Nonvertebrate Hemoglobins:
Functions and Molecular Adaptations

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Weber, Roy E., and Serge N. Vinogradov. Nonvertebrate Hemoglobins: Functions and Molecular Adaptations. Physiol Rev 81: 569–628, 2001.—Hemoglobin (Hb) occurs in all the kingdoms of living organisms. Its distribution is episodic among the nonvertebrate groups in contrast to vertebrates. Nonvertebrate Hbs range from single-chain globins found in bacteria, algae, protozoa, and plants to large, multisubunit, multidomain Hbs found in nematodes, molluscs and crustaceans, and the giant annelid and vestimentiferan Hbs comprised of globin and nonglobin subunits. Chimeric hemoglobins have been found recently in bacteria and fungi. Hb occurs intracellularly in specific tissues and in circulating red blood cells (RBCs) and freely dissolved in various body fluids. In addition to transporting and storing O₂ and facilitating its diffusion, several novel Hb functions have emerged, including control of nitric oxide (NO) levels in microorganisms, use of NO to control the level of O₂ in nematodes, binding and transport of sulfide in endosymbiont-harboring species and protection against sulfide, scavenging of O₂ in symbiotic leguminous plants, O₂ sensing in bacteria and archaeabacteria, and dehaloperoxidase activity useful in detoxification of chlorinated materials. This review focuses on the extensive variation in the functional properties of nonvertebrate Hbs, their O₂ binding affinities, their homotropic interactions (cooperativity), and the sensitivities of these parameters to temperature and heterotropic effectors such as protons and cations. Whenever possible, it attempts to relate the ligand binding properties to the known molecular structures. The divergent and convergent evolutionary trends evident in the structures and functions of nonvertebrate Hbs appear to be adaptive in extending the inhabitable environment available to Hb-containing organisms.
I. INTRODUCTION

A. Distribution and Location of Nonvertebrate Hemoglobins

Hemoglobin (Hb) is encountered in all five kingdoms of organisms. In the animal kingdom, apart from vertebrates, Hb occurs widely but sporadically in the phyla Platychelminthes, Nemertea, Nematoda, Annelida, Vestimentifera, Pogonophora, Echiura, Phoronida, Arthropoda, Mollusca, Echinodermata, and Chordata and is found in some 33% of the presently known animal classes (351, 542, 578). Over the last dozen years, single-chain globins have been found in nonleguminous plants, algae, and a number of prokaryotes, ranging from bacteria to cyanobacteria. Chimeric Hbs comprised of an NH2-terminal globin domain linked covalently to a flavoprotein have been found in bacteria and yeast. Very recently, an aerotaxis transducer in a bacterium and an archean was shown to have an NH2-terminal globin domain (240).

Nonvertebrate Hbs ranging from monomers to giant multisubunit structures occur in widely different anatomical sites, either in the cytoplasm of specific tissues (muscle, nerve and glial cells, gametes, etc.) or red blood cells (RBCs) or freely dissolved in vascular, coelomic, or pericentral body fluids. In this review Hb refers to all classes of O2 binding heme proteins ranging in size from single one-domain globins to the most complex ones, and myoglobin (Mb) is used nonexclusively to denote generally single one-domain Hbs occurring in muscle, nerve/glial, and other tissues as well as those found in unicellular organisms, including the IDO-like Mbs of abalone molluscs (525). Additionally, single one-domain globins occurring in symbiont-containing leguminous plants, in nerve tissue, and in cyanobacteria are denoted LegHbs, neuroglobins, and cyanoglobins, respectively. The term erythrocrurorin used in the older literature for all extracellular Hbs and some cytoplasmic invertebrate Hbs is no longer employed. Chlorocruorin (Chl) refers to a subgroup of hexagonal bilayer (HBL) Hbs that are greenish red as the result of a modified heme group and occur in four marine annelid families (see sect. A3C3). Chimeric Hbs comprised of covalently linked globin and flavoprotein domains and found in bacteria and yeasts (see sect. A3AI) are named flavoHbs (FHbs).

B. The Mb Fold is Common to All Hbs

Crystals of all the known vertebrate and nonvertebrate Hbs and Mbs exhibit a tertiary structure (the Mb fold) that consists of six to eight α-helical segments connected by short loops (50, 343). This structure forms a three-on-three helical sandwich able to bind heme with high affinity within a cavity lined by hydrophobic residues. The amino acid sequences of nonvertebrate Hbs and Mbs, now more than 170, including the sequences of all the known globin chains comprising the large and more complicated invertebrate Hbs whose crystal structures are not known, can be aligned quite reliably with the over 600 sequences of vertebrate Hbs and Mbs using the known crystal structures, mostly of monomeric globins (36, 289, 343, 394). Although the percent of amino acid identity varies widely and can be almost random, two features are conserved: the invariant residues, Phe and His at positions CD1 and F8, respectively, and the characteristic patterns of hydrophobic residues in each of the α-helical segments. The remarkable conservation of the Mb fold is consonant with the globin family having one of the highest conservation of residue-residue contacts among known protein families (82, 457). Phylogenetic trees based on the known sequences point to a common and quite ancient globin ancestor for all the known present-day globins (205, 394), possibly a primitive archaebacterium that developed 3,500 million years ago (20).

C. Variation in Structure and Function

Compared with the intensively studied vertebrate monomeric (17 kDa) Mb and tetrameric (64 kDa) Hb, nonvertebrate Hbs exhibit much broader variation in their primary and quaternary structures. Although nonvertebrates are phylogenetically more primitive than vertebrates, the high variability encountered in their Hbs reflects specialization and adaptation to a greater range of operating conditions than in vertebrates (630). However, compared with the vertebrate Hbs, much less is known about the relations between their physiological functions and their molecular structures at the atomic level.

The as yet incompletely investigated array of quaternary structures can be broadly subdivided into several distinct groups (618) (Fig. 1). 1) The monomeric, 17 kDa, one-domain, single-chain Hbs and Mbs, which can be intracellular (tissue or cytoplasmic or located within RBCs) or extracellular, form the largest group of nonvertebrate globins. In addition to the widely occurring muscle Mbs of molluscs, annelids, and nematodes and the monomeric Hbs in annelid RBCs, this group includes the “truncated” globins, which have some 30–40 fewer residues than normal globin chains. The latter comprise the 109-amino acid residue neuroglobin of a nemertean (602), the 116- to 121-residue protozoans Hbs (533), and the 118-residue bacterial cyanoglobin (438). 2) The second group contains dimers of bacterial Hbs (621), dimers and tetramers of intracellular RBC globins, such as the Hbs of the clam Scapharca (466), and higher complexes of single-chain globins, such as the polymeric intracellular Hb of Glyceria (612). 3) The third group comprises large...
multisubunit Hbs with masses ranging from 200 to 800 kDa, comprised of two-domain globin subunits (~35 kDa), found in arthropods (251) and nematodes (44). The fourth group contains multisubunit, multidomain Hbs, consisting of one or more chains of 4–20 covalently linked globin domains, encompassing the ~250-kDa Hb of brine shrimps (363), the 1,700- to 2,300-kDa snail Hbs (53), the largest known, polymeric Hbs (>8,000 kDa) found in clams (563), and the 124- and 153-kDa Hbs found in the hydrothermal vent polychaete Branchipolynoe (243).

The fifth group is the giant extracellular HBL Hbs (~3,600 kDa) of annelids and vestimentiferans comprised of 180–192 polypeptide chains of which about one-third are non-globin linker proteins (339). In addition to a wide variation in molecular size, nonvertebrate Hbs exhibit a broad spectrum of O$_2$ binding properties. Their O$_2$ affinities that may be dependent or independent of pH (due to the presence or absence, respectively, of Bohr effects) cover over five orders of magnitude, and cooperativity coefficients vary over a ~10-fold range (Table 2).

Although the physiological functions of vertebrate Hb are the transport of molecular O$_2$ and a role in nitric oxide (NO) metabolism, those of nonvertebrate Hbs are much more diverse. In addition to O$_2$ transport (see sect. uC3) and storage (see sect. uC4), they include facilitation of O$_2$ diffusion (see sect. uC5), reactions with sulfide and its transport (see sect. vB), complex and as yet incompletely elucidated roles in NO regulation and metabolism (see sect. wA1), maintenance of acid-base balance (see sect. va), O$_2$ scavenging (see sect. wA2), O$_2$ sensing (see sect. vD5), oxidase and peroxidase activities, the latter related to detoxification (see sect. vD1), vitellogenin-like function (see sect. vD3) and roles as light-shading pigments (see sect. wA4) and regulators of the buoyancy of aquatic insects (see sect. vD4). A salient characteristic of invertebrate Hbs is heterogeneity in molecular and functional properties, which can be extensive and which is likely to be beneficial to the organism (see sect. wA4). The two best-characterized cases are the extracellular larval Hbs of the insect Chironomus (see sect. wC1) and the intracellular Hbs of the bloodworm Glycera (see sect. wB3). Hbs with different O$_2$ binding properties may also occur in different sites providing a basis for intersite O$_2$ transfer (see sect. iv). The diversity in function of cytoplasmic Hbs has been surveyed in a unique review by J. Wittenberg (664).

D. Existing Reviews and Scope

Numerous reviews of specific groups of nonvertebrate Hbs and Mbs have appeared over the last three decades: Hbs in parasites (342), extracellular Hbs (13, 94, 351, 541, 609, 618), bacterial Hbs (434a, 647), crustacean Hbs (353), mollusc Hbs (53, 396, 445, 558), intracellular Hbs (351, 355, 463, 546, 558, 577, 629), symbiotic and nonsymbiotic plant Hbs (17, 18, 20, 21, 30, 167, 232), nematode Hbs (44) including Ascaris Hb (198, 199), Hbs in unicellular organisms (490, 533), cytoplasmic Hbs and Mbs (445, 664, 666, 668), Hbs of eukaryote/prokaryote symbioses (317a, 663, 668), extracellular Hbs (208, 342, 450, 451, 550, 578, 609, 618, 651) and Mbs (520). The respiratory functions of invertebrate Hbs have also been reviewed repeatedly (351, 353, 355, 356, 424a, 542, 550, 629, 630). The current knowledge of the crystallographic structures of the predominantly small nonvertebrate Mbs and Hbs has been reviewed by Bolognesi et al. (50).

In contrast to reviews on separate groups and types of nonvertebrate Hbs, we have attempted to provide here a comprehensive overview of their functional properties, structures, and adaptations and to identify the major adaptational and evolutionary strategies.
II. BIOLOGICAL ROLE

A. Organismic Significance

Although specific Hbs may be specialized for a particular function, a strict division between the roles in transporting $O_2$, storing $O_2$, and facilitating its diffusion is not feasible. In transporting $O_2$, Hb bridges wide and independent variations in $O_2$ tensions at the sites of $O_2$ loading and unloading, particularly in nonvertebrates subjected to highly variant ambient conditions. Although the $O_2$-transporting role of circulating Hbs can be readily established in larger organisms (from differences in $O_2$ saturations between the pre- and postbranchial/pulmonary circulations), doubt about their functional significance is provoked by the lack of correlation between its presence and the hypoxic/anaerobic tolerances in different species. However, apparent superfluosity under a given set of conditions does not exclude a vital role under another more stressful one (630). Furthermore, a capacity to live under anaerobic conditions does not exclude reliance on Hb, which “can function in $O_2$ transport only in the presence of $O_2$, not in its absence” (356). Several examples of the organismic role of Hb are described below.

The induction of Hb synthesis in many invertebrates under stressful conditions (hypoxia, temperature increase and CO poisoning) (59, 162, 307) attests to its role, as do inter- and intraspecific comparisons of animals with and without Hbs. Thus the mud-dwelling nematode *Enoplus brevis* that has pharyngeal Hb maintains higher feeding rates under hypoxia than the related, Hb-free *E. communis* (34). Analogously, CO blockade of Hb function drastically reduces filter-feeding in Hb-rich *Chironomus plumosus* larvae, but hardly affects that in Hb-poor *Endochironomus albipennis* specimens (623) and Hb-rich specimens of *C. plumosus* prepared with *C. plumosus* larvae survive progressive hypoxia longer than Hb-poor ones (624). Similarly, the Hb-bearing pulmonate snail *Planorbis corneus* shows greater diving potential, lower postdiving pulmonary $O_2$ tensions, and a greater exploitation of the pulmonary $O_2$ store than Hb-free *Lymnaea stagnalis* (274). At the tissue level, the biological significance of Hb is evident from a much longer duration of neural activity under anoxia (absence of free $O_2$) in cerebrovisceral tissues of the Hb-containing bivalve *Tellina alternata* than in the Hb-free tissues of *Tagelus plebeius* (144, 319). The biological advantages of hypoxia-induced increases in Hb concentration in the crustacean *Daphnia* are discussed in section wC7A.

Another illustration is the reduction in $O_2$ consumption rates following “CO poisoning” that blocks $O_2$ binding without inhibiting mitochondrial function. Figure 2 illustrates the contribution of Hbs to aerobic metabolism. In the case of the coelomic RBC Hb of the polychaete *Enoplobranchus sanguineus* and the extracellular Hb of *C. plumosus*, which burrow in marine and freshwater sediments, respectively, the contribution increases with decreasing ambient $O_2$ tension. In contrast, the contribution of the coelomic Hb in the clam *Noetia* increases with increasing tension, while that of the extracellular Hb of *Arenicola marina* is greatest at intermediate (50–100 Torr) tensions. The role of $O_2$-transporting proteins may vary with endogenous factors. In the polychaete *Sabella melanostigma*, blocking Chl function by NaNO$_2$ showed that the role of the Chl increases with body weight (292). CO-poisoning experiments require high values of the partition coefficient $M$ (the ratio of CO to $O_2$ affinities), which is markedly dependent on the species (351, 630). Hb structure imposes restrictions on CO binding. Compared with $M$ values of 200–250 and 20–50 characterizing mammalian Hbs and mammalian Mbs, respectively (12), the partition coefficient $M$ varies widely in nonvertebrates, from 0.08 in perienteric Hb of *Ascaris* to ~20,000 in *Glycera* intracellular Hb (Table 1). Generally, the $M$ values are low (2–50) in RBC and extracellular Hbs and even lower (<1) in cytoplasmic Hbs. The extremely high value (~20,000) found for the monomeric Hb of *Glycera dibranchiata* is a consequence of a correspondingly high CO-binding affinity (480). Analogously, the extremely low value for perienteric *Ascaris* Hb (191) tallies with its exceptionally high $O_2$ affinity, and the high $M$ value for *Branchioma* Chl (~540) (160) matches the very low $O_2$-binding affinities of Chls.$^1$

$^1$ In this review the $O_2$ binding affinities of Hbs are denoted as “low,” “moderate,” “high,” “very high,” and “extremely high” to indicate $P_{50}$ >20, 10–20, 1–10, 0.1–1, and <0.1 Torr, respectively.
TABLE 1. Values of the partition coefficient of selected nonvertebrate Hbs

<table>
<thead>
<tr>
<th>Species, Protein</th>
<th>M</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplastic, non-RBC Hbs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>0.1</td>
<td>294</td>
</tr>
<tr>
<td>Vitreoscilla (bacterium) Hb</td>
<td>0.97</td>
<td>414</td>
</tr>
<tr>
<td>Bradyrhizobium japonicum FixL</td>
<td>0.87</td>
<td>195</td>
</tr>
<tr>
<td>Hordeum sp. (barley) Hb</td>
<td>1.48</td>
<td>147</td>
</tr>
<tr>
<td>Glycine max (soybean) Hb</td>
<td>64</td>
<td>202</td>
</tr>
<tr>
<td>Gastrophilus intestinalis (insect) Hb</td>
<td>0.67</td>
<td>294</td>
</tr>
<tr>
<td>Ascaris lumbricoides (nematode) Hb</td>
<td>0.82</td>
<td>191</td>
</tr>
<tr>
<td>Mermis nigrescens (nematode) Hb</td>
<td>&lt;1</td>
<td>67</td>
</tr>
<tr>
<td>Explanatum explanatum (trematode) Hb</td>
<td>0.75</td>
<td>443</td>
</tr>
<tr>
<td>Paramphistomum epiclithum (trematode) Hb</td>
<td>0.25</td>
<td>443</td>
</tr>
<tr>
<td>Gastrothylax crumenifer (trematode) Hb</td>
<td>0.37</td>
<td>443</td>
</tr>
<tr>
<td>Aphroditus aculeata (annelid) Hb</td>
<td>167</td>
<td>660</td>
</tr>
<tr>
<td>Mammalian Hbs</td>
<td>19-51</td>
<td>12, 294</td>
</tr>
<tr>
<td>RBC Hbs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enoplobranchus sanguineus (polychaete) Hb</td>
<td>40-50</td>
<td>642</td>
</tr>
<tr>
<td>Glycera dibranchiata (polychaete)</td>
<td>20,000</td>
<td>480</td>
</tr>
<tr>
<td>monomeric Hb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyoneila geminata (echinoderm) Hb</td>
<td>24</td>
<td>512</td>
</tr>
<tr>
<td>H. Human Hb and blood</td>
<td>200-250</td>
<td>12</td>
</tr>
<tr>
<td>Extracellular Hbs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daphnia magna (crustacean) Hb</td>
<td>41</td>
<td>306</td>
</tr>
<tr>
<td>Moina macrocopa (crustacean) Hb</td>
<td>48</td>
<td>306</td>
</tr>
<tr>
<td>Ascaris (nematode) perienteric Hb</td>
<td>0.075</td>
<td>191</td>
</tr>
<tr>
<td>Marphysa sanguinea (polychaete) HBL Hb</td>
<td>50</td>
<td>636</td>
</tr>
<tr>
<td>Branchionmona vesiculosa (polychaete) Hb</td>
<td>540</td>
<td>160</td>
</tr>
<tr>
<td>HBL Ch</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Planorbis corneus (mollusc) Hb</td>
<td>40</td>
<td>160</td>
</tr>
</tbody>
</table>

M, partition coefficient (ratio of CO and O2 affinities); Hbs, hemoglobin; RBC, red blood cells; Chl, chlorocruorin; Mb, myoglobin; HBL, hexagonal bilayer.

B. Environmental and Endogenous Constraints on Hb Function

1. Oxygen

The low capacities of nonvertebrates for regulating their "milieu intérieur" imply that the operating conditions of their Hbs vary markedly in parallel with ambient conditions, particularly in internal parasites and in aquatic and burrowing species that routinely are subjected to anoxia, hypoxia (O2 shortage), or hyperoxia (O2 tensions exceeding atmospheric levels resulting from plant photosynthetic activity). Nematodes from vertebrate intestines face O2 tensions from zero in the lumen to 18–30 Torr at the mucosa (459). Higher tensions may prevail for Strongylus spp. that are embedded between crypts of richly vascularized duodenal villi supplied by O2-rich portal circulation, while the tracheal parasites like Syngamus trachea will experience near-atmospheric tensions (121, 460a). On the other hand, the larvae of the insect Gastrophilus intestinalis face intermittent deficiency in O2 within the semi-fluid contents of horse stomachs (294).

Internal O2 tensions PeO2, however, depend not only on external values PeO2 but also on cuticular O2 diffusion conductances (Gτ), as evident from profound differences between the ambient O2 tensions at which Hb transports O2 in vivo (from CO-poisoning experiments; see sect. IIIA) and the O2 tensions that half-saturate Hb in vitro (P50 values). Given that O2 uptake rate (MO2) equals Gτ(PeO2 – PiO2) (229), O2 transport by circulating Hbs is favored by ventilation and perfusion of the respiratory surfaces (gills, lungs, and skin).

2. Salinity and pH

Variations in salinity and pH in time and space may exert mandatory effects on Hb function in euryhaline invertebrates living in widely different salinities and lacking significant osmotic, ionic, and acid-base regulatory capacities. The in vivo body fluid pH values in polychaetes and other invertebrates studied range from 6.84 to 7.44 (360, 648) and predictably show greater variation under unfavorable conditions.

3. Sulfides and carbon monoxide

These compounds may block aerobic metabolism as well as Hb O2 binding and their effects may compound the impact of other environmental stresses, as in the polychaete Arenicola marina and clam Astarte borealis from marine sediments, where hypoxia increases sulfide-induced Hb autoxidation and the simultaneous liberation of hydrogen peroxide (1). Sulfide-rich habitats range from inter- and subtidal muds to deep-sea cold seeps, seagrass beds, sewage outfalls, and deep-sea hydrothermal vents. Bivalves, vestimentiiferans, and annelids like Alvinella pompejana from “white smoker” hydrothermal vents, encounter high concentrations of HS− (up to 1 mM) as well as relatively high CO concentrations (148, 345, 544). The CO/O2 partition coefficient M in Hbs from these animals appears not to have been measured. Many invertebrate groups living in sulfide-rich environments harbor symbiotic chemosynthetic bacteria that oxidize sulfide and fix CO2. The Hbs of these organisms bind sulfide without covalent modification of the heme groups ( unlike mammalian Mb and Hb) and may play a role in transporting sulfide or facilitating its diffusion and in protecting the tissues from sulfide poisoning (see sect. vB).

4. CO2

Variations in CO2 tension may affect the O2 binding properties through reversible CO2 binding to the Hb (as carbamino compounds), or indirectly through pH changes that affect the O2 binding affinity of the Hb (the Bohr effect). Compared with air breathers that are in a state of “compensated hypercapnic acidosis,” i.e., a state where CO2-induced pH decreases are compensated by high bicarbonate levels (133), aquatic species generally have low internal Pco2 values due to the high CO2 solubility in water. However, hypercapnia may occur in some aquatic
forms, such as the gutless, deep-sea hydrothermal vent tube worm Riftia pachyptila which uses symbiotic chemautotrophic bacteria for carbon fixing and is periodically exposed to CO₂ and sulfide-rich vent water, in which high internal PₐCO₂ values (up to 45 Torr) are associated with large base excesses in the coelomic and vascular fluids (580).

5. Temperature

The decrease in O₂ affinity with rising temperature mandated by the exothermic nature of heme oxygenation (ΔH ≈ -50 kJ/mol O₂) may jeopardize O₂ loading to Hb in ectothermic invertebrates (whose body temperature coincides with that of the environment) under hypoxic conditions and rising temperature. The temperature effect is, however, reduced by endothermic processes, including oxygenation-linked dissociation of Bohr protons and other ions (646, 674). The Hb of the intertidal polychaete lugworm, Arenicola marina, which functions under larger temperature variations than the Hb of the subtidal lugworm, Abarenicola claparedii, has a larger Bohr effect (φ = -0.9 and -0.3, respectively) and a lower temperature effect (ΔH = -22 and -66 kJ/mol, respectively) (627). Extreme thermal gradients occur in the deep-sea hydrothermal vent habitats resulting from random mixing of O₂-rich, cold (2°C) deep-sea and extremely hot (up to 320°C) anoxic vent waters (581). The hydrothermal vent polychaete Alvinella pompejana holds the record as the most thermotolerant known metazoan: in situ measurements of ambient temperature provided a mean of 68°C (76). In this species, a high temperature increased O₂ affinity (ΔH = -76 kJ/mol at pH 6.9) (Table 2, extracellular Hbs) appears compensated by an exceptionally high intrinsic O₂ affinity (P₅₀ = 0.3 Torr at 20°C) (581). Large thermal variations are also encountered by Hb-containing prokaryotes like the cyanobacterium Nostoc that extends from tropical to polar terrestrial environments (569).

C. Intrinsic Structural and Functional Characteristics

1. Molecular transitions

Hbs exhibit homotropic interactions (cooperativity between O₂ binding heme groups that causes the sigmoidal shape of the O₂ binding equilibrium curves and increase the O₂ turnover for a given change in PₐO₂) and heterotropic ones, like the Bohr effect (inhibitory interactions between proton binding sites and hemes that decrease O₂ affinity with falling pH and enhance O₂ unloading from Hb at the relatively acid pH in tissues). Tetrameric vertebrate Hbs, which accurately fine-tune O₂ transport to tissues through thermodynamic linkages between heme oxygenation and binding of a range of allosteric ligands, form a convenient reference point for reviewing structure-function relationships in nonvertebrate Hbs. The deoxygenated molecules occur in a low-affinity tense (T) conformation, constrained by intersubunit bonds that are disrupted upon oxygenation as the molecules shift to the high-affinity relaxed (R), oxygenated state. This shift is the basis for cooperativity and is reflected in the displacement between the lower and upper asymptotes of extended Hill plots (see sect. mC3b). In vertebrate Hbs, cooperativity requires the presence of two kinds of subunits associated as a tetramer (39), and the quaternary structural shift involves a 12–15° rotation of the α₁β₁-dimer relative to the α₂β₂-dimer, while the α₁β₂- and α₀β₀-contacts in the tetramer remain rigid (427). In vertebrate Hbs, proton binding responsible for the normal (“alkaline”) Bohr effect occurs mainly at the NH₂-terminal amino acid residues of the α-chains and the COOH-terminal histidines of the β-chains (428, 449, 464), whereas β143(H21)His appears to be implicated in the reverse “acid” Bohr effect (increase in affinity with falling pH seen at low pH). Hb-O₂ affinity is moreover decreased by chloride and by anionic organic phosphates that bind stereocchemically at specific cationic residues at the entrance to the central cavity between the two β-chains of deoxyHb.² Accordingly, structural mutations that strengthen the T state or favor effector binding decrease O₂ affinity, and those that favor the R state increase affinity. In vertebrates as well as invertebrates, hyperventilation that promotes excretion of CO₂ and other acidic wastes raises O₂ affinity of Hbs with a normal Bohr effect. In mammalian and lower vertebrate Hbs (592, 639), increasing proton and organic phosphate levels lower O₂ affinity by decreasing the O₂ association constant of the T state (K₉) without significantly affecting that of the R state (K₉).

