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Leech Extracellular Hemoglobin: Two Globin Strains That are Akin to Vertebrate Hemoglobin α and β Chains

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ABSTRACT—Leech (*Whitmania edentula*, *Haemadipsa zeylanica* var. *japonica* and *Erpobdella lineata*) extracellular hemoglobins are basically composed of three constituent subunits, a dimer (D_1 and D_2 chains) and two monomers (M1 and M2 chains). We isolated these four chains from respective species by a combination of reversed-phase chromatography on a Resource RPC column and gel-filtration on a Superdex 75 column. The apparent molecular masses of the four globin chains were estimated by SDS-PAGE analysis to be 13 kDa (M1), 16 kDa (M2; 19 kDa in its reduced form) and about 27 kDa for the dimer subunit (13 kDa for D_1; 15 kDa for D_2), regardless of the source. The amino (*N*)-terminal segments (21-30 residues) from twelve globin chains of the above three species were determined and aligned. It was found that the twelve sequences could be separated into two distinct globin groups A and B. This finding supports the original idea of "two globin strains in annelid hemoglobin", which was proposed without any evidence for leech hemoglobins. Comparing the sequences in the three classes of Annelida, Hirudinea, Oligochaeta and Polychaeta, we found two invariant amino acids, Cys and Trp, which are interposed by eleven amino acid residues. Furthermore, the globin chains belonging to strain A were readily discernible as they had three more invariants, Ser-13, Asp-16 and Trp-28, while the globin chains of strain B had two more invariants, Lys-12 and Arg-27. Consequently, we propose that each of the three classes of Annelida have two distinct groups of globin chains that are akin to vertebrate hemoglobin α and β chains.

INTRODUCTION

Hirudinea (or Achaeta), commonly called leeches or blood suckers, is one of the classes of Annelida, in which there are four orders and ten families (Harant and Grassé, 1959). Among them, Gnathobdellida and Pharyngobdellida have hemoglobin (Bourne, 1884; Sawyer and Fitzgerald, 1980). Indeed, leech hemoglobin was reported as red pigment in the circulatory system of non-rhynchobdellids by Lankester (1872). It has become clear that its physical and chemical properties are similar to those of other annelid extracellular hemoglobins: native leech hemoglobin is a giant extracellular protein whose sedimentation coefficient is estimated to be in the range of 55-60 *S*, corresponding to a molecular weight of about 3×10^6 (Svedberg, 1933; Andonian and Vinogradov, 1975; Andonian *et al.*, 1975; Shlom *et al.*, 1975; Vinogradov *et al.*, 1976; Wood *et al.*, 1976). In addition, electron microscopic studies of the molecules have shown that they possess a gross quaternary structure comprising two hexagonal structures with a diameter of about 30 nm and a total thickness of about 20 nm (Roche *et al.*, 1960; Andonian *et al.*, 1975; Kapp *et al.*, 1990; Ilan and Haroun, 1993a, b; Haas *et al.*, 1996). The properties of oxygen binding (Waxman, 1975; Ilan and Haroun, 1993a, b; Weber *et al.*, 1995) and absorption-spectrum (Needham, 1966a; Wood *et al.*, 1976) have also been studied. Of particular interest, however, are the reports of hemoglobin subunits: leech extracellular hemoglobin is markedly different in its constituent subunits, a dimer and two monomers, while the extracellular hemoglobins of oligochaetes and polychaetes are constituted of a trimer and a monomer (Shlom and Vinogradov, 1973; Gotoh and Kamada, 1980). A maximum entropy analysis of the electrospray ionization mass spectra revealed that the hemoglobin of a gnathobdellid leech, *Macrobdella decora*, was composed of three groups of subunit peaks: two monomer peaks at 17 kDa, three dimer peaks at 24 kDa and three linker peaks at 33 kDa (Weber *et al.*, 1995).

Gotoh *et al.* (1987) first showed that the constituent subunits of annelid extracellular hemoglobin are divided into two

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strains A and B, in which A is comprised of two trimer subunit chains and B is a monomer subunit and a chain of the trimer subunit (We reversed the original A and B because the present study demonstrated that globins belonging to group A have more sequence similarities to the human hemoglobin α chain than to the β chain). However, this idea needs to be verified by studies on leech extracellular hemoglobins. To date, information on the primary structure of leech hemoglobin is very limited: Kapp *et al.* (1990) demonstrated partial amino (*N*)-terminal amino acid sequences of four globin chains of *M. decora* hemoglobin. Comparing the primary structures of polychaetes, oligochaetes and hirudinean hemoglobins showed that only the sequence of the *M. decora* 2 chain was somewhat different. Hence, we decided to analyze, in particular, the structural properties of *N*-terminal segments of four globin chains from three leech species belonging to different families: Hirudidae (Gnathobdellida), Haemadipsidae (Gnathobdellida) and Erpobdellidae (Pharyngobdellida). We also intended to extend the original idea, "two globin strains A and B in Annelida", to leech hemoglobins.

