block of the native *Lumbricus* hemoglobin molecule and is not an artefact of SDS-polyacrylamide gel electrophoresis or of the method used in its isolation. Although we expect such a subunit to be the dominant subunit in all known oligochaete and polychaete extracellular hemoglobins, a non-disulfide-bonded trimer subunit may also be present in leech hemoglobins. Furthermore, disulfide-bonded tetramers of myoglobin-like polypeptide chains have been suggested to occur in chlorocruorins [30,31], the extracellular oxygen-binding proteins which contain a slightly altered heme group and which also have a low iron content and a very similar electron microscopic appearance and in at least one annelid hemoglobin, that of the polychaete *Nephthys incisa* [26], which is unusual in that it contains an additional subunit in the generally empty central cavity [32]. We are pursuing the isolation of disulfide-bonded and other subunits of annelid hemoglobins and chlorocruorins and the characterization of their heme

and carbohydrate contents. In addition, we are employing monoclonal antibodies to the various subunits of annelid hemoglobins and chlorocruorins as probes to determine the location and stoichiometry of the subunits by immunoelectron microscopy.

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