PRINCIPLES IN THE ASSEMBLY OF ANNELID ERYTHROCRUORINS

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ABSTRACT Erythrocruorins are giant extracellular respiratory proteins found freely dissolved in the blood of annelids. We present here results from our ultracentrifugation, electron microscopy, spectroscopy, and diffraction experiments on these erythrocruorins. These data are rationalized in terms of a three-dimensional model of the quaternary structure. The proposed structure is arranged in a hierarchy of symmetry. The implications of this structure for the assembly process are considered with special attention to uniqueness and self-limitation. The hypothesis is consistent with observations not used in its construction and it serves as a working hypothesis to focus further experimentation.

INTRODUCTION

The extracellular respiratory proteins found in the bloods of many invertebrates comprise complex assemblages of many subunits and have molecular weights in the millions (1). These giant molecules include the hemocyanins of molluscs (up to 160 oxygen-binding domains in 20 polypeptide chains for a total mass of 8–9 $\times$ 10^6d), the arthropod hemocyanins (up to 48 subunits for a molecular mass of 3.6 $\times$ 10^6 d) and several types of hemoglobins, otherwise known as erythrocruorins. The erythrocruorins of annelids are composed of some 200 subunits organized in a hierarchal symmetry that generates a characteristic 3.9 $\times$ 10^6 d structure.

We have undertaken a structural study of these giant respiratory proteins both to understand their cooperative oxygen-binding properties and to examine principles involved in the assembly process. Our immediate focus is on the annelid erythrocruorins and particularly on the earthworm protein. We report here on our initial experimental observations on erythrocruorins and present a hypothesis of the structural organization in these molecules. Deductions about the assembly process are intimately dependent on knowledge of the structure. Diffraction studies that we have underway should provide definitive structural information, but even now some aspects of the principles that govern assembly in these molecules can be examined. We expect that important features of assembly principles at work in the erythrocruorins will also pertain to questions of assembly in other biological systems.

Questions of Assembly

Large molecular aggregates play important roles in many biological structures. These include ribosomes, muscle filaments, viruses, multienzyme complexes, nuclear pores, and many others. Some, such as the fibrin clot, occur in a statistical distribution of sizes and configurations. Others, such as microtubules or deoxyhemoglobin S fibers, are well-defined in a repeating pattern but are not naturally limited to a specified length. However, the biological activity of many macromolecular assemblages demands that they be specific, precisely defined entities. Mechanisms must exist in the assembly process to avoid uncontrolled or unlimited aggregation. This is not a trivial problem when the ensemble is a complex structure that comprises dozens of subunits, many of which may be identical or very similar.

There is, of course, a simple solution to the design of unique structures from multiple copies of identical subunits. This is by the use of one of the closed-point symmetry groups—cyclic, dihedral, tetrahedral, octahedral, or icosahedral—as happens in many oligomeric proteins (2). Symmetry properties can have important functional consequences as in the Monod, Wyman, and Changeaux interpretation of cooperative interactions of proteins with ligands and substrates (3). Symmetry is also important in the economical packaging of genetic information in viruses. But, for most spherical viruses, simple point group symmetry does not suffice. More "identical" subunits exist than can be accommodated by the 60 equivalent positions of icoshahedral point symmetry. Caspar and Klug introduced the principle of quasiequivalence to solve this problem in their seminal paper on virus construction (4). High-resolution crystallographic structures of RNA plant viruses (5–7) have confirmed the spirit if not the literal predictions of this theory (8).

The icosahedral viruses provide a fascinating and instructive arena for the study of macromolecular assembly properties, especially in light of the theoretical foundation provided by Caspar and Klug. However, other important structures are very differently constructed and

other rules of assembly apply. These systems, often characterized by quasisymmetry and hierarchies of symmetry, also pose interesting assembly questions.

One such system is the acetylcholine receptor. Each receptor unit comprises five polypeptide chains in the stoichiometry of $\alpha_2\beta\gamma\delta$. The chains are homologous in sequence at the 40% level (9). Electron microscopy and labeling studies show that the protein subunits are arranged in a cylindrical wall about a central cavity in the cyclic order of $-\beta-\alpha-\gamma-\alpha-\delta-$ (10). This poses very interesting questions of how the two identical α-chains assemble to adapt specifically to distinctive yet similar environments.

Another significant class of proteins that present puzzles in assembly are the proton ATPases from mitrochondria and chloroplasts. The soluble catalytic F_1 factor of these enzymes consists of five kinds of polypeptide chains in the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$. Sequences of the large α and β chains are found to be highly homologous, whereas the minor chains are unique and smaller (11). Quite unexpectedly in light of the composition, F_1 molecules lie on twofold axes in crystals (12). These results raise questions about how identical chains achieve specialization and about how chemically distinct chains behave identically.

Phycobilisomes provoke another set of assembly questions. These light-harvesting pigments in cyanobacteria and red algae are large ($6–7 \times 10^6$ d) complexes of stacked discs arrayed around a central core. The elements of the disc are phycocyanins or phycoerythrins that have the composition $(\alpha\beta)_6$ and have D_3 point symmetry (13, 14). Each disc has two or three such units in structurally distinct sites. These are connected by "linker" polypeptides to the core allophycocyanins and to one another (15). The role of linker proteins helps to explain the assembly properties of phycobilisomes, but questions still remain about how specificity is achieved in this complex hierarchy of symmetries and how the unique linkers interact with the symmetric phycoproteins.

Many of the same assembly questions that are encountered in these and other systems also arise from work in the giant respiratory proteins of invertebrates.

Giant Respiratory Proteins

The molluscan hemocyanins appear to be organized with C_5 or D_5 symmetry, depending on the species, and have two polypeptide chains in the asymmetric unit. Each of these chains is a remarkable string of seven or eight domains that can be cleaved into functional 50,000-d oxygen-binding units. The assembly properties of these exceedingly large and complex molecules is certainly of interest, but relatively little is presently known about structural details (16).

The state of knowledge about arthropodan hemocyanins is more advanced. The simplest of these molecules is a hexamer of 70,000-d subunits related by D_3 point symmetry. The crystal structure of such a hemocyanin from the spiny lobster has recently been determined at 3.2 Å resolution by Hol and colleagues (17). Anthropodan hemocyanins are composed of heterogeneous subunits and these are associated in multiples of 1, 2, 4, or 8 hexamers depending on the species. Lamy and coworkers have found that the 24-mer of scorpion hemocyanin consists of eight electrophoretically and immunologically distinct subunits. These subunits occur in stoichiometric amounts and their positions in the molecule have been reproducibly located in electron micrographs of molecules labeled by specific F_{ab} antibody fragments (18, 19) as shown in Fig. 1. Amino-acid sequences indicate that the subunits within one species and also between different species are similar (20).

Clearly, if all subunits were identical, a molecule constructed in such a hierarchy of symmetries would not be self-limited. Scorpion hemocyanin has evolved an elegant solution to this problem. The distinctive roles of quasisymmetrically related subunits permit uniqueness through variations on a simple theme.

