

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/250540287>

ChemInform Abstract: Giant Hexagonal Bilayer Hemoglobins

ARTICLE *in* CHEMINFORM · APRIL 2010

Impact Factor: 0.74 · DOI: 10.1002/chin.199714332

CITATIONS

3

READS

28

6 AUTHORS, INCLUDING:



André Toulmond

Station Biologique de Roscoff

54 PUBLICATIONS 1,117 CITATIONS

SEE PROFILE



Roy E. Weber

Aarhus University

254 PUBLICATIONS 6,742 CITATIONS

SEE PROFILE



Serge N Vinogradov

Wayne State University

203 PUBLICATIONS 4,546 CITATIONS

SEE PROFILE

Giant Hexagonal Bilayer Hemoglobins

Jean N. Lamy,[†] Brian N. Green,[‡] André Toulmond,[§] Joseph S. Wall,^{||} Roy E. Weber,[⊥] and Serge N. Vinogradov^{*,#}

Laboratoire des Protéines Complexes, CNRS URA 1334, Université de Tours, 37032 Tours, France, Micromass UK Limited, 3 Tudor Road, Altrincham, Cheshire WA14 5RZ, UK, Equipe d'Ecophysiologie, Station Biologique, UPMC-CNRS-INSU, BP 74, 29682 Roscoff, France, Biology Department, Brookhaven National Laboratory, Upton, New York 11973, Department of Zoophysiology, Institute of Biological Sciences, Aarhus University, 8000 Aarhus C, Denmark, and Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan 48201

Received January 31, 1996 (Revised Manuscript Received August 2, 1996)

Contents

I. Introduction	3113
II. Physical and Chemical Properties	3113
A. Shape, Size, and Mass	3113
B. Iron, Heme, and Carbohydrate Contents	3114
C. Dissociation Defines a Hierarchy of Globin Subunits	3115
D. Globin Amino Acid Sequences	3115
E. Linker Amino Acid Sequences	3116
F. Mass Spectrometric Studies	3118
G. Phylogenetic Relationships	3119
III. Quaternary Structure	3119
A. Bracelet Model: Dodecamer vs Hexadecamer	3119
B. Three-Dimensional Reconstruction	3120
C. Effects of Group IIA Cations	3121
D. Metal Content	3121
IV. Equilibrium Oxygen Binding	3121
A. Do Linkers Play a Role in HBL Hb Cooperativity?	3121
B. Effects of Cations	3122
C. Lack of Alteration in Molecular Shape	3122
D. Stereochemistry of Ligand Binding	3122
V. Conclusion	3122
VI. References	3123

I. Introduction

The annelid phylum comprises over 8000 species, subdivided into terrestrial oligochaetes, aquatic leeches, and marine polychaetes. Most annelids have extracellular or intracellular hemoglobins (Hbs) and sometimes both.¹ The large extracellular Hbs of the polychaete *Arenicola* and the oligochaete *Lumbricus* were among the first proteins investigated by ultracentrifugation² and electron microscopy.³ They exhibit a highly symmetric HBL appearance in electron micrographs, masses in excess of 3×10^6 Da, acid isoelectric points, and high cooperativities of oxygen binding (Hill coefficients $n_{50} \approx 3$ –6 versus ~ 2.5 for

vertebrate Hbs.^{1,4–8} (Mass is in units of Daltons, where applicable.)

The discovery of hydrothermal vents on the Pacific Ocean floor at depths of up to 2500 m in 1977, and of the associated fauna, brought to light the existence in these sulfide-rich environments, of deep sea annelid species, e.g. *Alvinella* and of large, tube-dwelling vestimentiferans.^{9–11} The metabolic needs of vestimentiferan species such as *Riftia* and *Lamellibrachia*, are provided by symbiotic sulfide-oxidizing prokaryotes located in a special organ, the trophosome. Although the vascular (circulatory) compartment of the deep sea annelids contains only a HBL Hb,¹⁷ the vestimentiferans have the HBL Hb and $\sim 4 \times 10^5$ Hb; in addition, their coelomic compartment contains another $\sim 4 \times 10^5$ Hb. The deep sea Hbs bind oxygen as well as sulfide reversibly and with high affinity.^{12–16} Another group of small worms found at intermediate depths (~ 1500 m), the pogonophorans, appear to have only the smaller Hb.¹⁸

II. Physical and Chemical Properties

A. Shape, Size, and Mass

Numerous studies of negatively stained annelid Hbs by STEM have shown them to consist of two superimposed hexagons of pentagonally-shaped substructures and a central cavity, and similar dimensions, height ~ 20 nm and diameter ~ 30 nm.^{3,19} The EM appearance of the HBL Hbs from vestimentiferans and alvinellids is indistinguishable from that of annelid Hbs.^{20,21} Mass mapping studies with unstained, cryolyophilized annelid Hbs have been carried out since about 1987 using the STEM at Brookhaven National Laboratory; Figure 1 shows representative images of unstained and negatively stained *Lumbricus* Hb. The mass distributions of over 10^4 HBL particles in purified Hb and fresh blood have been analyzed recently.²² Although the resulting mass distributions are broad, they are also symmetrical and the mean masses are in good agreement with sedimentation equilibrium results, pointing to $(3.5$ – $3.6) \times 10^6$ as the most likely mass for *Lumbricus* Hb.²² The two techniques have also provided similar masses for leech (*Macrobdella*) Hb^{23,24} and *Eudistylia* Chl²⁵ (3-formyl instead of 3-vinyl). In the case of *Lumbricus* Hb, the masses determined for different preparations using the two techniques, vary over the range $(3$ – $4) \times 10^6$, suggesting that this heteromultimeric complex may not

* Please address correspondence to Dr. S. N. Vinogradov at the Department of Biochemistry, Wayne State University, 540 East Canfield, Detroit, MI 48201. Telephone: 313-577-1501. Fax: 313-577-2765. E-mail: svinogr@cms.cc.wayne.edu.

[†] Université de Tours.

[‡] Micromass UK Limited.

[§] Station Biologique, UPMC-CNRS-INSU.

^{||} Brookhaven National Laboratory.

[⊥] Aarhus University.

[#] Wayne State University School of Medicine.



Jean N. Lamy was born in La Châtre, France, in 1941. He received his B.Sc. and Ph.D. in Pharmacy at the University of Tours and a D.Sc. in Biochemistry from the University of Paris VI in 1982. He joined the faculty of the School of Pharmacy at the University of Tours in 1970 as Assistant Professor, was promoted to Associate Professor in 1975 and to Professor of Biochemistry in 1979. Since 1988 he has also been the Director of an Associated Research Unit (URA 1334) of the Centre National de Recherches Scientifiques. His research interest centers on the macromolecular structure of high molecular weight complex proteins, including hemocyanins, erythrocrucorins, α 2-macroglobulins, and immuno complexes determined from three-dimensional reconstruction of images observed by cryoelectron microscopy. He has published more than 90 papers and reviews in leading international journals.

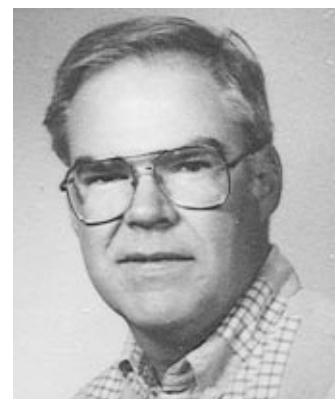


André Toulmond was born in Tourcoing, France, in 1938. He graduated from the Ecole Normale Supérieure de Saint-Cloud in 1963 and obtained a D.Sc. from the University of Paris in 1975. He joined the faculty of the Pierre-et-Marie-Curie University in Paris in 1964 and was promoted to Full Professor of Marine Biology in 1980. Since 1994, he has been the Director of the Station Biologique at Roscoff (W3:<http://www.sb-roscoff.fr>), a marine biological research station founded in 1872 and supported by the University and the Centre National de la Recherche Scientifique. His research interests center on the respiratory gas transfer systems of marine invertebrates living in extreme habitats, such as the intertidal zone and the deep sea hydrothermal vents. He is the author of over 50 publications in international journals.



Brian N. Green was born near Manchester, England, in 1933. He received a B.Sc. in General Science from Manchester University in 1955. He worked at AEI Scientific Apparatus as engineer, section leader, and technical manager, in the design and development of single and double focusing magnetic sector mass spectrometers for inorganic and organic applications from 1957 to 1972. He then joined VG Micromass (later VG Analytical) and worked in developing magnetic sector instruments principally for the organic applications market, becoming Technical Director in 1979. Since 1988, he has been involved with the design, development, and application of atmospheric pressure ionization techniques, including electrospray ionization, coupled to quadrupole instruments at VG Biotech (now Micromass UK Ltd.), where he is currently Applications Director. These techniques, either alone or combined with liquid chromatography, have vastly extended the applicability of mass spectrometry to the analysis of proteins with molecular weights ranging up to 200 000. He has over 90 publications and reviews in international journals and books.

have a constant subunit stoichiometry.²² The published masses for earthworm Hbs obtained using different methods range from 2.7×10^6 to 4.1×10^6 Da [mean $(3.4 \pm 0.5) \times 10^6$, $N = 19$] (Table 1). A. Riggs has suggested recently²⁶ that partial oxidation of the Hb may be responsible for the observed variation in mass. This appears to be unlikely for the following reasons: (1) the autoxidation rate ($\sim 0.005 \text{ h}^{-1}$)²⁷ is more than 10-fold slower than for vertebrate Mbs,²⁸ except for the L29F mutant,²⁹ possibly due to the presence of superoxide dismutase activity in the native *Lumbricus* Hb;³⁰ (2) metHb has the same sedimentation coefficient as the oxy form³¹



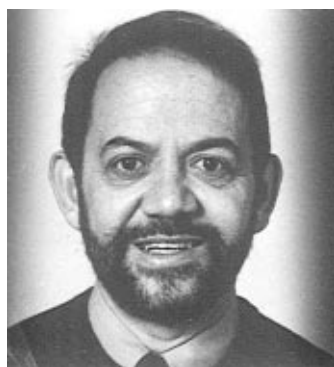
Joseph S. Wall was born in Madison, WI, in 1942. He obtained a Ph.D. in Biophysics from the University of Chicago in 1971 and has worked at Brookhaven National Laboratory since 1973; he is presently a Senior Biophysicist. His major interests include biological application of electron microscopy, instrument design and development, and techniques of specimen preparation. STEM1, designed and built by him at BNL and in service since 1977, operates as an NIH Biotechnology Resource used for mass mapping and absolute mass measurement. STEM3, completed in October, 1995, will also be used for elemental mapping at low dose. He has authored and coauthored over 200 publications.

and dissociates very slowly at neutral pH;^{31,32} (3) no significant difference was observed between the STEM mass mapping results obtained with fresh blood and with purified Hb.²²

B. Iron, Heme, and Carbohydrate Contents

The published iron and heme contents of Hbs from over 30 species of annelids range from 0.211 to 0.265 wt % (mean = 0.228 ± 0.013 , $N = 28$) and 1.83 to 3.64 wt % (mean = 2.60 ± 0.38 , $N = 29$).³³ The two mean values are in excellent agreement with each other, since 0.228 wt % iron is equivalent to 2.52 wt % heme. The iron and heme contents of other Hbs and Mbs are 0.31–0.37 and 3.4–4.1 wt %, respectively. The lower values for the HBL Hbs and Chls are due to the presence of about 30% of non-heme protein.