We report here on the isolation of four globin chains from *Whitmania edentula*, *Haemadipsa zeylanica* and *Erpobdella lineata*, respectively, followed by their *N*-terminal sequence analyses. Using computer-assisted alignments of the sequences of globin chains in *Lumbricus terrestris* (Oligochaeta) and *Tylorrhynchus heterochaetus* (Polychaeta), we clearly demonstrate the presence of two distinct types of globins in leech extracellular hemoglobins.

MATERIALS AND METHODS

Materials

Live leeches, *W. edentula* (WHITMAN), *H. zeylanica* var. *japonica* (WHITMAN) and *E. lineata* (O. F. MÜLLER) were collected in Gifu-Hashima (Gifu prefecture), the University Forest of The University of Tokyo (Chiba prefecture) and Fukushima (Fukushima prefecture), respectively. Acetonitrile, formic acid (90%), tri-*n*-butyl phosphine and 4-vinyl pyridine were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Trifluoroacetic acid (TFA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Separation columns, Resource RPC (3 ml packed with Source 15 RPC gel matrix) and Superdex 75 HR 10/30, were purchased from Pharmacia Biotec (Upsala, Sweden), and placed in a fast protein liquid chromatography (FPLC) system (Pharmacia Biotech). All other chemicals and solvents used were of the most purified grade available commercially.

Preparation and identification of leech hemoglobin

Hemoglobins of *W. edentula* and *H. zeylanica* were prepared by the method described previously for the preparation of oligochaete hemoglobin (Shishikura, 1996). *E. lineata* hemoglobin was prepared by the method of Ilan and Haroun (1993a, b). Globin chains were