Although lacking quaternary transitions, some monomeric invertebrate Hbs show pronounced Bohr effects based on protonation-linked T→R transitions. Thus conformational changes of the insect Chironomus (496, 637) and the pronounced, reverse (acidic) Bohr effect (φ = +0.8) in the Hb of the parasitic fluke from sheep liver Dicrocoelium dendriticum, (500). Analogously, pH-linked conformational changes (138, 482) appear to be responsible for the pH and lactate effect in monomeric vertebrate Mbs (185), and tertiary level fluctuating T states may occur within individual monomeric constituents of human Hb (55). Monomeric nonvertebrate Hbs showing heterotropic effects provide an ideal opportunity to analyze the roles

²The anionic organic phosphates are 2,3-diphosphoglycerate (DPG) in mammals, inositol pentaphosphate (IPP) in birds, and ATP and GTP in ectothermic vertebrates.
### Table 2. Representative O₂ binding properties

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Species, Protein</th>
<th>Pigment Type</th>
<th>$P_{50}$, Torr</th>
<th>$n_{50}$</th>
<th>pH</th>
<th>Temperature (°C) for $P_{50}$ and $n_{50}$</th>
<th>Bohr Factor ($\varphi$)</th>
<th>$\Delta H$, kJ/mol</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tissue Hbs/Mbs</strong></td>
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<td>Prokaryotes:</td>
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Vasc, vascular fluid (blood); coel, coelomic fluid; hl, in hemolymph; nf, measured in native fluid; hemol, hemolysate; M, monomer; D, dimer; heD, heterodimer; hoD, homodimer; T, tetramer; P, polymer; HBL, hexagonal bilayer; P_{50}, half-saturation O_2 tension; n_{50}, Hill cooperativity coefficient. * Previous name is C. mycoderma.

of individual Bohr groups compared with vertebrate Hbs, where these interactions are entangled with quaternary structural changes (500).

In mammalian Hbs, the “fixed-acid” Bohr effect resulting from proton binding is supplemented by a CO_2 Bohr effect attributable to oxygenation-linked binding of CO_2 at uncharged α-NH_2 groups. Curiously, a specific, positive effect of CO_2 on O_2 affinity, opposite to that in vertebrate Hbs, has been demonstrated in the extracellular Hbs of the polychaetes Arenicola marina (333), Neobalanus americanus (564), the oligochaete Megascolides australis (634) and the crustacean Artemia franciscana (124). The regulatory significance of any of these effects remains obscure.

Oxygenation-linked dissociation, first described in lamprey Hb (62), represents the simplest forms of homo- and heterotropic interactions. The cooperativity (decrease in affinity with falling O_2 saturation) of this Hb and the effect of pH on its affinity is determined by an equilibrium between low-affinity oligomers and a high-affinity monomer. Because proton binding stabilizes the aggregate, the Bohr effect represents a cooperative uptake of protons upon deoxygenation. A structural basis for the dimerization and the Bohr effect was recently provided by the crystal structure of lamprey deoxyHb determined by Heaslet and Royer (227). Oxygenation-linked dissociation has been inferred to occur in nonvertebrate Hbs exhibiting oligomerization, including cytoplasmic Hbs from the nemertean Cerbratulus (602), neuroHb of the bivalve Tellina (319), tracheal cell Hbs from the insect Anisops (652), and RBC Hbs of the sea cucumber Molpadia arenicola (454), the polychaete Glycera dibranchiata (391) and the bivalve A. broughtonii (171). The state of oligomerization of the larval Gastrophilus Hb remains unclear (56).
underlie the extremely high O\textsubscript{2} binding affinities of some nonvertebrate Hbs and Mbs. In one, exemplified by the perienteric Hb from the nematode parasite *Ascaris suum*, hydrogen bonding of the bound O\textsubscript{2} in the distal cavity to the B10Tyr is thought to be responsible for its very low O\textsubscript{2} dissociation rate, 4,500-fold smaller than in vertebrate Mb (\(k\textsubscript{off} = 0.004\) vs. 18 s\(^{-1}\), Table 3) (431). This low dissociation rate determines the extremely high O\textsubscript{2} affinity, which is 450-fold higher than Mb, even though the O\textsubscript{2} association rate of *Ascaris* Hb is 10-fold lower than Mb (\(k\textsubscript{on} = 1.5\) vs. 15 µM\(^{-1}\)·s\(^{-1}\), Table 3). The other type of kinetic mechanism found recently in trematode Hbs, where both distal residues at positions E7 and B10 are Tyr, combines a low dissociation rate (\(k\textsubscript{off} = 0.03–0.4\) s\(^{-1}\)) with a high association rate (\(k\textsubscript{on} = 100–200\) µM\(^{-1}\)·s\(^{-1}\), Table 3) to produce even higher O\textsubscript{2} affinities (442). However, the Hb of another trematode, *Dicrocoelium*, in which one of the two distal tyrosines is turned out of the heme pocket, binds O\textsubscript{2} rapidly (\(k\textsubscript{on} = 300\) µM\(^{-1}\)·s\(^{-1}\)), but has an O\textsubscript{2} affinity only 10-fold higher than Mb due to a 2-fold higher dissociation rate (137, 341).

Symbiotic plant Hbs, which have E7His and B10Tyr (9) and very high O\textsubscript{2} binding affinities, >10-fold higher than vertebrate Mb (Table 3), appear to be intermediate between the nematode and trematode Hbs. In contrast to the nematode and trematode Hbs, the B10Tyr does not interact with the bound O\textsubscript{2} to stabilize it. X-ray crystallographic studies imply hydrogen bonding to E7His and close contacts with E11Val (Leu in soybean) (225). Although the association rates of the plant Hbs are comparable, their dissociation rates are appreciably higher than the other two groups, resulting in 30- to 300-fold lower O\textsubscript{2} affinity (192) (Table 3). The recently discovered nonsymbiotic plant Hbs (9), which seem to have the same distal E7His and B10Tyr residues, have O\textsubscript{2} binding affinities as high as the nematode and trematode Hbs, due primarily to very low dissociation rates (31, 120, 147). In this case, the very high O\textsubscript{2} affinities are likely due to stabilization of the bound O\textsubscript{2} via a hydrogen bond to the E7His inferred to exist in barley Hb (120). Furthermore, the HisE7Leu mutation in rice Hb increases the O\textsubscript{2} dissociation rate by more than 1,000-fold (31).

In several nonvertebrate globins, the distal E7His is replaced by a nonpolar residue. Such substitutions in vertebrate Mb result in 10- to 100-fold reductions in O\textsubscript{2} affinity (460). The monomeric Hb from the polychaete *Glycera* has a distal Leu and very high rates of association and dissociation (\(k\textsubscript{on} = 190\) µM\(^{-1}\)·s\(^{-1}\) and \(k\textsubscript{off} = 1,800\) s\(^{-1}\), Table 3), which result in a ~10-fold lower affinity than Mb. Although the Mb from the gastropod mollusc *Aplysia* also has a nonpolar Val at E7, the bound O\textsubscript{2} appears to be stabilized by a hydrogen bond with the long and flexible E10Arg that rotates into the heme cavity; consequently, its O\textsubscript{2} dissociation rate is smaller than in *Glycera* Hb (\(k\textsubscript{off} = 70\) vs. 1,800 s\(^{-1}\), Table 3) and its O\textsubscript{2} affinity is higher (51, 661).

### Table 3. Kinetic constants for O\textsubscript{2} binding

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<th>Protein</th>
<th>Protein</th>
<th>(k\textsubscript{on} \text{ (µM}^{-1}\cdot\text{s}^{-1})</th>
<th>(k\textsubscript{off} \text{ (s}^{-1})</th>
<th>(K\textsubscript{d} = k\textsubscript{off}/k\textsubscript{on} \text{ (µM)})</th>
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\(K\textsubscript{d}\), O\textsubscript{2} binding affinity; \(k\textsubscript{on}\), association rate constant; \(k\textsubscript{off}\), dissociation rate constant. Other definitions are as in Table 1.

**2. The heme cavity and ligand binding kinetics**

Because the O\textsubscript{2} binding affinity is determined by the ratio of the O\textsubscript{2} association and dissociation rates (\(k\textsubscript{on} \text{ and } k\textsubscript{off}\), respectively), its variation can be due to alterations in only one or both rates. Table 3 shows the O\textsubscript{2} association and dissociation rates of a number of nonvertebrate Hbs and Mbs, compared with sperm whale Mb. The ligand binding kinetics of nonvertebrate Hbs are strongly influenced by the structure of the heme cavity, particularly the size and polarity of residues occupying the distal portion that exert steric and dielectric effects (50, 508).

Vertebrate Hbs and Mbs, with the exception of elephant Mb, have His and Leu at the distal positions E7 and B10, respectively, with the distal His able in some cases to hydrogen bond with the bound O\textsubscript{2} thus stabilizing the oxygenated structure (429). In many nonvertebrate globins, the E7His and B10Leu residues are replaced by Gln and Tyr, respectively, resulting in a tight cage for O\textsubscript{2} and much higher O\textsubscript{2} binding affinities relative to vertebrate Mb. Two types of kinetic mechanisms are now known to
Although our understanding of the roles played by residues close to the distal heme cavity underlying the differences in ligand binding kinetics has increased very substantially over the last decade, due mostly to the efforts of Olson and co-workers, it is far from complete. Thus, while single, double, and even triple mutants of vertebrate Mb display the qualitatively correct alterations in ligand binding kinetics, the effects are not quantitative, i.e., they do not reproduce the properties of the wild-type nonvertebrate Mbs and Hbs. A triple mutant of sperm whale Mb designed to simulate the ligand binding properties of_Aplysia _Mb (ArgCD3→Asp/HisE7→Val/ThrE10→Arg) (499) has much higher association and dissociation rates, resulting in a fivefold lower affinity than the native Mb ($k_{on} = 88 \mu M^{-1} \cdot s^{-1}$, $k_{off} = 2,300 s^{-1}$, $K = 0.039 \mu M^{-1} vs. \ 15 \mu M^{-1} \cdot s^{-1}$, 70 s$^{-1}$ and 0.21 $\mu M^{-1}$, respectively, Table 3). Another triple mutant designed to mimic_Ascaris _Hb (LeuB10→Tyr/HisE7→Gln/ThrE10→Arg) has an $O_2$ dissociation rate of 1 s$^{-1}$, >10-fold lower than Mb, but still 250-fold higher than the native Hb (694). The limited success achieved with engineered vertebrate Mb designed to simulate nonvertebrate Mbs and Mb ligand binding properties, stands in contrast to the much higher level of success achieved with mutants of human Hb designed to mimic the $O_2$ affinity of high-altitude geese Hb (270, 640) and the bicarbonate effect of crocodile Hb (314). The obvious explanation is that the differences in tertiary structures between vertebrate Hbs are much smaller than those between mammalian Mb and nonvertebrate globins, reflecting the much higher percent identity of sequences and much closer phylogenetic relationship among the vertebrates.

3. $O_2$ transport by circulating Hbs

Circulating Hbs occur commonly in metabolically active organisms. The conduits for $O_2$ transport in invertebrates vary widely: cellular and dissolved Hbs occur in blood (vascular fluids) and in hemolymph and coelomic and hemal fluids. Although invertebrates generally lack closed blood vascular systems, their extravascular fluids may be subjected to well-defined circulations that channel the internal distribution of Hb-bound $O_2$. In aquatic larvae of the insect_Chiromus_, a structured circulation of the Hb-rich hemolymph is secured by a well-developed system of internal septae that even extend into tubular appendages (425). Another interesting example is the scaleworm_Branchipolynoe_, which lives commensally in the mantle cavity of bivalves from deep-sea hydrothermal vents. In adaptation to its hypoxic, sulfide-rich microhabitat, its gills have very large surface areas and small $O_2$ diffusion distances. Unlike other polychaetes, its gills lack blood vessels but are perfused with Hb-rich coelomic fluid through the action of ciliary and myo-epithelial contractions (241).

Well-developed, closed vascular systems in invertebrates with distinct hearts, arteries, capillary networks, and veins that permit control of blood distribution are found only in annelids, vestimentiferans, and pogonophorans (377, 476). In large species such as the giant earthworm_Glossoscolex giganteus_ and the polychaete_Arenicola marina_, blood propulsion is aided by periodic contractions of the blood vessels and gills (271, 277, 351). Unlike polychaetes and oligochaetes, the closed blood vascular system of hirudineans (leeches) is derived from the greatly reduced coelome (377). Although lacking coelomes, nemerteans routinely have a closed system of vessels that are lined with endothelia as in vertebrates (377). Echinoderms (starfish, sea urchins, and sea cucumbers) have coelomic, water vascular, and hemal fluid systems whose interrelationships are not well understood, although each may contain RBCs (355, 377, 476).

$O_2$ transport in closed circulations may be quantified by the Fick equation, $M_{O_2} = V_{oh} (c_o - c_v)$, where $M_{O_2}$ is the rate of $O_2$ delivery to tissues, $V_{oh}$ is the fluid perfusion rate, and $(c_o - c_v)$ is the difference in $O_2$ content between “arterial” (postrespiratory surface) and “venous” (prerespiratory surface) fluids. The $O_2$ content difference thus increases proportionally with $O_2$ carrying capacity (Hb concentration) and depends on the “loading” tension at the respiratory surfaces (skin, gills, or lungs) and “unloading” tension in the respiring tissues, the Hb-$O_2$ affinity, and the shape of the equilibrium curve. The $O_2$ content difference $(c_o - c_v)$ is difficult to assess with available techniques in small and fragile invertebrates. For the lugworm_Arenicola cristata_, the pre- and postbranchial oxyHb saturation difference (0.06 ml $O_2$/ml blood), $O_2$ carrying capacity (130 ml/l), and $O_2$ consumption rate (0.158 ml · kg$^{-1}$ · min$^{-1}$) (352) indicate a cardiac output of 2.7 ml · min$^{-1}$ · kg$^{-1}$ at 22°C (575, 576).

4. $O_2$ storage

Heme-bound $O_2$ forms vital $O_2$ stores in invertebrates subjected to intermittent $O_2$ supply, particularly in cyclic-ventilating animals and gut parasites, and in nerve and muscle tissues that exhibit intermittent high-level activities (33, 96, 664). The duration of the $O_2$ stores obviously increases with a reduction in metabolic rates under hypoxic conditions, as occurs in “oxyconforming” species (629, 630).

Several studies illustrate the significance of tissue Hbs as $O_2$ stores. In the minute gastrotrichan_Neodasys_, which is below the “Harvey size limit,” the Hb-containing cells constitute 14% of the total body volume and are closely associated with nerve and muscle tissues; the heme concentration (18.5 mM) suffices for 17 min of $O_2$ consumption by an active animal under aerobic conditions (96). A detailed comparative electrophysiological study of several bivalve species with and without Hb in

3. Because diffusional transfer in tissues is extremely slow (>300,000 times slower than in air) (332), aerobic organisms that exceed
their nerve tissues (145, 319, 320, 322) showed that although there were no obvious electrophysiological differences between cerebrovisceral connectives with and without neuroHb, the connectives with neuroHb consumed much less O2 during action potential conduction than connectives and other nerves without neuroHb. Thus the neuroHb-containing connectives may effectively use the neuroHb O2 store to enable the organism to use continued neuromuscular activity under hypoxic conditions. O2 bound to the neural tissue Hbs in the clams *Tellina alternata* and *Spisula solidissima* and the nemertean worm *Cerebratulus lacteus* can support the O2 requirements of the nerves for up to 30 min during anoxic periods (145, 320, 602).

RBC Hbs may also serve as O2 stores. In the phoronid *Phoronis architecta*, the coelomocyte Hb functions as an O2 store for ~15 min (599), whereas coelomic PO2 values in the echiurid *Urechis caupo* indicate a longevity of the RBC Hb reservoir of up to 3 h (355, 439). Measurement of the rates of heat dissipation and O2 consumption in two terebellid polychaetes, *Enoplobrachus sanguineus* with Hb-containing coelomocytes and *Lysilla alba* lacking them, revealed a difference in metabolic response upon return to normoxic conditions after exposure to anoxic conditions: a much higher O2 consumption rate in the Hb-less species, indicating the repayment of an O2 debt incurred during the hypoxic period (145). Similar experiments with two bivalve species showed a different effect: under hypoxic conditions the rate of heat dissipation of the neuroHb-containing *Tellina alternata* remained high, while the metabolic rate cycles of the neuroHb-lacking *T. plebeius* disappeared (145). These studies show that while the response of marine invertebrates to hypoxic conditions can be complex and variable, the presence of Hb may play a role in the partitioning of metabolic flux into aerobic and anaerobic processes.

Extracellular Hbs may also have O2 storage roles. In the periodically ventilating tubiculous larvae of the insect *Chironomus*, the duration of the O2 stores corresponds well with the ventilatory pauses (624), which they thus may permit. The O2 carried in the blood and coelomic fluids of the hydrothermal vestimentiferan *Riftia pachyptila* (containing 3.5 and 1.9 mM heme, respectively) can support respiration at the constant and maximum rate for 15 min (87), whereas coelomic PO2 values and high Mb concentrations (279, 280). Monomeric Hbs may play important roles; although their diffusion rate is only 1/20 of that of free O2, their concentrations in tissues may greatly exceed that of free O2 (some 30-fold in working heart muscle and 10,000-fold in soybean root nodules) (668).

### III. OCCURRENCE AND FUNCTIONAL AND MOLECULAR PROPERTIES

This section briefly reviews the phylogenetic and anatomical distribution of Hbs and focuses on the functional and structural properties of nonvertebrate Hbs. Although Hbs tend to occur more generally in organisms facing lack of O2, their occurrence defies strict categorization in terms of phylogeny and environmental conditions. On the basis of their histological sites, quaternary structures, and physiological properties, nonvertebrate heme proteins can conveniently be categorized into 1) noncirculating cytoplasmic Hbs and Mbs that occur in unicellular organisms or in tissues of higher organisms, 2) RBC Hbs that occur in nucleated RBCs circulating in any fluid, and 3) extracellular Hbs that occur in solution in body fluids. The tremendous range in O2 binding properties encountered in nonvertebrate Hbs is illustrated in Table 2 and Fig. 3.

### A. Cytoplasmic Hbs and Mbs

Cytoplasmic Hbs and Mbs exhibit an even more intermittent phylogenetic distribution than circulating Hbs (520), occurring episodically in prokaryotes (bacteria), unicellular eukaryotes (yeasts, protozoans), flowering plants, and various tissue and cell types (muscles, nerves, gills, tentacles, and gametes) of phylogenetically diverse nonvertebrates animals. Although tissue Hbs are commonly 16- to 18-kDa monomers, dimeric 32- to 35-kDa
Mbs occur in radulae of some gastropod molluscs (63) and in annelids (553). Smaller chain, 11- to 12-kDa “miniHbs” are encountered in cyanobacteria, protozoans, and nemerteans (264, 533, 602).

Mbs are encountered in body walls and probosces of annelids (179, 553, 557, 644) and in radular, body wall, adductor, and stomach muscles of molluscs (445, 515, 520, 554, 558). Nerve Hbs are scattered among different taxons including nematodes (Ascaris) (180), annelids (Glycera and Aphrodite) (660), the echiuroid Urechis, lamellibranch molluscs (Tivela, Spisula), gastropod molluscs (Busycyon and Aplysia) (319, 475), nemerteans (603, 605), and arthropods (Daphnia, where ganglial Hb concentration rises in response to hypoxic conditions) (163). In molluscs and annelids, it occurs at millimolar concentration in glial cells surrounding the nerve cord (318, 322, 323). As in vertebrates, the O2 affinities of invertebrate Mbs are generally intermediate between those of circulating Hbs and mitochondrial cytochrome oxidase, thus suggesting that they form intermediate O2 transfer stations.

The physiological roles of cytoplasmic Mbs have received less attention than those of circulating Hbs. This inattention was based on a lack of cooperativity and the perceived absence of functional heterogeneity, conformational transitions, and sensitivities to pH and allosteric effectors. Recent studies have shown these perceptions to be invalid (11, 185, 301, 442, 482) and have brought to light a variety of potential physiological roles for the cytoplasmic Hbs and Mbs.

1. Prokaryote and unicellular eukaryote Hbs

A) FLAVOHEMOGLOBINS. In the last 10 years, members of a family of ~43 kDa two-domain (“chimeric”) flavohemoproteins (FHP) or FHb, comprising an NH2-terminal globin domain and a COOH-terminal flavin-binding domain, have been discovered in several microorganisms. Their functional significance is under intensive scrutiny. The Escherichia coli heme protein HMP was the first FHb to be sequenced by Poole and co-workers (604) in the course of an attempt to identify the genes encoding dihydropterdine reductase activity. Like the E. coli FHb, the FHb from the Gram-negative, hydrogen-oxidizing and denitrifying bacterium Alcaligenes eutrophus shows the highest sequence similarity to the homodimeric Hb from Vitreoscilla (107, 621); its COOH-terminal portion of 250 residues appears to belong to the ferredoxin reductase-like family of FAD-dependent oxidoreductases, despite low sequence identity. Although there is high sequence similarity between the globin domains of E. coli and Alcaligenes FHbs and the single-domain Hb from the bacterium Vitreoscilla (see sect. III B), the crystal structure of Alcaligenes FHb (150, 413) contained a tightly bound phospholipid in the heme cavity that precluded any

FIG. 3. O2 equilibrium curves illustrating the enormous variation in O2 binding affinities encountered in nonvertebrate Mbs and Hbs. A.l., Ascaris lumbricoides (nematode) extracellular perienteric Hb at 37°C (191); G.m., Glycine max (soybean) root nodule LegHb at 20°C, pH 6.5–7.0 (15, 262); E.e., Explanatum explanatum (trematode) cytoplasmic Hb B at 37°C, pH 7.2 (443); S.e, Siboglinum ekmani (pogonophoran) extracellular Hb at 20°C, pH 6.5 (650); A.m.Mbl and A.m.MblI, Arenicola marina (marine polychaete) Mbl and MblI, respectively, at 15°C and pH 7.5 (644) (R. E. Weber, unpublished results); N.c., Nostoc commune (cyanobacterium) Hb at 20°C, pH 7.5 (609); A.m.Hb, Arenicola marina hexagonal bilayer (HBL) Hb at 15°C, pH 7.5 (574); B.g., Biomphalaria glabrata (gastropod snail) extracellular Hb at 25°C and pH 7.46 (64); U.c., Urechis caupo (marine echiuran) RBC Hb at 20°C, pH 7.5 (178); A.a., Anisops assimilis (aquatic insect) tracheal cell Hb at 25°C, pH 6.9 and 29 mM heme concentration (652); E.c., Eupolyphenia crescentis (marine polychaete) HBL Hb at 10°C, pH 7.1 (365); E.v., Eudistylia vancouverii (marine polychaete) chlorocruorin (Chl) at 25°C pH 7.1 (259).
determination of protein-heme interaction at the distal side of the heme. Closely related FHbs have been found in other bacteria, *Erwinia chrysanthemi* (157), *Salmonella typhimurium* (109), *Bacillus subtilis* (336), and *Mycobacterium tuberculosis* (97, 246), as well as in the yeasts *Saccharomyces cerevisiae* (699) and *Candida* *nordensis* (266). Membrillo-Hernandez and Poole (380) have used a primer based on the consensus sequence of the foregoing FHbs to search for Hb-like genes in other bacteria. Such genes were found in *Campylobacter jejuni*, *Listeria monocytogenes*, *Rhizobium leguminosarum*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*.

Although the kinetics of O₂ binding with *Saccharomyces* and bacterial FHbs have not been determined, the *Candida* FHb has a very high O₂ affinity [dissociation constant (Kₐ) ≈ 0.02 μM] due to a high association rate and a low dissociation rate, 850 μM⁻¹s⁻¹ and 17 s⁻¹, respectively (416, 417).

*E. coli* HMP is expressed under aerobic and anaerobic growth conditions and has a high O₂ affinity (Kₐ ≈ 2.6 μM); it has been suggested that it could serve as an O₂ sensor by combining with intracellular O₂, thus limiting flavin reduction in the aerobic state (435, 436). Because it is also able to reduce Fe(III), cytochrome c, and the *Azotobacter* regulatory flavoprotein NifL (348, 436), it also has the ability to reduce Fe(III), cytochrome *c* and the *Azotobacter* regulatory flavoprotein NifL (348, 436), it might affect the redox status and contribute to the regulation of the *fur* gene (fumarate, nitrate respiration) and the *soxRS* genetic locus.

The expression of FHbs appears to be sensitive to alteration in O₂ concentration; it is enhanced by hyperoxic conditions in *Saccharomyces* (110, 696) and induced by lack of O₂ in the bacteria *Alcaligenes* and *Bacillus* (336, 440). Although the FHb deletion strains of *E. coli* do not appear to be sensitive to superoxide (378, 379), a *Saccharomyces* FHb deletion strain was found to be sensitive to oxidative stress (696).

Several recent results have indicated the involvement of FHbs in the metabolism of nitrogen oxides. Marked upregulation of *E. coli* HMP by NO was observed under aerobic and anaerobic conditions (435), and *Bacillus subtilis* FHb was found to be induced by nitrite (336). Crawford and Goldberg (109) showed that *Salmonella* FHb has a specific role in the protection from nitrosoglutathione, and presumably NO, that is independent of O₂. They also demonstrated that under anaerobic conditions, the inducible FHb protects *Salmonella* from acidified nitrite or an NO donor compound (108, 109). Likewise, Poole and co-workers (378, 379) showed that *E. coli* HMP expression was upregulated in the presence of NO and provided protection against oxidative stress. Anaerobic growth of an *Alcaligenes* FHb⁻ strain on nitrite was found not to accumulate nitrous oxide as a transient intermediate (107). Gardner et al. (177) isolated an O₂-dependent and cyanide-sensitive NO dioxygenase activity from NO-resistant *E. coli* and identified it as HMP, a result confirmed by Hausladen et al. (226). These results and more recent ones (512a) suggest that FHbs are protective against nitrosative stress. It is likely that FHbs have an additional function, since the O₂-dependent NO dioxygenase activity cannot explain the protection FHb affords against nitrosoglutathione in anaerobic *Salmonella* (109) or NO gas in anaerobic *E. coli* (177). Nor does it explain the benefit of increased FHb expression in anaerobic *E. coli* or *B. subtilis* grown with NO, nitrate, or nitrite (176, 336, 440). Kim et al. (299) have demonstrated that the *E. coli* FHb has NO reductase activity and have suggested that this activity may account for the anaerobic protection by FHbs.