Carbohydrate is present in the Hbs of *Lumbricus* (2.0 wt %)³⁴ and *Perinereis* (0.5 wt %).³⁵ ESI-MS has



Roy E. Weber was born in Berea, South Africa, in 1938. He obtained a B.Sc. (Honours) from the University of Cape Town in 1959 and a Dr.Phil. in Animal Physiology at the University of Leiden, The Netherlands, in 1965. He was Head of the Experimental Biology Department at the Netherlands Institute for Sea Research until 1972, Associate Professor at Aarhus University, Denmark, until 1978 and Professor of Ecophysiology at Odense University until 1988. Since then, he has been Professor of Zoophysiology at Aarhus University. He is a member of Academia Europaea, President of the European Society of Comparative Physiology and Biochemistry, and Director of the Centre for Respiratory Physiological Adaptation of the Danish Natural Science Research Council. His research is on the molecular mechanism of the adaptation of hemoglobin function in vertebrates and invertebrates. He has over 160 articles and chapters in international journals and books.



Serge N. Vinogradov was born in Beirut, Lebanon, in 1933. He received a B.A. in Chemistry in 1952 from the American University of Beirut and a Ph.D. in Physical Chemistry from the Illinois Institute of Technology in 1959. He was a Postdoctoral Fellow in the Chemistry Department, University of Alberta, Edmonton, Canada, until 1962 and then a Research Associate in the Biochemistry Department, Yale University School of Medicine. He joined the faculty of the Department of Biochemistry of Wayne State University School of Medicine in 1966, was promoted to Associate Professor in 1968 and to Professor of Biochemistry in 1971. His research interest centers on the structure-function relationships of invertebrate hemoglobins in general, including the large multimeric and hexagonal bilayer heteromultimeric globin complexes. He has published two books, over 120 papers in international journals, and contributed 16 book chapters.

shown that *Lumbricus* globin chain *a* and linker chain L1 are glycosylated (GlcNAc₂[Man]_{*n*}, *n* = 6–9).²² No carbohydrate has been detected by ESI-MS in *Eudistylia* Chl²⁵ or in *Arenicola*,³⁶ *Tylorrhynchus*,³⁷ *Riftia*,^{38,39} *Oligobranchia*,⁴⁰ and *Macrobdella*²⁴ Hbs. However, the $\sim 4 \times 10^5$ Hbs from *Riftia* do share a globin chain which exists in several glycosylated isoforms, similar to chain *a* in *Lumbricus* Hb.^{22,38,39}

C. Dissociation Defines a Hierarchy of Globin Subunits

Early studies using SDS PAGE showed that the giant annelid Hbs consisted of two types of polypeptide chains: four or more different $(16\text{--}17) \times 10^3$

globin chains and several $(24\text{--}32) \times 10^3$ nonglobin linker chains.^{41,42} The four globin chains (*a*–*d*) from *Lumbricus* Hb occur as a monomer (*d*) and a disulfide-bonded trimer (*a*+*b*+*c*), a situation commonly observed in oligochaete and polychaete Hbs. The leech (achaete) Hbs are comprised of monomers and disulfide-bonded dimers, whereas the Chls consist of disulfide-bonded dimers and tetramers.⁴² *Riftia* Hb consists of monomers and dimers, thus resembling the leech Hbs,^{38,39} while *Alvinella* Hb consists of monomers and trimers.¹⁷ The pogonophoran *Oligobranchia* Hb appears to comprise monomers and disulfide-bonded dimers and trimers of globin chains.⁴⁰

Following the determination of the smallest subunits and number of polypeptide chains by complete dissociation of *Lumbricus* Hb, we sought to identify larger subunits via partial dissociation of the HBL structure, at pH > 8,⁴³ at pH < 6,⁴⁴ and at neutral pH in the presence of urea, Gdm·Cl, and chaotropic agents.^{45,46} These studies provided unequivocal evidence for a functional $\sim 2 \times 10^5$ subunit having the stoichiometry [*a*+*b*+*c*]₃[*d*]₃, deficient in linker chains and which could be formed spontaneously upon mixing of the isolated trimer [*a*+*b*+*c*] and chain *d*.⁴⁶ Under similar conditions, the leech (*Macrobdella*) Hb produced a tetramer of globin chains and *Eudistylia* Chl dissociated into dodecamers and tetramers in equilibrium with each other.^{23–25} A recent study of the dissociation kinetics of *Lumbricus* Hb at alkaline pH and at neutral pH in the presence of urea, Gdm salts, and heteropolytungstate cations has demonstrated that the dodecamer is observed at all stages of the dissociation,³² the extent of dissociation decreasing in the order Gdm·SCN > Gdm·Cl > urea > Gdm·OAc and [BaAs₄W₄₀O₁₄₀]^{27–} > [SiW₁₁O₃₉]^{8–} > [NaSb₉W₂₁O₈₆]^{18–}. The kinetics appear to be heterogeneous, with at least three exponentials required to provide acceptable fits in the case of urea and Gdm·Cl and two in the case of the heteropolytungstates.³² Additional evidence for the importance of the dodecamer as a principal structural subunit has come from experiments on the reassembly of the dissociated Hb, which showed it to form rapidly both in the presence and absence of group IIA cations, conditions where the HBL structure is reassembled extensively and very little, respectively.³²

D. Globin Amino Acid Sequences

The first complete primary structure of a HBL Hb was provided by the amino acid sequences of the four globin chains and two linker chains of the Hb from the polychaete *Tylorrhynchus*, determined by Suzuki and Gotoh and their collaborators.^{47,48} Apart from the sequence of the monomer subunit (chain *d*),⁴⁹ the primary structures of the other three globin chains of *Lumbricus* Hb have been elucidated in the laboratory of A. Riggs.⁵⁰ The four globin sequences are shown in Figure 2 (chain *a* exists as four glycosylated isoforms²² and chain *d* as two major and several minor isoforms^{51,52}). The primary structures of the four globin chains from the 4×10^5 Hb of the vestimentiferan *Lamellibrachia* have been determined⁵³ as well as three of the globins from the pogonophoran *Oligobranchia*;⁴⁰ they are also shown in Figure 2. The amino acid sequences align well with the vertebrate globin chains^{54,55} and also group

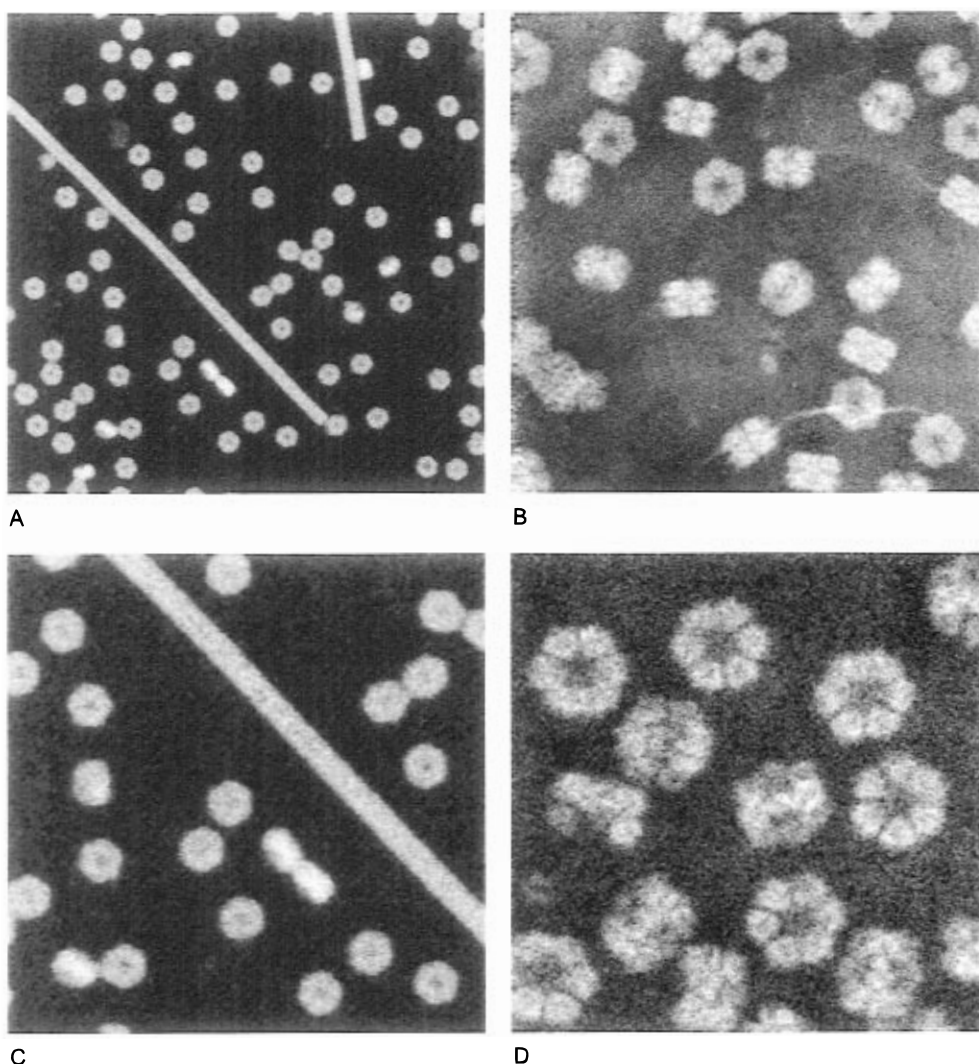


Figure 1. Scanning transmission electron microscope images of *Lumbricus* Hb: (A) unstained and cryolyophilized,⁹⁷ 5120 Å scan size; (B) negatively stained with uranyl acetate, 2560 Å scan size; (C) same as A but zoomed 2×, 2560 Å scan size; (D) negatively stained with methylamine vanadate, 1280 Å scan size. The images were obtained at the Brookhaven Scanning Transmission Electron Microscope Facility.