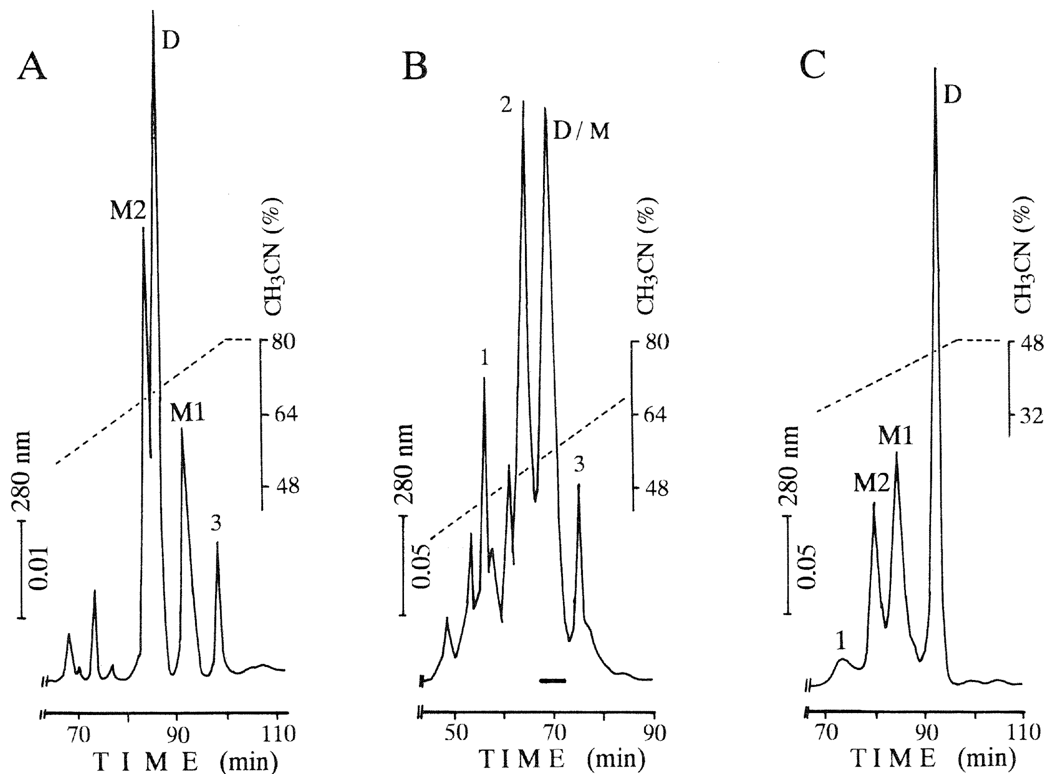

Fig. 1. Subunit separation of leech hemoglobin by reversed-phase FPLC chromatography. (**A**) *W. edentula* hemoglobin, (**B**) *H. zeylanica* hemoglobin, (**C**) *E. lineata* hemoglobin. Each sample was acidified by adding acetic acid (1/10 volume) and applied to a column (Resource RPC 3 ml packed with Source 15 RPC). Buffers were water containing 0.1% TFA (Buffer A) and 80% acetonitrile containing 0.08% TFA (Buffer B). For figures **A** and **B**, the gradient was 0% B to 100% B in 100 min, followed by 100% B for 20 min. For figure **C**, the gradient was 0% B to 40% B in 15 min, followed by 40% B to 60% B in 80 min, then 60% B for 20 min. Fractions pooled as indicated by a horizontal bar were used for further separation for globin subunits of *H. zeylanica*. The nomenclatures D, M1 and M2 are the same as described in the text.

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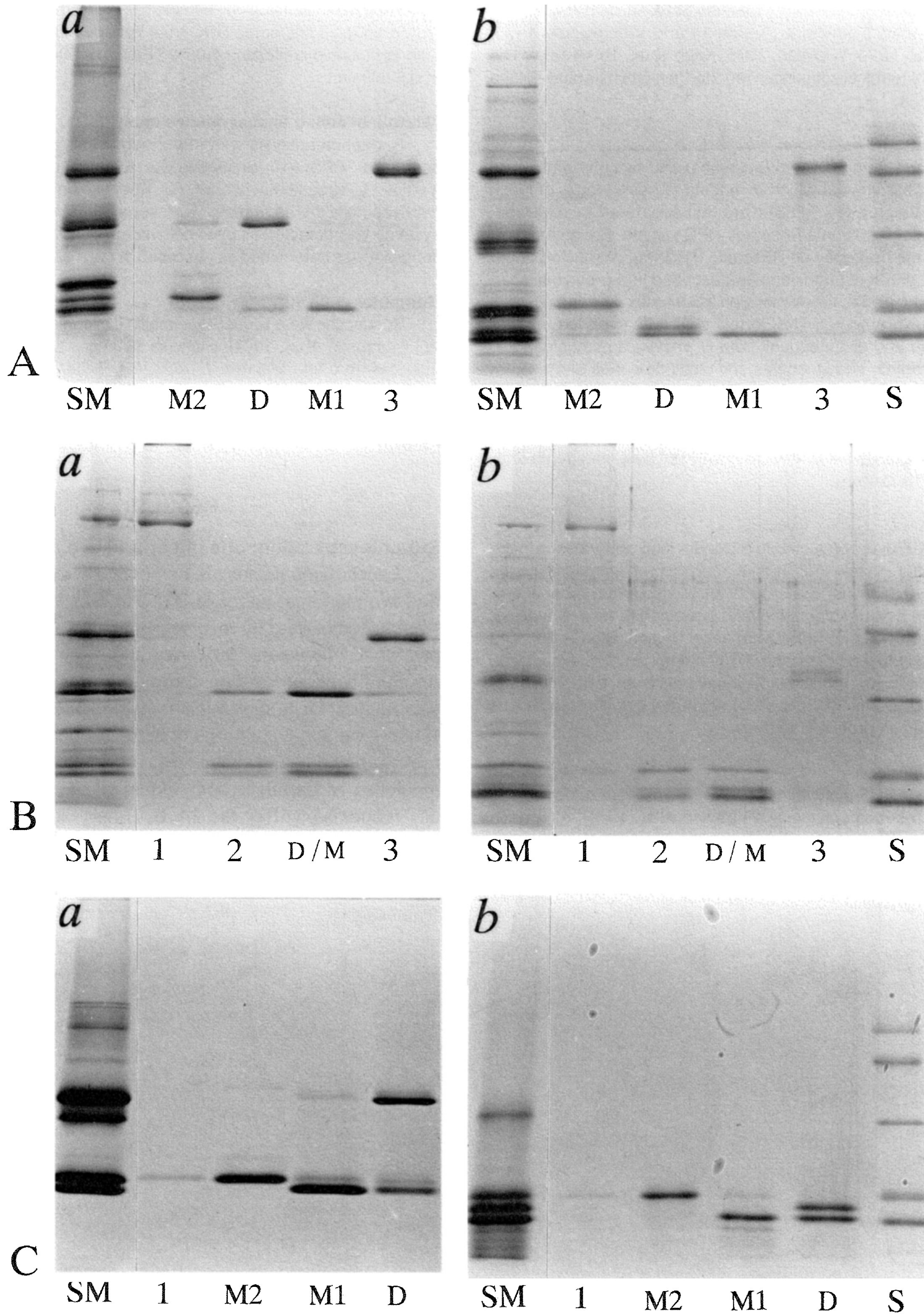