A very interesting point made by Gardner et al. (177) is that the agreement between the estimated origins of primeval Hb, blue-green algae, and free atmospheric O₂ at ~1.8, ~2.3, and ~1.8 billion years ago, respectively (699), suggests a plausible coevolution of Hb and NO dioxygenase functions. Linkage is possible because the dioxygenase activity of FHbs requires O₂ and because toxic NO is produced by an O₂-dependent oxidation of nitrogen compounds.

### b) Bacterial Hbs

The Gram-negative bacterium *Vitreoscilla*, an obligate aerobe, contains a dimeric Hb (621) whose sequence is more similar to the globin domains of FHbs and plant Hbs than other invertebrate Hbs (31). Its expression is upregulated at low O₂ levels (139, 140), and ~40% of it occurs in the periplasmic space (296). The Hb occurs as a stable oxy form, has a normal O₂ association rate and an unusually large dissociation rate of 5,600 s⁻¹ (414), and may play a role in the transfer of O₂ to the terminal oxidases under hypoxic conditions by facilitated diffusion (646, 668). The crystal structures of the ferriHb and of its azide complex have suggested hydrogen bonding of the distal B10Tyr hydroxyl via a water molecule to the O₂ ligand (537). The discovery of a NADH-dependent flavin reductase in *Vitreoscilla* suggests that the heme binding and flavin binding domains have separated in this bacterium during evolution (267).

*Vitreoscilla* Hb gene was found to be strongly expressed in *E. coli*, where its presence promotes growth under microaerobic conditions (295). In the absence of the two *E. coli* terminal cytochrome oxidases, cytochromes *o* and *d*, the expressed Hb behaves as a terminal oxidase (141). *E. coli* mutants lacking the *fur* gene product were unable to activate the *Vitreoscilla* Hb gene under microaerobic conditions (275). Bailey’s group (283, 586) has documented the involvement of *Vitreoscilla* Hb in enhancing the activity and efficiency of the electron transport chain in *E. coli* under hypoxic conditions. The beneficial effect of the heterologous *Vitreoscilla* globin gene expression in improving O₂ utilization by the host cells and their increased growth under hypoxic conditions is not limited to *E. coli*: it has been found to occur in other bacteria, in yeast, in a mammalian cell line (284), and in transgenic plants (237).
Two Hb genes glbN and glbO occur in the genome of *Mycobacterium tuberculosis* (97). The deduced amino acid sequences are similar to those of algal, protozoan, and cyanobacterial Hbs (106). Spectroscopic studies of the expressed HbN indicate the distal residue to be Leu, with Tyr in the B10 position (680). It has a substantial cooperativity (ν50 = 2), probably due to self-association of the deoxy form and an extremely high O2 binding affinity (P50 = 0.013 Torr at 20°C), resulting from fast association and slow dissociation rates of 25 μM⁻¹s⁻¹ and 0.2 s⁻¹, respectively (106). Mutation of the B10Tyr to Leu and Phe resulted in an increase in the dissociation rate constant by more than two orders of magnitude (45 and 30 s⁻¹, respectively) (106), indicating the importance of the stabilization of the bound O2 through hydrogen bonding with the Tyr hydroxyl. It is likely that the oxyHb has a role in protecting the *Bacillus* from NO similar to the role of pHb in *E. coli* (176, 177).

**c) Truncated Cyanoglobin.** Cyanoglobin is a single-chain Hb of 118 residues found in the photoautotrophic cyanobacterium *Nostoc commune*, which is capable of aerobic nitrogen fixation (438), and in *Synechocystis* (286). Both sequences have substantial identities with the truncated Hbs from the protozoans *Paramaecium* and *Tetrahymena*, 116 and 121 residues long, respectively. *Nostoc* cyanoglobin synthesis is induced by nitrogen starvation and anaerobic incubation; the positioning of its gene between two other genes essential for nitrogen fixation suggests it is involved in the latter function, perhaps as an O2 scavenger (10). Because protozoa and cyanobacteria often occur together and symbiosis between the two groups is known to occur, it is likely that protozoan Hbs are of cyanobacterial origin (569). A recent study (315a) showed that, in contrast to the Hb genes of *Paramaecium*, those of *Tetrahymena* and *Nostoc* lack introns. Cyanoglobin binds O2 with a rate (390 μM⁻¹s⁻¹) (570) that is among the highest known (Table 3); however, a fairly high O2 dissociation rate gives cyanoglobin an affinity (P50 ~0.55 Torr at 20°C; Fig. 3) intermediate between Mb and the LegHbs (570). Cyanoglobin has a higher rate of autoxidation than sperm whale Mb and a ~100-fold faster rate of hemin loss, due probably to the absence of a D helix evident from sequence alignment, which is known to destabilize the heme-apoMb complex (658).

**d) Truncated Protozoan Hbs.** Although the presence of heme proteins in ciliated protozoans was shown many years ago (293), little was known about them except for *Paramaecium* Hb, which exists as a monomeric globin with several different isoforms (6 and 12 components in *P. caudata* and *P. aurelia*) and an O2 affinity comparable to mammalian and *Nostoc* Mb (P50 ~0.6 Torr at 20°C) (233, 263, 501, 510, 588, 593–596). Shikama and co-workers (264, 265, 534) have sequenced the Hbs from *Paramaecium caudatum*, *Tetrahymena pyriformis*, and *T. thermophila* and found them to have 116–121 residues, with deletions occurring in the A- and D-helical as well as in the CD-interhelical regions. *Tetrahymena* Hb has an O2 affinity (~0.2 Torr) (316) comparable to that of the other truncated Hbs. Curiously, its rate of autoxidation is ~10-fold slower than that of *Paramaecium* Hb, and similar to that of sperm whale Mb. It is possible that the few additional residues relative to *Paramaecium* Hb and the even shorter, 109-residue nerve tissue Hb from the nemerteans *Cerebratulus* Hb (602), could be the reason for the greater stability of *Tetrahymena* Hb.

**e) Algal Hbs.** Recently, Guertin and co-workers (102) discovered three globin genes in the genome of the green unicellular alga *Chlamydomonas eugametos*; two genes were cloned, only one of which requires photosynthesis for expression (102). They code for 164- and 171-residue globins, which occur in the chloroplast at concentrations of up to 130 nM and are distributed between the proteinaceous ribulose diphosphate carboxylase-rich pyrenoid and the thylakoid membrane regions. Although the sequences exhibit the highest similarity to the *Paramaecium* (264), *Tetrahymena* (265), *Nostoc* (438), and *Synechocystis* (286) Hbs, they do not have the deletions associated with the truncated globins. The algal ferroHb forms stable complexes with O2 and CO, and the ferriHb forms complexes with thiols as well as with azide and cyanide (104). Spectroscopic and kinetic studies have established that the bond O2 forms multiple hydrogen bonds with the putative distal E7Gln and B10Tyr, residues, which are found predominantly in high O2 affinity invertebrate Hbs (104). The most recent spectroscopic study of *Chlamydomonas* Hb shows it to differ from the other E7Gln- and B10Tyr-containing Hbs, in that it is the B10Tyr and not the putative distal E7Gln and B10Tyr residues, which ligates the heme iron in the ferro form, aided by a strong interaction with E10Lys (119). The occurrence of a Hb in *Chlamydomonas* chloroplasts raises the question of its functional role in such a high O2 tension environment. Because of its small concentration, chloroplast Hb is unlikely to have a storage or facilitated diffusion function. In addition, the O2 dissociation rate being one of the slowest known (half time = 49 s), it is unlikely to support any function that requires dissociation of the bound O2. *Chlamydomonas* Hb has many ligand binding properties in common with the nonsymbiotic plant Hbs: slow dissociation of O2, a 6-coordinate low-spin Fe(III) form, ligand binding to a 6-coordinate low-spin Fe(II) form which requires prior dissociation of the sixth ligand, and fast autoxidation (103, 119).

The three-dimensional structures of *Paramaecium caudatum* Hb and of a truncated form of *Chlamydomonas eugametos* Hb have been recently solved by X-ray crystallography by Bolognesi (M. Bolognesi, personal communication). Both proteins display very similar tertiary structures, consisting essentially of helices B, C, E, G, and H of the conventional globin fold. Only one α-helical turn is found in the expected A- and F-helix regions. Particularly, almost the whole F helix is substituted by an extended protein loop, which may reflect specific se-
quence motifs (including Gly residues) in this molecular region. TyrB10 and GlnE7 residues are properly positioned to form direct hydrogen bonds to the distal site ligand.

2. Plant Hbs

In contrast to Hbs from the animal kingdom that have been known at least since Cain (First Book of Moses, chapter 4), “symbiotic” plant LegHbs in soybean root nodules were first described in 1939 by Kubo (334). The last decade has witnessed the discovery of another class of plant Hbs: the “nonsymbiotic” plant Hbs that are expressed at low concentrations in nonnodular, rapidly growing and metabolizing plant tissues and are widely distributed in both mono- and dicotyledonous plants (9, 31, 147, 232).

a) SYMBIOTIC HBS. LegHbs occur in nitrogen-fixing nodules formed as the result of infections of legume roots with one of four genera of prokaryotes, called rhizobia (17, 18); they belong to multigene families and comprise the most abundant soluble protein in the cytoplasm of the root nodules at local concentrations as high as 2–3 mM (167). The crystal structures of lupin (225) and of soybean LegHbs (222) have established the presence of His at both the proximal and distal heme sites.

Due to the combination of high O2 association rates and normal dissociation rates, 100–300 $\mu$M$^{-1}$s$^{-1}$ and 5–30 s$^{-1}$, respectively (192, 665), LegHbs have extremely high O2 affinities: $P_{50} = 0.04–0.07$ Torr at 20°C for three components of soybean Hb (Table 2, Fig. 3), and intermediate for a mixture of the components, indicating the absence of specific interactions, at least in dilute solutions (16). Direct equilibration measurements (A. Rashid and R. E. Weber, unpublished results) show a slightly lower affinity ($P_{50} = 0.14$ Torr at 25°C).

The kinetics of oxygenation of soybean LegHb distal histidine mutants support the hypothesis that the high affinity is determined mainly by enhanced accessibility and reactivity of the heme group (222). Contrary to the persistent notion that LegHbs act as O2 scavengers to prevent inhibition of nitrogen fixation, it is now clear that LegHbs function to provide an adequate supply of O2, albeit at low concentrations, to the terminal oxidases of the symbiotic bacteroids (17). This occurs at a stabilized free O2 concentration sufficient for the functioning of the oxidase but low enough to prevent inactivation of the nitrogenase enzyme also located in the bacteroids (18). The only nonlegume plant known to form a symbiosis with rhizobia is Parasponia andersonii, where a single gene appears to encode a Hb found in roots and root nodules (49). Although its O2 binding kinetics are similar to LegHbs (669), indicating a similar function, its sequence differs from other legHbs and is more similar to the nonsymbiotic Hbs. Symbiotic (actinorhizal) association between Frankia, a member of moldlike bacteria (actinomycetes) with a diverse range of dicotyledonous plants such as Casuarina glauca and Myrica gale, also results in the formation of nitrogen-fixing and Hb-containing root nodules (424, 498). The sequence of Casuarina Hb has been determined; its O2 affinity is similar to that of LegHbs (159).

b) NONSYMBIOTIC HBS. The nonsymbiotic Hbs of plants occur in the roots, stems, or germinating seeds of mono- and dicotyledonous plants at low concentrations, ~1–20 $\mu$M/kg wet tissue, and have amino acid and gene sequences quite distinct from those of LegHbs (9, 18, 29, 30, 232). Except for barley (539), two or more globin genes are expressed in diverse tissues of soybean (9), Arabidopsis (583), and rice (31), with the highest levels of expression occurring in metabolically active or stressed tissues (30).

Appleby et al. (20) were the first to propose an O2 sensing role for nonsymbiotic Hbs. The latter occur at very low concentration, ~100 nM, and could trigger an anaerobic response via formation of the deoxy form under lower than normal O2 concentrations. Based on the expression at moderate levels of nonsymbiotic Hb genes in soybean and Casuarina, Andersson et al. (9) proposed that the principal role of the nonsymbiotic Hb could be to facilitate intracellular diffusion of O2 to the mitochondria in metabolically active cells to meet an increased demand for oxidative respiration. Although Rhizobium-containing nodules (legumes or Parasponia) that fix nitrogen in the absence of cytoplasmic Hb have not been observed, some Actinorhizal symbioses do not require it for survival. Thus, in the former case, a facilitated flux of O2 via the Hb appears to be a necessary part of such symbioses (C. A. Appleby, personal communication). The kinetics of ligand binding determined recently with native and recombinant nonsymbiotic Hbs have shown that the high O2 binding affinities (7–1,800 $\mu$M$^{-1}$) are due to a moderate association rate (1–620 $\mu$M$^{-1}$s$^{-1}$) coupled with a very slow dissociation rate ($0.028–51$ s$^{-1}$) exceeded only by Ascaris Hb (31, 120, 147, 222, 583), probably due to stabilization of the bound O2 through hydrogen bonding to the distal E7His (120). These results do not provide support for either of the two proposed Hb roles: O2 sensing and facilitation of O2 transport.

Arredondo-Peter et al. (30) have proposed that nonsymbiotic plant Hbs could be involved in more than one metabolic pathway. The possible roles include, 1) O2 scavenging suggested by their high O2 affinity; 2) participation in electron transfer via interaction with a flavoprotein by analogy with the bacterial and yeast FHbs; 3) binding of O2, NO, and CO, which are known to be ligands, as part of a sensing mechanism including involvement in the regulation of cellular metabolism in response to fluctuation in the ligand level; and 4) binding to small organic molecules and function in fatty acid transport or participation in the synthesis of organic compounds under anaerobic conditions. In the case of barley, there is...
some evidence that the Hb is involved in some aspect of ATP metabolism (402). Barley Hb is very stable when liganded and oxidizes rapidly when ligand is removed (147). It has a redox potential of 180 mV, close to that of LegHbs, and is thus unlikely to act as an electron transfer protein (232). Recent work by Hill’s group (232, 507) on maize cells transformed with barley Hb strongly indicate a role for the Hb in glycolytic metabolism, perhaps as an oxygenase.

3. Platyhelminth Hbs and Mbs

Hbs and Mbs occur in representatives of the classes Monogenea (mostly ectoparasites of aquatic vertebrates), Trematoda (flukes), and Turbellaria (free-living flatworms) (35) and may also occur in the class Cestoda (tapeworms) as indicated by red patches in neck and scolex regions of several species (342).

After Wharton’s discovery in 1938 of Hb in Cer-corhichus robustus that parasitizes turtles (656), Hb has been recorded in a large number of trematodes from a diverse range of vertebrate and invertebrate hosts (e.g., *Isoparorchis hypselobagri* from the swim bladder of the catfish *Wallago attu*, *Explanatum explanatum*, *Paramphistomum epitilium*, and *Gastrothylax crumenifer* from the rumen and bile ducts of Indian buffalo *Bubalus bubalis*, *Gastrodiscoides hominis* from pig caeca, *Notocotylus triserialis* from duck digestive caeca, *Dicrocoelium dendriticum* from sheep liver, and *Paravortex scro-lularia* and *Proctoeces subtenuis* from the intestine and kidney, respectively, of the estuarine bivalve *Sero-bicularia plana* (218, 349, 432). The major forms of platyhelminth Hbs appear always to be monomeric (73, 444, 500), and disulfide-bonded dimer formation may be time dependent, as observed with *Isoparorchis hypselo-bagri* Hb (444).

Except for the recent work by Moens and co-workers (442) on trematodes, there is a paucity of information on the structure and properties of platyhelminth Hbs. The primary structures of the monomeric Mbs from *Paramphistomum epitilium* and *Isoparorchis hypselobagri* have been determined (442). Both Mbs exist in at least four isoforms and have Tyr residues in the B10 and E7 positions. Because trematodes do not have a coelom, their Mbs may represent the most primitive globin among multicellular organisms. The kinetics of O2 binding demonstrated very high rate constants for association, 108 and 205 μM−1s−1 for *Paramphistomum* and *Gastrothy- lax*, respectively (298), coupled with dissociation rate constants of 0.033 and 0.4 s−1, respectively, leading to O2 affinities higher even than that of *Ascaris* Hb (Table 2).

Although the association rates are similar to those of the LegHbs, the dissociation rates are more than an order of magnitude lower. A solution 1H-NMR study of *Paramphistomum* metHb heme cavity was unable to determine the orientation of the E7Tyr but indicated that the cavity was compact (695). The Hb of the liver fluke *Dicrocoelium dendriticum*, which has not been characterized as well, has a lower O2 affinity (P50 = 0.016–0.15 Torr at 25°C) (500). Although it also appears to have two Tyr residues in the distal cavity (341) and has a similar association rate, it has a normal dissociation rate (137). *Dicrocoelium* Hb exhibits a large reverse acid Bohr effect (φ = +0.96) that reflects proton uptake in the T→R transition, in contrast to monomeric *Chironomus* Hbs, which show only normal Bohr effects and vertebrate Hbs that possess a normal alkaline Bohr effect (due to proton dissociation upon oxygenation) and a reverse acid Bohr effect at low pH (500).

Trematode Hbs exhibit pronounced heterogeneity. *Dendriticum* Hb comprises two major (HbI and HbII) and one minor (HbIII) components (500), *Paramphistomum epitilium* has two, and *Explanatum explanatum* and *Gastrothylax crumenifer* Hbs consist of at least two and seven isoforms, respectively (443). The isoHbs from *E. explanatum*, *G. crumenifer*, and *P. epitilium* show extremely high O2 affinities (P50 ∼0.09 Torr at 37°C) and striking functional differentiation (an ~4-fold variation in O2 affinity among G. crumenifer isoHbs) (443). *Explanatum*, *Gastrothylax*, and *Paramphistomum* Hbs display very low M values: 0.8, 0.4, and 0.3, respectively (Table 1), resulting from a high O2 affinity rather than a low CO affinity (298). The high O2 affinity favors O2 transfer from the host, although it appears unfavorable for unloading O2. In confined specimens of the free-living trematode *Phaenocora unipunctata*, the oxyHb absorption bands appear and disappear reversibly in response to increase and decrease in O2 tensions (111).

The Hb from a metacercaria stage of a gymanophallid trematode, which parasitizes the nephridial sacs of the marine polychaete *Amphitrite ornata*, displays a low in vivo O2 affinity compared with mammalian Mb (P50 = 1.1 Torr at 20°C), pronounced cooperativity, and a marked Bohr effect (n = 2.2, φ = −0.35 below pH 7.4) (601). The extracted Hb appears to contain (~16 kDa) monomers and a large molecular weight (~2.5–3.0 MDa) fraction, which if confirmed in vivo would be unique among tissue Hbs.

*Fasciola hepatica* has an intracellular Hb that is located preferentially around the vitellaria and uterine coils and is highly antigenic (375).

4. Nematodes

A) Tissue Hbs. An early survey of nematodes indicated a majority to have Hbs (342). Blaxter (44) has grouped the Hbs occurring in at least 23 genera of the orders Strongylida, Ascaridida, Rhabditida, Spirurida, and Enop- lida into three molecular classes: 1) monomeric, intracel-lular Mbs found in the body wall and pharyngeal muscles; 2) tetrameric, extracellular, ~70 kDa Hbs occurring in the cuticle; and 3) extracellular, ~350 kDa octamers of two-
domain chains found in the perivascular or pseudocoelomic fluid. Although only a monomeric Hb has been found in the rhabditid Caenorhabditis elegans (514) and the trichostrongyloid sheep intestine parasite Trichostrongylus colubriformis (166), another trichostrongyloid Nippostrongylus brasiliensis and the ascarid Ascaris suum from pig intestines have all three Hbs (45, 410–412, 662). The pseudocoelomic Hbs are discussed in section III.

Ascaris Mb has the same B10Tyr and E7Gln residues that are considered to be determinants of the high O\textsubscript{2} affinity in the extracellular Hb (46). However, contrary to the extracellular Hb, the Mb can be deoxygenated in vivo (121) and has an ~50-fold lower O\textsubscript{2} affinity, resulting from a ~50-fold faster O\textsubscript{2} dissociation rate (191), suggesting that the B10Tyr may not form a hydrogen bond with the bound O\textsubscript{2} in this case.

The finding of a globin gene in the genome of Caenorhabditis elegans (514), a small (~1 mm) free living nematode not hitherto suspected of having a Hb because of a very low level of expression (302, 364), suggests that all nematodes may have globin genes. Despite substantial homology with the Ascaris Hb and Mb, it does not have a high O\textsubscript{2} affinity. There is some indication of the presence of an extracellular Hb as well (198).

Considerable evidence suggests a physiological role for nematode Mbs. Model analyses (203) indicate significant facilitation of O\textsubscript{2} diffusion by Ascaris Mb at low environmental O\textsubscript{2} tensions. In vivo deoxygenation of Ascaris Mb is accompanied by cessation of movement, indicating a role of the Mb in tissue O\textsubscript{2} supply, possibly as an O\textsubscript{2} store. A similar role was demonstrated for the Mb of the free-living nematode Enoplus brevis (33) and Nippostrongylus Mb (485). A comparative study of the feeding of two Enoplus species that share the same habitat of estuarine mud but differ in the possession of a pharyngeal Hb indicates that the presence of Hb permits grazing under conditions of low O\textsubscript{2} tension (34). Moreover, the higher O\textsubscript{2} affinities of the parasitic nematode Hbs than of the host Hbs (as in the turtle parasite Camallanus) (657) predicts O\textsubscript{2} transfer from the host's circulation. The ingestion of host oxy-Hb, which occurs in Strongyloids and Toxocara from horse and dog intestines (458), may provide a contributory source of molecular O\textsubscript{2} (630).

b) OCULAR PHOTORECEPTOR Hb. Among three general types of pigmented eye structure found in nematodes (70), adult females of the soil-dwelling nematode Mermis nigrescens have red anterior “chromotropes,” whose dense pigment is not a photopigment but an oxyHb, with high O\textsubscript{2} affinity that functions to shadow a photoreceptor (66, 67, 69, 71, 72). Burr, Moens, and colleagues (68) have determined the protein and gene structures of two related globins; both consist of 146 residues, with 84% identity and both have the distal E7Gln and B10Tyr. Although the two globins are expressed at low levels in the body wall tissue of Mermis, the eye globin accumulates to an extremely high concentration in the eye and epidermal cells of the head (67), similar to the accumulation of crystallins in the vertebrate eye. Mermis eye Hb is unique in forming true crystals and is the only known globin in eye cells.

5. Nemerteans

Hbs occur in the central nervous tissues of Amphiporus lactiflores (603), Lineus lacteus, L. ruber, and L. viridis (605) and in the body wall and neural tissues of Cerebratulus lacteus that lives in subtidal and intertidal habitats (602). With only 109 amino acid residues, the Cerebratulus body wall and neural Hbs are the smallest known globins. The sequence alignment indicates that the A, B, and H helices are about one-half the typical length (602). In addition to the distal E7Gln found in all the protozoan and bacterial Hbs and many invertebrate Hbs, the unusual presence of E11Thr in Cerebratulus could help stabilize the heme complex as suggested by the much decreased rate of hemin loss from the Met form of the ValE11Thr sperm whale Mb mutant (223). This feature could explain the decreased susceptibility to autoxidation relative to the 116-residue Paramecium Hb (264, 588). Cerebratulus body wall and neural Hbs show different affinities (P\textsubscript{50} = 4.1 and 2.9 Torr, respectively, at 15°C) and similar high cooperativities (n\textsubscript{50} = 2.9) that allow unloading of most of their O\textsubscript{2} at tensions close to their P\textsubscript{50} (602). Decreasing cooperativity with increasing dilution and O\textsubscript{2} saturation suggests deoxygenation-linked aggregation to dimers and perhaps tetramers, as seen in several other invertebrate Hbs (see sect. uC1). Similar oxygenation characteristics obtained for the body wall Hb in vivo and in vitro indicate the absence of affinity modulators in the tissues (602).

6. Gastrotrichans

The Hb occurring in cells closely associated with nerve and muscle bodies of the small (50 × 600 μm) Neodasys from intertidal beaches exhibits a moderately high affinity (P\textsubscript{50} between 0.4 and 1.5 Torr)(323) that appears to be tailored to provide an accessible O\textsubscript{2} store (see sect. uC4) under intermittent muscle and nerve activities (96).

7. Annelids

In contrast to human isoMbs, which exhibit similar oxygenation properties (461), the body wall Mb of the polychaete Arenicola marina consists of two major monomeric electrophoretic components, MBi and MBii, that exhibit markedly different O\textsubscript{2} affinities (329, 644) (Table 2, tissue Hbs/Mbs, and Fig. 3). This suggests that Mb-mediated transfer of O\textsubscript{2} from the vascular Hb to the mitochondrial combustion sites can occur over a broader range of O\textsubscript{2} tensions than with a single component (644). At least two additional minor electrophoretic components are also present (Weber, unpublished results). Primary struc-
tture analyses (301) reveal that MbI consists of two isoforms (Ia and Ib) and MbII of at least three major isoforms (IIa, IIb, and IIc), each having 145 residues. It furthermore appears that a single substitution (C6Asn→Asp) underlies the charge difference responsible for the electrophoretic separation of MbI and MbII and that the lower O2 affinity of MbII correlates with the E68Ser→Pro substitution that changes the surroundings of the E helix in the vicinity of the distal E7His residue in MbIb and the three MbII components (301).