Table 1. Molecular Mass of Earthworm Hb Determined by Different Methods

species ^a	mass, ×10 ⁶	method ^b	ref	species ^a	mass, ×10 ⁶	method ^b	ref
ND	2.73	SE	2	<i>Eisenia</i>	4.01	S&D	112
<i>Lumbricus</i>	3.45	LS	102	<i>Eisenia</i>	3.82 ± 0.05	SE	113
ND	2.5	GF	103	<i>Tubifex</i>	3.09 ± 0.15	SAXS	114
ND	3.1	LS	104	<i>Lumbricus</i>	3.68	SE	115
<i>Lumbricus</i>	3.23 ± 0.18	S&D	41	<i>Maoridrillus</i>	3.2	GF	116
ND	3.84	SE	105	<i>Glossoscolex</i>	3.1	S&D	117
ND	2.92 ± 0.1	LS	106	<i>Lumbricus</i>	3.8 ± 0.3	EM	118
<i>Lumbricus</i>	3.86 ± 0.09	SE	107	ND	3.34 ± 0.51	GF	119
<i>Lumbricus</i>	3.68 ± 0.17	SE	108	<i>Lumbricus</i>	3.41 ± 0.39	SE	22
<i>Pheretima</i>	3.07	S&D	109		3.56 ± 0.13	STEM	
<i>Lumbricus</i>	3.95 ± 0.15	SAXS	110				
<i>Lumbricus</i>	4.1	S&D	111	mean	3.42 ± 0.44		

^a ND, not determined. ^b Abbreviations: SE, sedimentation equilibrium; LS, light scattering; GF, gel filtration; S&D, calculated using the Svedberg equation; SAXS, small-angle X-ray scattering; EM, electron microscopy; STEM, mass mapping by scanning transmission electron microscopy.

naturally into two types of chains A and B as predicted by Gotoh,⁵⁶ much like the vertebrate Hbs. Note the strikingly conservative residue variation at the 37 solvent inaccessible positions and the fact that His residues occupy both the distal and proximal positions.

E. Linker Amino Acid Sequences

The primary structures of two linker chains of *Tylorrhynchus* and of single linker chains of *Lamel-*

librachia and *Neanthes* Hbs have been determined.^{48,57,58} The sequences of linker *L1*⁵⁹ and the remaining three linkers of *Lumbricus* Hb have also been established (A. Riggs, personal communication). The linker sequences share 40 residue Cys-rich motifs, whose sequences are similar to comparable motifs in the N-terminal regions of human and *Xenopus* LDL receptor proteins and C9 complement.^{57–59} The role of the linker subunits is

	1 5 10 15 1 5 10 15 1 5	
Mb fold	AAAAAAAAAAAAAAAAA	BBBBBBBBBBBBBBBBCCCCCCCCDDDD
Phys	*****VLSEGEWQLVHVWAKVEA*****DVAGHGQDILIRLFKSHFETLEKDFDRF	
Position	10 20 30 40 50 60	
Tyl I	*****TDCGILQRIKVKQQAQVYSGV***ESRTDFAIDVFNNFFRTNPD*RSLFN**	
Tyl IIA	*****SSDHGQLQRLKVKQQAQVYSGV***HERVELGIALWKSMPAQNDARDLDFK**	
Lum b	*****KKQCGVLQGLKVKSEWGRAYGSG***HDEAFSQAIWRATFAQVPESSRLFK**	
Lum d	*****ECLVTESLKVKLQWASAFGHA***HERVAFGLELWRDIIIDHPEIKAPFS**	
Lum BI	*****DCNIIQLRLKVKMQWAKAYGFG***AERAKFGNSLWTSIFNYAPDARELDF**	
Lum BIII	*****YECGFLQRLKVKRWAEAYGSG***NDREDFGHFIWTHVFKDAPSAARDLDFK**	
Oli 1	*****VCNRLQILVKRWQAQSYGEA***ENRAAFSRDLFSESLFIQGSRLALFS**	
Oli 3	*****DCTSLNRLVKRWAEAYGEG***TSRELLGNRIWEDLFANMPDARGLFS**	
Consen A	*****C*LRLKVK*QWA*AYG*G*****R**FG*LW***F***P**R*LF**	
Tyl IIB	*****DDCCSAADRHEVLDNWKGISAEF*TGRRVAIGQAIQOELFALDPNAKGVFG**	
Tyl IIC	*****DTCCSIEDRREVOALWRSIWSAED*TGRRTLIGRLLEFEIDGATKGLFK**	
Lum a	*****ADDEDCCSYEDRREIRHIWDDVWSSSF*TDORVAIVRAVFDDLFKHYPTSKALFE**	
Lum c	*****DEHEHCCSEEDHRIQKQWDILWRDTESSKIIGFGRLLTKLAKDIPDVNDLDFK**	
Lum BII	*****SSNSCTTERDEMQLMWANVWSAQ*TGRRLAIAQAVFKDLFAHVPAAIGLDF**	
Lum BIV	*****SKFCEGSDATIVIKQWNQIYNAGISAGSRLTMGNKIFSTLFKLPKESLALFS**	
Oli 2	*****SSCCSNDRANVMHNWDAWSAAYS*RRVALAQAVFASLFSRDAQAQLFV**	
Consen B	*****CCS*EDR**V***W***WSA***T*RR**IG**F***LF***P***LF**	
	1 5 1 5 10 15 20 1 5 10	
Mb fold	CC CC. . . D . . . E . . . P .FFFF	
Phys	DD DDDDDDDDEEEEEEEEEEEEEEEEEEEF FFFFFFFFPGGGG	
Position	70 80 90 100 110 120	
Tyl I	**RVN**GDNVYSEPFKAHMMVRVFAFGFDILISVLDD**KPVLDQALAHYAAPHKQ***F	
Tyl IIA	**RVNH**GEDVHSPAFEAHMAVRVFNGLDRVVISLTD**EPVLNAQLEHRLRQOHK***L	
Lum b	**RVH**GDDTSHPAFIAHAERVVLGGLDIAISTLDQ***PATLKEEDHLQVQHEGR***	
Lum d	**RVN**GDNVYSEPFKAHMMVRVFNGLDRVVISLTD**EPVLNAQLEHRLRQOHK***L	
Lum BI	**SRVK**SEEMQSPQFKAHVARVIGGLDRVVISLTD**EPVLNAQLEHRLRQOHK***L	
Lum BIII	**RVN**GDNVYSEPFKAHMMVRVFNGLDRVVISLTD**EPVLNAQLEHRLRQOHK***L	
Oli 1	**GV***DDMNSAFAHCLRVLTGALNRLISQLDQ***QATINVSIAHLAAGQHASR***	
Oli 3	**RVN**GNDIDSSEFQAHSRLVLGGLDMCVASLDD**VPVLNALLARLNSQHSR***	
Consen A	**RV***GD***SP*F*AH**RVLGGLD**IS*LDD*****L***LAHL**QH**R***	
Tyl IIB	**RVN**V*DKPSEADWKAHVIRVINGLDLAVNLLED**PKALQEEKLHLARQHRRER***	
Tyl IIC	**RVN**V*DDTHSPPEFAHVLVRVINGLDLIGVLGD**SDTLNLSLDHLAEQHKAR***	
Lum a	**RVK**I*DEPESGEFKSHLVRVINGLDLILNLLDD**TLVLQSHLGLHLAQHQR***	
Lum c	**RVD**I*EHAEGPKFSAHALRILNGLDLAINLLDD**PPALDADLHLAQHQR***	
Lum BII	**RVH**GTDVNSNEFKACIRVINGLDLIGVLGD**PSTLNDQLHLAQHQR***	
Lum BIV	**NVN**V*ANMSSGAFHAHTVRVLSGLDMGINYLND**AATLSTLSLHLAGQHASR***	
Oli 2	**GVS**ADNPDADFAHCVRVINGLDLAVNLLED**PAVLNEQLAHLAQAQHQR***	
Consen B	**RV*****S**F*AH**RVNGLD**IN*L*D*****L***L*HLA**QH**R***	
	1 5 10 15 1 5 10 15 20 25	
Mb fold	F*F. HHHHHHHHHHHHHHHHHHHHHHH	
Phys	G*GGGGGGGGGGGGGGGGGGGGG HHHHHHHHHHHHHHHHHHHHHHH	
Position	130 140 150 160 170 180	
Tyl I	GTI***PFKAFGQTMFQTIAEHI*****HGADIGAWRACYAEQIVTGITA*****	
Tyl IIA	*GITGHMFLNMRGTGLAYVLPALQGL**RC**FDKEAWAACWDEVIYPGIKHD*****	
Lum b	K*IPDNVFDAPKTAHLHVVAALQGL**RC**YDEAWDACIDHIEDGKGGH*****	
Lum d	N*LKPEFFDIFLKHLLHVLGDLRG*****THDFGAWHDCVDQIIDGK*****	
Lum BI	*GLDALNFVAFKALFATVGGKFGV**C**FDLPWESCYKVIKAGTGTGNDMFN*****	
Lum BIII	*GVSAAGQDVVEHVSVMGVHEIGQN*V**FDKDAWQACLDVITGGIQGN*****	
Oli 1	*NLDAENFAAMGQAVMSVVPVTHL**D*C**FNQHAWGECYERIASGISG*****	
Oli 3	*GIPAAGYPAFVASAISAVRATVGA*R**SFNDANWSCMNQIVSGISG*****	
Consen A	*****F**F*****V***LG*****FD**AW**C***I**GI*G*****	
Tyl IIB	SGVKAVYFDEMEKALLKVLPO*VS***SHFNSGAWDRCFTRIADVIAKALP*****	
Tyl IIC	AGFKTVYFKEFGKALNHLVPE*V*AS*C**FNEPAWNHCFDGLVDVISHRIDG*****	
Lum a	KGVTKEYFRGIGEAFAFARVLPQ*VL*S*C**FNVDANWRCFHLRVARIADKDL*****	
Lum c	EGVQKAHFKFGEILATGLPQ*VL***DDYDALAWKSCGLKGLTKESSRLNA*****	
Lum BII	AGVTKGGFSATIAQSFLRVMPO*V*AS*C**FNPDAWNSRCFNRITNGMTEGLA*****	
Lum BIV	TGLKAVYFDAMGKVLMTVLPALI*****DNFNPDANWRCNLLPLKSAIAEGLP*****	
Oli 2	AGVAAAHFDVMAEFAFVMPQ*VS*S*C**FSSDSWNRFCFARIANGISAGL*****	
Consen B	**V*****F*****I**VLPQ*V**S*****FN**AW**CF**I**I*****	

Figure 2. Alignment according to Kapp *et al.*,⁵⁴ of the amino acid sequences of sperm whale Mb and of globin chains from the HBL Hbs of the annelids *Tylorrhynchus* (I, IIA–C)⁴⁷ and *Lumbricus* (a–d)^{50,52} and from the $\sim 4 \times 10^5$ Hbs of the vestimentiferan *Lamellibrachia* (BI–BIV)⁵³ and the pogonophoran *Oligobranchia* (1–3).⁴⁰ The Mb-fold indicates the canonical secondary structure;⁹⁸ the 37 solvent inaccessible positions⁹⁹ are in bold type. The distal and proximal residues are indicated by D and P, respectively. Note that not all of the 182 positions required to align the 700 globin sequences⁵⁴ are used here: the 84 positions common to all globins are underlined. The sequences group into two types.⁵⁶ The identical residues in the consensus sequences for each group are in boldface.