The erythrocruorins found freely dissolved in the bloods of annelids bind oxygen at the iron atoms of heme groups as in hemoglobin. However, unlike their mammalian counterparts, these molecules are very large and not all of the protein subunits bear hemes. Functionally, they are highly cooperative, with Hill coefficients as high as 5.5. There is an abundant literature on erythrocruorins and many features are clear, but there is also considerable confusion. Details will be dealt with in the presentation of results. By way of introduction, let it suffice to give a brief overview. These are giant molecules with a mass of nearly four million daltons each. They are arranged in two apposed layers of six-membered rings that can be dissociated into twelve major subunits. The 1/12th particles can in turn be further dissociated into a few kinds of 15–20,000 d chains. Some of these chains are like the mammalian globins, but others appear to be nonhelical and without heme. It seems clear that the annelid hemoglobins are organized in an

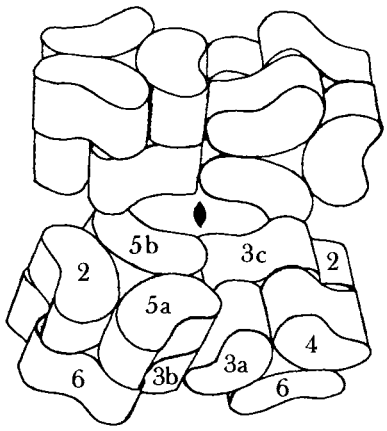


FIGURE 1 A schematic representation of the quaternary structure of scorpion hemocyanins. The locations of distinctive subunit types as identified by immune microscopy are identified. The hidden subunits within hexamers are of type 4 on the left and type 5a on the right. Adapted from Lamy et al. (19).

intricate hierarchy of symmetries, but the actual configuration of the 200-odd subunits in this assemblage and the mechanisms by which spontaneous, self-limited assembly is achieved remain obscure.

In following sections we present our experimental observations on annelid erythrocruorins and elaborate a structural hypothesis. These experimental results have not been reported previously, although in some cases we have had the data for some time. We use these results and related findings published by others to illustrate pertinent properties of the erythrocruorins. The essential features of the theoretical work have been presented before in the proceedings of a conference on invertebrate oxygen carriers (21). The model is certainly too simplistic and it may well be wrong even in some fundamental respects. However, it remains compatible with the more recent experiments and it serves as a working hypothesis to focus thinking and experimentation. More importantly in the present context, it also illustrates principles in the assembly of molecules organized in a heirarchy of symmetries.

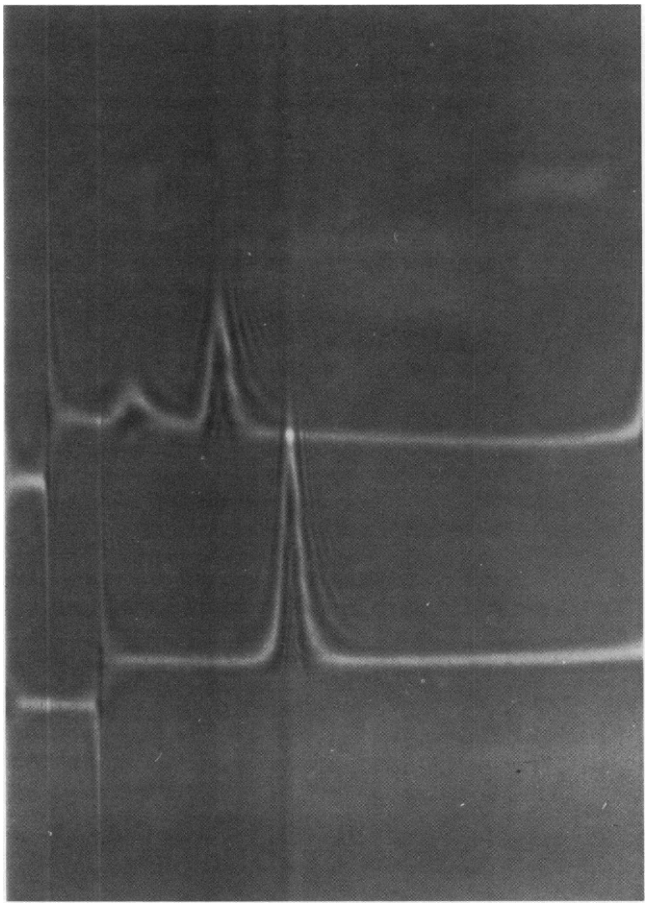


FIGURE 2 Schlieren pattern at 16 min after achieving 60,000 rpm in a sedimentation run on a Beckman Model E analytical ultracentrifuge. *Upper* trace: *Amphitrite* erythrocruorin in 0.5 M NaCl. The two components are sedimenting with apparent velocities of $S_{20,s}$ = 24 and 48. *Lower* trace: *Arenicola* erythrocruorin in 0.5 M NaCl. This protein is sedimenting with a velocity of $S_{20,s}$ = 52.

BIOPHYSICAL CHARACTERIZATION

Ultracentrifugation

Annelid erythrocruorins were among the first samples examined by Svedberg in his pioneering applications of the ultracentrifuge in the physical characterization of macromolecules. Hemoglobins from *Arenicola marina* and from *Lumbricus terrestris* were found to sediment with a velocity of roughly 60S and at alkaline pH (>9) they were dissociated into 11S particles (22). These results suggested that the subunit structure of annelid hemoglobins could be investigated by controlled dissociation. We have carried out a series of ultracentrifuge studies with this purpose in mind. This work was conducted at the Marine Biological Laboratory with a Beckman Model E ultracentrifuge (Beckman Spinco Co., Palo Alto, CA). The erythrocruorins from two annelids indigenous to Woods Hole (and still available when this work was done in 1965) were examined. These species are the lugworm *Arenicola cristata* and the marine terebellid *Amphitrite ornata*.

These experiments showed a rich variety of dissociation products. Both native hemoglobins sediment with velocities similar to those observed by Svedberg, but they behave somewhat differently under dissociating conditions. Sedimentation profiles from a typical centrifuge run are shown in Fig. 2. *Arenicola* erythrocruorin is stable even at dilute protein concentrations, and hence it was possible in this case to obtain an accurate sedimentation constant extrapolated to infinite dilution. The result was $S_{20,w}$ = 55.3 Svedberg units. In the presence of 1 M $MgCl_2$, a known dissociating agent, these molecules sediment with S_{20} = 27.6. *Amphitrite* erythrocruorin is more labile and it dissociates into a variety of species as the protein is diluted or the salt concentration is reduced. Most cases result in multicomponent systems so that the determination of accurate intrinsic sedimentation constants is not possible. Nonetheless, these results suffice for a qualitative assessment of aggregation states and their approximate populations as is shown in Table I.

The correspondence between sedimentation velocity and molecular mass is, of course, complicated by other hydrodynamic parameters such as the frictional coefficient and partial specific volume. However, with recourse to plausible symmetry of division and theoretical relationships regarding shape factors, it is possible to make meaningful interpretations. This is done in Table II with reference to the structural model discussed in a later section. This framework of classification also encompasses the earlier results on earthworm (*Lumbricus*) erythrocruorins (22) and results published later on these and other annelid hemoglobins (23–29). All of the dissociation products seen in any of these cases are represented among the components identified in this work on *Arenicola* and *Amphitrite* erythrocruorins.