Fig. 2. SDS-PAGE patterns of peaks obtained from Fig. 1. (**A**) *W. edentula* hemoglobin, (**B**) *H. zeylanica* hemoglobin, (**C**) *E. lineata* hemoglobin. Non reducing gradient gel (panel *a*) and reducing gradient gel (panel *b*) between 10 and 20% (top to bottom) were used. Numbers (1, 2 and 3) and names (D, D/M, M1 and M2) of lanes in both panels *a* and *b* correspond to those of peaks in Fig. 1. SM, starting material; S, molecular mass markers, 66, 45, 29, 18.4 and 12.4 kDa from top to bottom.

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identified by comparing them with patterns of annelid globin chains on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) described elsewhere (Andonian and Vinogradov, 1975; Andonian *et al.*, 1975; Waxman, 1975; Kapp *et al.*, 1990) as well as similarities to *N*-terminal sequences reported in a previous study (Kapp *et al.*, 1990).

Chain separation

The dimer subunit (D_1 and D_2 chains) and two monomer subunits (M1 and M2 chains) which contribute to basic blocks of leech hemoglobin (Vinogradov, 1985) were first separated by reversed-phase chromatography on a Resource RPC column. For further purification, rechromatography on Resource RPC was conducted under more shallow gradient conditions as described in a previous report (Shishikura *et al.*, 1987). Gel-filtration on a Superdex 75 column, which had been equilibrated in 8.75% formic acid, was used to separate each monomer and dimer subunit from *H. zeylanica* hemoglobin.

To separate D_1 and D_2 chains, the disulfide-bonds of the dimer were cleaved by reduction and *S*-pyridylethylation, followed by separating each of the two constituent chains on Resource RPC from water containing 0.1% TFA to 80% acetonitrile containing 0.08% TFA. All fractions were monitored at 214 nm or 280 nm by a spectrophotometer (Model 116, Gilson).

SDS-PAGE

SDS-PAGE was conducted in a Hoeffer slab apparatus with the Laemmli's buffer system (1970) on a 10-20% gradient gel. Samples were incubated in 1% SDS at 100°C for 90 sec in the presence or absence of 1% β-mercaptoethanol and subjected to electrophoresis. The slab gel was stained with Coomassie Brilliant Blue R 250 and destained in a solution containing 5% methanol and 7% acetic acid. Bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa) and cytochrome *c* (12.4 kDa) were used as protein standards.

Protein modification

Reduction and *S*-pyridylethylation of globins were performed by the method described previously (Friedman *et al.*, 1970). After each reaction, the modified protein was dialyzed against 0.1 M ammonium bicarbonate and lyophilized. Finally, the remaining reagents were completely removed from the sample by reversed-phase column chromatography on Resource RPC from water containing 0.1% TFA to 80% acetonitrile containing 0.08% TFA. Flow rates were maintained at 0.5 ml/min.

***N*-terminal amino acid sequence analysis**

Sequence analysis was performed using a gas phase protein sequencer, PPSQ-10 (Shimadzu Co., Kyoto, Japan), equipped with a class LC-10 amino acid analyzer. Phenylthiohydantoin (PTH)-derivatives from the sequencer were separated and quantified. PTH-cysteine was detected as pyridylethylated-PTH-cysteine, whose elution point was determined as described in the manufacturer's manual.

Computer analysis

Sequences were aligned by a multiple alignment program, Clustal W (Thompson *et al.*, 1994). Pairwise distances among 26 globin sequences from four leeches, *L. terrestris*, *T. heterochaetus* and human hemoglobins were analyzed using a computer program Protdist under the Dayhoff PAM matrix option of the PHYLIP package (Felsenstein, 1993).

RESULTS

Subunit separation: one dimer and two monomers

Leech hemoglobins are comprised of a dimer subunit (D) and two monomer subunits (M1 and M2), whose separation were accomplished by reversed-phase column chromatography for *W. edentula*, *H. zeylanica* and *E. lineata* hemoglobins. An additional gel-filtration chromatography on Superdex 75 was needed for *H. zeylanica* hemoglobin. Figures 1A, B and C show the elution patterns of reversed-phase column chromatography on a Resource RPC column for *W. edentula* hemoglobin, *H. zeylanica* hemoglobin and *E. lineata* hemoglobin, respectively. Figures 2A, B, C and Fig. 3*b*, *c* show the results of SDS-PAGE analyses of the elution peaks in Fig.