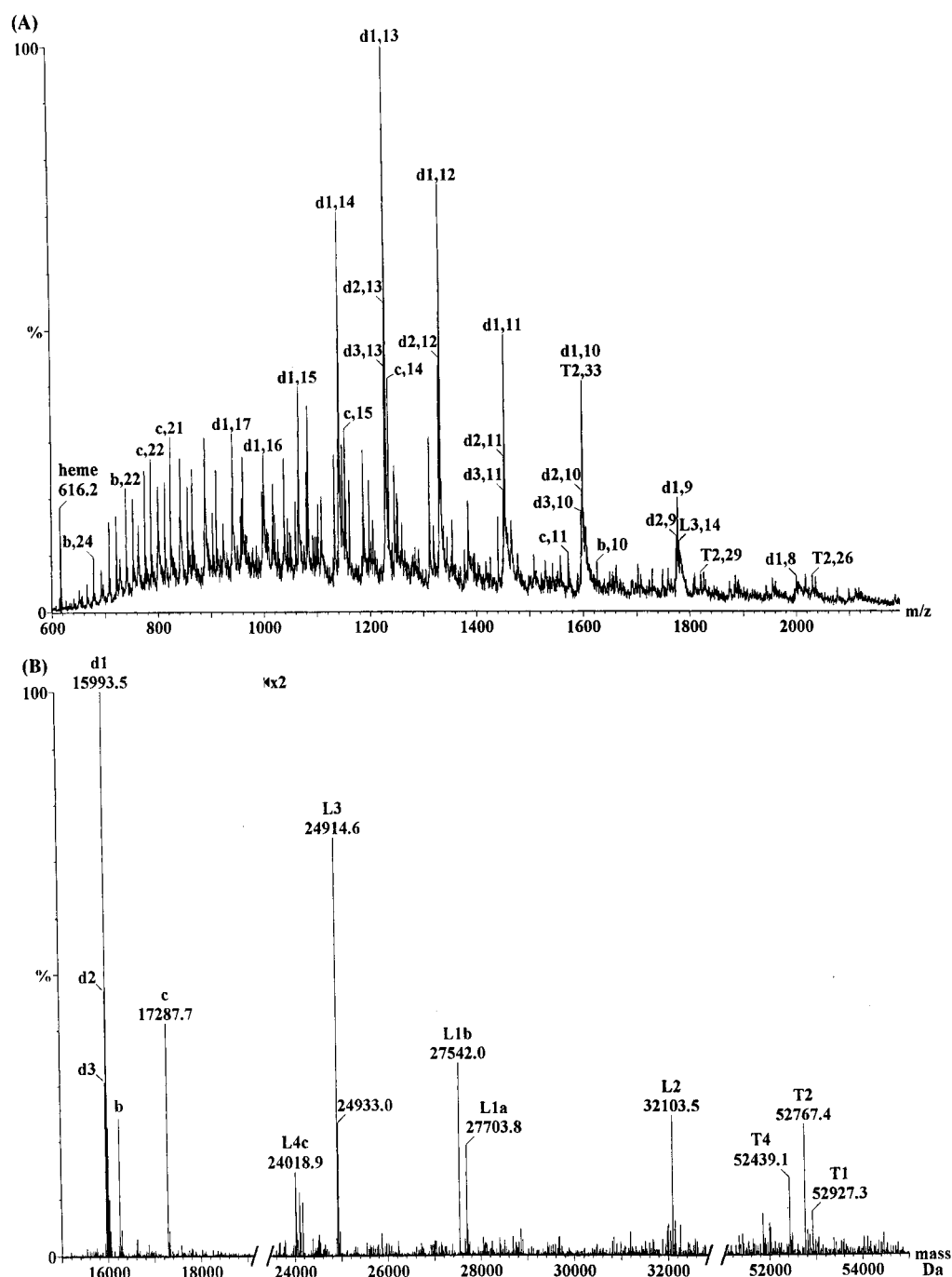


Figure 3. The electrospray ionization mass spectra of native *Lumbricus* Hb:²² (A) the baseline-subtracted, smoothed, multiply-charged spectrum, and (B) the corresponding zero charge (molecular mass) spectra obtained with the MaxEnt program.¹⁰⁰ The identifications of the multiply-charged peaks in A provide the subunit or chain and the positive charge: thus, d1,13 is $d1 + 13H^+$. Note that only chain *d* appears in the spectra, since chains *a*–*c* form a disulfide-bonded trimer in the native Hb.

predominantly structural, since in their absence, the functional dodecamer/tetramer subunits do not reassemble into HBL structures. In both *Riftia* and *Lamellibrachia* Hbs an additional role as sulfide binding site has been suggested.^{38,53,60}

F. Mass Spectrometric Studies

Very recently, one of us (B.N.G.) was able to use maximum entropy deconvolution of the raw ESI-MS of several native and chemically modified annelid,^{22,24,36,37} vestimentiferan,^{38,39} and pogonophoran⁴⁰ Hbs, to obtain complete zero charge spectra of all their constituent subunits and polypeptide chains and determine their masses with an undreamed of

accuracy of ± 1 – 3 Da (for subunits ranging from 16×10^3 to 50×10^3). Figure 3, A and B, shows the raw ESI-MS of *Lumbricus* Hb and the resulting deconvolution on a true mass scale, using the MaxEnt program. The mass spectra of the native Hbs in combination with the spectra of reduced, reduced, and carbamidomethylated and unreduced carbamidomethylated forms permit the determination of the number of Cys residues as well as the numbers of free Cys and disulfide bonded Cys residues. ESI-MS has also provided unambiguous determination of the nature of glycosylation, whether of globin or linker subunits, generally $(HexNAc)_2(Hex)_n$ ($n = 6$ – 9). Furthermore, if the ionization efficiencies of all the

Table 2. ESI-MS Masses of Globin Chains^a

<i>Lumbricus</i> ^b			<i>Arenicola</i> ^d			<i>Tylorrhynchus</i> ^e			<i>Macrobdella</i> ^f			<i>Riftia</i> ^g			<i>Oligobranchia</i> ^h		
CHAIN	MASS	CYS	CHAIN	MASS	CYS	CHAIN	MASS	CYS	CHAIN	MASS	CYS	CHAIN	MASS	CYS	CHAIN	MASS	CYS
<i>d1</i>	15992.4	3	<i>a1</i>	15952.5	3	<i>I</i>	15575.4	2	<i>A</i>	16770.1	2	<i>b</i>	16133.5	3	<i>a1</i>	14861.6	1
<i>d2</i>	15978.0	3	<i>a2</i>	15974.8	3							<i>c</i>	16805.9	2	<i>a2</i>	14937.2	1
<i>d3</i>	15962.1	3										<i>d</i>	15578.5	3	<i>a3</i>	15040.9	1
<i>b</i>	16254.4	3	<i>b1</i>	15920.9	3	<i>IIA</i>	16773.1	3	<i>B</i>	16841.9	2	<i>e</i>	16149.1	4	<i>a4</i>	15070.8	1
			<i>b2</i>	16020.1	3							<i>f</i>	16368.1	3	<i>a5</i>	15310.8	3
			<i>b3</i>	16036.2	3												
<i>c</i>	17289.2	3	<i>c</i>	16664.8	3	<i>IIB</i>	16851.6	3	<i>P</i>	16052.2	3	<i>a^c</i>	15933.4	ND	<i>b</i>	15174.8	4
<i>a^c</i>	17524.0	4	<i>d1</i>	16983.2	5	<i>IIC</i>	17022.2	4	<i>Q</i>	16537.3	3	<i>a1</i>	17637.4	2	<i>c</i>	15608.0	5
<i>a1</i>	19389.9	4	<i>d2</i>	17033.1	5				<i>R</i>	16666.7	3	<i>a2</i>	17800.0	2	<i>d</i>	14776.2	ND
<i>a2</i>	19227.4	4							<i>S</i>	16792.9	3	<i>a3</i>	17962.4	ND			
<i>a3</i>	19065.3	4										<i>a4</i>	18452.1	ND			
<i>a4</i>	18902.9	4										<i>a5</i>	18613.7	ND			

^a Mass in Da; estimated error of measurement ± 1.0 Da. ND, not determined. ^b From Martin *et al.*,²² chains *a*–*c* form disulfide-bonded trimers. ^c *N*-Deglycosylated; exists only in glycosylated forms with (HexNAc)₂(Hex)_{*n*}, *n* = 6–10. ^d From Zal *et al.*,³⁶ chains *b*–*d* form disulfide-bonded trimers. ^e From Green *et al.*,³⁷ chains *IIA*–*C* form disulfide-bonded trimer. ^f From Weber *et al.*,²⁴ chain *P* forms three heterodimers with chains *Q*, *R*, and *S*. ^g From Zal *et al.*,^{38,39} the HBL Hb consists of chains *b*, *c*, *d*, and *e* and a heterodimer *d*+*e*. The vascular and coelomic 4×10^5 Hbs have no linkers and share globin chains *b*, *c*, *e*, *f*, and *a1*–*a5* and a heterodimer *e*+*f*; they differ in that the vascular Hb also has chain *d* and heterodimer *e*+*f*. ^h From Yuasa *et al.*,⁴⁰ heterodimer *b*+*c* and trimer *b*+*c*+*d* are also present.

Table 3. ESI-MS Masses of Linker Chains^a

<i>Lumbricus</i> ^b			<i>Arenicola</i> ^d			<i>Tylorrhynchus</i> ^e			<i>Macrobdella</i> ^f			<i>Riftia</i> ^g		
chain	mass	CYS	chain	mass	CYS	chain	mass	CYS	chain	mass	CYS	chain	mass	CYS
<i>L1a</i>	27702.4	10	<i>L1</i>	25174.1	12	<i>L1</i>	28202.2	12	<i>C</i>	24340.1	10	<i>L1</i>	23505.2	12
<i>L1b</i>	27540.8	10												
<i>L1^c</i>	25837.5													
<i>L2</i>	32104.3	ND	<i>L2</i>	26829.7	12	<i>L2</i>	26317.2	12	<i>D</i>	24398.6	10	<i>L2</i>	23851.4	ND
<i>L3</i>	24912.9	ND				<i>L3</i>	25326.9	10	<i>E</i>	24420.0	9	<i>L3</i>	26342.4	ND
<i>L4a</i>	24169.9	ND				<i>L4</i>	24835.4	10				<i>L4</i>	27425.8	ND
<i>L4b</i>	24102.3	ND				<i>L5</i>	23233.8	10						
<i>L4c</i>	24019.0													

^a Mass in Da; estimated error in mass measurement ± 2.0 Da. ND, not determined. ^b From Martin *et al.*,²² linkers *L1a* and *L1b* have (HexNAc)₂(Hex)_{*n*}, *n* = 9 and 8. ^c From *N*-deglycosylated Hb; exists only in glycosylated form. ^d From Zal *et al.*,³⁶ linker *L1* forms a homodimer and a heterodimer with linker *L2*. ^e From Green *et al.*,³⁷ linker *L2* forms a homodimer. ^f From Weber *et al.*,²⁴ ^g From Zal *et al.*,^{38,39}

chains are comparable, the relative intensities of the observed peaks provide a quantitative measure of the amount of each component. Tables 2 and 3 shows the detailed polypeptide chain inventories of several HBL Hbs. This cornucopia of detailed mass information has allowed the verification of the known globin and linker sequences and has allowed us to propose suitable models of the quaternary structures with much greater confidence than heretofore possible.^{22,24,36–38}

The average ESI-MS mass of the four globin chains of *Lumbricus* Hb is 17.8×10^3 , corresponding to an iron content of 0.31 wt %, which should be compared to the experimentally determined values for the Hb, 0.221–0.230 wt %:^{41,43,61} it is clear that globin chains account for only 70–74% of the total Hb mass. The ESI-MS results²² also show that chain *a* and linker *L1* have glycan side chains, providing a calculated carbohydrate content of 2.1% (on the basis of 36 chain *a* and 12 *L1*) in good agreement with the earlier value (section II.B).