TABLE I
DISSOCIATION PRODUCTS OF ANNELID ERYTHROCRUORINS COMPOSITION IN SEDIMENTATION COMPONENTS

Species and conditions	2S	4S	11S	15S	28S	60S
Arenicola						
oxy (0.1M NaCl)						100%
oxy (1.0M NaCl)					100%	
oxy (8M Urea)	100%					
oxy (5M Gn HCl)	100%					
Amphitrite						
oxy (Sea water)						100%
oxy (0.5M NaCl)					30%	70%
oxy (0.1M NaCl)			20%		65%	15%
oxy (1.0M $MgCl_2$)				40%	60%	
oxy (28mM PO_4)			60%		40%	
deoxy (28mM PO_4, dithionite)			100%			
deoxy (5.5M Urea)		100%				

TABLE II
SEDIMENTATION VELOCITY OF DISSOCIATION PRODUCTS FROM ANNELID ERYTHROCRUORINS

Aggregate	Model mass ($d \times 10^3$)	Trion units	Calculated velocity		Observed velocity (S)					
			Equivalent sphere*	Kirkwood theory‡	Aren.§	Amph.	Lumb.	Spir.	Euni.	Haem.
One chain	18	1/3	1.7		~2		2.3		~2	2.2
Two chains		2/3	2.7							
Trion	54	1	3.5	3.5		~4	3.5			4.2
Ditrion		2	5.6	5.2						
Tritron		3	7.3	7.0						
Hexatrion	324	6	11.6	11.7		~11	10.1	~10	~10	
Ring Dimer		12	18.3	17.7						
Stack Dimer		12	18.3	18.0		~15		~15	~16	
Tetramer		24	29.1	28.4	27.6	~25				
Ring Hexamer		36	38.2	33.7						
Dodecamer	3,888	72	60.6	57.8	55.3	56	61.1	57.5	57.0	59.1

*$S_n = n^{2/3} S_1$

‡$S_n = (1 + \sigma_n) S_1$ where $\sigma_n = (1/n)\, r_1 \Sigma_i \Sigma_{j \neq 1} d_{ij}^{-1}$ where r_1 is the hydrated sphere radius of a unit and d_{ij} is the distance between the i^{th} and j^{th} units of the model shown in Fig. 7.

§Erythrocruorin sources are identified here by an abbreviation of the animal's genus name. The reported data are from the first of the references listed below in ultracentrifugation studies on these hemoglobins: *Arenicola* (this work, 20, 21), *Amphitrite* (this work, 26), *Lumbricus* (23, 20, 22), *Spirographis* (27), *Eunice* (25), *Haemopsis* (24).

Electron Microscopy

Objects as large as annelid erythrocruorins can be readily visualized in the electron microscope. Roche et al. (30) and Levin (31) first obtained electron micrographs of these molecules and demonstrated their characteristic double-layered structure of six-membered rings. Our own 1965 micrographs and other later results (32–35) conform to this general picture.

Face-on images of the six-membered rings tend to exhibit sixfold symmetry. This has prompted several workers to use rotational averaging in image processing (34). However, theoretical considerations (21), as discussed in a later section, suggest that the true particle symmetry might be D_3 rather than D_6 as previously supposed (1, 34–36). While the proposed model could have approximate sixfold symmetry in projection, significant departures would not be unexpected. We have recently undertaken a new electron microscopic study with a view toward testing this hypothesis.

Electron microscopy of earthworm erythrocruorin was carried out together with Harold Erickson at Duke University. Specimens were prepared from solutions of hemoglobin freshly dissolved into 0.1 M NaCl buffered with phosphate to pH 7.5 from Type II crystal (see below). Grids were negatively stained with uranyl acetate and examined in a Phillips 301 electron microscope (Mahwah, NJ). Representative areas from two of the micrographs are shown in Fig. 3 and a few selected images are shown in Fig. 4.

Several characteristic views of the molecules are found reproducibly in these micrographs. About 45% of the full molecules occur in the face-on "hexagonal" view; another 25% lie in side views of two kinds (possibly corresponding to on-edge and on-point hexagons); 10% are found in an oblique view; and 20% could not be characterized. Typical stained images measure ~260 Å between hexagonal edges and ~160 Å in height in the side views. Many of the images show considerable sub-structural detail including distinctive densely staining centers in the 1/12th subunits as seen both in "hexagons" and side views. Occasional dissociation products are also seen. These are primarily "square" one-third molecule arrays of four subunits and isolated 1/12th particles.

Many of the "hexagons" appear actually to have threefold rather than sixfold symmetry. Examples are included in Fig 4. The distinctions are somewhat subtle, but they are particularly evident in the pattern of staining in the septa that divide the six projected molecular sections. We judge from a survey of 123 "hexagons" that 45% of the images show threefold character.

Spectroscopy

Erythrocruorin is the dominant protein in annelid blood and, because of its large size, it can readily be purified by repeated ultracentrifugation. Despite high purity these molecules give a relatively low ratio of heme-specific to protein-specific absorption. We measured a ratio of $OD_{540}/OD_{280} = 0.28$ for *Arenicola* oxyhemoglobin. A similar heme-to-protein ratio (0.27) was found for earthworm hemoglobin (37). The comparable ratio for human hemoglobin is 0.40. Both *Arenicola* and *Lumbricus* hemoglobin have an aromatic content similar to that in human hemoglobin (23, 37). Given that these erythrocruorins consist of 18,000-d chains, it thus appears that only about two of every three possesses heme.

Recently, with help from Dr. B. A. Wallace, we have measured and analyzed circular dichroism data from earthworm erythrocruorin. Type III and type II crystals of *Lumbricus* erythrocruorin (see below) were freshly dissolved into 10 mM phosphate buffer at pH 7.5 that had been bubbled with carbonmonoxide gas. Spectra were recorded on an AVIV (Lakewood, NJ) model 60DS Circular Dichroism Spectropolarimeter that had been calibrated with d-10-camphor sulforic acid at 290 nm and at 192.5 nm. Spectra were taken from five samples of dissolved single crystals by averaging six scans measured every 2 Å in the range from 190–240 nm.

The composition of secondary structure in the erythrocruorin was determined from these data. This was done by a nonlinear (constrained) least-squares fitting to the experimental spectra with normalization as described by Mao et al. (38) to a reference set of CD data (39). This procedure does not depend on precise knowledge of concentration. Excellent fits were obtained. The analysis gave values of 47.4 ± 5.7% for α-helix and 32.0 ± 5.3% for β-sheet in the erythrocruorin, whereas a similar analysis on horse myoglobin gives values of 78.9% and 0.7% (B. A. Wallace, personal communication). Representative spectra are shown in Fig. 5. Others have also found a low helix content in erythrocruorins (40–42, 26–29), but the β-sheet composition has not been reported before. These results are consistent with about two of every three chains being largely helical as in mammalian hemoglobins, while the third is primarily a β-sheet subunit.