An in vivo functional differentiation between *Arenicola* isomBs is witnessed by distinct, paramagnetically shifted $^1$H-NMR signals (that reflect the cellular oxygenation state of the muscle tissue) (330). The $^1$H-NMR signals indicate a critical intracellular P O$_2$ value (below which cellular O$_2$ consumption decreases significantly) of <1 Torr in *Arenicola* tissue and a much greater extracellular/intracellular P O$_2$ gradient than in mammals [~140:1 and 15:1, respectively (331)]. The high affinities of *Arenicola* isomBs I and II (0.2 and 0.6 Torr, respectively, at 15°C, and 0.31 and 0.69 Torr at 20°C) (330, 630, and Weber, unpublished results) indicate extremely high O$_2$ affinities in its cytochrome oxidase. In mammals, the affinity of isolated mitochondrial cytochrome oxidase far exceeds that of the Mb (P$_{50}$ 0.5 Torr at 20°C) is found in *Arenicola marina* body wall Mbs (above) and *Glycera* (557).

Interestingly, a ~30-kDa dimeric Mb exhibiting marked cooperativity ($n = 2.0, P_{50} = 0.4$ Torr at 20°C) is found in the ophelid polychaete *Travisia foetida* (553).

The nerve Mb from the polychaete annelid *Aphrodite aculeata* exists as a homodimer in solution and has fast O$_2$ association and dissociation rates and a ~2.5-fold lower O$_2$ affinity than vertebrate Mb (Table 3) (135a, 660). The Mb has 150 residues, shows a 31–32% identity with the intracellular polypeptide Hbs from the polychaete annelid *Glycera*, and has a normal rate of autoxidation (135a). Because of its abundance, it is likely to function as an O$_2$ store, similar to the gastrotrich *Neodasys* (96).

8. *Molluscs*

* A) *MUSCLE MBS. I) Amphineurans.* With the exception of *Liolophura japonica* Mb that consists of three 17 kDa monomers (517), the radular Mbs of the primitive Amphineuran molluscs consist of both monomers and disulfide-bonded dimers (63, 445, 504, 558). The disulfide-linked dimeric Mbs of *Amaurochiton glaucus* and *Sipharochiton pelliserpentis* show distinct cooperativity ($n ~1.4$) (63, 504) (Table 2, tissue Hbs/Mbs).

II) *Gastropods.* Mbs have been isolated from some 37 species of Gastropods whose radular Mb concentrations may be two- to threefold that in human cardiac and skeletal muscles (445, 520). Gastropod Mbs exist as monomers (e.g., *Aplysia* and *Dolabella* Mbs) (515, 660) or cooperative dimers [e.g., *Buccinum undulatum* (554) and *Nassa mutabilis* (99, 181)]. The distal E7 His is conserved in *Neptuna, Busycon, Nassa,* and *Cerithidea* Mbs but is replaced by valine in *Aplysia, Dolabella,* and *Bursatella* Mbs (372, 535).

*Aplysia* has hemocyanin as an extracellular O$_2$ binding protein (183) in addition to the Mb that occurs in the radular muscle and the triturating stomach at concentrations comparable to the Mb in mammalian muscle. The presence of a distal Val residue has been confirmed by its crystal structure (51). Shikama and Matsuoka (489) have investigated the spectroscopic properties of a number of Mbs finding that the extinction characteristics of the Soret peak provide a useful criterion for predicting substitution of the distal His residues.

Homodimeric Mbs in the muscle cells of the several gastropod sea snails of the prosobranchia subclass have been characterized. Although the Mbs of *Busycon canaliculatum* and *Cerithidea rhizophorum* do not exhibit cooperativity in their O$_2$ binding (478, 535), *Nassa mutabilis* Mb has a $P_{50}$ of 5 Torr and a $n_{50}$ of 1.5 (181). Curiously, the *Nassa* Mb sequence has a high percent identity to the other two, 63% with *Busycon* and 46% with *Cerithidea* Mb (421). Recent investigations of the mechanism of O$_2$ binding by Coletta et al. (99) have shown it to be quite different from that of *Scapharea homodimeric* Mb; its kinetic behavior is different, and the control of cooperativity appears to be exerted predominantly through the ligand-linked variation of the ligand association rates.

In contrast to other molluscan Mbs that lack allosteric modulation (e.g., by hydrogen ions, CO$_2$, etc.) (558), NaCl increases the O$_2$ affinity of *Buccinum undulatum* Mb (555). The biological significance of a higher O$_2$ affinity in polyhaline environments (if any) is not clear.

Although molluscan Mbs exhibit some electrophoretic heterogeneity, two dimers in the gastropod *Nassa mutabilis* (181), and two monomers in the clam *Mercenaria mercenaria* (315), functional differentiation between the isomBs has not been established.

B) Evolution of MB function from indoleamine dioxygenase. In 1989, Suzuki and co-workers (525, 531) found a 41-kDa Mb in the buccal mass of the gastropod *Sulculus diversicolor* (abalone) whose sequence had no significant similarity to Hbs and Mbs but showed 35% identity to the vertebrate heme-containing, tryptophan-degrading enzyme, indoleamine dioxygenase (IDO). The distribution of the IDO-like Mb was found to be surprisingly wide; it occurs in the gastropod molluscs *Nordotis madaka* (516), *Omphalinus* (291), *Turbo* (521), and *Chlorostoma* (520). Although IDO forms an unstable oxygenated reaction intermediate and cannot serve as an O$_2$ carrier, *Sulculus* Mb binds O$_2$ reversibly, with a lower affinity than vertebrate...
Mbs (P_{50} = 3.8 Torr at 20°C) and no cooperativity or Bohr effect. It also does not have any IDO enzymatic activity. Thus it appears that the IDO-like Mbs represent a case of functional convergence (520, 531). The recent finding of NO dioxygenase activity of yeast and bacterial FHbs (see sect. III A1) emphasizes the close link between Hbs and dioxygenases.

c) BIVALVE GILL HBS. Cytoplasmic Hbs occur in the gills of symbiont-harboring clams from the families Solemyidae, Lucinidae, and Vescimyidae, in the mussel Bathymodiolus (Mytilidae), and in the deep-sea gastropod Alviconcha (667). These Hbs bind O_2 and sulfide, are an almost ubiquitous feature of the symbiosis between mussels and chemosautrophic bacteria, and may play a role similar to the Hbs of nitrogen-fixing plants (666). Their reactions with sulfide are discussed in section vB.

In the Puerto Rican clam Lucina pectinata where individual isoHbs appear specialized to supply either O_2 or sulfide for metabolism (see sect. vB), Hbs I, II, and III bind O_2 and sulfide cooperatively and with similar, very high affinities (P_{50} ~ 0.1–0.2 Torr at 20°C) (324) (Table 2, tissue Hbs/Mbs). Whereas HbI invariably appears to be monomeric, concentrated equimolar mixture of Hbs II and III associate, indicating the existence of noninteracting (HbII)_2 (HbIII)_2 tetramers in the tissues (324). In the clam Myrtea spinifera, a homodimeric Hb and a fraction containing three Hb subunits isolated from symbiont-harboring gills both bind O_2 with pronounced cooperativity (n = 2), whereas sulfide appears to bind to a non-Hb protein present in the gills (113). The Hb of the clam Solemya reidi comprises three components with high O_2 affinities (P_{50} = 0.3–0.5 Torr at pH 7.5 and 20°C) and similar O_2 dissociation rates (~10 s^{-1} at 10°C) (321). While slightly increasing the O_2 dissociation rate of Hbl, sulfide drastically decreases the dissociation rates of HbII and HbIII, suggesting that sulfide itself or a rapidly formed oxidation product may be a factor controlling O_2 delivery (321).

d) NERVE HBS. The maintenance of ion gradients in nervous tissue requires aerobic metabolism and readily available of O_2. The role of Hb found in the nervous systems appears to be well documented in a few bivalve molluscs (144). In Tellina alternata, nerve action potential conduction ceases when the Hb is deoxygenated under anoxia (319), and in Spisula solidissima and Tellina alternata, the O_2 consumption rates of Hb-rich cerebro-visceral connectives closely match the rates of O_2 unloading from Hb in situ (320). The Hb in nervous tissues appears to be situated largely in glial cells (and is absent in the neurons in the bivalve Tellina alternata Hb), thus providing a possible O_2 store for use by the neurons (144). For homodimeric Hbs of Tellina alternata and Spisula solidissima, in situ measurements on the cerebrovisceral connectives indicate P_{50} values of 1.3 and 2.3 Torr, respectively. These data indicate marked cooperativities (n = 3.7 and 2.1), which appear to ensure constancy in the rates of O_2 unloading from the neural Hbs during hypoxic conditions (144, 319).

Among gastropods, nerve Hbs occur only in glial cells in Limnaea stagnalis and Planorbis corneus and only in the neurons in Helix pomatia and Cepaea nemoralis (475). Aplysia neural Hb occurs particle bound at high concentration and has been suggested to have a photoreceptive function (30). O_2 storage in glial cells has been reviewed by Wittenberg (664).

9. Arthropods

Hbs occur sporadically in insect tissues, including specialized “tracheal” (fat body) cells, the parietal muscular and hypodermis of larval horse botfly Gasterophilus intestinalis (294), and tracheal/fat-body cells of backswimming insects like Buenoa confusa (40), Anisops pellucens, and A. assimilis (385, 652). Globin synthesis in Buenoa occurs in fat-body cells (40), as in the case of the extracellular chironomid Hbs (584).

The dimeric (34 kDa) Gasterophilus Hb occurs in at least two isoforms (2, 135). The Hbs from Buenoa and Anisops appear to consist of three fractions and exhibit an oxygenation-linked dissociation: the deoxy form occurs in a complex association-dissociation equilibrium ranging from ~17-kDa monomers to ~112-kDa hexamers, while the oxyHb is predominantly monomeric (40, 652).

In contrast to the moderately high O_2 affinity of Gasterophilus Hb (P_{50} = 4.9 Torr, n = 1.0) (294), Anisops assimilis Hb has a low affinity (P_{50} ~ 40 Torr) and exhibits its heterophasic cooperativity. It has very high cooperativity (n_{50} ~ 5.2) (Table 2, tissue Hbs/Mbs) at concentrations (~20 mM heme) resembling those in the living cells (652) and strongly increased O_2 affinity and reduced cooperativity at low Hb concentration, indicating ligand-linked dissociation. In life, Anisops Hb appears to release O_2 into the tracheal system during dives, thus lowering the depletion rate of the gaseous store used for maintaining neutral or positive buoyancy. Accordingly, mean dive durations in Anisops fall drastically in the presence of CO (385, 386, 652).

In addition to extracellular Hbs found in hemolymph of their larval stages (discussed in sect. mCl), the ovaries and eggs of chironomid insects have three and four Hbs, respectively, that appear to be cytochemically similar to the hemolymph Hbs. Together with the decrease in egg Hbs during development, this indicates that hemolymph Hbs taken up and stored in the developing oocytes may provide a possible O_2 store for use by the developing embryos (584).

The hemeproteins occurring in the saliva of the blood-sucking hemipteran beetle Rhodnius prolirxus and the bedbug Cimex lectularius and perhaps also those observed in eggs of the louse Pediculus humanus, which had been thought to be Hbs (418), are vasodilatory nitrophorins that act as a store and transporter of NO (622).

A very unexpected recent development was the iden-
tification by Hankeln and his collaborators of a 153-amino acid globin in the genome of the dipteran *Drosophila melanogaster* (65a). The sequence shows identities of 39% with *Gastrophilus* Hb (135) and 29% with *Chironomus* HbVI (135, 204).

10. Cephalochordates

Within the phylum Chordata, members of the subphyla Urochordata and Cephalochordata share a common ancestry with the members of the third subphylum Vertebrata (591); unlike the latter, however, they appeared to have no Hb. However, a ~38-kDa cytoplasmic Hb was recently isolated from the notochord cells of the amphioxus *Branchiostoma* (42). Its high O₂ affinity (P₅₀ = 0.27 Torr) and absence of cooperativity (42) indicate a possible role in facilitating diffusion of O₂ into the notochord cells (668).

B. RBC Hbs

1. General features

Nucleated RBCs circulating in body fluids are encountered in six invertebrate phyla: Phoronida (apparently in all species), Annelida (in 5 polychaete families), Nemertina, Echiura, Mollusca (in 2 classes, Bivalvia and the primitive Solenogastres), and Echinodermata (in 1 of the 3 orders of the Ophiuroidea and 2 of the 6 orders of Holothuroidea) (355). Implicit scenarios are that RBCs either evolved once before differentiation into phyla or that they arose separately on several different occasions.

In striking contrast to vertebrates, the invertebrate RBCs are nucleated and mostly extravascular, commonly occurring in coelomic fluid. Exceptions are the phoronids where the RBCs circulate in a closed vascular system (180) and nemerteans where they are found in at least partially closed circulations equipped with pulsatile vessels and valves to direct flow (95, 355, 377). The extravascular location of invertebrate RBCs may be due to their higher viscosity relative to an equimolar extracellular Hb solution, which would be disadvantageous in a closed cardiovascular system with a relatively weak heart (351, 356, 505). Interestingly, RBCs are totally lacking in arthropods.

Although the invertebrate RBC Hbs generally having low molecular masses (~17 kDa), some exhibit moderate aggregation. Their O₂ binding affinities are moderately high but lower than those of the cytoplasmic Hbs; they exhibit low cooperativities (in accordance with the small number of interacting hemes per molecule), small Bohr effects, and insignificant sensitivity to allosteric effectors like NaCl and organic phosphates (356).

2. Phoronids

The phoronids live in intertidal and subtidal sediments and have RBCs, which circulate in the blood vascular fluids, in contrast to other invertebrates. The vessels penetrate contractile tentacles that function as hearts and are bathed by RBC-free coelomic fluid, indicating a blood-to-coelomic fluid O₂ transfer (149). The two species, *Phoronopsis viridis* (180) and *Phoronis architecta* (600), that have been investigated each have four unique globin chains, two of which are monomeric and two associate at least to dimers. The Hb also functions as an O₂ store [lasting ~15 min (600)]. *Phoronis architecta* Hb shows higher in cellulo than in vitro O₂ affinity (P₅₀ = 1.3 and 0.7 Torr at 20°C, respectively) and substantial cooperativity (n = 2.7 and 2.8, respectively) (600).

3. Annelids

A) Functional properties. Among annelids, RBCs appear to be restricted to the polychaetes where they are encountered in the families Capitellidae, Glyceridae, Ophelidae, Terebellidae, and Scalibregmidae. In some polychaetes like the glycerids, coelomic RBCs circulate through gills without significant mixing of oxygenated and deoxygenated cells, and through the coelome by body wall contractions that also ventilate the burrow (353, 354). In others, like the terebellids *Amphitrite ornata* and *Pista pacific*, RBC-rich coelomic fluid bathes branchial efferent blood vessels that carry extracellular Hbs (351, 362), allowing for intersite (blood to coelome) O₂ transfer (547, 642).

Annelid RBC Hbs may be monomeric (terebellids), dimeric (capitellids and ophelids), tetrameric (*Glycera robusta* and Scalibregmidae), or occur in combinations of different aggregational states (354, 630).

The organic phosphates ATP and 2,3-diphosphoglycerate (DPG) that depress Hb O₂ affinity in vertebrate RBCs do not exert physiologically significant allosteric effects on annelid RBC Hbs (cf. Refs. 224, 354, 362, 551, 635, 642, 645). Minor effects observed in the predominantly tetrameric *Glycera gigantea* Hb (P₅₀ changes <1–2 Torr) (628) may represent nonspecific electrostatic interactions, similar to those observed with NaCl and invertebrate Mbs (555).

The monomeric Hbs of the terebellids *Enoplobranchus sanguineus* and *Amphitrite ornata*, which live side by side in O₂-depleted muds, differ in their O₂ affinities, P₅₀ = 1.4 and 2.8 Torr, respectively (642). The higher affinity of the former species, which lacks a vascular system and has short unbranched parapodial gills, correlates with lesser anatomical differentiation compared with the latter species, which has highly developed gills and vascular Hb (362). Both the RBC Hb and the Mb from the ophelid *Travisia foetida* are dimeric and exhibit pronounced cooperativity (n = 1.8–2.0) in their O₂ binding (555). Compared with the small variation in RBC O₂ affinities observed with other annelids (<4 Torr), the closely related capitellid genera *Capitella* and *Capitomastus* show large affinity differences (9 Torr) that may...
be related to differences in thermal environmental conditions (355).

b) EXCEPTIONAL HETEROGENEITY OF GLYCERA HB STRUCTURES. Although pronounced Hb heterogeneity and polymorphism is common among nonvertebrate Hbs, it has been documented most extensively in two cases: the RBC Hbs of the polychaete G. dibranchiata and the extracellular Hbs from Chironomus thummi larva.

In addition to heterogeneity, glycerid Hbs show a striking variation in quaternary structure. Although the Hb of Glycera robusta is tetrameric (552), those of G. gigantea, G. rouxi, and G. americana have a major tetrameric fraction and a smaller dimeric or monomeric fraction (635, 638, 645). G. dibranchiata RBCs contain a monomeric as well as polydisperse polymeric Hbs (236, 357, 612, 645), both forms consisting of 17-kDa chains (612). The monomeric Hb has a slightly higher O2 binding affinity than the polymeric Hb (P50 = 4–6 vs. 9–11 Torr) and lacks cooperativity, whereas the polymeric Hb shows small cooperativity that appears to be due to reversible oxygenation-linked alterations in aggregation (56, 391). The two Hbs are synthesized in comparable amounts (483) and were found to consist each of at least a dozen components (101, 285, 684, 685).

The sequences of G. dibranchiata Hbs have shown that while the polymer Hbs have the normal distal E7HIs (684, 685), the monomeric Hbs have a distal Leu (6, 261, 566) whose presence has been verified by high-resolution crystal structure studies of a monomeric Hb (23, 60). The crystal structure shows the typical globin “fold” with no D helix and the heme inserted in the reverse orientation compared with vertebrate hemes, as was observed in Chironomus Hb (511). A recent electrospray ionization mass spectrometric (ESI-MS) study of the Glycera pooled Hbs has shown at least 18 peaks attributable to monomer Hbs (14,500–15,200 Da) and an approximately equal number of polymer Hbs (15,500–16,400 Da) (212). Blood from individual worms had generally fewer than six monomer and six polymer components; in a couple of cases there was a complete absence of polymeric Hb. Taking into account possible fragmentations of the known globin sequences, a conservative estimate of the number of different monomeric and polymeric Hbs is about 10 each.

4. Nemerteans

The properties of RBC Hbs found in (partially) closed circulatory systems of at least four genera of nemerteans (355, 606) appear not to have been studied.

5. Echiuroids

The echiuroids are phylogenetically close to annelids (376). Although they have a closed circulatory system, there are no respiratory proteins in the blood, and Hb is found in coelomic cells, coelomic epithelium, body wall muscles, and nerve cord (546). The inter- and subtidal “fat innkeeper worm” Urechis caupo has abundant Hb-containing cells in the coelomic fluid, which has 1.6–5.0 g Hb/100 ml and an oxygen-carrying capacity of 27–72 ml/l (446). The Hb-containing cells function to transport O2 to the Mb in the muscles used in burrow ventilations, since the Mb has a higher O2 affinity than the Hb (439, 446). The Hb is tetrameric and comprises one major fraction (F-I), which consists of identical chains and two minor ones (F-II and F-III), both of which are heterogeneous, with at least five components (178). The homotetrameric Hb has a moderate affinity (P50 = 12 Torr at 20°C), a very small Bohr effect, and is noncooperative (178). Its crystal structure showed the molecules to have an “inside-out” quaternary structure with the G and H helices at the surface facing the solvent, and tightly bound water molecules help mediate intersubunit interactions (312, 313). Although this structure is reminiscent of the structure found for the homodimeric HbI of the clam Scapharca inaequilabi (see sect. A6), the absence of cooperativity may result from lack of contacts among the F helices, which together with E-helical contacts mediate cooperativity between the closely packed hemes in S. inaequalbi Hb (463, 468).

The only other echiuroid Hb investigated is that of Thalassema (Lissomyema) mellita, which inhabits the tests of dead sand dollars, Mellita pentapara. It has three coelomic cell Hbs consisting of three globin chains; the two major components are a homo- and a heterodimer, and the third is monomeric, sharing a globin chain with the heterodimer (619). All three isolated Hbs have a higher affinity (P50 = 1–2 Torr) and lower cooperativity (n50 = 1.0–1.3) than the coelomic cell suspension and the lysates (P50 = 2.5–3.0 Torr and n50 = 1.5–1.9) (619) (Table 2, RBC globins).

6. Molluscs

a) GENERAL MOLECULAR PROPERTIES. Arcid bivalve RBCs commonly have dimeric Hbs. Noetia ponderosa has a major heterodimer and a minor homodimer (165, 472). Anadara and Scapharca have dimeric and tetrameric Hbs (58, 85, 143, 409, 463). In Anadara broughtoni, A. ovalis, and Scapharca inaequilabi, HbI is homodimeric (γ2) and HbII is tetrameric consisting of two different subunits (αβ), which is rare among invertebrates (463). The tetrameric HbII of A. broughtoni polymerizes (mainly to dodecamers) upon deoxygenation (169–171). The deep-sea, thermal vent clam species Calyptogena, the only heterodont clam with circulating RBCs, also exhibits congeneric variation in its Hbs; although C. magnifica has a tetrameric Hb (560) that may exist in three forms, viz., αγ, αβγ, and (γ2) (691), C. soyoae has two homodimeric Hbs (527).

The arcid clam Barbatia reeveana is unique in having a ~430-kDa polymeric Hb in addition to a tetrameric one (214). Furthermore, the former is a dodecamer of two-
domain, ~35-kDa subunits, and its state of aggregation is not affected by ligand binding (215). It is the largest known intracellular Hb. The two domains show ~80% identity; the corresponding globin gene has two novel introns, a “precoding” intron and a “bridge” intron that separates the two domains (397). Suzuki et al. (522) have determined the sequences of the Hbs from several other Barbatia species. B. virescens has a heterodimeric Hb in addition to the polymer, while two subspecies of B. lima differ in their Hb contents: one has only a αβ₄-tetramer, and the other has the tetramer, a homodimer (δδ), and a different type of polymeric ~290-kDa Hb comprising both two-domain subunits as well as the γ-chain (522). The δ-chain has 71–74% identity to the two domains and appears to be their ancestral single-domain globin. Although the O₂ affinity and cooperativity of the B. reeveana tetramer are higher than that of the polymeric Hb (P₅₀ = 19 Torr and n₅₀ = 2.2 at 20°C compared with 33 Torr and 1.8, respectively), both Hbs exhibit no Bohr effect over the pH range 6.8–7.0 (215). b) O₂ BINDING. Bivalve RBC Hbs generally have lower O₂ affinities (P₅₀ = 8–17 Torr at 20°C) (558) than stripped vertebrate Hbs. The affinities of dimeric isoHbs exceed those of the tetrameric ones (Table 2, RBC globins); both dimers and tetramers exhibit cooperativity (n₅₀ = 1.5–1.8 and 1.6–3.0, respectively) and small or no Bohr effects (463, 558). Despite the absence of Bohr effects in A. broughtonii Hbs I and II, their circular dichroism spectra and the reactions with ligands indicate the occurrence of an R→T transformation upon O₂ binding. Thus the absence of Bohr effects may be related to a lack of proton ionizing groups (169–171). A. broughtonii and S. inequivalvis Hb tetramers polymerize to form larger complexes on deoxygenation; in Scapharca inequivalvis, the polymerization is anion linked and decreases O₂ affinity (48, 85, 171). Unpublished findings (R. E. Weber and J. S. Djangmah, unpublished data) fail to confirm an earlier report of reverse Bohr effects in A. senilis Hbs (143).

In Noetia ponderosa, the major, heterodimeric and minor, homodimeric Hbs that have different affinities (P₅₀ = 16.8 and 8.7 Torr, respectively, at pH 7 and 25°C) (Table 2, RBC globins) do not form larger complexes upon deoxygenation (472). The polymeric and tetrameric Hbs of Barbatia reeveana have remarkably low affinities (P₅₀ = 33 and 19 Torr, respectively, at 20°C) with substantial cooperativities (n₅₀ = 1.8 and 2.2, respectively) and no Bohr effects (214, 215). Because bivalve Hbs have lower affinities than Mbs in juxtaposed tissues (see below), O₂ transfer from RBC to Mb may occur.

Data in the literature indicate widespread structural and functional polymorphism in bivalve RBC Hbs. Whereas Hb from Noetia ponderosa found in Virginia (United States of America) is an electrophoretically single dimer, the Hbs from Noetia found in the Gulf of Mexico exist as homo- as well as heterodimers and exhibit lower O₂ affinities (P₅₀ = ~5 and ~7.5 Torr, respectively) (358). Again, although the tetrameric component of Anadara trapezia Hb is invariant, the dimeric Hb occurs as one of two homozygotes or as a heterozygote that shows a geographical cline from low to high frequency over ~1,000 km of the southeastern Australian coastline (408a).

A low temperature dependence of O₂ affinity, that will safeguard O₂ loading at high temperatures, has been reported in several bivalve RBC Hbs. Compared with Mbs (ΔH = −55 to ~−68 kJ/mol) (12), the molluscan Hbs exhibit low overall heats of oxygenation; ΔH is ~−9 kJ/mol in Noetia ponderosa (165) and ~12 and ~23 kJ/mol in the polymeric and tetrameric Hbs, respectively, of Barbatia reeveana (215). Inverse temperature effects (O₂ affinities increasing with rising temperature) reported to occur at temperatures above 20°C in three species of Anadara (98) were not confirmed by measurements with hemolysates of Anadara senilis that show a linear van’t Hoff plots (ΔH = −35 kJ/mol between 5 and 30°C) (R. E. Weber and J. S. Djangmah, unpublished data). Viewed in conjunction with the insensitivities of these Hbs to protons and other effectors, which reduce the temperature effect through endothermic dissociation of effectors upon oxygenation, the low temperature effects suggest lower intrinsic heats of oxygenation in bivalve Hbs than in other Hbs and Mbs.