G. Phylogenetic Relationships

Phylogenetic trees based on the globin sequences of the annelids *Tylorrhynchus* and *Lumbricus*, the vestimentiferan *Lamellibrachia*, and the pogonophoran *Oligobranchia* suggests a very close phylogenetic relationship between the annelids and the two other groups,^{40,60} in agreement with the results of other

studies based on elongation factor-1 α ⁶² and 28S ribosomal DNA sequences.⁶³ The globin gene duplication events responsible for the appearance of two group of globin sequences⁵⁶ (Figure 2) must have occurred prior to the divergence of the annelids, vestimentiferans, and pogonophorans.

III. Quaternary Structure

A. Bracelet Model: Dodecamer vs Hexadecamer

On the basis of the observation of a dodecamer lacking linker chains as a product of *Lumbricus* Hb dissociation, we proposed a bracelet model for its quaternary structure, consisting of 12 dodecamers ($12 \times [a+b+c]_3[d_3]$), decorating a bracelet or framework of 30–40 linkers.^{45,46} On the basis of the most recent Hb mass of 3.55×10^6 , the 12 dodecamers ($2.13 \times 10^5 \times 12 = 2.56 \times 10^6$), account for ~72% of the total mass, in excellent agreement with the iron and heme contents (sections II.B and II.F). This model also accounts for the low α -helical content deduced from CD spectra (~40%)^{4,6,64} and appears to hold for all annelid and vestimentiferan Hbs for which ESI-MS and STEM mass data have become available recently.^{22,24,36–38} It should be noted that in the case of *Lumbricus* Hb, even with the ESI-MS masses of its subunits, it is impossible to be sure of the number of linker subunits, since the two likely possibilities, 36 and 42, both provide masses within the experi-

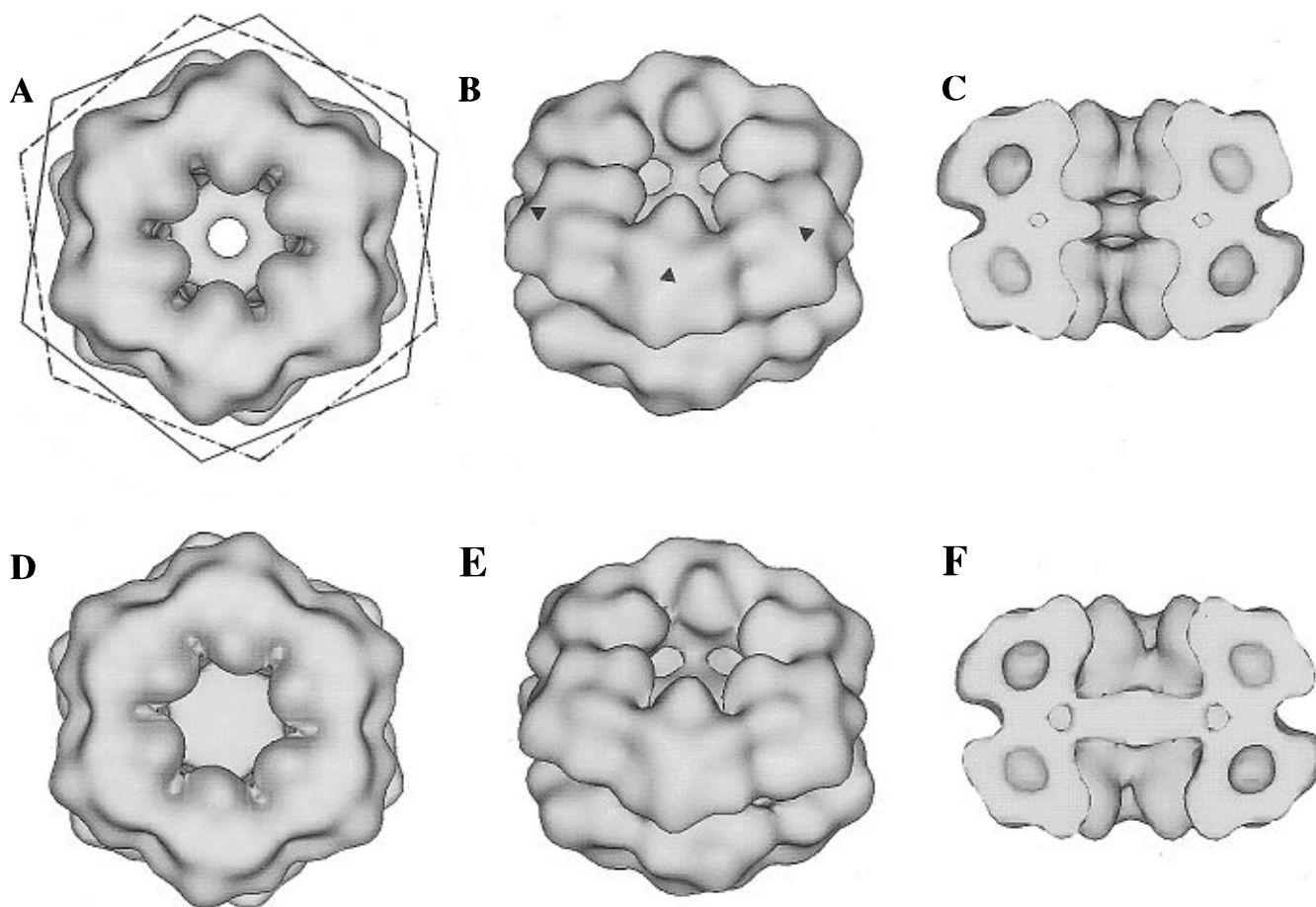


Figure 4. Surface representations of the 3D reconstruction volumes of *Macrobdella decora* (leech) (A–C) and *Lumbricus terrestris* (oligochaete) (D–F) computed by the method of Penczek *et al.* from TEM images of frozen, hydrated specimens.¹⁰¹ (A, D) top views; (B, E) 45° views; (C, F) whole molecule cut in half by a plane passing through the 6-fold axis. Due to the position of the 2-fold axes, the two hexagonal layers appear to be rotated by ~15° relative to each other as indicated by the positions of the two hexagons in A. In B, the triangles indicate the position of the local pseudo-3-fold axes of the hollow globular substructures (HGS) each containing a dodecamer of globin chains. The main difference between the two Hbs is the presence of a hole in the central mass of *Macrobdella* Hb that is well visible in A and C. In B and E, one of the 12 connections between the central mass and the rest of the molecule is visible in the opening of the volume. In C and F, the central cavities of the four HSG's are apparent in the section surface. The 3D reconstructions of *Riftia* Hb⁷⁰ (vestimentiferan) and *Eudistylia*⁶⁸ (polychaete) Chl are quite similar to those of *Macrobdella*⁶⁹ and *Lumbricus* Hbs. The illustrations were prepared by F. De Haas.

mental error of the total Hb mass.²² Because each dodecamer is expected to have at least three contacts with neighboring dodecamers, 36 linkers is conceptually the more satisfying number.

Riggs and his collaborators isolated the trimer and monomer subunits from *Lumbricus* Hb dissociated at pH > 9 and upon mixing the two at neutral pH obtained a reassociation product, whose sedimentation velocity behavior they interpreted to be that of an octamer of globin chains $[abcd]_2$.⁶⁵ Believing the latter to be the principal functional subunit of the Hb, they proposed a model consisting of 12 hexadecamers of globin chains ($12 \times [a+b+c+d]_4$) and 24 linker chains, on the basis of measured globin:linker ratio (84:16) and heme content (3.04%).^{66,67} It should be noted that earlier work on the reassociation of the alkaline pH dissociation products of the Hb has demonstrated the formation of a $\sim 3 \times 10^5$ product which was incapable of reassociating further to a native-like HBL structure.⁴³ The relevance of a globin-containing subunit obtained by reassociation of subunits obtained under relatively harsh conditions, to the quaternary structure of *Lumbricus* Hb remains unclear. Furthermore, the Riggs model is based on appreciably higher than the normal values

of heme and iron content (section II.B)^{6,33} and provides a total mass in excess of 4×10^6 , substantially higher than the latest values obtained by STEM mass mapping and sedimentation equilibrium (section II.A).

B. Three-Dimensional Reconstruction

Recent 3D reconstructions from cryoelectron microscopic images have demonstrated that the structures of *Eudistylia* Chl⁶⁸ and the Hbs of *Macrobdella*,⁶⁹ *Riftia*,⁷⁰ and *Lumbricus*⁷¹ are very similar at 30–40 Å resolution, thus supporting the notion of a common quaternary structure and the applicability of the bracelet model. Figure 4 shows some representative results. The reconstructions indicate that each of the 12 hollow globular substructures has a local 3-fold axis of symmetry, in agreement with the molecular symmetry of *Lumbricus* dodecamer crystals.⁷² The occurrence of a local 3-fold symmetry suggests that the hexadecamer model proposed by Ownby *et al.*⁶⁷ is unlikely to be correct.

The 3D reconstructions also demonstrated that the two hexagonal rings were rotated relative to each other by ~15° from the fully eclipsed conformation.