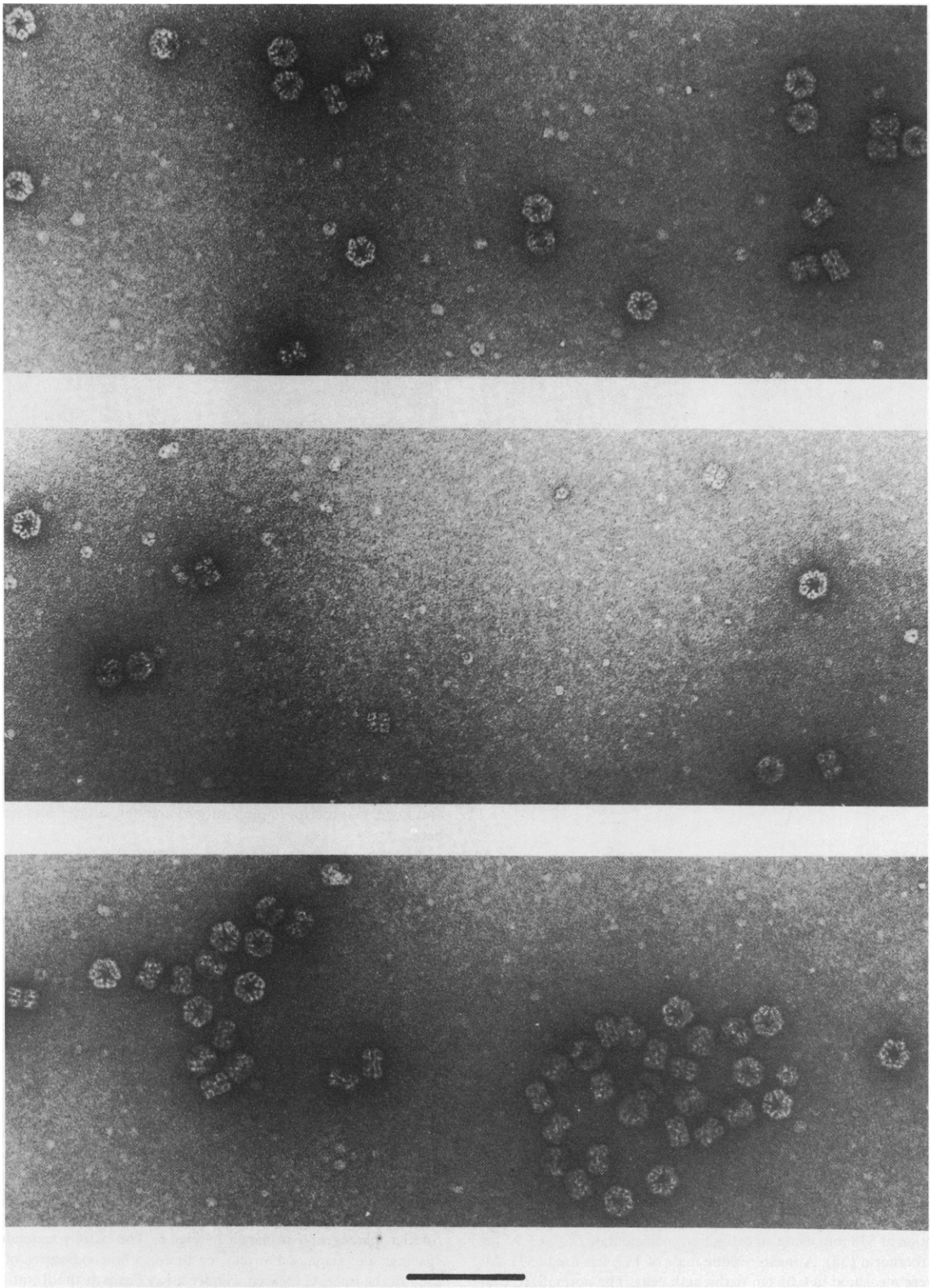


FIGURE 3 Portions of electron micrographs of negatively stained erythrocruorin molecules. The *Lumbricus* erythrocruorin was freshly dissolved into 0.1 M NaCl at pH 7.5 from Type IIb crystals, then dropped onto carbon-coated grids, and negatively stained with uranyl acetate. The bar corresponds to 1,000 Å. The micrograph was taken by H. P. Erickson at Duke University.

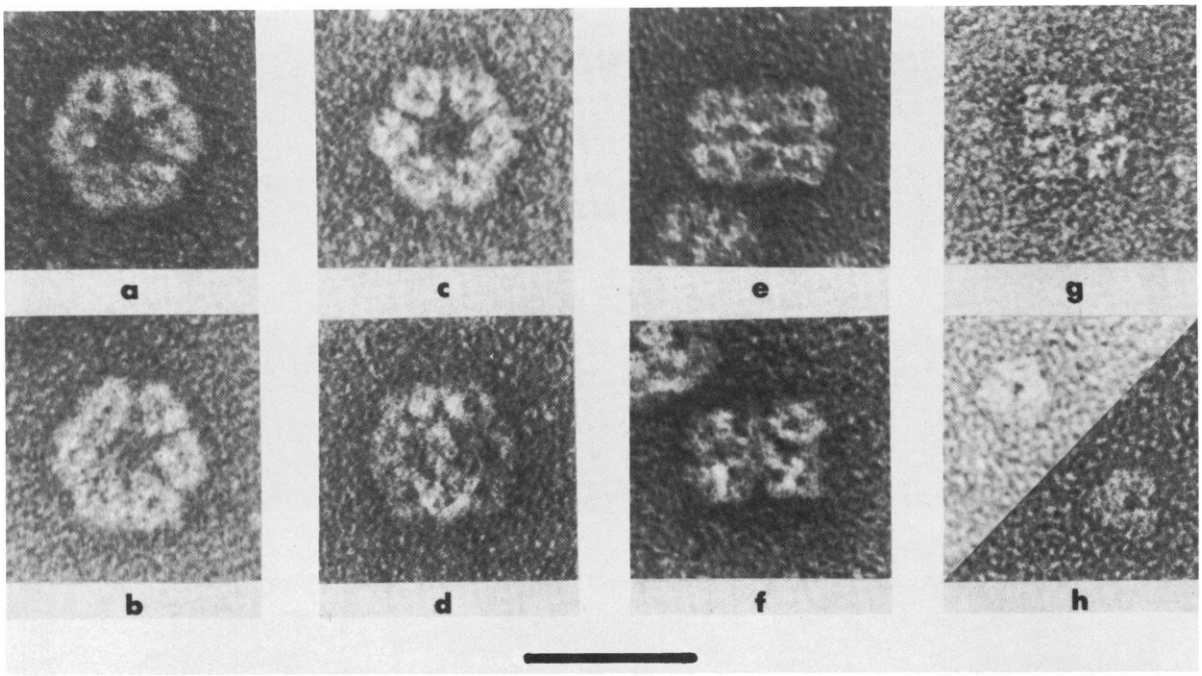


FIGURE 4 A gallery of electron micrographs of erythrocruorin molecules from *Lumbricus terrestris* enlarged from specific areas of micrographs such as those shown in Fig. 3. The bar corresponds to 300 Å. (*a–b*): Face-on views showing threefold character; (*c*) face-on view; (*d*) oblique view; (*e*) side-on-point view; (*f*) side-on-edge view; (*g*) tetramer view; and (*h*) 1/12th particles.