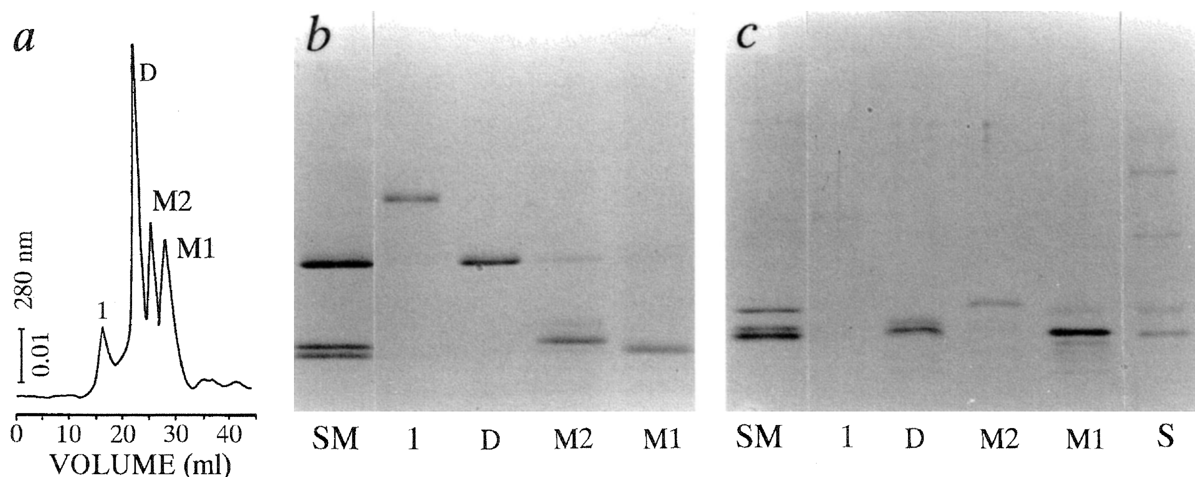

Fig. 3. Gel-filtration chromatography of *H. zeylanica* globins (panel *a*) and SDS-PAGE analyses (panels *b* and *c*). Panel *a*, pooled fractions (indicated by the horizontal bar in the Fig. 1B) were applied on a Superdex 75 column which had been equilibrated with 8.75% formic acid and eluted at a flow rate of 0.5 ml/min. Panels *b* and *c*, under non reducing gradient gel and reducing gradient gel, respectively. Numbers and names of lanes correspond to those of peaks in panel *a*. SM, starting material of the globin fractions of *H. zeylanica*; S, molecular mass markers, 66, 45, 29, 18.4 and 12.4 kDa from top to bottom.

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1A, B, C and Fig. 3*a*, respectively. Panel *a* of Fig. 3 shows a typical gel-filtration pattern of pooled globin fraction of *H. zeylanica* (indicated by a horizontal bar in Fig. 1B), resulting in the dimer and two monomers. Several protein bands were also detected in the lanes designated SM in Fig. 2. Among them, the polypeptides designated 1 and 3 were analyzed by SDS-PAGE (Fig. 2).

Separation of two constituent chains of the dimer subunit

After reduction and *S*-pyridylethylation of each dimer subunit, D_1 and D_2 chains were purified by reversed-phase chromatography on Resource RPC, whose elution patterns are shown in Fig. 4. After sequencing *N*-terminal segments of D_1 and D_2 chains, the names D_1 and D_2 were finally designated based on sequence similarities to the previously grouped globin chains of *L. terrestris* and *T. heterochaetus* hemoglobins: D_1 belongs to strain B and D_2 belongs to strain A. These elution positions of *W. edentula* D_1 and D_2 are reversed compared with those of the two other species (Fig. 4).

All the globin chains isolated and purified are shown in Fig. 5 as SDS-PAGE patterns. The apparent molecular masses of these chains were estimated by migration distances of these proteins *vs* those of standard proteins (Table 1). It is noteworthy that the mobility of M2 polypeptides of the three species differs markedly when treated with β-mercaptoethanol or by reduction and *S*-pyridylethylation (Fig. 2 and Fig. 3*b*, *c*).

N-terminal amino acid sequencing and alignment

Figure 6 shows the *N*-terminal amino acid sequences aligned by the Clustal W multiple alignment program and compared with those of *M. decora*, an oligochaete (*L. terrestris*) and a polychaete (*T. heterochaetus*) hitherto reported. Together with the results of computer analysis by Protdist under the Dayhoff PAM matrix option, all globin chains studied were divided into two distinct strains A and B, which are akin to human hemoglobin α and β chains.