Table 4. Oxygen-Binding Parameters of Some Annelid, Vestimentiferan, and Pogonophoran Hbs

Hb/SUBUNIT	<i>T</i> , °C	pH	<i>P</i> ₅₀ , Torr	<i>n</i> ₅₀	ϕ^a	ΔH , kJ mole ⁻¹	ref
<i>Lumbricus</i> Hb	25	7.0	15.2	3.6			65
+100 mM Na ⁺			15.4	3.5			
+100 mM Na ⁺ + 25 mM Ca ²⁺			11.6	4.2			
<i>Lumbricus</i> trimer	15	7.2	4.0	1.3	-0.35		81
monomer			0.6	1.1	0.0		
<i>Lumbricus</i> Hb dodecamer	25	7.1	11.8	3.3			46
			11.7	2.1			
<i>Lumbricus</i> blood	25	7.2	11.4	5.2	-0.42	-45	82
+100 mM Mg ²⁺			5.7	7.0	-0.62		
+100 mM Ca ²⁺			4.2	6.0	-0.53		
Hb	25		13.6	4.5	-0.24	-50	
+100 mM Mg ²⁺			6.3	5.8	-0.56		
+100 mM Ca ²⁺			4.8	5.7	-0.49		
dodecamer	25		15.4	1.8	-0.19	-56	
+100 mM Mg ²⁺			7.9	2.6	-0.61		
+100 mM Ca ²⁺			5.9	2.0			
<i>Arenicola</i> Hb ^b	15	7.4	5.7	2.5			83
+120 mM Na ⁺			4.7	2.6			
+120 mM Mg ⁺			1.0	3.4			
<i>Macrobdella</i> Hb	25	7.3	4.8	4.0	-0.38	-44	24
tetramer			2.7	1.4	-0.30	-40	
monomer		7.5	0.3	1.0	0.0	-37	
<i>Eudistylia</i> Chl	25	7.1	145	6.9	-0.44		80
+100 mM Mg ²⁺			107	7.7			
dodecamer			58	1.7	-0.22		
+100 mM Mg ²⁺			55	1.8			
tetramer			43	1.4	-0.21		
<i>Alvinella</i> Hb ^c	20	7.3	0.3	3.0	-1.18	-89	17
<i>Riftia</i> Hb FI ^d	30	7.0	0.5	2.4	-0.35	-68	14
<i>Riftia</i> Hb FII ^d			0.3	1.6	-0.04	-69	
<i>Siboglinum</i> Hb	20	7.0	0.3	2.0	+0.25	-13	18

^a Bohr effect = $\Delta \log P_{50}/\Delta \text{pH}$ over the range pH 6–8, since the large majority of annelid HBL Hbs (but not Chls) dissociate at pH > 8. ^b Deionized Hb. ^c In the presence of 50 mM Mg²⁺ and 10 mM Ca²⁺. ^d In the presence of 31 mM Mg²⁺ and 11 mM Ca²⁺. The O₂ affinities of *Riftia* FI (HBL Hb) and FII ($\sim 4 \times 10^5$) are unaffected by sulfide. The affinity for sulfide is comparable to the O₂ affinity; half-saturation of blood occurs at [HS⁻] \approx 11 mM.

Furthermore, 3D reconstructions of reassembled HBL structures from *Lumbricus* Hb subunits missing one of the linkers (*L2*) in one case or a globin subunit (chain *d*) in the other⁷³ has permitted the localization of the positions of these subunits in the HBL complex (F. De Haas and J. Lamy, unpublished observations). These results imply that formation of a HBL structure does not require all the subunits, in consonance with our suggestion of a variable subunit stoichiometry,²² supported by the kinetic heterogeneity of Hb dissociation.³²

C. Effects of Group IIA Cations

The stability of the HBL structure is strongly dependent on the presence of group IIA cations (Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺).^{4,6,7,74–76} Although the concentration of Ca²⁺ is amazingly constant in the blood of vestimentiferans and annelids, at 7–13 mM, including the deep sea species, that of Mg²⁺ varies from 2 to 44 mM,^{77,78} while the pH is generally in the range 7.0–7.5. Exposure of HBL Hbs to a chelating agent such as EDTA leaves 40–60 Ca²⁺ per molecule;^{22,79} even complete dissociation of *Lumbricus* Hb still leaves some 25–30 Ca²⁺ present.²² A recent detailed study of its dissociation and reassembly³² has shown that the optimal cation concentration for both processes is \sim 10 mM. These results suggest that there are several different types of Ca-binding sites with different binding affinities.

D. Metal Content

In addition to the heme iron and the protein-bound Ca²⁺, *Lumbricus* and *Tylorrhynchus* Hbs contain 1–4

Cu²⁺ and Zn²⁺ per mole.⁷⁹ The presence of Cu and Zn appears to be related to the existence of superoxide dismutase activity in *Lumbricus* Hb, thought to reside in one of the linker subunits.³⁰

IV. Equilibrium Oxygen Binding

A. Do Linkers Play a Role in HBL Hb Cooperativity?

Table 4 presents the oxygenation parameters of several HBL Hbs and Chls and of their functional subunits. The variation in affinity is about 1000-fold, with the deep sea Hbs having much higher affinities than the sea level Hbs. *Lumbricus* dodecamer has the same affinity of oxygen binding as the native Hb but only two-thirds of its cooperativity (*n*₅₀ \approx 2.1 vs 3–4).⁴⁶ The functional tetramer of *Macrobdella* Hb and dodecamer/tetramer of *Eudistylia* Chl have higher affinities and lower cooperativities than the native Hb and Chl.^{24,80} The small monomeric subunits have the highest affinities and exhibit no cooperativity.^{24,65} We believe that the low cooperativity of the functional subunits (*n*₅₀ \approx 1.6–2.0) is due to globin–globin interactions and that the added cooperativity of the native HBL structures is due to additional interactions between dodecamer/tetramer and linker chains. The essential role of linker chains is underscored by the fact that the 4×10^5 Hb from *Riftia*, which consists only of globin chains, has a substantially smaller cooperativity than the HBL Hb¹⁴ (Table 4). Globin–linker interactions could entail interactions between the dodecamer/tetramer and one or more of the linker chains or linker-

mediated interactions between neighboring dodecamers/tetramers, or both. Furthermore, these interactions could be equatorial, i.e. within the plane of a hexagonal ring or axial, i.e. between neighboring dodecamers/tetramers from each of the two hexagonal rings.

Fushitani and Riggs have found the affinity and cooperativity of oxygen binding of their putative $[abcd]_2$ reassociation product to be equal to those of the native Hb at pH 6.8;⁸¹ however, at higher pH up to 8, the affinity was higher and the cooperativity lower than those of the native Hb. This result is in general agreement with the results of a recent oxygen binding study of *Lumbricus* blood, Hb and dodecamer:⁸² (1) the affinities were similar at pH 6.5–6.7 and increased in the order dodecamer < Hb < blood at higher pH, reaching maximum deviation at pH 8; (2) although the cooperativities of the Hb and blood were similar at neutral pH, they deviated at higher pH with that of the blood reaching a maximum at pH 7.5–8; (3) the difference in the cooperativity of Hb vs dodecamer increased with increase in pH. Although differences in cooperativity were observed for dodecamer preparations obtained by dissociation of the Hb in urea and in the presence of heteropolytungstates, which appear to be related to the Ca content of the dodecamer, the highest cooperativity for a dodecamer was 2.7, while the cooperativities of the Hb and blood varied over the range 3–6 and 3–9, respectively.⁸² In view of the lack of documentation for the complete absence of linker chains in the $[abcd]_2$ preparation of Fushitani and Riggs, it appears that the weight of experimental evidence is in favor of a role for the linker chains in the full manifestation of the cooperativity of oxygen binding in *Lumbricus* and other annelid HBL Hbs.

B. Effects of Cations

In contrast to vertebrate Hbs, the affinity and cooperativity of oxygen binding of HBL Hbs and Chls while unaffected by organic phosphate anions, such as DPG and ATP, are substantially increased in the presence of group IIA cations at concentrations to 100 mM (Table 4).^{24,65,75,80–84} Although monovalent cations such as Na^+ , also increase affinity and cooperativity, they do so to a much lesser extent.^{24,65,75,80–84} There exists the possibility that other divalent cations may also exert structural and functional effects.⁷⁵ The recent observation that *Lumbricus* blood had a substantially higher affinity and cooperativity than the Hb purified by gel filtration⁸⁵ in the pH range 7–8⁸² (Table 4) suggests the need to consider the presence of an unknown organic/inorganic allosteric effector other than monovalent and divalent cations.

C. Lack of Alteration in Molecular Shape

SAXS studies of *Lumbricus* Hb and its dodecamer show no experimentally detectable alteration in shape or size in going from oxy to deoxy form.^{82,86} On the basis of the angular displacement of $\sim 15^\circ$ between the two hexagonal layers observed in the 3D reconstructions, we propose that relative rotation of the two hexagonal rings may underlie the cooperativity due to dodecamer/tetramer–linker interactions.

It is unlikely that relative rotation of the two layers in a HBL structure attendant upon ligand binding or dissociation would lead to alteration in shape or size.

D. Stereochemistry of Ligand Binding

In contrast to vertebrate Hbs whose cooperativity of ligand binding is due to ligand binding-induced alterations in the stereochemistry of the heme iron-to-proximal His bond,⁸⁷ the cooperativity of ligand binding of HBL Hbs and Chls appears to have a different structural basis. The EPR spectrum of nitrosyl Hb from the earthworm *Octolasmus* corresponds to that of a hexacoordinate structure and unlike the vertebrate Hb derivatives, remains unaffected by variation in pH from 4.7 to 7.4 and presence of Ca^{2+} up to 100 mM.⁸⁸ Resonance Raman studies of the deoxy forms of *Potamilla* Chl and of *Travisia* Hb and its $1/12$ subunit (~ 240 kDa) showed that the spectra of the native Chl and Hb and the $1/12$ th were the same,^{89,90} with the Fe–His stretching at $219\text{--}222\text{ cm}^{-1}$ being similar to the vertebrate deoxyHbs.⁹¹ The deoxyChl line was unaffected by the presence of 5 mM Mg^{+2} , when the oxygen affinity was increased 3-fold.^{89,90} A more recent resonance Raman study of the deoxy, oxy, and CO forms of *Lumbricus* Hb at pH 7 and 9.2 revealed minimal differences in the region of the Fe–His stretching vibration and the π -electron distribution in the heme group,⁹² with the spectral frequencies being similar to those observed in R-state vertebrate Hbs; the HBL Hb heme groups appear to behave more like the heme in Mb.