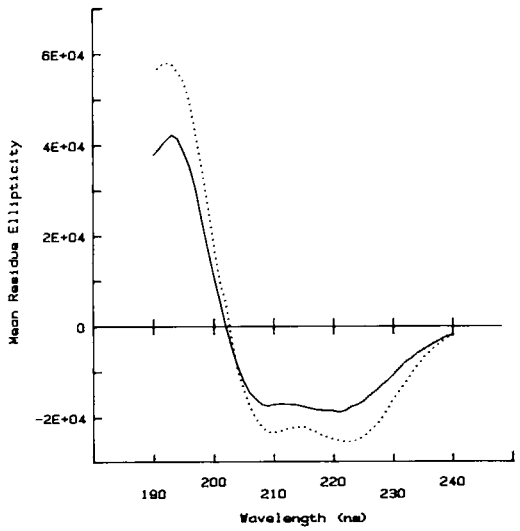


FIGURE 5 Normalized circular dichroism spectrum of earthworm erythrocruorin (—) plotted along with that from horse myoglobin (· · ·). Note the difference not only in the magnitudes of the curves, but also in the shape. An average helix length of 13.4 residues was used in both fits. The erythrocruorin concentration was calculated to be 0.18 mg/ml based on absorption at 538 mm using an extinction coefficient of $E_{1\%}^{1cm} = 5.97$ for CO erythrocruorin (38). A mean residue mass of 117 was used (39). All spectra were measured in 0.05 cm pathlength cells. The normalized best fit suggested the concentration was actually 0.165 mg/ml. The normalized spectra gave a helix content of 53% and β sheet content of 30%, while use of unnormalized spectra would give α helix content of 49%, β sheet of 27%. The normalized standard deviation (38) was 0.032 for this erythrocruorin spectrum, and 0.030 for the myoglobin spectrum.

Diffraction

Erythrocruorin from the earthworm (*L. terrestris*) has been crystallized in a form suitable for diffraction analysis. Four different types of crystals have been grown, two of which are obvious variants of one another. An account of one of these, Type IIb, has been given previously (36) although the reported lattice parameters are somewhat in error. A full description of the various crystals will be published elsewhere (Royer, Hendrickson and Love, manuscript in preparation) and only a brief summary is given here.

Type I crystals were grown from a purified protein sample at pH 5.6 using polyethylene glycol (PEG) as a precipitant. These crystals have not been fully characterized. Type IIb crystals are also grown from PEG, but from whole blood at higher pH (7.5) and in the presence of >0.1 M phosphate. These crystals are triclinic with one whole molecule in the unit cell. The diffraction pattern extends to ~8 Å spacings and appreciable mosaic character is evident. Type II*a* crystals exhibit a curious tripling of the *a*-axis. They grow when the phosphate concentration is lower but conditions are otherwise similar. Type III crystals are also grown from whole blood, but in this case from a high concentration of phosphate (1.8 M).

The Type III crystals are well-formed prisms that grow quite large (0.5 × 0.5 × 1.0 mm). They are in space group $C222_1$ and have unit cell dimensions of a = 503, b = 299 and c = 348 Å. The asymmetric unit contains a half molecule, and packing considerations confine the structure to a pseudo-hexagonal array compatible with the C-centered unit cell. We have recently measured a complete set of diffraction data from these crystals at the Stanford Synchrotron Radiation Laboratory. A typical rotation photograph is shown in Fig. 6. The pattern extends to 3.8 Å spacings and remained strong for three to four exposures of 3–10 min each in the intense, focused wiggler x-ray beam at the Rotation Camera Facility. Somewhat inferior photographs required 72 h of exposure on a conventional x-ray source with double-mirror focusing. A structural analysis based on the Type III diffraction data is in progress.

Diffraction studies have also been useful in molecular weight determinations. Until recently molecular weight estimates had been near 3.0

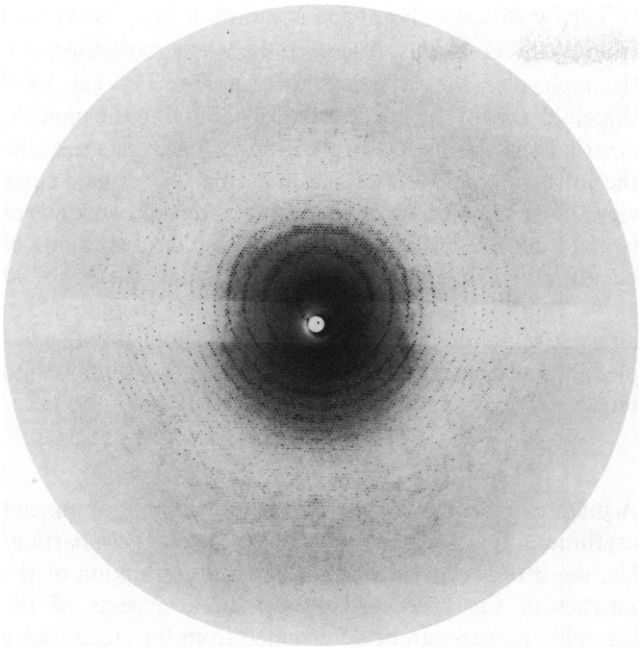


FIGURE 6 Rotation photograph of a Type III crystal of earthworm erythrocruorin. The crystal was oriented with the *a*-axis along the rotation axis (horizontal) and was rotated through 0.8° with the x-ray beam directed at ~2° from the *c*-axis. The face centering produces a roughly hexagonal net. However, we note a few violations of the C-centering. Reflections can be seen that correspond to 3.8 Å spacing.

million. However, Pilz et al. (43) found a molecular mass of 3.9 million daltons from small-angle x-ray scattering. Recent sedimentation equilibrium results also conform to the higher value (26).

Chemical Composition

Although we have not ourselves attempted to characterize the polypeptide chain composition of erythrocruorins, it is important here to summarize the findings of others. Attempts at understanding the structural properties of annelid erythrocruorins have been frustrated by confusing, complicated and often conflicting chemical characterizations.

Polypeptide chain separations under denaturing conditions generally show 2–4 bands in the 12–19,000 d range (23, 26, 28, 37, 44–46) but higher molecular weight components are also present in most cases. Interchain disulfide linkages (23, 44, 45) and other aggregation properties tend to prevent complete separation. In particular, in the absence of reducing agents all or much of the material is found at positions corresponding to ~54,000 d.

Recently, progress has been made in the chromatographic separation of native functional heme-containing subunits in the presence of dithiothreitol (46–48). In these studies, nearly all of the protein is found in 18,000-d components that each contain heme. This is puzzling since it conflicts with spectroscopic results on specific absorption ratios and the overall heme content, which is typically found to be one heme per 26,000 d of erythrocruorin (26, 28, 33, 37, 40, 44).

Amino-acid sequence determinations have been reported for three polypeptide chains. Garlick and Riggs determined the sequence of a 157-residue chain from *Lumbricus* erythrocruorin (49) and found it to be distinctly, though weakly, homologous to mammalian hemoglobins. Suzuki and coworkers have sequenced 139-residue and 149-residue chains from *Tylorrhynchus* erythrocruorin and also found homology with other globins (48, 50). About 25% of the residues are the same in any pair of these sequences. In earthworm erythrocruorin, Garlick and Riggs found equimolar amounts of three chains, all of which are similar in length. Two chains, including the one that has been sequenced, are similar in amino-acid composition, but are markedly different in composition from the third, suggesting that the third might be a nonglobin chain (49). However, the situation appears to be more complex since there is also evidence for a fourth kind of chain in this molecule (37; K. Fushitani, personal communication).