No information appears to be available on functional and allosteric properties of Hbs from solenogaster molluscs.

c) NOVEL MECHANISM OF COOPERATIVITY IN SCAPHARCA HOMODIMERIC HB. The homodimeric HbI and heterotetrameric HbII of the bivalve lamellibranch Scapharca inequivalvis have different functional properties. HbI has a constant O₂ affinity (P₅₀ = 7.8 Torr) over the pH range 5.5–9.5 and a cooperativity (n₅₀ = 1.5) that is high for a dimer. HbII has a lower affinity (P₅₀ = 9.1 Torr), which increases at low pH due to an acid Bohr effect, where protons are exposed in mammalian Hbs) face each other in close proximity. Although large alterations in tertiary structure attend ligand binding, the changes in quaternary structure
are small. The $O_2$ affinity of each subunit appears to be determined by the position of the Phe97 side chain, which is located within the proximal moiety of the heme cavity. Its expulsion into the interface between the two subunits upon ligand binding leads to alterations in the interactions between the two hemes and the residues in the heme cavity providing the functional linkage for direct heme-heme communication (86, 462). The high-resolution crystal structures of CO HbI and its deoxy form determined by Royer et al. (464, 469) showed that the movement of the two Phe residues in turn displaces 6 of the 17 well-ordered water molecules forming a cluster in the interface between the two subunits of the homodimer. Interestingly, like human Hb, the deoxyHbI is more tightly associated than the liganded state, and subunit dissociation leads to increase in $O_2$ affinity (465). The small alterations in the homodimer structure upon ligand binding are consonant with the retention of the cooperativity of $O_2$ binding in the crystal (385). Chiancone and co-workers (47, 118) have studied in great detail the structural and functional properties of the various forms of Scapharca HbI. Mutation of the distal His to Val increases ligand affinity and abolishes cooperativity (217). Likewise, mutation of Thr72, whose hydroxyl group forms a hydrogen bond to the intersubunit water cluster, results in 40-fold reduction in $O_2$ affinity and substantial loss of cooperativity (420).

7. Echinoderms

Hb occurs in Ophiuroidea and Holothuroidea. In the holothurians (sea cucumbers), RBCs may be found in one, two, or each of the fluid compartments, the coelomic, water vascular, and hemal systems, of which only the last-mentioned exhibits clearly unidirectional flow, and appear to pass freely between these systems (356). Very little is known about Ophiurid Hbs. Hemipholis elongata Hb consists of a major monomeric and a minor dimeric form and is almost fully oxygenated at 9 Torr (219).

Holothurian Hbs from Cucumaria miniata and Caudina (Molpadia) arenicola are dimeric in oxygenated state and aggregate to predominantly tetrameric forms upon deoxygenation (54, 56, 548). The four major globin chains A-D of Caudina arenicola RBC Hb assemble into seven different homo- and heterodimeric forms (374). The single chains exhibit slight but measurable cooperativity and high affinity ($P_{50}$ = 2–3 Torr). Affinity falls and cooperativity increases markedly when the D chain is added to any of the other three chains (300).

The atomic structures of the cyan-Met derivative of the dimeric HbD of Caudina arenicola (390) resemble the structure of Scapharca inequivalvis homodimeric HbI (466) despite only 22% amino acid identity. Although this suggests a mechanism for cooperativity similar to that in Scapharca HbI, the intersubunit contact regions in Caudina Hb lack the key residues implicated in the Scapharca mechanism (300).

C. Extracellular Hbs

These Hbs are invariably synthesized intracellularly and are then secreted; they occur in solution in the hemolymph of six “protosome” invertebrate phyla (Annelida, Vestimentifera, Pogonophora, Nematoda, Arthropoda, and Mollusca) (541, 578). Because they lack the cellular microenvironment where effector levels may be regulated as in vertebrates, their operating conditions are more dependent on the vagaries of ambient conditions than the Hbs enclosed in ionoresorbing cells.

The extracellular occurrence of Hbs is predicated on their large molecular size that prevents excretory loss through membranes. An exception is the Hb from chironomid (insect) larvae that consists of mono- and dimers. We postulate that these small structures may be permitted by the possession of Malpighian tubules as excretory organs in insects, instead of filtration-reabsorption type nephridia/kidneys found in other invertebrates that would result in loss of freely dissolved globins. Chironomid Malpighian tubules moreover appear to be specialized for the breakdown of Hb and release of the catabolic products (268). The correlation between extracellular location and absence of filtration-type excretory organs appears to extend to the giant, ~3,600 kDa extracellular, HBL Hbs of annelids. These Hbs are intravascular, with exception of nephthyd polychaetes where Hb also occurs freely dissolved in the coelomic fluid and the excretory organs are pronephridia rather than nephridial coelomoducts (626, 629). No information appears to be available on the excretory organs of the deep-sea, hydrothermal vent polynoid polychaete Branchipolynoe, which contains ~124- and ~153-kDa Hbs freely dissolved in the coelomic fluid (243).

1. Chironomids

A) EXCEPTIONAL HETEROGENEITY OF HBS. The Chironomidae represent one of the largest insect families with $\geq$10,000 species (37). They exhibit stage-specific and tissue-specific single-chain globin synthesis throughout the four larval and the pupal stages. The Hbs are synthesized in the larval fat body and are then secreted into the hemolymph (41, 471). The Hbs have been found to be potent human allergens (37).

Chironomid Hbs appear to fulfill clear physiological roles of transporting and storing $O_2$ in the larvae that burrow in polluted and hypoxic muds (418, 623, 624) and to be important in the absorption of $O_2$ from ambient water at partial $O_2$ pressures ranging from 10 to 50 Torr (see Fig. 2 and sects. iA and iC4). Their Hbs allow the larvae to maintain aerobic metabolism under hypoxic conditions (693). A possible but undefined role has been
proposed for them in the metabolism of xenobiotics in the frequently polluted, hypoxic environments in which Chironomus flourish (418).

Braunitzer and co-workers (204) isolated, identified, and sequenced 12 different Hbs from the hemolymph of Chironomus thummi thummi (CTT) in the 1970s; five were found to exist as monomers, six as homodimers, and one in a monomer-dimer equilibrium (204). The monomer Hb CTT-III was the first invertebrate Hb whose high-resolution crystal structure was determined (247, 511). In the crystal structure the heme group is rotated by 180°, and the heme cavity in the deoxy form has an unusual open gate conformation with the distal HisE7 able to swing out of the cavity (511). This Hb has been the subject of numerous thermodynamic, kinetic, and spectroscopic studies (138, 470).

A recent ESI-MS study of the Hb from the fourth instar larvae demonstrated the presence of more than 20 components ranging in mass from 14,417.3 to 17,356.5 Da (209a). Studies of globin gene sequences in several Chironomus species carried out in the laboratories of Schmidt and Bergtrom have indicated that their number is likely to be >40 (216, 220, 221, 287, 288). All but one of the 15 major components observed by ESI-MS could be assigned to known genomic sequences; it appears therefore that not all of the globin genes are expressed in the fourth instar larvae of Chironomus.

The Hb from the Japanese midge Tokunogayusurika akamussii consists of at least 11 components (168), which fall into two approximately equal groups, one having a distal His (N type) and the other a distal Leu (L type). The former has a higher percent of identity (40–48%) with the Chironomus Hb sequences than the latter (26–27%), since all the Chironomus Hbs have a distal His. The L-type Hb has an O₂ affinity comparable to mammalian Mbs and exhibits a Bohr effect. Spectroscopic studies indicate this Hb to have a unique structure in the distal heme cavity (5, 317).

The pronounced heterogeneity characterizing chironomid Hbs may be adaptive to exogenous and endogenous factors. In CTT, the relative contribution of dimeric Hbs is markedly (~70%) higher in summer larvae than in the spring larvae (344), and in C. tentans, the isoHbs patterns reveal selective expression of individual globins during larval development (568). Although C. thummi Hbs are monomeric and dimeric, C. tentans Hbs are exclusively monomeric and show no evidence of subunit aggregation (567). C. plumosus Hb is predominantly dimeric (532), and C. strenzkei Hb is tetrameric (495). Comparison of amino acid sequences and antibody cross reactions indicate that the mono- and dimeric Hbs originated from a common ancestor that diverged near the base of chironomid evolution and that present-day insect taxa lacking Hb might contain unexpressed globin pseudogenes (204, 572). Whereas C. pallidivittatus and C. tentans express at least 8 and 10 electrophoretic compo-

nents, respectively, hybrids of these species inherit Hb patterns of both parents (571). Bergtrom and collaborators (288) have proposed that an increased number of globin genes has been positively selected as a mechanism to achieve the high Hb concentration presumed to be desirable for survival of the larvae.

b) O₂-binding properties. C. thummi isoHbs bind O₂ noncooperatively with high affinity (P₀₅₀ at 20°C, pH 7 = 0.4–1.3 Torr for monomeric Hbs I, III, and IV and 0.3–0.7 Torr for dimeric Hbs II, VI, VIIb, IX, and X), with varying pH sensitivity (φ = 0 to −0.37 for the monomeric Hbs and −0.50 to −0.94 for the dimeric Hbs) and marked temperature sensitivities (ΔH = −40 to −80 kJ/mol) whose pH dependencies do not correlate uniformly with the magnitudes of (endothermic) Bohr effects (637).

The occurrence of a Bohr effect in a monomeric Hb is unusual. The Bohr effect expressed in monomeric Chironomus Hb may be controlled by a single O₂-linked proton dissociating group (470, 495) that has been assigned by NMR to be the HisG2. In C. thummi Hb III and IV at low pH, this residue forms a salt bridge with the COOH-terminal carboxyl group of MetH22 that stabilizes the low ligand affinity state (470). In HbIII, where the heme can assume two distinct orientations, heme orientation transitions occur in the same region as the conformational changes responsible for the Bohr effect (138, 338), and the Bohr effect is solely attributable to the pH dependence of O₂ dissociation rates (182).

2. Two-domain nematode Hbs

The pseudocoelomic (perivascular) Hb of Ascaris is a very abundant protein; it is developmentally regulated upon entry into the intestines of its host the pig and is more abundant in the larger females (44). The Hb is a ~350-kDa octamer of two-domain globin chains. The sequences of the pseudocoelomic Hbs from Ascaris (125, 487) and the closely related Pseudoterranova decipiens (142) have been determined. Substantial evidence supports a model of the quaternary structure of Ascaris Hb to consist of two layers, each of four two-domain subunits stacked in an eclipsed orientation (i.e., with the upper and lower layers directly superimposed) (116, 117) as earlier suggested for Parascaris equorum Hb (587) (Fig. 1). Nematode Hbs appear to be highly antigenic: both Trichostrongylus and Pseudoterranova Hb were found as the result of a search for immunogenic parasite proteins in sheep and gray seal, respectively (142, 166).

The tandem globin domains have a unique COOH-terminal polar zipper region, which was proposed to be responsible for the oligomerization (125, 430). Goldberg and collaborators (388, 389) have confirmed this hypothesis by showing that the expressed COOH-terminal domain but not the NH₂-terminal domain could form an octamer. Furthermore, they established the COOH-terminal tail to be necessary but not sufficient for octamer
formation and that it plays no role in the stabilization of the oligomeric structure; rather, it functions as an intramolecular chaperone promoting the octamer assembly (387).

The pseudocoelomic Ascaris Hb has one of the highest known O2 affinities (P50 = 0.001–0.004 Torr at 20°C; Table 2, extracellular Hbs; Fig. 3). Its affinity for O2 exceeds that for CO (P50 = 1 Torr), resulting in an extremely low M value (0.036) (412) and is ~10,000-fold higher than that of the host Hb (Table 1). Studies of the kinetics of ligand binding of Ascaris, Parascaris, and Pseudoterranova Hbs indicated that the high affinity is due to an extraordinarily low rate of O2 dissociation (100, 190, 191) (Table 3). Furthermore, the O2 affinities of the individual domains were found to be identical to the affinity of the native Hb (304). The crystal structures of individual domains show that the unusual B10Tyr is within the distal heme cavity and that its hydrogen bond to the bound O2, also hydrogen bonded to the distal E7Gln, is probably responsible for the very slow O2 dissociation rate (126, 303, 679). In support of this concept, the mutation LeuB10Tyr abolished the high affinity (303). An engineered triple mutant of sperm whale Mb (LeuB10→Tyr/ HisE7→Gln/ThrE10→Arg) designed to mimic Ascaris Hb did not evince a comparable O2 affinity (694).

Several possible functions have been proposed for the high-affinity pseudocoelomic Hb of Ascaris Hb. It could serve as an O2 scavenger or sink to prevent access of O2 to the fully anaerobic mitochondrial oxidation pathway of Ascaris (44), similar to the role played by LegHbs in the nitrogen-fixing nodules of legumes, as a store of nonglobin, linker chains in an ~2:1 ratio, and represent a summit of complexity for heme proteins binding O2 reversibly (339, 609). In the recently described deep-sea orbiniid polychaete Methanoaricia dendrobranchiata living in association with mussel communities from the Gulf of Mexico cold seeps, ~3,500 kDa HBL Hb occurs together with a 210-kDa Hb (S. Hoursez, R. E. Weber, and C. R. Fisher, unpublished data).

Early electron microscopic studies of annelid extracellular Hbs revealed them to consist of two superimposed layers of six pentagonally shaped subassemblies, ~20 nm high and ~30 nm in diameter, surrounding a central cavity (455, 610). In several cases, material was observed in the central cavity in the marine polychaetes Oenone fulgida (598), Nephtys incisa (381, 651), Euzonus mucronata (565), and Ophelia bicornis (77, 184) as well as in the oligochaete Eophysa tellini (78). Several studies used small-angle X-ray scattering to investigate the molecular shape and mass of HBL Hbs (381, 432a, 433a, 566a, 659b). Krebs et al. (291) have reviewed the results obtained with Hb from the oligochaete Lumbricus terrestris and compared them to those obtained by electron microscopy. More recently, three-dimensional reconstructions at ~3-nm resolution of Lumbricus Hb were obtained by the groups of Van Heel and Lamy (128, 474) (Fig. 4). The structures revealed no protein density in the central cavity and a local threefold axis of symmetry was observed for each of the 12 subassemblies forming the 2 layers, and the latter were found to be rotated by ~16° (128). Extensive additional work by Lamy’s group showed very similar quaternary structures for the Hbs from the leech Macrobdella decora, the vestimentiferan Riftia pachyptila, the deep-sea polychaete Alvinella pompejana, and the Chl from Eudistylia vancouverii (127, 129–131), except for the absence of rotation in A. pompejana Hb (131). This observation was repeated in the latest cryoelectron microscopic studies of several other...
polychaete Hbs, notably that of *Arenicola marina*, suggesting that polychaetes, including those inhabiting the deep-sea hydrothermal vents but excluding the four families which contain Chl, have the HBL structure with absence of rotation, and all the other annelids and vestimentiferans have the rotated HBL structure (J.-C. Taveau and J. Lamy, unpublished data).

HBL Hbs have a sedimentation coefficient of ~60S, an acidic isoelectric point, and unusually low iron and heme contents corresponding to a stoichiometry of 1 mol heme/20–26 kDa (532, 610, 614). The latter property is accounted for by the presence of 16- to 17-kDa heme/heme/20–26 kDa (532, 610, 614). The globin subassembly was determined to be a dodecamer D \([bac]_3[d]_3\), consisting of three copies each of the trimer T \((bac)\) and a monomer M \((d)\), giving a composite \(M_3T_3\) (613). Furthermore, an overall mass of 3.56 ± 0.13 MDa determined by scanning transmission electron microscopy (STEM) mass mapping and sedimentation equilibrium measurements was found to be compatible with either 36 or 42 linker chains forming the central scaffolding complex (370). The dodecamer subassembly is an obligate intermediate in both the dissociation of the HBL Hb structure as well as its reassembly (484). The essential correctness of the bracelet model was demonstrated by the recent three-dimensional reconstruction using cryoelectron microscopy of *Lumbricus* Hb (128, 474). The local 3-fold axis of symmetry present in every 1 of the 12 subassemblies forming the two layers is in agreement with the symmetry found for the dodecamer subassembly crystals (369) and expected on the basis of the \(M_3T_3\) structure of the subassembly.

The presence of a dodecamer subassembly has been demonstrated in *Eudistylia* Chl (441) but not in the Hb of the leech *Macrobdella decora* (290). The latest three-dimensional reconstruction of *Lumbricus* Hb at 2.2-nm resolution by Taveau et al. (538) revealed all 144 globin chains organized into 12 dodecamer subassemblies and 42 linker subunits. At the same time, Green and co-workers (209) determined the masses of the isolated dodecamer subassemblies of *Lumbricus* and *Arenicola* Hbs by ESI-MS to be in excellent agreement with the calculated masses for \(M_3T_3\). Furthermore, Green (personal communication) was also able to observe the dodecamer subassemblies directly by ESI-MS of *Lumbricus* and *Arenicola* Hbs at neutral pH. Finally, the just completed crystal structure of *Lumbricus* Hb at a resolution of 5.5 Å has confirmed all the features of the three-dimensional reconstructions obtained by cryoelectron microscopy (469a). The three-dimensional reconstructions obtained by Lamy’s group imply that all HBL Hbs have the same quaternary structure, at least at low resolution. It is interesting to note that although all the structural results obtained so far point to a symmetric HBL structure, a recent determination of the dipole moments of *Lumbricus* Hb and its dodecamer subassembly found them to be 17,300 and 1,400 Da, respectively (536).

An alternative structure for the *Lumbricus* Hb and its globin subassembly was proposed by Riggs and collaborators. Based on trimer and monomer subunits isolated by dissociation of the Hb at alkaline pH and their reassociation at neutral pH, they have proposed the subassembly to be a hexadecamer of globin chains \([abc]_4[d]_4\) (419, 697, 698). According to their view, the native Hb thus consists of 12 \([abc]_4[d]_4\) units and 24 linker chains with a total calculated mass of 4,108 MDa. This value is an outlier compared with over a dozen masses for earthworm Hbs.
found in the literature obtained using a variety of experimental methods (339) whose overall mean of ~3.7 MDa is close to the value proposed by Martin et al. (370). The latest ESI-MS results and the three-dimensional reconstructions obtained by cryoelectron microscopy provide no support for the foregoing model.

**B) GLOBIN AND LINKER AMINO ACID SEQUENCES.** The first complete primary structure of a HBL Hb was provided by the sequences of the four globin and two linker chains of the Hb from the marine polychaete *Tylorrhynchus heterochaetus* determined by Suzuki, Gotoh, and co-workers (518, 526). The sequences of chains a, b, and c (174) and the two variants of chain d (350, 494, 675) of *Lumbricus* Hb have been determined. These globin sequences and an additional dozen or more from other HBL Hbs align well with each other and vertebrate globin sequences (289, 394, 618). Furthermore, they can be separated into two distinct groups (207, 208, 492, 493).

The linker subunits are necessary for HBL formation (206, 335, 698). The amino acid sequences of linkers from several HBL Hbs have been determined (523, 524, 526). All the linker chains have a single 42-residue cysteine-rich domain (CRD) in the NH2-terminal moiety. This domain contains six disulfide-bonded Cys residues similar to the CRDs found in members of the scavenger receptor cysteine-rich superfamily including the low-density lipoprotein (LDL) receptor protein family and other diverse proteins. The structure of the ligand-binding CRD out of the seven CRDs found in LDL receptor protein has been determined (43, 156); it requires Ca$^{2+}$ for folding and has a high Ca$^{2+}$ affinity, ~70 nM. The latter property is likely shared by all HBL Hb linkers, thus explaining the universal stabilization of the HBL structure by Ca$^{2+}$ and its requirement for reassembly (335). A recent study of *Lumbricus* HBL structure reassembly (from dodecamers and individual linkers as well as various combinations of linkers) has shown that the four types of linkers are structurally interchangeable (335). Furthermore, this study showed that the presence of at least 1 mM Ca$^{2+}$ is necessary for HBL reassembly to produce its maximum yield, in agreement with earlier observations obtained with *Glossoscolex* Hb (52). In most of the known HBL Hbs, the globin subassemblies are noncovalent complexes of monomeric and disulfide-bonded subunits forming a noncovalent complex with linker subunits, which in some cases are themselves disulfide bonded. In *Nephtys* Hb, however, all the subunits, globin and linker, are disulfide-bonded to each other (617).

Over the last several years, maximum entropy deconvolution of the ESI-MS of several HBL Hbs by Green and co-workers has provided detailed enumerations of the globin and linker chains as well as disulfide-bonded subunits, with masses known to ±1–3 Da over the mass range 17–50 kDa. In addition to verifying the masses calculated from the known sequences, this method provides the number of free and disulfide-bonded Cys residues as well as an indication of posttranslational modification, such as glycosylation. The ESI-MS of the Hbs from *Lumbricus* (370), *Macrobodella* (641), *Riftia* (688), *Alvinella* (686), other deep-sea alvinellids (687a), *Arenicola* (687), *Tylorrhynchus* (213), and *Haemopis* (210) and the Chi from *Eudistylia* (211) showed that in addition to monomeric globins there was a broad variation in the type of disulfide-bonded subunits present. Thus, while *Lumbricus*, *Alvinella*, *Arenicola*, and *Tylorrhynchus* Hbs have disulfide-bonded trimers, *Macrobodella*, *Haemopis*, and *Riftia* Hbs have disulfide-bonded dimers and *Eudistylia* Chi has disulfide-bonded tetramers, in agreement with the results of early electrophoretic studies (608, 616). The polypeptide chain and subunit masses when used to calculate the masses based on the bracelet model of 12 dodecamers (144 globin chains) and 36 or 42 linker chains provide generally good agreement with the experimental masses of the native Hbs. Furthermore, simple glycosylation [(GlcNAc)$_p$(Man)$_n$, $n = 6–9$] was observed only in *Lumbricus* (~2.1 wt%) (370) and *Haemopis* Hbs (210). Although the annelid Hbs that have been examined do not appear to have free Cys residues, *Eudistylia* Chi has a cysteinylated Cys in one of its six globin chains (211).

Studies on *Perinereis aibuhitensis* Hb indicate a high affinity of linker chains for the globin subassemblies, a low affinity for the abc trimers, and no affinity for the monomeric subunits (206). The linker chains were inferred to hold the subassemblies together in the two-tiered structure by connecting the carbohydrate side-chain groups of the globin chains (“carbohydrate gluing”) (677, 678). In view of the relatively rare occurrence of glycosylation in annelid Hbs, such a role for the linker subunits appears to be unlikely.

**C) O2 BINDING PROPERTIES AND ALLOSTERIC TRANSITIONS.**

**I) O2 affinities.** Extracellular annelid Hbs occur at high in vivo concentrations and provide large blood O2-carrying capacities, up to 200 ml/l blood (as in humans) in the giant earthworm *Glossoscolex giganteus* and ~135 ml/l in *Arenicola marina* (272, 575, 576), compared with the low solubility of O2 (5–7 ml/l) in air-equilibrated body fluids. They generally exhibit moderately high O2 affinities ($P_50 = 2–10$ Torr at 20°C) (94, 351, 582, 629) (Fig. 3; Table 2, extracellular Hbs) that appear to be adaptations to low ambient O2 tensions in aquatic species, and low internal O2 tensions associated with the general lack of specialized respiratory surfaces (lungs) in terrestrial ones. In contrast to the tissue and RBC Hbs that show either low sensitivities to effectors or none, the extracellular Hbs exhibit highly variable expression of allosteric interactions in different species and in the same species under different physicochemical conditions (see Fig. 5) (94, 582, 629). The span of the functional properties is illustrated by the high O2 affinity and cooperativity and large Bohr effect in *Arenicola marina* Hb ($P_50 ≈ 2$ Torr at 20°C, $n_{50} = 5$, $\varphi = -0.77$) (576) (Fig. 3) and the low corresponding values in the terrebellid *Eupolymina crescentis*. 
drothermal vents are characterized by very high O₂ affinities (P50 = 0.3–0.4 Torr at pH 7.25 and 20°C) (581) (Fig. 5). In the absence of data on in situ O₂ tensions and pH, it is not known whether these are adaptations to hypoxia. Moreover, given the high heats of Hb oxygenation (ΔH = −59 kJ/mol), the in vivo blood O₂-binding affinities in animals from the hot (50°C) water surrounding the hydrothermal vent chimneys may be considerably lower (544). The in vivo affinities are decreased further by low pH values and large Bohr factors (φ = −0.9 and −1.2 in Alvinella pompejana and A. caudata, respectively, at 30°C) (579, 581). The HBL Hb from the cold-seep oribindii polychaete Metanoaricia dendrobranchiata similarly has a high O₂ affinity and marked pH and temperature effects (P50 = 0.6 Torr, φ = −0.44 at 20°C, ΔH = −58 kJ/mol) (S. Hourdez, R. E. Weber, and C. R. Fisher, unpublished data).