V. Conclusion

The results obtained over the last 10–15 years lead to several significant conclusions. (1) The HBL Hbs and Chls are heteromultimeric complexes, with masses close to 3.5×10^6 , consisting of 4–6 different globin chains accounting for 70–74% of the total mass and 2–4 groups of linker chains. (2) Dissociation studies have clearly delineated the hierarchical organization of the heme-containing subunits, ranging from dodecamer to monomer. (3) A bracelet model consisting of 144 globin chains arranged in two hexagonally symmetric layers of 12 dodecamers decorating a framework of 36–42 linker chains fits the ESI-MS and molecular mass data for annelid Hbs and Chls as well as a vestimentiferan Hb. (4) Cryoelectron microscopic 3D reconstructions demonstrate the essential similarity of annelid and vestimentiferan HBL structures, support the bracelet model, and show that each of the 12 globular substructures have a local 3-fold axis of symmetry, in agreement with the crystallographic molecular symmetry found for *Lumbricus* Hb dodecamer. (5) The 3D reconstructions of *Lumbricus* Hb and reassembled HBL structures suggest that not all globin or linker subunits are necessary for the formation of a HBL structure. (6) SAXS studies suggest that there is little or no alteration in the shapes of *Lumbricus* Hb and its dodecamer subunit in going from oxy to deoxy state. (7) In addition to about 40–60 tightly bound Ca^{2+} , HBL Hbs have small amounts of Cu and/or Zn. Maximum protection of the HBL structure and promotion of its reassembly occurs at $\sim 10\text{ mM Ca}^{2+}$,

Mg²⁺, and Sr²⁺. The latter also increase the oxygen binding affinities and cooperativities of the HBL Hbs and their functional subunits at concentrations up to 100 mM. (8) The functional subunits have either similar or lesser oxygen binding affinities than the native HBL Hb and lower cooperativities, implying that there are two levels of cooperativity: one due to globin–globin interactions and another, based on globin–linker interactions. (8) The absence of alterations in the EPR spectra of the ligated deoxy forms and in the resonance Raman spectra of deoxy and ligated forms of HBL Hbs and Chls, with pH and in the presence of Ca²⁺ and Mg²⁺, suggest that ligand binding is not accompanied by alterations in the stereochemistry of the heme iron–proximal His bond.

The mechanisms underlying the cooperativity of oxygen binding at the functional subunit level due to globin–globin interactions and at the level of the native HBL structure due to globin–linker–globin interactions are bound to be quite different from the two presently well-delineated mechanisms, one in vertebrate Hbs^{87,93} and the other in the dimeric and tetrameric Hbs of the mollusk *Scapharca*.^{94–96} Furthermore, the globin–linker interactions make the HBL Hbs a paradigm for multiprotein and multienzyme complexes where allosteric interactions occur between unlike proteins.

Abbreviations

Hb	hemoglobin
HBL	hexagonal bilayer
Chl	chlorocruorin
STEM	scanning transmission electron microscopy
ESI-MS	electrospray ionization mass spectrometry
Gdm	guanidinium
Mb	myoglobin
SDS	sodium dodecyl sulfate polyacrylamide gel electrophoresis
PAGE	trophoresis
CD	circular dichroism
3D	three-dimensional
DPG	2,3-diphosphoglycerate
ATP	adenosine triphosphate
SAXS	small-angle X-ray scattering

VI. References

- Mangum, C. *Adaptation to Environment: Physiology of Marine Animals*; Newell, P. C., Ed.; Butterworth's: London, 1976; p 191.
- Svedberg, T.; Erickson, I. *J. Am. Chem. Soc.* **1933**, *55*, 2834.
- Roche, J. *Studies in Comparative Biochemistry*; Munday, D. A., Ed.; Pergamon Press: Oxford, 1965; p 62.
- Antonini, E.; Chiancone, E. *Annu. Rev. Biophys. Bioeng.* **1977**, *6*, 239.
- Weber, R. E. *Physiology of Annelids*; Mill, P. J., Ed.; Academic Press: London, 1978; p 393.
- Chung, M. C. M.; Ellerton, H. D. *Prog. Biophys. Mol. Biol.* **1979**, *35*, 53.
- Vinogradov, S. N. *Comp. Biochem. Physiol.* **1985**, *82B*, 1.
- Gotoh, T.; Suzuki, T. *Zool. Sci.* **1990**, *7*, 1.
- Jones, M. L. *Oceanol. Acta Spec.* **1988**, *8*, 69.
- Childress, J. J.; Fisher, C. R. *Oceanogr. Mar. Biol.* **1992**, *30*, 337.
- Gaill, F.; Hunt, S. *Rev. Aquat. Sci.* **1991**, *4*, 107.
- Toulmond, A. *Symp. Soc. Exp. Biol.* **1985**, *39*, 164.
- Arp, A. J.; Childress, J. J.; Vetter, R. D. *J. Exp. Biol.* **1987**, *128*, 139.
- Arp, A. J.; Doyle, M. L.; Di Cera, E.; Gill, S. J. *Respir. Physiol.* **1990**, *80*, 323.
- Fisher, C. R.; Childress, J. J.; Sanders, N. K. *Symbiosis* **1988**, *5*, 229.
- Toulmond, A.; De Frescheville, J.; Frisch, M. H.; Jouin, C. *Oceanol. Acta* **1988**, *8*, 195.
- Toulmond, A.; El Idriss Slitine, F.; De Frescheville, J.; Jouin, C. *Biol. Bull.* **1990**, *179*, 366.
- Terwilliger, R. C.; Terwilliger, N. B.; Hughes, G.; Southward, A. J.; Southward, E. C. *J. Mar. Biol. Assoc. U.K.* **1987**, *67*, 219.
- Vinogradov, S. N.; Kapp, O. H.; Ohtsuki, M. *Electron Microscopy of Proteins*; Harris, J., Ed.; Academic Press: New York, 1982; Vol. 3, p 135.
- Terwilliger, R. C.; Terwilliger, N. B. *Biol. Soc. Wash. Bull.* **1985**, *6*, 273.
- Terwilliger, R. C.; Terwilliger, N. B.; Bonaventura, C.; Bonaventura, J.; Schabtach, E. *Biochim. Biophys. Acta* **1985**, *829*, 27.
- Martin, P. D.; Kuchumov, A.; Green, B. N.; Oliver, R. W. A.; Braswell, E. H.; Wall, J. S.; Vinogradov, S. N. *J. Mol. Biol.* **1996**, *255*, 154.
- Kapp, O. H.; Qabar, A. N.; Bonner, M. C.; Stern, M. S.; Walz, D. A.; Schmuck, M.; Pilz, I.; Wall, J. S.; Vinogradov, S. N. *J. Mol. Biol.* **1990**, *213*, 141.
- Weber, R. E.; Malte, H.; Braswell, E. H.; Oliver, R. W. A.; Green, B. N.; Sharma, P. K.; Kuchumov, A.; Vinogradov, S. N. *J. Mol. Biol.* **1995**, *251*, 703.
- Qabar, A. N.; Stern, M. S.; Walz, D. A.; Chiu, J. T.; Timkovich, R.; Wall, J. S.; Kapp, O. H.; Vinogradov, S. N. *J. Mol. Biol.* **1991**, *222*, 1109.
- Zhu, H.; Ownby, D.; Riggs, C. K.; Riggs, A. F. International Conference on Supramolecular Organization and Functional Regulation of Invertebrate Dioxygen Binding Proteins, July 1–6, 1996, Padova, Italy.
- Suzuki, T.; Kobayashi, M.; Furukohri, T.; Gotoh, T. *Comp. Biochem. Physiol.* **1983**, *75B*, 17.
- Brantley, R. E.; Smerdon, S. J.; Wilkinson, A. J.; Singleton, E. W.; Olson, J. S. *J. Biol. Chem.* **1993**, *268*, 6995.
- Carver, T. E.; Brantley, R. E.; Singleton, E. W.; Arduini, R. M.; Quillin, M. L.; Phillips, G. N.; Olson, J. S. *J. Biol. Chem.* **1992**, *267*, 14443.
- Liochev, S. I.; Kuchumov, A. R.; Vinogradov, S. N.; Fridovich, I. *Arch. Biochem. Biophys.* **1996**, *330*, 281.
- Ascoli, F.; Rossi Fanelli, M. R.; Chiancone, E.; Vecchini, P.; Antonini, E. *J. Mol. Biol.* **1978**, *119*, 191.
- Sharma, P. K.; Kuchumov, A.; Chottard, G.; Martin, P. D.; Wall, J. S.; Vinogradov, S. N. *J. Biol. Chem.* **1996**, *271*, 8754.
- Vinogradov, S. N.; Sharma, P. K.; Walz, D. A. *Comp. Biochem. Physiol.* **1991**, *98B*, 187.
- Shishikura, F.; Mainwaring, M. G.; Yurewicz, E. C.; Lightbody, J. L.; Walz, D. A.; Vinogradov, S. N. *Biochim. Biophys. Acta* **1986**, *869*, 314.
- Ebina, S.; Matsubara, K.; Nagayama, K.; Yamaki, M.; Gotoh, T. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7367.
- Zal, F.; Green, B. N.; Lallier, F.; Vinogradov, S. N.; Toulmond, A. *Eur. J. Biochem.* **1996**, in press.
- Green, B. N.; Suzuki, T.; Gotoh, T.; Kuchumov, A. R.; Vinogradov, S. N. *J. Biol. Chem.* **1995**, *270*, 18209.
- Zal, F.; Lallier, F. H.; Wall, J. S.; Vinogradov, S. N.; Toulmond, A. *J. Biol. Chem.* **1996**, *271*, 8869.
- Zal, F.; Lallier, F.; Green, B. N.; Vinogradov, S. N.; Toulmond, A. *J. Biol. Chem.* **1996**, *271*, 8875.
- Yuasa, H. J.; Green, B. N.; Takagi, T.; Suzuki, T.; Vinogradov, S. N.; Suzuki, T. *Biochim. Biophys. Acta* **1996**, *1296*, 235.
- Shlom, J. M.; Vinogradov, S. N. *J. Biol. Chem.* **1973**, *248*, 7904.
- Vinogradov, S. N. *Respiratory Pigments in Animals*; Lamy, J. N.; Truchot, J. P.; Gilles, R., Eds.; Springer Verlag: Berlin, 1985; p 9.
- Kapp, O. H.; Polidori, G.; Mainwaring, M. G.; Crewe, A. V.; Vinogradov, S. N. *J. Biol. Chem.* **1984**, *259*, 628.
- Mainwaring, M. G.; Lugo, S. D.; Fingal, R. A.; Kapp, O. H.; Vinogradov, S. N. *J. Biol. Chem.* **1986**, *261*, 10899.
- Vinogradov, S. N.; Lugo, S.; Mainwaring, M. G.; Kapp, O. H.; Crewe, A. V. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 8034.
- Vinogradov, S. N.; Sharma, P. K.; Qabar, A. N.; Wall, J. S.; Westrick, J. A.; Simmons, J. H.; Gill, S. J. *J. Biol. Chem.* **1991**, *266*, 13091.
- Suzuki, T.; Gotoh, T. *J. Biol. Chem.* **1986**, *261*, 9257.
- Suzuki, T.; Takagi, T.; Gotoh, T. *J. Biol. Chem.* **1990**, *265*, 12168.
- Shishikura, F.; Snow, J. W.; Gotoh, T.; Vinogradov, S. N.; Walz, D. A. *J. Biol. Chem.* **1987**, *262*, 3123.
- Fushitani, K.; Matsuura, M. S. A.; Riggs, A. F. *J. Biol. Chem.* **1988**, *263*, 6502.
- Xie, Q.; Donahue, R. A.; Fushitani, K.; Linse, K.; Riggs, A. F. Abstract LB52, ASBMB/DBC-ACS Joint Meeting, May 21–25, 1995, San Francisco, CA.
- Maier, C.; Arbogast, B.; Hahn, U.; Kuchumov, A.; Vinogradov, S.; Walz, D.; Deinzer, M. L. *J. Am. Soc. Mass Spectrom.*, submitted for publication.
- Takagi, T.; Iwaasa, H.; Ohta, S.; Suzuki, T. *Structure and Function of Invertebrate Oxygen Carriers*; Vinogradov, S. N.; Kapp, O. H., Eds.; Springer Verlag: New York, 1991; p 245.
- Kapp, O. H.; Moens, L.; Vanfleteren, J.; Trotman, C. N. A.; Suzuki, T.; Vinogradov, S. N. *Protein Sci.* **1995**, *4*, 2179.
- Moens, L.; Vanfleteren, J.; Van de Peer, Y.; Peeters, K.; Kapp, O. H.; Czeluzniak, J.; Goodman, M.; Blaxter, M.; Vinogradov, S. N. *Mol. Biol. Evol.* **1996**, *13*, 324.
- Gotoh, T.; Shishikura, F.; Snow, J.; Ereifej, K.; Vinogradov, S. N.; Walz, D. A. *Biochem. J.* **1987**, *241*, 441.
- Suzuki, T.; Takagi, T.; Ohta, S. *J. Biol. Chem.* **1990**, *265*, 1551.
- Suzuki, T.; Ohta, T.; Yasa, H. J.; Takagi, T. *Biochim. Biophys. Acta* **1994**, *1217*, 291.