STRUCTURAL HYPOTHESIS

In an effort to rationalize the diverse experimental results on the erythrocruorins from annelids, we have developed a hypothetical structural model. The available data are not yet definitive with respect to structure; however, they do constrain the possibilities. An important premise of the model is that a universal architectural pattern is likely to pertain to all annelid erythrocruorins. Thus we make use of data from the proteins of various species. Still, the lack of definitiveness precludes a strictly deductive description. Instead we present the model as a postulate and then compare its properties with the observations.

Subunit Constitution

We postulate that the erythrocruorin molecule is composed of equal amounts of three kinds of polypeptide chains. Two of the chains (α and β) have the myoglobin fold and contain heme. The third chain (γ) is radically different; it has no heme, little if any helical secondary structure, and appreciable β sheet content. Each of these subunits has a molecular mass of 18,000 d.

The model was influenced by the seemingly simple subunit compositon of earthworm (37) and *Amphitrite* (28) erythrocruorins for which only three kinds of chains appeared to be present and in about equal amounts. This presents the most stringent condition for the assembly process; additional subunit diversity can readily be accommodated.

Hierarchical Association

We propose that the folded chains are organized in a hierarchy of associations:

(*a*) In the first hierarchy of association, two hemoglobin chains (not necessarily one of each kind) and the unique hemeless chain (γ) form a tight trimeric complex which we call a "trion."

(*b*) In the second hierarchy of association, six of the three-chains (trions) form specific hexameric aggregates (hexatrions).

(*c*) In the final level of hierarchy twelve hexatrions associate to complete the molecular assembly.

This model has a total of 216 chains ($3 \times 6 \times 12$) of which 144 contain heme. It has a molecular mass of 3,888,000 d, which compares well with experimental values. The calculated heme concentration is one heme per 27,000 d as typically observed. If we assume 70% helical content for the α and β subunits and no helical structure in the γ subunit, the net helix content is 47% as seen by circular dichorism. A subunit with nearly total β-sheet secondary structure would explain our CD results (~32% sheet).

The subunit structure in this model is like that of Wood et al. (24) in the last two stages and it is rather like that of Garlick and Riggs (37) in all these stages. However, we give an explicit description of secondary structure in the γ-chain to account for the CD data.

Quarternary Structure

A detailed description of the structure clearly requires crystallographic analysis. However, the ultracentrifugation and electron microscopy results also place important constraints on the three-dimensional models. Other constraints derive from the assumption that two-thirds of the subunits have a myoglobin fold. Based on these and other clues, we propose the following simplified model, which can be visualized most readily with reference to Fig. 7.

(*a*) The three-chain units (trions) can be inscribed in spherical envelopes such that interspherical contacts adequately describe actual intersubunit contacts between trions.

(*b*) The hexamers of three-chain spheres (hexatrions) are arranged as trigonal antiprisms with D_3 point symmetry.

(*c*) The whole molecule also has D_3 symmetry, but its asymmetric unit comprises two hexatrions.

(*d*) The symmetry elements are ordered in a hierarchy to produce a closest packing of spheres arrangement.

One of many conceivable arrangements for three chains within a trion unit is shown in Fig. 7 *a*. This drawing has the two globin chains related as in the $\alpha_1\beta_2$ dimer of human hemoglobin and the unique γ chain fills in the third portion. Perhaps an $\alpha_1\beta_1$ association is more probable. Indeed the γ chain might be analogous with the heavy chain of haptoglobin that binds to an $\alpha_1\beta_1$ dimer in the haptoglobin:hemoglobin complex (52). Haptoglobin heavy chains are homologous with the serine proteases (53) which are nonheme, β-structure proteins of a size appropriate to the proposed γ chain.

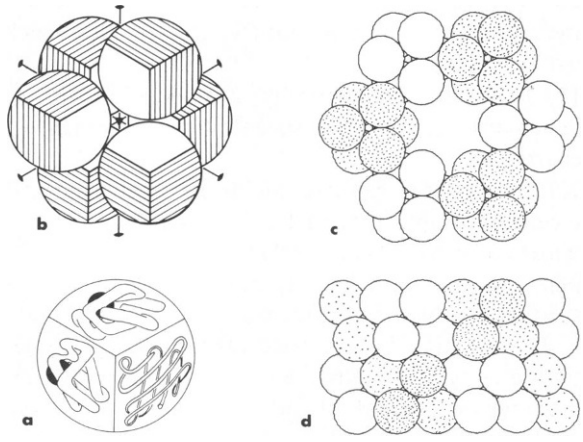


FIGURE 7 A model for the structure of annelid erythrocruorins: (*a*) schematic of a conceivable single trion; (*b*) a hexatrion; (*c*) triad view of a whole molecule; and (*d*) diad view from the front of *c*. (Reproduced, with permission, from reference 21.)

The hypothetical hexatrion is shown in Fig. 7 as viewed from its threefold axis. A view of the whole molecule down the molecular threefold is shown in Fig. 7 *c*. The local threefold axes of the hexatrions are parallel to the molecular triad and this naturally predisposes these units to make the intra-ring contacts of the molecule. A perpendicular view, down a molecular diad looking at one of two kinds of twofold contacts, is shown in Fig. 7 *d*. The two kinds of hexatrions (plain and stippled) are related by local twofold axes. This diagram shows the two layers staggered. An alternative eclipsed arrangement with stippled over stippled is inconsistent with the observed disassembly into tetrahexatrions (Table II).

ASSEMBLY CONSIDERATIONS

A unique description of the course of assembly of annelid erythrocruorins is surely impossible at present. In particular, too little is known even to begin a description of the kinetics of the process. However some aspects of the assembly process can be determined from the steady state condition. These are an integral part of the structural model and must be addressed. The assembly of different macromolecular aggregates is achieved in various ways and there are no fixed governing rules. Despite the variety, some general principles usually apply. These include the following:

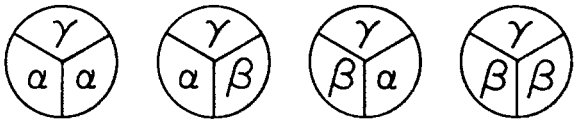
Spontaneous and unique assembly. While there are assembly processes that require scaffoldings or enzymatic attack, simpler mechanisms more often apply. Usually, the structure of the elementary components and assembly intermediates themselves are such that nonproductive pathways are avoided.

Self-limited aggregation. The molecular design must be such that association does not continue indefinitely. This is not a problem for simple, closed symmetry groups because equivalent bonding sites are then internally saturated. However, for assemblages constructed in a hierarchy of symmetries, unlimited propagation might not be inherently precluded.

Genetic and biochemical economy. Simplicity of design is to be expected. Proliferation of heterogeneous components places a burden on the genome, and complicated processes are apt to require excessive biochemical energy. These are not likely to provide selective advantage without good reason.

It is instructive to consider these principles in relation to the proposed hierarchal model for annelid erythrocruorins. If all trions were identical (e.g., always $\alpha{:}\beta{:}\gamma$ in one specific arrangement or with only one kind of hemoglobin chain), then the same bonding surfaces used in and between the six-membered rings would also be exposed on the molecular surfaces. This would naturally cause further propagation. Similarly, if the two kinds of hexatrions were identical, inter-ring bonding surfaces used in one configuration would be presented to the exterior in another. This would allow aberrant side propagation.