Although the O₂ binding cooperativities of Chls (n50 ~3.3) are similar to those found in HBL Hbs, Chls have much lower O₂ affinities (P50 ~150 Torr at neutral pH) (Fig. 3), which decrease drastically at low pH due to pronounced Bohr effects, indicating that the upper part of their O₂ binding curves is not exploited in life (14, 94, 187, 259, 260, 383, 549, 649). The presence of a distal Phe observed in the sequence of a Eudistylia Chl globin chain (L. Moens, personal communication) correlates with the low O₂ affinity of the native complex since a distal Phe substitution in HbA leads to decrease in affinity (297). Studies of Eudistylia Chl structure using chemical disassociation and ESI-MS (211, 441) have shown it to consist of disulfide-bonded dimers and tetramers of several globin chains, which form noncovalent tetramers and dodecamer subassemblies, respectively. The isolated subassembly has a higher affinity and a lower cooperativity than the native Chl (P50 ~60 Torr and n50 ~2.1) (259). According to the Monod, Changeux, and Wyman (394a) model, the low O₂ affinity is attributable to low values of both K₇ and Kₓ, it is an intrinsic property of the protein and does not originate from a bias in the allosteric equilibrium toward the T state (259). Unlike Lumbricus Hb, Eudistylia Chl does not show any effect of assembly size (down to tetramers) on the ligand binding kinetics (188, 189). Curiously, chlorocruorohemes and protohemes coexist in the HBL heme protein present in the vascular fluid of the polychaete Serpula vermicularis (549). It is not known whether the two hemes share a common HBL structure or each heme is associated with different HBL structures. This mix results in a relatively high, pH-independent Hb-O₂ affinity (P50 = ~8 Torr at 20°C) and a low, pH-dependent Chl-O₂ affinity (P50 ~40 Torr at pH 7.1, φ = −0.8) (549).

A major factor modulating the temperature dependence of O₂ affinity is O₂-linked proton binding (see sect. uB5), which varies greatly in HBL Hbs. The lower overall heat of oxygenation (ΔH) of the intertidal Arenicola marina Hb relative to that of the subtidal Abarenicola claripedi Hb correlates with a larger Bohr effect in the former (627). Analogously, ΔH of Perinereis Hb falls from −47
kJ/mol at pH 6.6, where the Bohr effect is small, to −20 to
−27 kJ/mol at pH 7.4–8.2, where the Bohr effect is max-
imum (589). The reduction in ΔH in Potamilla Chl in the
presence of Mg2+ (from −16.3 to −2.9 kJ/mol) (260)
indicates that cation binding to HBL is endothermic and
safeguards O2 loading at high temperatures. A similar
effect of Ca2+ is observed in Arenicola marina Hb at pH
<7 (R. E. Weber, R. Birkedal, and A. Fredsted, unpub-
lished data).

Potamilla Chl exhibits an interesting effect related
to the heats of oxygenation for binding successive O2 mole-
cules. In contrast to Hbs from the Octolasion complan-
tatum (473), Macrobdella decora (641), and Arenicola ma-
rina (Weber, unpublished results), where the upper and
lower asymptotes of the Hill plots show similar tempera-
ture-induced shifts, the ΔH1 and ΔH∞ values (the oxygen-
ation heats for the first and last O2 molecules bound)
differ drastically in the Chl (+6.3 and −69 kJ/mol) (260),
implying the domination of endothermic processes in the
initial stages of the oxygenation process. This is reminis-
cent of CO binding to trout HbI that shows a reverse
temperature effect at low saturation due to endothermic
conformational changes conditioned to the subunits in
the T state (674).

II) Allosteric transitions. A resonance Raman spec-
troscopic study of Lumbricus Hb showed that in contrast
to vertebrate Hbs, ligand binding did not result in spectral
alterations involving the iron-proximal His stretching
mode (607). This behavior, analogous to that of Mb, sug-
gests that the mechanism(s) of cooperativity in HBL Hbs
is likely to be very different from the mechanisms of
cooperativity in vertebrate Hbs (427, 429) and in the
isolated dodecamer subassembly showed no observable
alterations in their molecular shapes in going from one
form to the other and back (326, 328). This result suggests
that ligand binding does not produce an alteration in
quaternary structure larger than an ∼1% (−2–3 Å in height,
respectively) change in molecular shape, the
latter being the limit of the technique.

In contrast to mammalian Hb where Hill coefficients
are typically constant at levels of O2 saturation between
10 and 90% over a wide range of pH values (12), those of
the HBL Hbs vary greatly from species to species (94, 186,
578, 630) and intraspecifically with O2 saturation, pH,
inorganic cation concentration, and temperature (248,
333, 405, 581, 630) (Fig. 5). Cooperativity is commonly
maximal under physiological pH conditions, as in Areni-
cola marina where it peaks at pH 7.2–7.6 (20°C) and in
the hydrothermal vent alvinellids Alvinella ovalata and
A. pompeii, where peak values near pH 6.9 appear to
 correlate with low blood pH (581, 582). However, mis-
matching between in vivo pH and pH of maximum coop-
erativity is seen in Lumbricus terrestris where blood
cooperativity increases from ∼3 at low pH to a maximum
of 9.5 near pH 7.8 (326). Likewise, the cooperativities
of the Hbs from the oligochaetes Phereetima hilgendorfi
(404) and Eisenia fetida (248) and leeches Hirudo me-
dicinalis (255) and Macrobdella decora (256, 641) are
maximal at pH above 8.0. Marked temperature depend-
ence is illustrated by the thermotolerant Alvinella pom-
pejana Hb, whose nmax at pH 7.25 falls from 3 at 20°C to
1.2–1.5 at 10 and 40°C (581) (Fig. 5). This indicates pro-
nounced differences in the overall heats of oxygenation of
the deoxygenated and oxygenated forms of the Hb.

The cooperativity of Hb in fresh blood appears to be
higher than that of purified Hb, e.g., in Octolasion com-
planatum (473) and Lumbricus terrestris (326). This may
result from loss of cations during purification, which in-
creases O2 affinity at high saturation (see below) and
stabilizes the HBL structure. It should be pointed out that
ESI-MS revealed no additional component in Lumbricus
blood compared with the Hb in solution (B. Green and S.
Vinogradov, unpublished results).

Among annelids, large Bohr effects that permit O2
unloading in tissues at high tension but hinder O2 loading
in stagnant and acidic environments commonly charac-
terize species with intense ventilatory or swimming activ-
ity (e.g., Arenicola marina), whereas small effects com-
monly occur in species that lack well-defined burrows
[e.g., the oligochaete Alma emini (38) and polychaetes
Travisia pupa (366) and Neptys hombergii (625)]. This
is in accordance with the principle that pH-induced O2
unloading at the destination tissue is an adaptive option
only if O2 loading at the respiratory surface is adequate.
Lower pH values in prebranchial than in postbranchial
blood, a prerequisite for a functional Bohr effect, has been
demonstrated in Arenicola cristata (352). Conceivably,
large Bohr effects may compensate for small pre- and
postbranchial pH differences. Apart from lactic acid and
CO2, annelids produce succinic, propionic, and acetic
acids under conditions of O2 shortage, resulting in
decreased tissue pH (437, 477).

Among the Hb-containing intertidal eunicid polychaetes, Marphysa sanguinea inhabits stagnant bur-
brows, whereas Diopatra cuprea inhabits impermeable,
vigorously ventilated tubes. The higher O2 affinity and
smaller Bohr effect in Marphysa Hb relative to Diopatra
Hb correlate well with lower O2 dissociation and higher
CO2 association rates and a lower pH dependence of these
reactions (636). A study of the kinetics of ligand binding
by the Hb from the polychaete Cirriformia grandis (659)
showed that the origin of the strong Bohr effect was
entirely due to the strong pH dependence of O2 dissocia-
tion rates, which decrease 800-fold in going from pH 9
to 6 while the rates for O2 and CO2 association remain
invariant.

In Arenicola Hb, increases in the Bohr effect and in
cooperativity with increased O2 saturation (in accordance
with the pH dependence of KIR, see below) enhance O2
loading in the gills (631) and increase O\textsubscript{2} extraction (which may exceed 85%) from the ventilatory water flow (582). Exploitation of the upper part of the equilibrium curve under normoxic conditions enhances the O\textsubscript{2} diffusion gradient to tissue Mb and mitochondria and ensures a high “venous” O\textsubscript{2} reserve (578, 582). Correlations seen between the magnitude of cooperativity and that of the Bohr effect in individual Hbs and within the same Hb under specific physicochemical conditions (e.g., pH, O\textsubscript{2} saturation) suggest similarity of the underlying hetero- and homotropic transitions or implication of the same allosteric transitions.

D) CATIONIC EFFECTORS. Blood electrolyte levels in annelids vary greatly due to the varying ambient salinity and the absence of well-defined osmoregulatory capacities in the aquatic species and the potential for dehydration in the absence of well-defined osmoregulatory capacities in the terrestrial forms. In osmoconforming *Arenicola marina*, blood O\textsubscript{2} affinity increases with water salinity (333) resulting from the facilitating effect of divalent inorganic cations on O\textsubscript{2} binding (151, 631). Similar effects are seen in other HBL Hbs and Chls.

Effect modulator of annelid Hbs differs radically from that in vertebrate Hbs. In the latter, organic anions (like DPG and ATP) decrease the O\textsubscript{2} affinity by preferentially decreasing the O\textsubscript{2} association equilibrium constants in the deoxygenated T state (right shifting the lower asymptote of extended Hill plots). In contrast, inorganic cations (Na\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+}, Mg\textsuperscript{2+}) increase the affinity of *Arenicola* Hb by preferentially increasing the binding constants in the oxygenated (putative) R state (left shifting the upper asymptote); the divalent cations exert a greater effect than monovalent cations (631) (Fig. 6). Analogously, in the physiological pH range, the Bohr effect of *Arenicola* marina Hb is primarily due to increases in K\textsubscript{R} with rising pH, whereas the Bohr effect in vertebrate Hbs generally results from increases in K\textsubscript{R} with K\textsubscript{T} remaining relatively constant (631). In the annelid Hbs, the changes in K\textsubscript{R} result in increases in cooperativity and the Gibbs free energy for heme-heme interaction (\(\Delta G\)) with increased pH and cation concentration.

The changes in K\textsubscript{R} indicate that the oxygenation-linked binding of protons and divalent cations to HBL protons occurs late in the oxygenation process (173, 631), thus coinciding with high in vivo O\textsubscript{2} saturations, as observed in *Arenicola*. Modulation of K\textsubscript{R} by divalent cations and pH has been documented in a number of species, including the Hbs of oligochaetes, *Megascolecides australis* (634), *Octolasion complanatum* (473), *Lumbricus terrestris* (173), *Eisenia fetida* (248, 405), the polychaete *Perineis aibukhitenis* (589), the leech *Macrobdella decora* (641), and the Chl of *Potamilla leptochaeta* (260). Modulation of K\textsubscript{R} implies the existence of at least two high-affinity, “relaxed” states. For *Lumbricus* Hb, the pH and cation dependence of K\textsubscript{R} indicates at least one state at low pH that is independent of the presence of salt and others occurring at high pH and/or high salt concentrations (173).

Curiously, a different control mechanism appears to operate in the Hb of the deep-sea polychaete *Alvinella pompejana*, where 0.1 M Ca\textsuperscript{2+} at pH 7 increases affinity by raising K\textsubscript{R} without significantly affecting K\textsubscript{T} (R. E. Weber and F. Zal, unpublished results). Affinity modulation via K\textsubscript{T} in this species is also reflected in the large Bohr effect in the (almost completely) deoxygenated Hb and an insignificant effect in the (almost fully) oxygenated Hb (581).

Although the effects of divalent group IIA cations on O\textsubscript{2} affinity of HBL Hbs exceeds that of monovalent ions, the cation sensitivities in *Lumbricus* Hb (Ba\textsuperscript{2+} > Ca\textsuperscript{2+} > Sr\textsuperscript{2+} > Mg\textsuperscript{2+} > Li\textsuperscript{+} > Na\textsuperscript{+} > K\textsuperscript{+}) not only depends on ionic strength but reveal effects of ionic radius within each valence class (173). This accords with greater effects of Ca\textsuperscript{2+} than Mg\textsuperscript{2+} in *Amphitrite ornata* (84), *Eisenia fetida* (248, 405), *Phereetima hilgendorfi* (404), and *Hirudo medicinalis* (255) Hbs as well as a greater effect of Ba\textsuperscript{2+} than other divalent cations in *Macrobodella decorra* Hbs (256). However, O\textsubscript{2} binding to *Tubifex* (408) and *Marphysa sanquinea* Hbs (R. E. Weber and J. Bonaventura, unpublished results) appears to be insensitive to Ca\textsuperscript{2+} and Mg\textsuperscript{2+} concentrations, as is observed with *Eurythoe complanata* Hb and Mg\textsuperscript{2+} (250). Also, Ca\textsuperscript{2+} and Mg\textsuperscript{2+} exert the same effects on the affinity of *Glossoscolex paulistus* (368) and *Arenicola marina* Hbs (151) (T. Ochiai and R. E. Weber, unpublished results). Furthermore, the pH decreases observed upon cation addition to *Arenicola* Hb solutions are in accord with their ion
contributions calculated from changes in the local Debye–Hückel distributions of ions at the negatively charged surface, indicating the dominance of ionic charge (643).

Little is known about effects of other metal cations. Hbs from the oligochaetes *Lumbricus terrestris*, *Tubifex tubifex*, and *Tyloorrhynchus* contain 1–4 Zn and Cu atoms (509). In *Phretilma hilgendorfii*, where the blood contains 0.9 mM Zn$^{2+}$, and 1–2 Zn atoms per 164 Fe atoms that cannot be removed by dialysis, addition of Zn$^{2+}$ increases affinity and almost obliterates cooperativity by raising $K_T$ (407), suggesting a different mechanism and/or different binding sites compared with Ca$^{2+}$ and Mg$^{2+}$. Cooperativity of *Glossoscolex* Hb is higher in the presence of Mn$^{2+}$ than with Ca$^{2+}$ and Mg$^{2+}$, despite similar O$_2$ affinities induced by these cations (368). Curiously, specific rabbit antiserum to *Glossoscolex paulistus* Hb increased its O$_2$ affinity, whereas nonspecific serum and serum albumin decrease its affinity (74).

The cation effects on HBL Hbs may have ecophysiological implications. In *Arenicola marina*, where blood Ca$^{2+}$ and Mg$^{2+}$ levels are 8 and 60 mM, respectively (333), the cation sensitivity of $K_T$ conceivably increases Hb oxygenation at low tide when burrow ventilation ceases and hypoxic conditions coincide with the residence of dense, saline, high-tide water in the burrows (631).

E) Common origin of pH and cation effects. Considerable evidence suggests a common origin for the observed pH and cation effects on the affinity and cooperativity of O$_2$ binding, based probably on competition for the same sites within the HBL Hbs. Cation addition to solutions of *Arenicola marina* Hb decrease pH in the bulk solution, indicating displacement of protons and that cations and protons bind to the same sites (643). Two and one protons appear to be released per oxygenated heme for each bound Ca$^{2+}$ and Na$^+$, respectively, in *Lumbricus terrestris* and *Eisenia fetida* Hbs (173, 248). The highly polar pre-A helix NH$_2$-terminal extensions of chains a and c in *L. terrestris* trimer may provide the cation binding site (174). In the oligochaete *Octolasilus complanatum*, the Bohr effect appears to be wholly attributable to O$_2$-linked binding of allosteric effectors, and the O$_2$ affinity varies within a well-defined range, set by the “cation-free” (low affinity, low cooperativity) and “cation-saturated” (high affinity, high cooperativity) states of the Hb (473).

Further evidence comes from similar dose-response relationships. Apart from their major effects on $K_T$ at moderate concentrations and pH values (that span physiological conditions), high cation or pH values also raise $K_T$ in the Hbs of *Arenicola marina* (631), *Lumbricus terrestris* (173), *Eisenia fetida* (248), and *Macrobdella* (641) as well as in *Potamilla leptochaeta* Chl (260).

There is substantial evidence that the integrity of the HBL structure depends on a minimal concentration of group IIA divalent cations (52, 84, 405, 434, 473). Most reported instances of quaternary structure stabilization have been semiquantitative observations of reduction in the extent of dissociation of the HBL structure at pH >8 in the presence of 1–100 mM cation. The following typical effects were observed with *Lumbricus* Hb: 10–20 mM Mg$^{2+}$, Ca$^{2+}$, and Sr$^{2+}$ were equally effective in decreasing dissociation from 70 to 10% at pH 9 (434). Likewise, almost complete dissociation in 4 M urea at neutral pH in the presence of 1 mM EDTA after 144 h decreased to ~50% dissociation in the presence of 10 mM Ca$^{2+}$ (484).

Furthermore, the extent of reassembly of *Lumbricus* Hb from isolated dodecamer subassembly and isolated linker(s) is absolutely dependent on the presence of Ca$^{2+}$, albeit at levels of ~1 mM (335). Illustrative examples of cation effects on the affinity and cooperativity of O$_2$ binding are seen in oligochaete *Eisenia* Hb, where $P_{50}$ and $n_{50}$ values at pH 8.0 are 3.3 Torr and 4.2, respectively, in native Hb, 2.6 Torr and 6.3 in 1 mM Ca$^{2+}$, 1.8 Torr and 8 in 10 mM Ca$^{2+}$; 3.2 Torr and 5 in 1 mM Mg$^{2+}$, and 2.7 Torr and 6.6 in 10 mM Mg$^{2+}$ (405), and in polychaete *Perinereis* Hb (589), where $P_{50}$ and $n_{50}$ values of 15.5 Torr and 2.7, respectively, observed in the native Hb at pH 7.4 and 25 °C, changed to 12.1 Torr and 4.1 in 25 mM Mg$^{2+}$ and to 10.9 Torr and 4.9 in 100 mM Mg$^{2+}$.

It is evident that the presence of 36–42 linker chains, each with a CRD domain known to have high affinity for Ca$^{2+}$ (see sect. II A(B)), provides *Lumbricus* Hb and by implication most HBL Hbs with a range of Ca$^{2+}$-binding sites. There is some evidence for some Ca$^{2+}$ binding in the dodecamer subassemblies as well (334a). More importantly, ES-MS of isolated linker L3 in the presence of Ca$^{2+}$ showed it to be able to bind up to 6–8 Ca$^{2+}$ (334a). It appears likely that structural stabilization is effective at low, ~1–10 mM, cation concentrations, while the effects on O$_2$-binding affinity and cooperativity occur at higher cation levels, from ~10 to 100 mM.

F) O$_2$ binding properties of globin subassemblies and subunits. An intriguing issue is the effect of assembly size on functional properties, or to put it another way, to what extent the functional properties manifested in the HBL structures are expressed in the constituent subassemblies and subunits. The quest for the “minimum functional subunit” is complicated by the fact that it depends on the functional criterion (O$_2$ affinity, cooperativity, Bohr effect, cation or temperature effects, etc.), specific physicochemical conditions (pH, effector concentration, temperature, etc.), the species, and the point of reference (whole blood or purified Hb).

The high $n_{50}$ values encountered in HBL Hbs (9.5 in *Lumbricus terrestris* at pH 7.8; Fig. 5) (326) suggest cooperative interaction between at least 10 hemes according to the MWC model. Early studies on alkaline dissociation products of HBL Hbs indicate similar O$_2$ affinities and cooperativities in the ~10S subassemblies as in the intact ~60S molecules for *Arenicola marina* (625) and lumbricids (122, 186) Hbs, but lower cooperativity for the subassemblies from *Abaren-i-cola affinis* (92, 93) and from *Eisenia fetida* (166a) Hbs. The dodecamer
subassemblies of *Lumbricus terrestris*, prepared by mild dissociation (at neutral pH in 4 M urea), show similar P50 values (11.5–11.9 Torr at 25°C and pH 7.5) and the same Bohr effect and Mg2+ and Ca2+ sensitivities as the intact Hbs, but lower cooperativity (n50 ~2–3 compared with ~5 in Hb and ~9.5 in whole blood, at pH 7.8) (326, 613). Further evidence comes from the cold-seep orbiniid *Methanoaricia* (see sect. III C3A), where 210-kDa Hbs, which resemble HBL subassemblies in size, exhibit lower homotropic and heterotropic interactions than HBL Hbs (n = 2.0 and 1.2, respectively; φ = −0.44 and −0.13, respectively, at 20°C) although their O2 affinities are the same at pH 7.4 (S. Hourdez, R. E. Weber, and C. R. Fisher, unpublished data).

Investigation of the functional properties of the trimer and monomer subunits of *Lumbricus* Hb (175) found P50 = 3 Torr and n50 = 1.4 for the abc trimer and 1.6 Torr and unity, respectively, for the d monomer. In contrast to the monomer subunit, the O2 affinity of the trimer is modulated by pH and Ca2+, and a putative [abc-d]2 complex exhibited the same P50 and n50 as the whole molecules at pH 6.8 in the presence of Ca2+. An effect of assembly size was also observed in ligand binding kinetics of *Lumbricus* Hb and its subunits (189).

Another case is the Hb of the leech *Macrobdella decora*, where the intact HBL molecules, and the tetramer and monomer subunits show neatly increasing affinities (P50 = 4.4, 1.9, and 0.3 Torr, respectively, at pH 7.5 and 25°C) and decreasing Bohr factors (−0.38, −0.30, and 0, respectively) and cooperativities (n = 3.1, 1.4, and 1.0, respectively) (641) (see Fig. 7). For *Eudistylia vancouveri* Chl, the CO binding kinetics of dodecameric and tetrameric fragments are like that of the holoprotein, suggesting that the tetramer is the major cooperative unit (188). However, some extended interactions occur, as evidenced by the fact that the dodecamer subassembly exhibits lower cooperativity and Bohr coefficient than the native Chl (259).

Analysis of precise O2 binding data in terms of the MWC model provides an alternative approach to delineating functional subunits. Graphical analyses indicate six interacting sites in *Perineries aibuhitensis* Hb (589) and *Potamilla leptochaeta* Chl (260) and between 5 and 12 in *Lumbricus terrestris* (173). By fitting the number of interacting O2 binding sites together with the other MWC parameters to O2 equilibrium data, Weber et al. (641) find the number that gives the best possible fit to be ~10 for *Macrobdella decora* Hb. This value is close to the number of hemes per dodecamer and to the highest values of n observed in HBL Hbs but much greater than the number of hemes in the smaller dissociation products. *Lumbricus* subassemblies exhibit equally large free energies of heme-heme interaction as the whole molecules at pH 7.7 (326) (Weber and Vinogradov, unpublished results). Together, these considerations confirm the early conclusion (122, 186, 613, 625) that dodecamer subassemblies are the principal functional subunits of HBL Hbs, although full cooperativity appears to be dependent on the presence of the complete HBL structure (613).

Additional evidence derives from the KT and KR values of dissociation products. Given that finite bond energies constrain the molecules in the T (tense) states, the difference in the KT values (that can be determined experimentally with greater accuracy than KR values) indicates the bond energy difference associated with dissociation to subunits (641). The difference in T-state bond energies between the dodecamer subassembly and the native HBL structure of *Lumbricus* Hb [calculated as

![Fig. 7. A: O2 equilibrium curves of the extracellular HBL Hb of the leech *Macrobdella decora* and its tetrameric (T) and monomeric (M) subunits. B: extended Hill plots of O2 equilibria. C: pH dependence of half-saturation O2 tensions (P50) and of Hill’s cooperativity coefficient at half-saturation (n50). [Data from Weber et al. (641).]](http://physrev.physiology.org/)

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$\Delta G_T = RT \ln(K_{T,D}/K_{T,Hb})$, where $R$ is the gas constant and $T$ the absolute temperature] is small (~1 kJ/mol heme) (326), suggesting that assembly of the dodecamer subassemblies into the HBL structure imparts very little constraint on the dodecamers in the deoxygenated form. In *Macrobdella decora* Hb, the higher $O_2$ affinity of the tetramer subunit than that of the native HBL similarly correlates with increased $K_T$ (Fig. 7). The $K_T$ values for the HBLs and tetramers, 0.28 versus 0.037 Torr$^{-1}$, respectively (at pH 7.26 and 25°C), reflect a bond energy difference of ~5 kJ/mol heme, corresponding to the loss of approximately one salt bridge per heme group upon dissociation into tetramers (641). It should be noted that at least in the case of *Lumbricus* and *Macrobdella* Hbs, the Hill plots are not symmetrical (326, 613, 641). Consequently, $n_{50}$ is generally found to be lower than $n_{max}$. In view of the complex quaternary structure of HBL Hbs, analyses in terms of “nested” MWC models are desirable.

**g) Vestimentiferan Hbs.** This section deals with the structure and $O_2$ binding properties of vestimentiferan Hbs; the reactions of these and other Hbs with sulfide are discussed in section vBI.

Vestimentiferans, like the hydrothermal vent worm *Riftia pachyptila*, lack an alimentary canal and derive energy from symbiotic, chemolithoautotrophic, endosymbiotic, sulfide-oxidizing bacteria that occur in cells of a specialized organ, the trophosome. *Riftia* blood has high $O_2$-carrying capacity (2.3–4.6 mM heme), may carry 1.3 times as much sulfide, and stores and transports $O_2$ and sulfide to the symbionts that in turn satisfy the worm’s nutritional needs (25, 26, 87, 196).

*Riftia* possesses three extracellular Hbs: a vascular, ~3,500 kDa Hb V1, a vascular, ~400 kDa Hb V2, and a coelomic, ~400 kDa Hb C1. Hb V1 is an HBL Hb that consists of six different globin chains, some of which form disulfide-bonded dimers and several nonglobin linker chains. The ESI-MS of the Hb (688) looks very similar to the ESI-MS of the leech Hbs, *Macrobdella* (641) and *Haemopis* (210). Its three-dimensional reconstruction obtained by cryoelectron microscopy (130) shows a quaternary structure very similar to the of *Lumbricus* and other HBL Hbs. Hbs V2 and C1 consist only of globin chains (688), which they share with the HBL Hb V1.