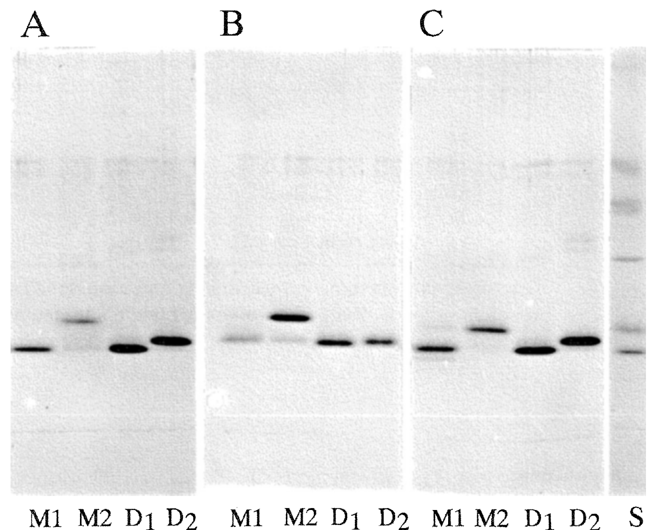

Fig. 5. SDS-PAGE analyses of purified M1, M2, D_1 and D_2 of the three species. Globin polypeptides were reduced, *S*-pyridylethylated and applied on a gradient gel between 10 and 20% (top to bottom). (**A**) *W. edentula* globin chains, (**B**) *H. zeylanica* globin chains and (**C**) *E. lineata* globin chains. The nomenclature of M1, M2, D_1 and D_2 are the same as described in the text. S, molecular mass markers, 66, 45, 29, 18.4 and 12.4 kDa from top to bottom.

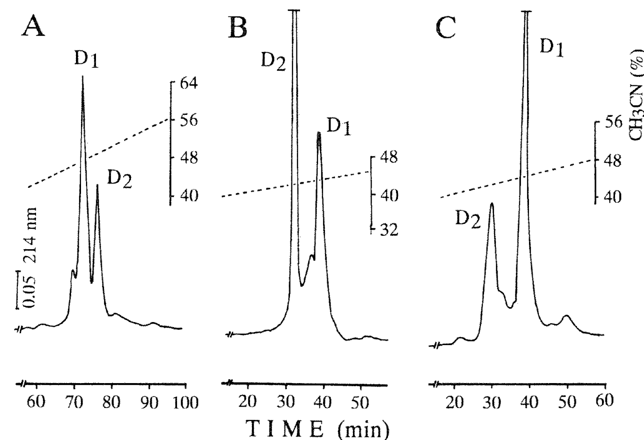

Fig. 4. Chain separation of D_1 and D_2 from the respective dimers of the three species by reversed-phase chromatography. (**A**) *W. edentula* dimer subunit, (**B**) *H. zeylanica* dimer subunit, (**C**) *E. lineata* dimer subunit. Buffers were the same as used in Fig. 1. For **A**, the gradient was 0% B to 30% B in 15 min, followed by 30% B to 80% B in 100 min. For **B**, the gradient was 0% B to 50% B in 15 min, followed by 50% B to 65% B in 90 min. For **C**, the gradient was 0% B to 50% B in 15 min, followed by 50% B to 70% B in 80 min. The nomenclatures of D_1 and D_2 are the same as described in the text.

DISCUSSION

Chain separation

The separation of the four constituent polypeptides which comprise leech extracellular hemoglobin was accomplished by separating the individual subunits, a dimer and two monomers, from native hemoglobin preparation, and then by isolating each of the two dimer chains (D_1 and D_2), whose disulfide-linkages were cleaved prior to separation by reduction and *S*-pyridylethylation. According to the results of reversed-phase column chromatographies (Figs. 1, 4) and SDS-PAGE analyses (Figs. 2, 5), the Resource RPC column provided excellent resolution in separation and purification of the constituent polypeptides. To separate the globin subunits of *H. zeylanica* hemoglobin, however, a more suitable method was needed: As shown in Fig. 3*a*, Superdex 75 for gel-filtration matrix in a column equilibrated with 8.75% formic acid provided excellent results. When higher concentrations of formic acid were used, such as 17.5% or 35%, which previously yielded good results in separating globin subunits of oligochaete hemoglobins (Shishikura *et al.*, 1987; Shishikura, 1996), however, M1 and M2 were co-eluted from the column (data not shown). With evidence showing that all M2 polypeptides

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Table 1. Apparent molecular masses of the constituent globin chains of leech hemoglobins