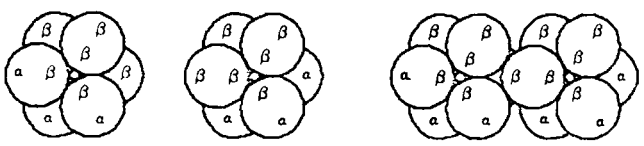
In the present model there are two kinds of hemoglobin chains. If we assume an approximate symmetry between α and β chains such that they are interchangeable within a trion, then there are four possible trion isomers:



If there were three kinds of hemoglobin chains, nine such isomers could exist. Then each three-chain unit in a hexatrion could be unique, but some isomers would be left over. Additional variation might arise from conformational heterogeneity. Although the compact globin units would not be expected to produce distinctive conformational isomers, alternative subunit contacts within trions that could switch depending upon the environment within the hexatrion would be plausible. However, our immediate purpose here is to ascertain the minimal requirements in assembly properties. For this, it suffices to consider four trion isomers composed from three chain types as above.

Each of the chain sites in a trion must have a unique position in the hexatrion. The chains in these positions then offer certain surfaces for specific intersubunit contacts. If α and β chains are endowed with differing bonding potentials at the various contact surfaces, then it is possible to form hexatrions that have the necessary properties for unique, spontaneous and self-limiting assembly. For simplicity in self-assembly we assume that α, β, and γ chains occur in equimolar amounts and further that the four isomers are equally likely. Since the four isomers are taken six at a time, this means that there must be at least two kinds of hexatrions. This is consistent with the two kinds of hexatrions in the structural model shown in Fig. 7.

As a sort of proof of existence, we have constructed one of many possible assembly models. This model gives γ-chains the property of a central glue that holds hexatrions together but does not involve them in inter-hexatrion contacts. One of the hemoglobin positions has exposed surfaces that are involved in axial inter-ring bonds on one side of the hexatrion and ones that are nonbonding on the quasisymmetric opposite site. The other hemoglobin position has surfaces that form the equatorial intra-ring bonds. The two hexatrions of this specific model are pictured at left below:



Hidden positions on the underside near the threefold axis are occupied by α chains. If α-chain surfaces are thought of as anti-bonding and β-chain surfaces as bonding units, then these structures would have the desired unique assembly properties. A bonded dimer is shown on the right above. These dimers can naturally be perpetuated into a ring of six hexatrions, and two rings related by diads and bonded by axial β units produce a unique whole molecule. The proposed configuration precludes abortive intermediates and has self-limited assembly properties.

VERIFICATIONS AND RAMIFICATIONS

The proposed model for the structure and assembly of annelid erythrocruorins explains several experimental observations that were not used in its construction. Some of these have been described previously (21). Let it suffice here merely to summarize: The molecular dimensions agree very well with those seen in electron micrographs. A packing arrangement of the simple hard-sphere model reproduces the triclinic Type IIb lattice parameters to within 3 Å and 4 Å. Relative sedimentation velocities (Table II) and the existence of tetramers of hexatrions are consistent with this model (and difficult to rationalize with D_6 symmetry). Observed heterogeneity in undenatured 1/12th particles and 54,000 d units (23) is consistent with the proposed isomeric composition. The unusual filled-hexagon structures of certain erythrocruorins (53) can be explained by a seventh hexatrion with appropriate bonding surfaces. And finally, threefold character found in micrographs of hexagonal views is consistent with the proposed symmetry.

On the other hand, some newer data seem to be at odds with the model. There are recent suggestions that all purified chains contain heme and that disulfide-bridged trimers of such chains occur (48; K. Fushitani, personal communication). Perhaps there are both all-hemoglobin and hemoglobin/γ-chain trions. However, these characterizations are certainly incompatible with spectroscopic results in helicity and heme content.

Additional factors not considered directly here may also have implications for the assembly of erythrocruorins. First, at least in some cases, hydrogen ions and divalent cations are required to maintain the assembled structure (24, 28, 54, 55). Secondly, the cooperative character of oxygen binding to these molecules must be considered. Alterations in quaternary structure are very likely involved and the presence of disulfide-bridged trimers suggests that these alterations may be quite different from those in mammalian hemoglobins. While a role for interactions between 1/12 particles has not been ruled out, there are indications that such interactions are not essential for full cooperative binding (56). In fully cooperative (n = 5.5) *Octolasium* erythrocruorin, a fit of oxygen-binding data to the MWC model yields 12 interacting heme groups (55). This corresponds to the number of heme groups in the 1/12th particle in our present model.

A definitive resolution of structural questions about annelid erythrocruorins must await analysis of the diffraction data. Reconstructions from electron micrographs of the molecules (35) are of inadequate resolution to answer many of the interesting questions with respect to assembly.

Such results will however, be important in optimizing parameters of a molecular envelope model for initiation of the diffraction analysis. The model as it stands has aspects that are solidly grounded in experiment and others that are uncertain. The molecular dimensions are well established from the microscopy and crystal results. The dissociation data clearly demonstrate a hierarchal organization and point strongly to less than D_6 symmetry because 4 × (1/12th) particles are stable. The hydrodynamic data also indicate a rather compact structure for the 1/12th particles. It is quite secure that all chains are of a size like that postulated in the model and it seems inescapable that nonhelical and nonheme protein is present. However, details of the structural organization within the 1/12th particles is not well established. By using spherical trions, we strive to introduce as little unfounded information as possible while attempting to capture the essence of the geometric construction.

Although details of this particular model may be wrong, it is a concrete and useful construction for exploration of the principles of assembly that govern molecules organized in a hierarchy of symmetries. It is clear from the arthropod hemocyanin example (19) that specialized roles for approximately equivalent subunits can be important in this regard. The erythrocruorins seem to present an analogous situation. Within certain ground rules for bonding topologies, the implications of various isomeric configurations for spontaneous and self-limited assembly can be examined. When the true structure and true bonding topologies are known, similar considerations will apply to understanding the actual assembly process. Indeed the analysis for any assemblage of multiple components depends upon details of the geometry, but the logic of assembly considerations stays much the same.

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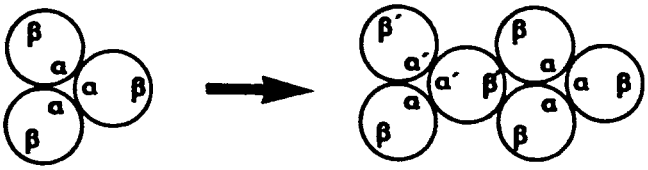
DISCUSSION

Session Chairman: Adrian Parsegian
Scribes: Madeline A. Shea and Dinakar Salunke

STUBBS: I am very impressed with this model. I would like to ask how you deal with the possiblity of aberrant hexatrion forms. Your model uses all four trion isomers in equal amounts, so no problem arises at that level, but there are many hexatrion isomers that are not used. Why would these isomers not form? In particular, what about an all-β isomer? Such an isomer would permit unlimited branched molecules to grow.

HENDRICKSON: I think the problem with having a hexatrion composed of a single type of chain can be illustrated by considering a model that would allow only two kinds of trions. Using the same symmetry suggested in our paper, we would be able, with such trions, to construct only one kind of hexatrion that would be a closed two-layer structure. When assembled, the structure could be extended laterally from the ring indefinitely by addition of similar hexatrions, violating the self-limited assembly principle. A model with four kinds of trions permits us to construct assemblies that leave no exposed bonding surfaces. To construct the two kinds of hexatrions, we must break the strict three-dimensonal symmetry in these hexatrions. If an all β hexatrion does form, it must be a low-energy, low-probability assembly.