The deep-sea, vestimentiferan tube worm *Lamellibrachia* has a ~3,000-kDa, extracellular HBL Hb that has four heme-bearing chains (AI-AIV) and two linkers (AV and AV1) and a ~440-kDa Hb that has four chains (BI-BIV) (528–530). The amino acid sequences of the eight heme-bearing chains (AI-AIV and BI-BIV) show high homology with those of annelid HBL Hbs. However, the *Lamellibrachia* chains possess an extra cysteine residue (Cys-74) that is considered to be a potential sulfide-binding site (529). The $O_2$ and sulfide binding properties of *Lamellibrachia* Hbs have not been studied.

Whole *Riftia* blood binds $O_2$ cooperatively ($n_{50} = 2.8$) with a moderately high affinity ($P_{50} = 2.7$ Torr at 14°C, pH ~7.4) and low pH sensitivity ($\varphi = -0.12$) (25). A 1,700-kDa vascular Hb fraction, which could represent partially dissociated Hb V1 and the 400-kDa vascular Hb V2, showed lower cooperativities ($n = 1.9$ and 1.3, respectively) and marked differences in $O_2$ affinities ($P_{50} = 1.3$ and 0.9 Torr at 40°C) and pH sensitivities ($\varphi = -0.35$ and $-0.04$, respectively) but similar high overall heats of oxygenation (~$-69$ kJ/mol) (28) (Table 2, extracellular Hbs). The $O_2$ association rate of *Riftia* Hb shows temperature independence, in contrast to pronounced temperature dependence of the $O_2$ dissociation rate, which indicates compensation by a large decrease in the value of the conformational equilibrium constant, a behavior similar to that of the electrochemically cathodic HbI of trout (674).
three-dimensional reconstructions using cryoelectron microscopy was possible with binary and ternary linker combinations as well as with single linkers (335) (J.-C. Taveau and J. Lamy, personal communication). This finding implies that each linker has globin and linker-binding domains. Consequently, a model of HBL reassembly was proposed starting with 12 globin dodecamer subassemblies and 36 linker chains (335) (Fig. 8), based on the idea that the binding of a linker dimer (but not a monomer) causes conformational alterations in the linker-dimer bound two trimer subunits permitting lateral self-association between the dodecamer subassemblies. Given the threefold symmetry of the latter, lateral self-association of dodecamer-linker subassemblies DL2L and DL2L-LL2D dictates the formation of either a single hexagonal complex (DL2L)6 (which can then dimerize) or a complete HBL structure (DL2L-LL2D)6. Although this model provides a rational explanation for why an HBL structure is formed, it does not say anything about the packing of the linker chains. Furthermore, this model suggests that it is the formation of a threefold symmetric dodecamer subassembly that is required for an HBL structure to be formed. Thus the next question is why would a dodecamer subassembly M3T3 be elaborated?

4. A new annelid quaternary structure comprised of four-domain globins

A diverse panoply of Hbs was found recently by Hourdez and co-workers (243, 244) in the coelomic fluid of the polynoid polychaete Branchipolynoe (scaleworm) existing within the pallial cavity of the mussel species Bathymodiolus, which occurs at the deep-sea hydrothermal vents. In addition to a minor component that has a mass of ~3 MDa and could represent an HBL Hb, two major components of 153 and 124 kDa and a minor one of ~23 kDa were observed (243, 244). ESI-MS of the two major components showed them to be trimers and dimers of 57,996- and 57,648-Da chains, respectively, each chain comprising four globin-binding domains. The littoral species of the family Polyonidae has not been found to contain Hbs except in the case of Aphrodite and Halosyndra, which have limited amounts of Hb in nerve tissues (660). The two major Hbs of Branchipolynoe have moderately high O2 affinities (P50 = 1.4 and 2.3 Torr at pH 7 and 20°C) and low cooperativites (n50 ranging from 1.0 to 1.9 over the pH range 6.5–8) (243). Unlike the vestimentiferans, alvinellid polychaetes and vesicomycid clams inhabiting the hydrothermal vent environment, Branchipolynoe does not host a sulfur-oxidizing symbiont. The high affinity and low cooperativity, reminiscent of Mbs, and the presence of gills with a large surface area and small diffusion distances (241) are not inconsistent with a possible O2 storage role for the Hbs, which would enable the worm to sustain an aerobic metabolism in spite of progressive O2 depletion occurring in the host mantle cavity during periods of shell closure (244).

5. Pogonophorans

Relatively little is known about pogonophore Hbs. Siboglinum fiordicum and Oligobrachia mashikoi occur buried in marine sediments and have ~400-kDa Hbs that are probably similar to the V2 and C1 Hbs found in Riftia (562, 683). Both Hbs consist of 14- to 15-kDa globin chains. Surprisingly, a low heme-to-protein ratio similar to HBL Hbs (1 heme/21.5-kDa protein) has been reported for Siboglinum Hb (562), although there is no evidence for nonglobin linker chains. Oligobrachia Hb consists of monomers and disulfide-bonded dimers and trimers (683) like the monomers and trimers encountered in oligochaete and polychaete Hbs and the monomers and disulfide-bonded dimers in leech and Riftia HBL Hbs (339). The Oligobrachia globin sequences exhibit high similarities with those from oligochaete (Lumbricus), polychaete (Tylorrhinchus), and vestimentiferan (Lamellibrachia) Hbs (683). This supports recent genetic and embryological evidence for including vestimentiferans and pogonophorans in the annelid phylum (57, 311, 376, 682).

S. atlanticum Hb occurs at high in vivo concentration (~0.6 mM), has a low isoelectric point (pI = 4–5) like the HBL Hbs, and comprises two electrophoretically distinct components (367). The Hb and blood of S. ekmani (650) and S. fiordicum and S. atlanticum (562) show high O2 affinities (0.4–1 Torr at 15–20°C), marked cooperativi-
ties ($n = 2–3.3$), and a small Bohr effect ($\varphi = +0.18$ in $S. fiordicum$) that may be related to recurring low ambient $O_2$ tensions and the need to transport $O_2$ to the buried posterior ends of the body that harbor autotrophic endosymbiotic bacteria (562).

The $\sim 400$ kDa mass of the pogonophore Hbs and the C1 and V2 Hbs of $Riftia$ suggests that they consist of $\sim 24$ globin chains. Although they could be dimers of dodecamer subassemblies, it should be noted that the latter require nonglobin linker chains to form HBL structures and when isolated do not self-associate. Furthermore, the lower cooperativities of these Hbs than those of HBL Hbs underscore the putative role of the linker subunits in enhancing the cooperativity of the dodecamer subassembly upon formation of the HBL structure.

6. Molluscs

Molluscs have two classes of large extracellular Hbs: 1) the 1.65- to 2.25-MDa Hbs found in freshwater, pulmonate gastropod snails of the family Planorbidae (e.g., Planorbis, Biomphalaria, Helisoma, Indoplanorbis and Planorbella), and 2) the 8- to 12-MDa Hbs occurring in the heterodont bivalve families Astastidae and Carditidae (including Astarte and Cardita) (53, 543, 558, 608). Features shared by the mollus extracellular Hbs, as well as their alternate copper-containing oxygen carrier hemocyanin, are large multidomain polypeptide chains, comprised of 10–12 and 18–20 globin domains in pulmonates and bivalves, respectively (543, 558).

A) Planorbis gastropod Hbs. The 1.65- to 2.25-MDa pulmonate Hbs are comprised of large, 175–200 kDa, 10–12 domain chains that form disulfide-bonded dimers of $\sim 350–400$ kDa (7, 24, 230, 231, 406, 556) (Fig. 1). The quaternary structure of these Hbs remains unclear, mainly because of the lack of sequence information. Although Daniel and collaborators (254, 433) have proposed a slightly ellipsoidal shell structure with tetrahedral symmetry consisting of 12 subunits, Herskovits and Hamilton (230, 231) have proposed a compact two-layer ring structure of 10 decamer subunits. Biomphalaria Hb is a glycoprotein that contains 3% sugars (3). In addition to the extracellular Hb, a muscle Mb is also found in Biomphalaria (136).

The hemolymph of Biomphalaria glabrata shows a moderately high $O_2$ binding affinity ($P_{50} = 6$ Torr at 25°C, pH 7.7) (64, 597) and exhibits pH-dependent cooperativity ($n_{50} = 1.2–2.0$). It shows similar “fixed acid” and “$CO_2$” Bohr effects ($\varphi = -0.2$ to $-0.5$; Fig. 5), indicating the absence of a specific $CO_2$ effect and exhibits novel allosteric control mechanisms (64). As in the annelid HBL Hbs, cations increase $O_2$ affinity (with the following relative effects: $Ca^{2+} > Mg^{2+} > Na^+ > K^+$), and the Bohr effect is predominantly attributable to pH-induced increase in $K_R$. However, in contrast to annelid Hbs where cations raise $K_R$, $Ca^{2+}$ increases $K_I$ in Biomphalaria Hb. Thus $\Delta G$ rises with increasing pH and falls with increasing cation concentrations (64). This indicates that cations bind to Biomphalaria mainly in the initial stages of the oxygenation process and protons dissociate predominantly at high $O_2$ saturation levels, whereas both processes occur late in the oxygenation process in annelid HBL Hbs. Dose-response curves for the cation effects indicate binding of 0.17 $Ca^{2+}$ per oxygenated heme in Biomphalaria (64). The absence of significant pH changes following $Ca^{2+}$ addition underscores a different cation binding mechanism compared with Arenicola HBL Hb (643) (see sect. wC3). Analysis in terms of the MWC model indicates 6–9 interacting hemes in the functional subunit of Biomphalaria blood/Hb at pH 7.2–7.8. Nonlinear van’t Hoff plots of Hb reflect a decrease in temperature sensitivity of $O_2$ binding with increasing temperature that suggests changes in the heat capacity of the system resulting from the dissociation of salt bridges and hydrogen bonds that attends the $T \rightarrow R$ conformational alteration (155).

Helisoma trivolvis Hb has a low cooperativity and a marked Bohr effect ($n_{50} = 1.5$ and $\varphi = -0.37$, respectively, at pH $>7.5$) but appears to lack cation sensitivity; its $O_2$ affinity is unaffected by $Mg^{2+}$, $Na^+$, and $K^+$ and is only slightly increased by 0.25 M $Ca^{2+}$ (545, 561). Polypeptide chain fragments corresponding to one, two, and more domains obtained by partial proteolysis lacked cooperativity and Bohr effects, indicating that the 10-domain (175 kDa) chains are required for full expression of homo- and heterotrophic interactions in multidomian pulmonate Hbs (556, 561).

b) Giant Hbs of heterotodont bivalves. The 8- to 12-MDa Hbs found in the two families Astastidae and Carditidae hold the record among invertebrate Hbs in terms of size (563, 676). These physically heterogeneous Hbs appear to be comprised of 240- to 390-kDa polypeptide chains containing 14–24 globin domains. Negatively stained electron microscopic images show cylindrical structures varying in length from 36 to 120 nm (541, 558). Cardita borealis Hb has a high $O_2$ affinity ($P_{50} = 4.5$ Torr at 20°C) and lacks homo- and heterotrophic interactions (563). The lack of cooperativity and similar $O_2$ affinities obtained for the intact molecules and the monomorphic domain fraction isolated after subtilisin digestion indicate that the monomers are fully functional entities whose properties are not altered by their integration into the giant native structures (563). Recent findings (R. E. Weber and D. Abele-Oeschger, unpublished results) show a distinct Bohr effect in C. borealis Hb ($\varphi = -0.4$ near pH 7.5) and that $Ca^{2+}$ raise the $O_2$ binding affinity (as with extracellular annelid and gastropod Hbs), whereas $Mg^{2+}$ has no significant effect. Astarte castanea Hb shows a slight cooperativity and moderate Bohr effect ($n_{50} = 1.2$, $\varphi = -0.39$). In the absence of $Ca^{2+}$ at pH 9, it dissociates into single ($\sim 335$ kDa) protomers, each consisting of a linear sequence of $\sim 20$ globin domains (676).
7. Crustaceans

Polymeric extracellular Hbs occur in the hemolymph of five crustacean classes: 1) the predominantly freshwater Branchiopoda, that include anostracans (Artemia, Parartemia, and Streptocephalus), conchostracans (Cyzicus, Caen estheriella and Caenestheria), cladocerans (Daphnia and Moina), and notostracans (Triops and Lepidurus); 2) Ostracoda; 3) Copepoda; 4) Cirripedia; and 5) Malacostraca (115, 163, 541). A recent study has documented the occurrence of Hb mRNA in fat cells and in epithelial cells of the epipodites of Daphnia, providing the only known evidence regarding the sites of Hb synthesis in crustaceans (200).

A) Branchiopods. Branchiopod Hbs assume highly diverse quaternary structures and include 220- to 300-kDa proteins occurring in anostracans and conchostracans, and larger and more variable (420–670 kDa and 600–800 kDa, respectively) ones in cladocerans and notostracans (cf. Refs. 253, 541).

I) Two-domain Hbs. Despite molecular masses ranging from 220 to 800 kDa (251), the extracellular Hbs of Cyzicus (Conchostraca), Daphnia and Moina (Cladocera), and Lepidurus (Notostraca) are all comprised of ~35 kDa, two-domain globin chains (251). The two domains of the water flea Daphnia Hb, D1 (176 residues) and D2 (154 residues), have been sequenced (573), and a hexadecameric bilayered eclipsed quaternary structure has been proposed (257). When Daphnia Hb sequences are compared with other two-domain Hbs, such as the clam Barbatia (397), the percent of identity of D1 to D1 and D2 to D2 is 80–90%, whereas comparison of any D1 to any D2 provides only some 20% amino acid identity. Thus it is likely that the two-domain Hbs originated in an ancient tandem duplication via unequal crossing over of two single-domain globin genes; the ancestral two-domain globin gene then evolved with much later occurring serial gene duplications (T. Gorr and F. H. Bunn, personal communication). Daphnia is currently the focus of investigations aimed at the structure and evolution of its globin genes (135b, 227a, 299a) as well as of its physiological adaptations (424a, 433b, 433c).

II) Anostracan nine-domain Hb. In contrast to two-domain Hbs from other branchiopod groups, the 260-kDa Hb of the anostracan Artemia is a complex of two 130-kDa covalent polymers of nine globin domains (303, 392). Each polymer is encoded by a gene representing nine successive globin domains that have different sequences and are presumed to be the result of repeated duplication of an ancestral single-domain gene, possibly involving chains of three domains at some stage (269, 373). Two different polymers T and C exist as the result of a complete duplication of the nine-domain gene, allowing the formation of either homodimers or heterodimers; both have the same number of residues and differ by an average of 12% of the residues; within either polymer, the domains are more divergent, differing at 61–77% (585).

Molecular models proposed on the basis of negatively stained electron microscopic images and dissociation studies visualize the ~494-kDa Hb of the cladoceran Daphnia magna as sixteen 31-kDa chains grouped in two 8-sided layers stacked in eclipsed orientation (a hexadecameric bilayer) (257), and the ~302-kDa structures of the conchostracan Caenestheria inopinata as ten 30-kDa subunits arranged in two pentagonal layers stacked in an eclipsed orientation (253). The Hb of the anostracan Artemia appears in electron micrographs as two stacked disks (393).

III) Induction of Hb during hypoxia. The synthesis of Hb in cladocerans and anostracans is regulated by the ambient O2 concentration (164, 307). The biological advantages of increased Hb concentration under hypoxia are manyfold. In the case of the water flea Daphnia, these include greater viability in hypoxic water, higher swimming activity, increased egg production (164), increased feeding rates, and exploitation of food resources (481). Furthermore, it demonstrably governs the dependence on aerobic metabolism (596) and even affects thermal preference. As recently documented (659a), Hb-rich specimens of D. carinata show a lower metabolic rate than control animals under normoxic conditions, and a higher preferred temperature than control animals under hypoxic conditions.

An intriguing question is whether these advantages are imparted solely by increased O2 concentration (carrying capacity) or whether alteration in the in vivo oxygenation properties are implicated as well. The latter possibility is indicated by the higher ambient O2 tensions required to half-saturate Hb in vivo in Hb-poor than in Hb-rich specimens of D. magna (310), the positive correlation between O2 affinity and Hb concentration (307), and the higher overall heats of oxygenation in Hb from dark red specimens compared with pale ones (ΔH = −29 and −61 kJ/mol, respectively; Ref. 310). This suggests lower endothermic contributions from oxygenation-linked reactions in the Hb-poor specimens. Hb-rich Daphnia produce more eggs containing threefold higher Hb concentration (309), implying that the advantages of hypoxic acclimation are passed on to the developmental stages. Furthermore, O2 affinity of Hb isolated from eggs of Hb-rich animals is much greater than that of the Hb from eggs produced by Hb-poor animals (P50 = 2.9 and 7.2 Torr, respectively) (309). In Artemia, the synthesis of Hb III is stimulated under hypoxia and inhibited under high ambient O2 tensions (136a, 228). Because it shows the highest O2 affinity and lowest pH and temperature effects (see below), preferential synthesis of this component may favor aerobic metabolism under hypoxia.

IV) O2 binding properties. The relatively high O2 affinities of cladoceran Hbs (P50 = 3.5 and 2.1 Torr, respectively) in Daphnia magna and Moina macrocopa
at pH 7.2 and 20°C (513) suggest an O₂ transporting function at low ambient tension. This is in accord with results of CO poisoning experiments that indicate a maximum role for D. magna Hb in O₂ transport at ~30 mmHg (239) (Fig. 2). Four major Hb fractions of D. pulex, that may consist of at least 12 isoHbs with pI values of 5.0–6.5, show similarly high affinities (P₅₀ = 1.4–2.7 Torr at pH 6.9, 20°C), no significant Bohr effects, and low cooperativity (n₅₀ = 1.4–2.3) (673). The eggs of D. magna contain at least eight Hb components, including specific embryonic ones that have higher pI values than those in adults (309). Exposure to hypoxic conditions increases the proportion of Hb components with high pI values. An analysis of O₂ equilibria in terms of Adair’s model (310) suggests that a multiple Hb system that shows an inverse relationship between the mass and P₅₀ as in mammalian Hbs (124). Hb II obtained from Artemia displays distinct functional heterogeneity; the three components, Hbl (CC), HbII (CT), and HbIII (TT), have different O₂ affinities (P₅₀ = 7.3, 5.1, and 2.6 Torr, respectively) and lower cooperativities (n₅₀ = 3.2, 3.3, and 1.4, respectively) than the hemolymph (n₅₀ = 4.3) (672) (Table 2, extracellular Hbs). Hbl is the most abundant component (64–91%), and Hbl or HbIII is absent in strains from some geographical regions (China and Brazil, respectively) (671). The O₂ affinity of Hb of Artemia, which tolerates extremely high salinity, is slightly decreased by Cl⁻ and increased by CO₂ at high pH, suggesting that CO₂ is bound to free NH₂ groups as in mammalian Hbs (124). Hb II obtained from Artemia populations living in lakes with high levels of sulfate exhibits the same functional properties as that from high Cl⁻ salterns. Curiously, however, its O₂ affinity is decreased by sulfate but not affected by high (1 M) chloride concentration (123).

An interesting effect of assembly size was observed on the O₂ binding properties of Artemia Hb fragments containing one, two, three, and four heme-binding domains obtained by partial proteolysis (672). The fragments had higher affinities (P₅₀ = 0.7–1.5 Torr at 18°C and pH 7.5) than either the individual chains (P₅₀ = 3.5 Torr) or the native Hb (P₅₀ = 6.2 Torr) and exhibited a linear relationship between the mass and P₅₀. Furthermore, moderate cooperativity (n₅₀ = 1.7) was only observed with the native Hb.

The wide spectrum of oxygenation properties encountered in branchiopods is illustrated by the exceptionally high O₂ affinity of the Hb from the conchostracan Cyzicus cf. hierosolymitanus from high-altitude ponds that may become hypoxic at night (P₅₀ = 0.035 Torr and n₅₀ = 2.3) (22), the intermediate affinities of the Hbs from the conchostracans, Caenesthesiella setosa and C. inopinata (P₅₀ = ~6 and n₅₀ = 1.2–2.5) (115, 252), and the unusually low affinities of the Hbs from the notostracans Lepidurus lynchi, L. couesi, and L. bilobatus (P₅₀ ~20 Torr at 20°C) that express substantial cooperativity and a Bohr effect (n₅₀ = 2, φ = −0.2) (114, 115). Interestingly, a 14- to 16-kDa fraction of Lepidurus bilobatus Hb obtained by partial proteolysis lacked a Bohr effect and showed much higher O₂ affinity (P₅₀ ~2 Torr) and higher cooperativity (n₅₀ = 3), indicating that expression of cooperativity in the subunits is suppressed by their association into the whole molecules (114). The O₂ affinity of the notostrachan Triops longicaudatus Hb (P₅₀ = 6.8 Torr, at 22°C, pH 7.1, n₅₀ = 1.4–2; φ = −0.23) is increased by Mg²⁺ and Ca²⁺ (238), as seen in extracellular amnelid and gastropod Hbs (see sect. III, C3 and C6).

b) other crustaceans. Hb from the recently described copepod Benthoxynus speculifer living in association with the vestimentiferan, hydrothermal-vent tube-worm Ridgida piceasae, is a 208-kDa protein composed of seven 14.3- and seven 15.2-kDa globins. In accordance with the highly variable environmental conditions that include extremely low O₂ tensions and high CO₂ and sulfide levels, the Hb exhibits a very high O₂ affinity (P₅₀ = 0.05 and 0.13 at 10 and 20°C) and lacks cooperativity and a Bohr effect (245).

Very little is known about the Hbs of the other crustacean classes (the Malacostraca, Ostracoda, and Cirripedia) (163, 541). The only malacostracan Hb known so far studied is that of the amphipod Cyamus scammoni, an obligatory ectosymbiont of the gray whale (540). It has a mass of 1,800 kDa and an unusual chevron-like appearance in electron micrographs (540, 541). The smallest subunit observed is 175 kDa, suggesting that the Hb is comprised of 10-domain polypeptide chains. Furthermore, a low heme-to-protein ratio indicates that not all domains bind heme or that some domains lose heme easily. Its low affinity (P₅₀ = 14–24 Torr) appears to match the high loading tensions prevailing in well-oxygenated Pacific waters traversed by the whales (540).

Hb has been reported in a number of parasitic rhizopelcian cirripeds, including Septosacculus cuenotti (426) and Pellogaster curvatus and Parthenopea subterranea (161). The cirripedian barnacle Briarosaccus callosus, which parasitizes the king crab, has abundant Hb that appears to have masses >1,000 kDa and is physically heterogeneous (491, 564). It differs from branchiopod Hbs in having 17- to 19-kDa subunits when dissociated and has a very unusual springlike appearance in negatively stained electron micrographs, ~15 nm wide and 35–125 nm long (541). The structures and properties of ostracod Hbs have not been investigated.

IV. INTRA- AND INTERSITE FUNCTIONAL DIFFERENTIATION

A. Role of Hb Heterogeneity

As evident from section III, B3, C1, and C7, invertebrates abundantly display Hb heterogeneity that involves
both multiplicity (different isoHbs occurring in the same individual organisms) and polymorphism (different Hb components, Hb patterns, or relative concentrations occurring in different genetic strains). Whereas functionally different Hbs occurring in the same site implies intrasite heterogeneity, their occurrence in different juxtaposed sites provides a basis for intersite O2 transfer (362, 547, 644). The organismic advantages of heterogeneity are manifold (632). Intrasite functional heterogeneity implies that the composite Hb functions in O2 transport, O2 storage, and facilitation of O2 diffusion over a greater range of O2 tensions than possible with a single Hb component. By permitting a division of labor between Hb components, it extends the range of conditions under which they can function and thus enlarges the organism’s habitable environment (632). Hb multiplicity may alleviate the effects of mutational change, since deleterious effects in one gene would not affect the expression of other similar genes. Additionally, the implicit differentiation in isoelectric points may extend a protein’s capacity for regulating free ion levels (448). The two cases of functional isoform multiplicity that have been the most comprehensively documented are the extracellular Chironomus Hbs (see sect. μC) and the Glycera RBC Hbs (see sect. μB3). Other explicit examples are the Hbs of Paramecium (263, 501), trematodes Gastrothylax and Explanatum (443), and Daphnia and Artemia (670, 673).

A related question is whether cytoplasmic and RBC isoHbs occur in the same cells and whether they interact in a functionally significant manner when they are in the same medium. Although the O2 affinities of individual G. dibranchiata RBCs show a normomodal distribution, indicating that both forms are in the same cells (359), there is little evidence for interactions between co-occurring components in invertebrate Hbs. In vitro mixing of the monomeric and polymeric Glycera dibranchiata Hb preparations whose O2 affinities differ by a factor of two results in an intermediate affinity (645), contrasting with an earlier observation that mixing low molecular and high molecular fractions produces a lower O2 affinity than either fraction (236). An interesting intramolecular functional differentiation given the much lower affinities of Chl than of annelid HBL Hb (see sect. μC3) appears in the polychaete Serpula, where hemes and chlorocruorohe-mes occur within the same or very similar HBL complexes (549).

B. O2 Transfer Systems

Compared with the simpler (Hb→Mb) O2 transfer occurring in vertebrates, those identified in invertebrates appear to show striking adaptive variation within the constraints imposed by the anatomical and molecular structures. While the relative O2 affinities in annelids like Travisia pupa indicate a vascular HBL Hb → coelomic RBC Hb → muscle Mb O2 transfer (366), the remarkable adaptability is illustrated in the burrowing echiurid Aplysia fungicola where O2 uptake occurs through the body wall and the O2 affinities reveal a muscle Mb → coelomic Hb transfer (366). Fascinatingly, the properties of Mbs from molluscs with hemocyanin-rich hemolymph, like Siphonaria (655), Aplysia and Dolabella, and Buccinum undulatum reveal a Hc → Mb O2 transfer, despite the low O2 affinity in some molluscan Mbs (P50 = 13 Torr in Buccinum Mb) (555).