	W. edentula		*H. zeylanica*		*E. lineata*	
Globin chains	−βME	+βME	−βME	+βME	−βME	+βME
	kDa	kDa	kDa	kDa	kDa	kDa
M1	13.3 ± 0.3	13.8 ± 0.7	14.0 ± 1.0	13.8 ± 0.7	13.5 ± 1.0	12.8 ± 0.2
M2	15.8 ± 0.7	19.5 ± 0.5	15.7 ± 1.3	19.4 ± 0.1	15.5 ± 0.5	18.9 ± 0.1
D	27.3 ± 0.2	–	26.8 ± 0.2	–	27.3 ± 0.5	–
D_1	–	12.8 ± 0.2	–	13.6 ± 1.4	–	13.3 ± 0.3
D_2	–	14.3 ± 0.2	–	15.4 ± 0.6	–	15.6 ± 0.4

The molecular masses of the subunits and their constituent chains were determined by SDS-PAGE with or without β-mercaptoethanol (βME). The values expressed are the average of two to eight determinations. The deviations given are average deviations.

```
Globin Strain A
Hirudinea                     1        10        20        30
 M.decora 2(1)                        DE VA D KX-----W EF
 W.edentula M2           DLHGDHDHEVQ CS AG D GALVQEE W DHL W EN
 H.zeylanica M2           DVHVEDHDEL CS GG D GNIVVED W NQL W EG
 E.lineata M2                DHHLSDE CS AG D RHIVQEQ W STL W HT

 M.decora IIB(1)                 DHH CS IE D IRLV
 W.edentula D2                 SDDGE CS AA D GQQVLHD W THL W ED
 H.zeylanica D2                  DYH CS IE D IRDIQHD W QFT W GD
 E.lineata D2                     DH CS VE D IHEVQNY W NEI W GR

Oligochaeta
 L.terrestris c(2)            DEHEHC CS EE D HRIVQKQ W DIL W RD
 L.terrestris a(2)            ADDEDC CS YE D RREIRHI W DDV W SS

Polychaeta
 T.heterochaetus IIB(3)          DDC CS AA D RHEVLDN W KGI W SA
 T.heterochaetus IIC(3)          DTC CS IE D RREVQAL W RSI W SA
                                      *     *         *
 Human α chain(4)                  VL S PA D KTNVKAA W GKV G AHAGE Y GAE
 Human β chain(4)                 VHL T PE E KSAVTAL W GKV N VDEVG G EAL

Globin Strain B
Hirudinea                     1        10        20        30
 M.decora 1(1)                   SHE C NLLHRF K F
 W.edentula M1                    NE C GLLQKF K FFKQ W SEV F GVGEQ R VEF
 H.zeylanica M1                 DPHQ C GLLEKF K FYKQ W TEV F GLGEQ R IEF
 E.lineata M1                    DDK C TMIERF K IHNQ W ERA F GYGED R IVF

 M.decora IIA(1)                 SEV C EDLHAI K V
 W.edentula D1                   SHV C PELIAI K VHTQ W RVA Y ADSAD R VAL
 H.zeylanica D1                  THV C PELSAI K VQTQ W REA Y ADSSD R VAL
 E.lineata D1                     SQ C TDLDKI K VMTQ W RQA Y SSGQD R IDF

Oligochaeta
 L.terrestris I(5)                 E C LVTEGL K VKLQ W ASA F GHAHQ R VAF
 L.terrestris b(2)               KKQ C GVLEGL K VKSE W GRA Y GSGHD R EAF

Polychaeta
 T.heterochaetus I(3)             TD C GILQRI K VKQQ W AQV Y SVGES R TDF
 T.heterochaetus IIA(3)         SSDH C GPLQRL K VKQQ W AKA Y GVGHE R VEL
                                                    *
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Fig. 6. Alignment of the *N*-terminal amino acid sequences of globin chains of leech hemoglobins in comparison with those of oligochaete and polychaete hemoglobins. The alignments of globin chains belonging to strain A or B were achieved by a multiple alignment program, Clustal W (Thompson *et al.*, 1994). By postulating the five-unit deletion shown here the *M. decora* 2 chain can be made to have the invariant amino acid residue, Trp, at position 24 in the alignment. The residues indicated by boxes are invariant amino acid residues in strain A or B. The residues indicated by asterisks are homologous amino acid residues with those of the human α chain or β chain which are representative of vertebrate globin superfamily. (1), Kapp *et al.*, 1990; (2), Fushitani *et al.*, 1988; (3), Suzuki and Gotoh, 1986; (4), Braunitzer *et al.*, 1961; (5), Shishikura *et al.*, 1987.

had a rather unusual shift in relative electrophoretic mobility under reduced and non-reduced conditions (Fig. 2 and Fig. 3*b*, *c*), it can be said that the nature of the M2 polypeptides of the three species are somewhat different from those of other globin polypeptides.

Molecular size and subunit structure