- (59) Suzuki, T.; Riggs, A. F. *J. Biol. Chem.* **1993**, *268*, 1354.
- (60) Suzuki, T.; Takagi, T.; Okuda, K.; Furukohri, T.; Ohta, S. *Zool. Sci.* **1989**, *6*, 915.
- (61) Vinogradov, S. N.; Kosinski, T. F.; Zak, B. *Anal. Biochem.* **1982**, *182*, 111.
- (62) Kojima, S.; Hashimoto, T.; Hasegawa, M.; Ohta, S.; Seki, H.; Okada, N. *J. Mol. Evol.* **1993**, *37*, 66.
- (63) Williams, N. A.; Dixon, D. R.; Southward, E. C.; Holland, P. W. H. *J. Mar. Biol. Assoc. U.K.* **1993**, *73*, 437.
- (64) Hendrickson, W. E.; Royer, W. E., Jr. *Biophys. J.* **1986**, *49*, 177.
- (65) Fushitani, K.; Imai, K.; Riggs, A. F. *J. Biol. Chem.* **1986**, *261*, 8414.
- (66) Fushitani, K.; Riggs, A. F. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 9461.
- (67) Ownby, D. W.; Zhu, H.; Schneider, K.; Beavis, R. C.; Chait, B. T.; Riggs, A. F. *J. Biol. Chem.* **1993**, *268*, 13539.
- (68) De Haas, F.; Taveau, J. C.; Boisset, N.; Lambert, O.; Vinogradov, S. N.; Lamy, J. N. *J. Mol. Biol.* **1996**, *255*, 140.
- (69) De Haas, F.; Boisset, N.; Taveau, J. C.; Lambert, O.; Vinogradov, S. N.; Lamy, J. N. *Biophys. J.* **1996**, *70*, 1973.
- (70) De Haas, F.; Taveau, J. C.; Boisset, N.; Lambert, O.; Zal, F.; Toulmond, A.; Lamy, J. N. *J. Mol. Biol.* **1996**, in press.
- (71) Schatz, M.; Orlova, E. V.; Dube, P.; Jäger, J.; van Heel, M. *J. Struct. Biol.* **1995**, *114*, 28.
- (72) Martin, P. D.; Eisele, K. L.; Doyle, M. A.; Kuchumov, A. R.; Walz, D. A.; Arutyunyan, E. G.; Vinogradov, S. N.; Edwards, B. F. P. *J. Mol. Biol.* **1996**, *255*, 170.
- (73) Kapp, O. H.; Mainwaring, M. G.; Vinogradov, S. N.; Crewe, A. V. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7532.
- (74) Chiancone, E.; Vecchini, P.; Verzili, P.; Ascoli, F. *J. Mol. Biol.* **1984**, *172*, 545.
- (75) Ochial, T.; Hoshina, S.; Usuki, I. *Biochim. Biophys. Acta* **1993**, *1203*, 310.
- (76) Harrington, J. P. *Comp. Biochem. Physiol.* **1994**, *109A*, 799.
- (77) Oglesby, L. C. *Physiology of Annelids*; Mill, P. J., Ed.; Academic Press: London, UK, 1978; p 555.
- (78) Sanders, N.; Childress, J. *Comp. Biochem. Physiol.* **1991**, *98A*, 97.
- (79) Standley, P. M.; Mainwaring, M. G.; Gotoh, T.; Vinogradov, S. N. *Biochem. J.* **1988**, *249*, 915.
- (80) Imai, K.; Sharma, P. K.; Vinogradov, S. N. *Comp. Biochem. Physiol.* **1996**, *113B*, 618.
- (81) Fushitani, K.; Riggs, A. F. *J. Biol. Chem.* **1991**, *266*, 10275.
- (82) Krebs, A.; Kuchumov, A.; Sharma, P.; Braswell, E. H.; Zipper, P.; Weber, R. E.; Vinogradov, S. N. *J. Biol. Chem.* **1996**, *271*, 18695.
- (83) Weber, R. E. *Nature* **1981**, *292*, 386.
- (84) Santucci, R.; Chiancone, E.; Giardina, B. *J. Mol. Biol.* **1984**, *179*, 713.
- (85) Vinogradov, S. N.; Sharma, P. K. *Methods Enzymol.* **1994**, *231*, 112.
- (86) Krebs, A.; Zipper, P.; Vinogradov, S. N. *Biochim. Biophys. Acta* **1997**, in press.
- (87) Perutz, M. F. *Nature* **1970**, *228*, 726.
- (88) Desideri, A.; Chiancone, E. *J. Inorg. Biochem.* **1985**, *25*, 225.
- (89) Yoshikawa, S.; Mochizuki, K.; Chihara, S.; Hagihara, B.; Kitagawa, T. *Biochim. Biophys. Acta* **1984**, *786*, 267.
- (90) Kitagawa, T.; Chihara, S.; Fushitani, K.; Morimoto, H. *J. Am. Chem. Soc.* **1984**, *106*, 1860.
- (91) Friedman, J. M. *Science* **1985**, *228*, 1273.
- (92) Vidugiris, G. J. A.; Harrington, J. P.; Friedman, J. M.; Hirsch, R. E. *J. Biol. Chem.* **1993**, *268*, 26190.
- (93) Perutz, M. F. *Q. Rev. Biophys.* **1989**, *22*, 139.
- (94) Royer, W. E., Jr.; Hendrickson, W. A.; Chiancone, E. *Science* **1990**, *249*, 518.
- (95) Chiancone, E.; Verzili, D.; Boffi, A.; Royer, W. E., Jr.; Hendrickson, W. A. *Structure and Function of Invertebrate Oxygen Carriers*; Vinogradov, S. N., Kapp, O. H., Eds.; Springer Verlag: New York, 1991; p 139.
- (96) Royer, W. E., Jr. *J. Mol. Biol.* **1994**, *235*, 657.
- (97) Wall, J. S.; Hainfeld, J. F. *Annu. Rev. Biophys. Chem.* **1986**, *15*, 355.
- (98) Lesk, A. M.; Chothia, C. *J. Mol. Biol.* **1980**, *136*, 225.
- (99) Gerstein, M.; Sonnhammer, E. L. L.; Chothia, C. *J. Mol. Biol.* **1994**, *236*, 1067.
- (100) Ferrige, A. G.; Seddon, M. J.; Green, B. N.; Jarvis, S. A.; Skilling, J. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 707.
- (101) Penszek, P.; Radermacher, M.; Frank, J. *Ultramicroscopy* **1992**, *40*, 33.
- (102) Rossi Fanelli, M. R.; Chiancone, E.; Vecchini, P.; Antonini, E. *Arch. Biochem. Biophys.* **1970**, *141*, 278.
- (103) Wiechelman, K.; Parkhurst, L. J. *Biochemistry* **1972**, *11*, 4515.
- (104) Harrington, J. P.; Pandolfelli, E. R.; Herskovitz, T. T. *Biochim. Biophys. Acta* **1973**, *328*, 61.
- (105) David, M. M.; Daniel, E. *J. Mol. Biol.* **1974**, *87*, 89.
- (106) Herskovitz, T. T.; Harrington, J. *Biochemistry* **1975**, *14*, 4972.
- (107) Wood, E.; Mosby, L.; Robinson, M. *Biochem. J.* **1976**, *153*, 589.
- (108) Vinogradov, S. N.; Shlom, J. M.; Hall, B. C.; Kapp, O. H.; Mizukami, H. *Biochim. Biophys. Acta* **1977**, *492*, 136.
- (109) Ochial, T.; Enoki, Y. *Biochim. Biophys. Acta* **1979**, *579*, 442.
- (110) Pilz, I.; Schwartz, E.; Vinogradov, S. N. *Int. J. Biol. Macromol.* **1980**, *2*, 279.
- (111) Vinogradov, S. N.; Shlom, J. M.; Kapp, O. H.; Frossard, P. *Comp. Biochem. Physiol.* **1980**, *67B*, 1.
- (112) Ochial, T.; Enoki, Y. *Comp. Biochem. Physiol.* **1981**, *68B*, 275.
- (113) Frossard, P. *Biochim. Biophys. Acta* **1982**, *704*, 524.
- (114) Theuer, M.; Pilz, I.; Schwarz, E.; Wilhelm, P.; Mainwaring, M. G.; Vinogradov, S. N. *Int. J. Biol. Macromol.* **1985**, *7*, 25.
- (115) Ellerton, H. D.; Chen, C.; Lim, A. *Comp. Biochem. Physiol.* **1987**, *87B*, 1011.
- (116) Ellerton, H. D.; Bearman, C. H.; Loong, P. C. *Comp. Biochem. Physiol.* **1987**, *87B*, 1017.
- (117) Costa, M. C. P.; Bonafe, C. F. S.; Meirelles, N. C.; Galembeck, F. *Braz. J. Med. Res.* **1988**, *21*, 115.
- (118) Vinogradov, S. N.; Kolodziej, P. *Comp. Biochem. Physiol.* **1988**, *91B*, 577.
- (119) Barnikol, W.; Burkhard, O.; Pötzchke, H. *J. Chromatogr.* **1989**, *497*, 231.

CR9600058