In Amphitrite ornata, vascular (HBL Hb) → coelomic (RBC) Hb transfer involves similar total amounts of both heme compounds and a 60-fold higher vascular than coelomic Hb concentration, which implies that, at maximum exchange efficiency, only a small volume of blood needs to perfuse the gills to oxygenate the voluminous coelomic fluid, thus permitting a drastic restriction of gill size (642). Curiously, the coelomic monomeric Hb from the hydrothermal-vent polychaete Alvinella shows very similar, pH-dependent O2 affinities as in the HBL vascular Hb. This indicates a bidirectional O2 transfer between the two Hbs in the perioesophageal pouch that maintains oxygenation of the brain (242). The occurrence of marked cooperativities (n = 2–3) in the coelomic Hb under the same pH and temperature conditions where the Bohr effects are pronounced suggests the implication of deoxygenation-linked aggregation.

Despite a physical separation, polychaete coelomic and circulatory compartments are in close contact (suggesting that the coelomic fluid is an ultrafiltrate of blood) (398). The occurrence of apparently identical HBL Hb molecules in the vascular and the coelomic fluids of Neptys hombergii (273, 626) indicates that the barrier is not absolute in this species.

V. OTHER FUNCTIONS, REACTIONS, AND ACTIVITIES

Apart from its roles in O2 binding (transport, storage, and scavenging of O2), protecting microorganisms from NO, and use of NO to control levels of O2 in nematodes such as Ascaris discussed above, Hbs may be involved in a number of other functions and reactions, that may be integrally linked with its O2 binding reactions (e.g., acid-base regulation and sulfide binding).

A. Acid-Base Balance

Hbs occurring at high concentrations may play an important part in body fluid acid-base balance (578) that is intimately linked with their gas-binding properties. Hbs, which are the main proteins in invertebrate body fluids, buffer H+ from carbonic acid, allowing formation of bicarbonate ions. Due to (negative) linkage between O2 and proton binding, Hbs with normal Bohr effects will more-
over transport protons from acid tissues to the respiratory surfaces. Marked Haldane effects (higher bicarbonate levels and pH values in deoxy than in oxy blood that are a direct consequence of Bohr effects) have been demonstrated in invertebrates with extracellular Hbs, including those in Arenicola marina (575, 576), Neoamphitrites figulus (654), and the giant Gibbsland earthworm Megacolides australis (634). In intertidal Arenicola, the transient acidosis that follows Hb oxygenation upon high-tide immersion is dampened by oxygenation-dependent buffering by the Hb, which is maximum at pH 7.48 and may result from the unmasking of an imidazole group (578).

The role of RBC Hbs in acid-base balance appears not to have been studied in invertebrates but is implicit in the greater buffering capacity of “true plasma” (the plasma that contains suspended RBCs) relative to RBC-free plasma in mammals (133).

B. Reactions With Sulfide

In contrast to vertebrate Hbs and Mbs that react with sulfide to form covalently modified heme groups of the green sulfoHb and sulfoMb (81), invertebrate Hbs and Mbs react with sulfide in several different ways, without concomitant covalent modification of the heme group. Mbs react with sulfide in several different ways, without concomitant covalent modification of the heme group. 

1. Extracellular Hbs

The earliest reaction observed was the oxidation of sulfide by the vascular, extracellular HBL Hb of the marine polychaete Arenicola, resulting in the formation of hematin (ferriheme hydroxide) (422, 423). However, in the case of the closely related Abarenicola Hb, no reaction with sulfide was observed and the O2 binding affinity of the Hb remained unaltered (653).

The elucidation of additional reactions of invertebrate Hbs with sulfide has come about mostly as the result of the discovery of symbioses of invertebrates with sulfur-oxidizing chemoautotrophic prokaryotes, which coincided with the discovery in 1977 of hydrothermal vents occurring at isolated sites on the ocean floor at depths of up to ~2,500 m and an associated thriving fauna including deep-sea vestimentiferan tube worms (e.g., Riftia and Lamellibrachia), polychaetes (Alvinella), and vesicomyid clams (e.g., Calyptogena) (590). Since then, symbioses have been found in diverse sulfide-rich habitats, ranging from deep-sea cold seeps to seagrass beds and sewage outfalls; the symbionts fix carbon from the oceanic CO2 and supply most of the organic carbon required by the host (88, 158, 400). Adult vestimentiferans do not have a digestive tract, and the sulfide-oxidizing, prokaryote endosymbionts on which they depend for supplying their nutritional requirements are localized within a highly vascularized organ (trophosome) located within the trunk of the tube worm (579). The trophosome comprises up to one-third of the host and is surrounded by noncircuiting coelomic fluid containing the ~400-kDa Hb C1, in equilibrium with the circulating vascular blood, containing the HBL HbV1 and another ~400-kDa Hb V2 (559, 577) (see sect. mC3). The host supplies the symbiont with inorganic carbon, O2, sulfide, and nitrate. It is significant that sulfide and O2 have to be segregated to avoid spontaneous sulfide oxidation (479). Sulfide acquisition occurs via HS− rather than via H2S, and that of inorganic carbon occurs via diffusion of CO2 rather than via bicarbonate transport (196, 197). The coelomic fluid and, to a lesser extent the blood of Riftia, is characterized by a pronounced base excess that permits retention and storage of large quantities of CO2, up to 50 mM, compared with 7.5 mM in Arenicola. However, unlike Arenicola, only a small fraction of the total CO2 appears to be associated with the Hb (580). In addition to binding O2 with high affinity (28), Riftia Hbs share the ability to bind sulfide reversibly and independently of O2 (27). The amino acid sequence of a globin chain common to all three Hbs has revealed a free Cys residue (692), which is able to bind sulfide (690). In addition, the Cys residues present in the linker chains of Hb V1 are able to combine with sulfide to form persulfides, thus accounting for the much higher sulfide-binding capacity of Hb V1 relative to Hb V2 and Hb C1. Free Cys residues have also been found in the globin chains of the HBL Hbs of the vestimentiferan Lamellibrachia (528) and the polychaetes Alvinella (686) and Arenicola (687), which inhabit sulfide-rich environments.

The terebellid polychaete Alvinella living in organic tubes on active sulfide chimneys walls at hydrothermal vent sites (134) is called the “Pompeii worm” in reference to its existence under a constant precipitation of mineral particles at temperatures higher than any other known metazoan (mean in situ ambient temperatures of 68°C with spikes up to 105°C!) (76). In contrast to vestimentiferans, the alvinellids have a gut and no endocellular prokaryotic symbionts. However, they exist in obligate association with epibiotic bacteria (134). Alvinella has a coelomic intracellular Hb in addition to its vascular HBL Hb (278). One or both of these Hbs may participate in the detoxification of sulfide, either by binding it and transporting it to the epibionts associated with the surface of the worm or by direct reaction with sulfide (134). Although both Hbs were reported not to bind sulfide (371), it is not absolutely clear that this point has been resolved (134).
HBL Hbs and the vestimentiferans *Riftia* and *Lamellibrachia* (683). Like the latter, but not the former, they have two free Cys residues, which may be involved in sulfide binding. Two interesting and related points are the presence of the ~400-kDa Hbs in vestimentiferans and the absence of an HBL Hb in the pogonophorans. Because the subunit and quaternary structures of *Riftia* HBL Hb V1 (130, 688, 689) are very similar to those of the leech *Macrobdella* Hb (127, 290, 641) and all three of *Riftia* Hbs share common globin chains, it appears logical to consider the smaller Hbs in both *Riftia* and *Oligobrachia* to be dimers of globin chain dodecamer subassemblies. If this is the case, then one can view the vestimentiferan HBL Hb as a means of increasing the sulfide-binding capacity of the vestimentiferan blood, a property not required by the much smaller pogonophorans.

2. **Cytoplasmic Hbs**

O₂ and sulfide binding coexist in Hbs from symbiont-harboring gills of the clams *Solemya* and *Lucina* (321, 324). The sulfide binding Hbs exist in an oxy form, which is partly converted to a ferric sulfide derivative (146, 324). This differs from those cases described in the preceding section, where the sulfide is bound to free Cys residues on globin and linker chains and heme is not involved. The sulfide reactive Hb may facilitate diffusion of sulfide through the cytoplasm to provide the symbiont with the nessesary supply (664, 666).

An intriguing adaptive differentiation appears in the symbiont-harboring Puerto Rican clam *Lucina pectinata* whose gill Hb consists of three components: the sulfide reactive monomeric Hbl and O₂-reactive Hbs II and III which remain oxygenated in the presence of H₂S and self-associate in a concentration-dependent manner to tetramers when mixed (324, 325). Although the three Hbs have similar, high O₂ binding affinities (P₅₀ ~0.1–0.2 Torr at 20°C), Hbl has a high affinity for sulfide (K_D ~3.4 nM) and a slow dissociation rate (k_off = 0.00022 s⁻¹), indicating that sulfide delivery by simple dissociation is unlikely. A possible mechanism could be the reduction of the ferric sulfide derivative near the bacterial surface leading to the ferrous Hb and rapid dissociation of sulfide (664). The amino acid sequences of O₂-reactive HBII and HBIII that form a noncooperative tetramer are known (234, 235). Because of slow O₂ dissociation rates, these components may not deliver sufficient O₂ and may function as a terminal oxidase by accepting electrons from the symbiont (664). The crystal structures of the aquomet and sulfide-bound forms of Hbl have been determined (452, 453). The unique sulfide binding property appears to be predicated on an unusual distal heme cavity environment, wherein an E7Gln is surrounded by B10Tyr and E11Phe. Although the corresponding triple mutant of sperm whale Mb was found to have a ~700-fold higher sulfide binding activity than wild-type Mb, its activity was still approximately sevenfold lower than *Lucina* Hbl. Comparison of the crystal structures indicates that the higher affinity of Hbl could be due to a significantly larger ligand-binding site than in the mutant, which thus is able to better accommodate the large ligand (401). Detailed spectroscopic studies by López-Garriga and collaborators (79, 347, 399, 497) have indicated that there may be in addition, a unique orientation of the heme 2-vinyl groups stabilizing the heme Fe(III) state and that the lack of hydrogen bonding of the heme propionates to the polypeptide chain may provide the heme group with a rocking freedom that facilitates sulfide binding and that ligand stabilization occurs via interactions with the B10Phe and E11Phe. A detailed comparison of the spectroscopic properties of *Ascaris* perienteric Hb and *Lucina* Hbl has suggested that the much higher O₂ affinity of the former is due to a strong hydrogen bonding network between the bound O₂, E7Gln and B10Tyr, forming a tight cage for the bound ligand. In *Lucina* Hbl, this hydrogen bonding network is more tenuous, and the distal cavity is more accessible to large ligands than in *Ascaris* Hb (431).

Yet another effect of sulfide occurs in the case of the *Solemya reidi* cytoplasmic Hbs (146), which have high O₂ binding affinities (P₅₀ ~0.3–0.5 Torr) and comparable O₂ dissociation rates (~10 s⁻¹) (321). Although there does not appear to be any alteration in the optical absorption spectra of the Hbs in the presence of sulfide, the O₂ dissociation rates of two of the three Hbs decrease by 5- to 15-fold in the presence of 600 μM HS⁻ (equivalent to PH₂S = 0.43 Torr). The physiological function of such an effect would be the conservation of the intracellular O₂ store with increase in sulfide concentration and/or a decrease in the O₂ level (321).

The deep-sea vesicomyid clams, e.g., *Calyptogena*, have large symbiont-harboring gills and voluminous blood, which can account for 13–22 and 24–44% of total body weight, respectively (400). The host supplies the symbiont with inorganic carbon, O₂, hydrogen sulfide, and nitrate; in particular, O₂ and sulfide have to be segregated to avoid spontaneous sulfide oxidation (479). A cytoplasmic Hb is present in the gill at concentrations of up to 250 μmol/kg wet wt (663), and the blood contains intraerythrocytic dimeric and tetrameric Hbs of moderate O₂ affinity (P₅₀ ~7.6 Torr) (89, 90, 560). The sequence of the dimeric Hb has been determined (527). Given that a Zn-containing, large-molecular-weight serum protein was shown to bind and transport sulfide in this species (90), the role of the Hbs remains unclear. The dimeric Hb is similar to the cytoplasmic Hbs of the lucinid clam *Myrtea*, which bind O₂ cooperatively but do not bind sulfide; the latter role is performed by a nonHb protein in the gill (113). Thus vent bivalve Hb may serve to transport or store O₂ (559), as demonstrated in a nonvent bivalve (124a).
C. Autoxidation and Hemichrome Formation

The autoxidation of the ferrous form of O₂ binding heme proteins is an important measure of their stability in the performance of their function (508). This topic is only a part of the extensive subject dealing with oxidation of Fe(II) complexes (see Chem Rev 94, issue 3: “Metal-Dioxygen Complexes,” 1994). The autoxidation of mutant mammalian Mbs has been extensively investigated by Olson, Phillips, Jr, and collaborators (61, 75, 508). Mutation of the distal E7(64)His has been shown to result in dramatic increases in the autoxidation rate in the pH range 5–9 in the order Val > Gly > Leu > Gln > His (wild type). The fact that the Mb of the prosobranch mollusc Cerithidea, which has a distal His (535), has an autoxidation rate similar to that of the Mb from the opistobranch mollusc Aplysia, which has a Val (51), underscores the difficulty of explaining the properties of invertebrate Hbs and Mbs based on mutagenesis of vertebrate Mb. The autoxidation reactions of vertebrate and nonvertebrate Hbs and Mbs have been reviewed recently by Shikama (488). In the case of Mbs, the autoxidation rate can vary by two orders of magnitude. Comparison of autoxidation rates found in the literature for nonvertebrate globins is complicated by differences in the experimental methods used to determine them. Two types of mechanisms have been considered in the autoxidation of oxygenated ferrous heme proteins: 1) electron transfer from Fe(II) to the bound O₂, followed by dissociation of the protonated superoxide into the oxidized protein and free superoxide radical, and 2) a bimolecular reaction between noncoordinated O₂ and either a pentacoordinated or a hexacoordinated deoxyheme, the sixth group in the latter case being a water molecule or a ferric anionic ligand such as azide, fluoride, or chloride. In Mb and its mutants still having the distal His, autoxidation occurs via a combination of superoxide dissociation and the bimolecular reaction between O₂ and deoxyMb containing a weakly coordinated water molecule (61). The introduction of bulky hydrophobic residues at positions E11 and B10 decreases accessibility of the Fe to solvent water molecules and reduces autoxidation rates by up to 10-fold (75). A recent study of the autoxidation of Rhizobium FixL and Aplysia Mb, both of which are known to have a pentacoordinate ferric form, has shown that they autoxidize primarily via the bimolecular reaction of O₂ with an intermediate having water coordinated to the heme Fe(II), indicating that a hexacoordinate aquomet species is not required (202).

A number of nonvertebrate Hbs and Mbs exhibit the unusual formation of a hemichrome at alkaline pH instead of the usual hydroxy-Met form upon autoxidation. This was found for Paramecium Hb (588), the intracellular Hb from the sea cucumber Caudina (390), and the extracellular Hbs from the earthworms Octalasium (32) and Glossoscolex (4). The formation of a hemichrome instead of a Met form upon oxidation also occurs in the case of LegHbs (19). A recent crystallographic study (222a) shows that the active site of ferric rice HbI differs significantly from those of other hemoglobins in that the proximal and distal His residues coordinate directly to the heme iron, forming a hemichrome with spectral properties similar to those of cytochrome b₅. Hemochrome formation at alkaline pH has been observed in the case of the extracellular HBL Hb from Arenicola (281, 282). The oxygenated Cerithidea Mb exists as homodimers in solution and upon oxidation forms a monomeric hemichrome over the pH range 5–11 instead of the usual MetMb (372). This reaction is reversible in Cerithidea Mb, unlike the case with Paramecium Hb (588). It has been suggested that the formation of hemo- and hemichromes could have a functional role (4, 390, 588). In humans, hemichrome formation appears to favor the formation of Heinz bodies and the subsequent destruction of RBCs (447).

A recent report of spontaneous hemin release from Lumbricus Hb (503) purports to provide evidence for facile heme loss. Measurements of the rate of heme loss from completely oxidized human HbA, Lumbricus Hb, soybean LegHb and horseradish peroxidase C in the presence of apoMb provided the following rate constants: 7.7, 19, 7.1, and 0.24 × 10⁵ min⁻¹, respectively. Since no evidence of heme loss from the native Lumbricus oxyHb was provided, the ~2.5-fold higher rate of hemin loss from Lumbricus metHb relative to HbA appears to be of very little if any relevance to the stability of the native oxyform. The autoxidation of Lumbricus oxyHb is a very slow process whose rate is ~0.01/h at pH 7 and 20°C (697), and the dissociation of the met form is known to be very slow (484). Riggs and collaborators (697) have concluded that “Lumbricus Hb is at least as stable as human Hb tetramers or dimers.” These observations emphasize that the so-called facile heme loss from the Met form to apoMb (503) has little if any relevance to the stability of native Lumbricus oxyHb.

D. Minor Activities and Specialized Functions

1. Oxidase and peroxidase-like activities

Human Hb is known to exhibit monooxygenase activity characteristic of cytochrome P-450, as exemplified by the catalysis of aniline p-hydroxylation (384). The monooxygenase activity appears to be confined primarily to the β-subunits. The same activity is displayed by the extracellular Hb from Lumbricus (J. Mieyal, personal communication). The oxidase activities of cytoplasmic Hbs have been discussed by Wittenberg et al. (664, 668).

Lebioda and collaborators (337, 340) have recently isolated a dehaloperoxidase from the marine polychaete Amphitrite ornata: its amino acid sequence and its crystal structure show it to have the typical Mb fold. Although the complete sequence showed greatest similarity with
4. Buoyancy regulation

The insect backswimmers Anisops and Buena are two hemipteran genera that synthesize Hb in both the larval and adult forms (40). As indicated in sections 3C4 and mA9, the low O2 affinity of the Hb that is concentrated in abdominal tracheal cells permits unloading of O2 into the tracheal system to regulate the insect buoyancy (385, 386, 652).

5. Oxygen sensing

The best-documented case of an O2-sensing heme protein is FixL, a homodimer of two polypeptide chains each comprising an NH2-terminal heme-binding domain and a COOH-terminal histidine kinase domain (193–195). The sensing of low O2 concentration by FixL and its transduction by FixJ, the response regulator protein, are required for the expression of the nif and fix genes for nitrogen fixation by the soil bacterium Rhizobium meliloti associated with root nodules formed in its plant host Medicago sativa (alfalfa). The phosphorylating ability of the kinase domain of FixL is dependent on the spin state of the heme Fe in the other moiety: high-spin but not the low-spin forms are active kinases (194). Because the binding of O2 to ferrous FixL converts it to a low-spin state, FixL may function as a sensor for other ligands, such as NO or CO. The heme-binding domain of FixL has an amino acid sequence, which cannot be easily aligned with Hbs and Mbs. Although its O2 and CO dissociation rates are comparable to those of Mbs, the very slow association rates account for its 30- to >100-fold reduced affinities for these ligands (195). Gilles-Gonzalez and collaborators (201) have obtained the crystal structures of a high-spin Met form and a low-spin cyanometh form of a monomeric Bradyrhizobium FixL domain. Unlike the Hbs and Mbs, it consists of a helix and a five-stranded antiparallel β-sheet with a hydrophobic distal cavity. Comparison of its heme cavity structure with that of the polychaete Glycera Hb (23) reveals a surprising similarity in the positions of the distal Leu and Ile with the annelid E7Leu and E11Ile residues. In addition, another hydrophobic group Ile215 is close enough to interact with bound ligands. In contrast to Hbs and Mbs, the small hydrophobic distal cavity in FixL does not have any polar side-chain groups capable of hydrogen bonding with bound ligands.

In addition to the O2 sensing heme protein FixL and the O2 sensors for the eukaryotic hypoxic response, other nonglobin heme proteins that sense NO (guanylate cyclase) and CO (Rhodospirillum CooA) are under active investigation (65, 456).

While the present manuscript was under review, Alam and collaborators (240) have described a new class of O2-sensing, heme protein aerotaxis transducers in the archean Halobacterium salinarum and the bacterium Bacillus subtilis. These are ~50-kDa chimeric proteins comprising an NH2-terminal globin domain which exhibits ~14% identity with sperm whale Mb and has the invariant amino acid sequence, which cannot be easily aligned with Mbs, the very slow association rates account for its 30- to 100-fold reduced affinities for these ligands. Although its O2 and CO dissociation rates are comparable to those of Mbs, the very slow association rates account for its 30- to >100-fold reduced affinities for these ligands (195).

6. Bacterial glutamate racemase

The glutamate racemase from the bacterium Pediococcus pentosaceus represents a unique and curious case: it is a 265-residue protein whose sequence comprises a stretch of 92 residues (92–183), of which 27 are common to bovine Mb (91). The high sequence similarity in the regions corresponding to the E and F helices, which form the heme-binding cavity, explains the formation of a 1:1 complex with hemein, leading to loss of racemase activity. Although the aspartate racemase sequence (681) is similar to the glutamate racemase, it has less similarity to the Mb and does not bind heme.
VI. CONCLUDING REMARKS

Present-day globins that are presumed to have descended from a single ancestral globin reveal a spectacular diversity in structure and function that often is manifested within individual categories of cytoplasmic, RBC, and extracellular nonvertebrate Hbs. The divergence, which is seen interspecifically as well as in multiple Hbs within the same individual organisms, correlates with wide variation in the physicochemical conditions under which the globins operate in vivo and may represent a greater shift of the regulatory burden toward the molecular level than in vertebrates, which may compensate for a lesser development of nonvertebrates at the organ level (630).

Mangum (356) has recently argued that the evolutionary history of the oxygen carrier proteins among invertebrates is not as capricious as it appears superficially. Cytoplasmic Hbs that are commonly monomeric or dimeric and show no or little cooperativity exhibit a 4,000-fold variation in $P_{50}$ (from 0.01 to 40 Torr). This variation may be important to their roles in the storage of $O_2$ and the facilitation of its diffusion (when at high concentration) and may allow them to serve in Hb-mediated oxidative phosphorylation (when at low concentration) (cf. Ref. 664). The intracellular RBC Hbs, which have fairly similar physiological properties, exhibit only limited pH sensitivity and no significant sensitivity to ions and intracellular organic modulators, are generally associated with simple circulatory systems, such as the open coelomic circulation in marine invertebrates and appear broadly to serve intersite $O_2$ distribution. The $O_2$ content of RBC Hbs can sustain reduced levels of metabolism in aquatic invertebrates during ventilatory pauses in intertidal species during low-tide emersion. The low cooperativities of these Hbs imply release of $O_2$ over a wider span of $O_2$ tensions than in Hbs with high cooperativity. With the monomeric chironomid Hbs as a prominent exception, the extracellular Hbs are commonly complex multimeric structures that function as $O_2$ carriers in open and closed circulatory systems with primitive heart function. They exhibit large variations in homotropic interactions ($n = 1–9$) and heterotropic ones (effects of pH and divalent cations) even in closely related species. Exceptions to these generalizations "confirm the rule" in that they generally correlate with specific, identifiable exogenous (environmental) conditions or endogenous factors (e.g., habit, ventilation pattern, anaerobic capability, excretory organ type, etc.).

In contrast to the divergences, the occurrence of similar functional properties in different categories of Hbs and in Hbs of phylogenetically diverse organisms reveal repeated instances of adaptive and evolutionary convergences in $O_2$ binding properties. Thus Hbs from some 23 unrelated plant families achieve extraordinary high $O_2$ affinity through high $O_2$ association and low dissociation rates. In contrast, the low $O_2$ affinities of cytoplasmic Hbs in three animal phyla are achieved by radically different mechanisms, suggesting that when the need arose for special functional properties, the necessary molecular mechanisms were evolved.

Although the ability of large organisms to survive and thrive may depend critically on Hbs, it should be borne in mind that the functional properties of these proteins are only part of a symphony of mutually complementary adaptations manifested at organismic, cellular, and molecular levels, each of which may become critically implicated under extreme conditions. Thus, apart from possessing Hb with specific $O_2$ binding characteristics, the adaptations securing tissue oxygenation in the annelid Alvinella from high temperature hydrothermal vent habitats include highly developed gills with large specific surfaces and small diffusion distances for $O_2$ and a branchial circulatory system whose complexity parallels that observed in fish gills (276, 581).

Whereas studies of structural and molecular properties of nonvertebrate Hbs have advanced tremendously in the last decade as a result of the deployment of an arsenal of newly available techniques (site-directed mutagenesis and cloning, X-ray crystallography, mass spectrometry, cryoelectron microscopy), our understanding of the physiological significance of Hbs appears to have lagged behind, commonly through lack of information on in vivo conditions (of $O_2$ tension, pH, and ion levels) and technical difficulties associated with their experimental assessment in small and delicate animals. Additionally, the often observed differences in the functional properties of Hbs in situ and in vitro, which may reflect changes in quaternary structure or loss of effectors, suggests caution in attributing organismic functions to Hbs on the basis of in vitro studies of isolated Hbs.

The recent findings of a human neuroglobin (65b), of simple globins in the insect Drosophila (65a) and in the bacteria whose genomes have been determined (43a), of truncated Hbs in a substantial number of bacterial groups (43a), of chimeric globins in bacteria and archaea (240) and the apparently widespread occurrence of plant symbiotic and nonsymbiotic globins (9, 583), including a globin in moss (31a), indicate that globins occur much more widely among nonvertebrates than hitherto suspected. This raises the possibility that Hbs may be ubiquitous and that their apparent absence may be due simply to very low levels of expression, as exemplified by nonsymbiotic plant Hbs (9) and the Hbs from the nematode Caenorhabditis (302, 364) and the insect Drosophila (65a).

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REFERENCES


April 2001

NONVERTEBRATE HEMOGLOBINS 621


R UPH, ALTERMÜLLER AG, AND GEHRONDE K. Preparation and char-