STUBBS: That seems reasonable. However, I'd like to propose an alternative to this as shown in the figure below.



Here, the α subunits have the function of forming the hexatrions and the β subunits normally have the role of forming higher-order oligomers. This model postulates that conformational switching rather than special positioning of isomers determines the ultimate arrangement and limits growth.

When one hexatrion binds to the other in a polar way, one of them switches. The second kind of hexatrion is capable of bonding in a second direction but not in a third, thus limiting the assembly.

HENDRICKSON: You have proposed a kind of model that we intentionally did not consider: one that involves conformational distinctiveness. This certainly may happen. However, we know experimentally that there are at least three different polypeptide chains. This is sufficient to satisfy the criteria of unique and self-limited aggregation, and has the appeal of genetic and biochemical economy. Certainly we know that conformational switching is an important regulator in some systems, and it could create subunit differences. However, the origin of chain distinctions (chemical or conformational) is immaterial to our argument. I believe your alternative model focusses on dynamic intermediates in the process of assembly. Although such species are important and interesting, we barely understand the final (equilibrium) products of assembly in this system.

STUBBS: This alternative model of hexatrion forms and assembly suggests different roles for the most fundamental protein elements rather than a kinetic model.

CASPAR: Your analysis indicates that the annelid hemoglobin assembly has 32-point group symmetry, and that each asymmetric unit consists of 12 copies of three different types of molecules. Some kind of switching must be involved to control the assembly of such a structure. The subunits could be chemically modified to bond in different ways; or, as Gerald Stubbs has just suggested, they could be modified by conformational switching. In other structures of comparable complexity, such as icosahedral viruses, the parts can often be reassembled into polymorphic aggregates which display the variable bonding properties of the subunits. Can the annelid hemoglobins be taken apart and is it possible to reassemble subsets of the components?

HENDRICKSON: On the specific point of assembly intermediates or minimal assemblies, there have not been carefully controlled reconstitution studies that directly address this. No one has disassembled the particles keeping chains in their native states and there are no reservoirs of pure subunits available to allow mixing experiments.

CASPAR: What about the possibility of cloning the genes to get pure products?

HENDRICKSON: Clones are not available. But I'd like to respond to one of your other remarks by emphasizing the point of departure for this study. Switching could well be involved in the erythrocruorins, but I don't see it as essential. An illustration of an assembly regulated by chemical contacts is scorpion hemocyanin, shown in Fig. 1. Lamy and co-workers (see reference 18) have shown that it consists of four each of subunit types 2, 4, 5*a*, and 6, and two each of types 3*a*, 3*b*, 5*b*, and 3*c*. The subset of known chain sequences are similar but not identical; however, each has a very distinctive role. These molecules have very close to three-dimensional symmetry. Inner contacts are conserved as well as surfaces that may be used in further assembly of the 24-mers. In this case, chemical differences rather than conformational switches are known to control the assembly.

GLAZER: Presumably there are several ways of knowing whether you have any chemical variants of these chains that might arise from post-translational modification or other mechanisms. Can you partially reconstitute the assemblies? If so, what is the smallest particle that can be obtained that meets the criterion of morphologically uniform particles? If such reconstitution is impossible, it suggests that there are steps in the assembly pathway of the final materials that are not accessible under the in vitro conditions investigated.

HENDRICKSON: Erythrocruorin assemblies can be dissociated down to the level of the 1/12th particle (the hexatrion) and reassembled very easily. I am not aware of any studies that have allowed reversible disassembly of the subunits on a finer scale (trions or chains). The only similar studies were conducted by converting the hemoglobin chains to the hemichrome form; they were irreversibly chemically modified by the procedure.

SCHUSTER: In studying Tobacco Mosaic Virus (TMV), we have found that dynamic assembly properties are the most sensitive indicators of polymorphism. Have any such studies been done for these annelid erythrocruorins?

HENDRICKSON: I am not aware of any conducted under suitably controlled conditions. We know that these assemblies are polymorphic in the sense that there are several kinds of polypeptide chains. However, these have not been isolated, so proper reassembly studies cannot be conducted. In our case, we look to Bill Royer's diffraction experiment as the best hope for a clearer picture.

FRANK: Very subtle differences in images may be resolved using averaging and multivariate statistical analysis. In fact, the scorpion hemocyanin you showed is an example of where these methods have been successfully applied to examine subtler levels of architecture. Such an approach could confirm some of your predictions for the erythrocruorins. Referring specifically to your micrographs showing particles in axial projection, I wonder why they should sometimes express sixfold symmetry and sometimes threefold? How does this change in appearance occur? We conducted some pilot studies of these same assemblies and found only one cluster.

A multivariate statistical analysis of projections having threefold symmetry aligned in arbitrary sixfold-related orientations would yield two clusters. However, detection of the two clusters would depend on changes that would occur. Do you have a feeling for what changes to expect?

HENDRICKSON: The model that we have presented would have sixfold symmetry in a projection. Distinctions could come about because of bonding differences between the two kinds of pairwise stack-dimer interactions in the structure (see Fig. 7*c*, *d*). Moreover, the micrographs might show differences depending on the uniformity of staining in the two layers.

Correspondence analysis is critically important to do and we're anxious to have that done. However, we also hope to learn the answer to that independently from the diffraction analysis that is currently underway in our lab. Electron microscopy should provide us with a molecular envelope for the particle to then be used in the diffraction analysis.

FRANK: In the electron micrographs that you show (in Figs. 3 and 4), was a single-layer or a sandwich preparation used?

HENDRICKSON: A single-layer preparation was used.

BAYLEY: I admire the boldness of your interpretation of the circular dichroism studies. You believe that one trion chain may be totally lacking helical structure. Would you comment on the structures that would be predicted on the basis of the sequences that are available? Do you know how these sequences compare with those of other known hemoglobins?

HENDRICKSON: Comparison of a known earthworm erythrocruorin sequence with those of human β chain, lamprey hemoglobin, bloodworm hemoglobin, and leghemoglobin (legume hemoglobin) shows that it contains characteristic features of the myoglobin fold. The common, high helix content in the three-dimensional structures of these molecules leads us to believe that the globins of erythrocruorin are also highly helical. The boldness of our CD interpretation reflects our conviction that an indication of 30% β sheet is significantly different from the value of 3% found for myoglobin. Thus, there must be another chain that has a significantly different conformation.

RHODES: By controlling conditions, presumably pH and divalent cation concentrations, you have apparently generated stable assembly intermediates such as the ring dimer. Couldn't you selectively label either the α or β chains and verify the proposed model using a direct visualization technique such as electron microscopy?

HENDRICKSON: I should first clarify that when we have a dimer we don't know whether it is a ring or a stacked dimer. Perhaps you're thinking of antibody or biotin labels for specific chains. The problem is that this system is even more complicated than the scorpion hemocyanin assembly (Fig. 1). Even if we could selectively decorate α chains in each trion, we would not expect to be able to distinguish among the several kinds of interactions in which the α chains participate. Though it would give some information, it is unlikely to permit resolution of some of the key predictions and unique features of our model.