The globin polypeptides of various species of leeches have been studied by SDS-PAGE under the presence and absence of β-mercaptoethanol. There is some controversy concerning their number and size (Andonian *et al.*, 1975; Andonian and Vinogradov, 1975; Shlom *et al.*, 1975; Wood *et al.*, 1976). In the present work, we were able to fractionate, purify and partially sequence the four basic constituents. The molecular masses of the four chains, M1, M2, D_1 and D_2, have been estimated to range from 13-19 kDa in reduced forms, regardless of the leech species (Table 1). The correspondence of the four globin chains examined in the present study to the previously-studied globin chains of polychaetes (Suzuki and Gotoh, 1986; Matsubara *et al.*, 1996) and oligochaetes (Shishikura *et al.*, 1987; Fushitani *et al.*, 1988; Shishikura, 1996; Shishikura and Nakamura, 1996) was deduced by comparing their *N*-terminal sequences. Therefore, all four chains are analogous to the tetramer of globin chains of polychaete and oligochaete hemoglobins. The controversy over the number and size of polypeptides is probably due to the occurrence of linker chains (or non-reducible chains, Kapp *et al.*, 1990) in other annelid hemoglobins (Vinogradov, 1985). Linker chains would not be isolated by our separation method, however, they may have been eluted in the preceding position of reversed-phase column chromatography as previously observed in the separation of globin chains and linker chains of *Pheretima communissima* and *Pontodrilus matsushimensis* hemoglobins (Shishikura and Nakamura, 1996).

In addition, the peaks designated 3 in Fig. 1A, or B, were neither globins nor linker chains because the *N*-terminal sequences (data not shown) differed from those of the globins or linker chains of annelids (Fushitani and Riggs, 1988; Suzuki *et al.*, 1990). These polypeptides may have been contaminated when the sample was collected because no homologous polypeptides were observed in *E. lineata* hemoglobin

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(Fig. 1C). Hence a tetramer of globin chains, which can occur in a disulfide-bonded dimer and two monomer subunits as indicated previously (Vinogradov, 1985; Gotoh and Suzuki, 1990; Kapp *et al.*, 1990), is one of the basic building blocks of leech hemoglobins.

Two distinct globin strains in leech hemoglobin

Previously, Gotoh *et al.* (1987) proposed a novel hypothesis of "two strains of globin chains" in the multisubunit hemoglobins of annelids. However, this hypothesis lacks crucial support as to whether it can be extended to leech hemoglobins. Kapp *et al.* (1990) were the first to report on the partial *N*-terminal sequences of four chains of *M. decora* hemoglobin, but they did not evaluate the hypothesis well enough. In this study, we provided the amino acid sequences of *N*-terminal segments of twelve globin chains from three species of leeches and aligned them with a computer assisted Clustal W multiple sequence alignment program (Thompson *et al.*, 1994). The alignment shown in Fig. 6 confirmed the "two strains of globin chains" hypothesis. In addition, we compared amino acid residues in the sequences to those of previously sequenced globin chains of *M. decora* (Kapp *et al.*, 1990), an oligochaete (Shishikura *et al.*, 1987; Fushitani *et al.*, 1988) and a polychaete (Suzuki and Gotoh, 1986). Consequently, we found two structural characteristics in their sequences: strain A can be distinguished from B by the presence of five invariant amino acid residues, Cys-Ser, Asp, Trp and Trp, in the first 30 *N*-terminal sequences, as clearly seen in Fig. 6, while strain B can be distinguished from A by Cys, Lys, Trp and Arg. In addition, it was very surprising to find that three invariant residues, Ser, Asp, and Trp, in strain A (indicated by asterisks) were conserved not only in annelids but also in the human α chain. Moreover, an invariant residue, Trp, in both globin strains (asterisk) also showed a similar correspondence with the Trp of the *N*-terminal segment of the human β chain. This is why we would like to rename A of original grouping as B and B as A.

Glossiphoniidae and Piscicolidae belong to the more primitive order Rhynchobdellida and have white colorless blood (Bourne, 1884). Needham (1966b) isolated a water-soluble yellow pigment from three glossiphoniids and a piscicolid, but the nature of the pigment is not known. Shlom *et al.* (1975) reported the presence of hemoglobin in *Placobdella*, a member of Glossiphoniidae. These studies suggest the possibility of the presence of hemoglobin in Rhynchobdellida, but systematic reinvestigations on the distribution of hemoglobin in primitive leeches may be necessary to solve this confusion.

In conclusion, although the formation of disulfide-bonded dimers or trimers seems to occur separately after the branching of Hirudinea and Polychaeta-Oligochaeta from the ancestral species of Annelida, our results provide strong support not only for the structural similarities of globin chains among the three classes, but also for extending the original idea of "two strains of globins (Gotoh *et al.*, 1987)" to the extracellular hemoglobin of Hirudinea.

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