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Erythrocruorin from the Aquatic Snail *Helisoma trivolvis*. Quaternary Structure and Arrangement of Subunits

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ABSTRACT: The subunit structure of erythrocruorin from the planorbid snail *Helisoma trivolvis* was studied. The native protein was found to have a sedimentation coefficient of 34.7S and a molecular weight, as determined by sedimentation equilibrium, of 2.25×10^6. Iron and heme determinations gave 0.270 and 3.21%, corresponding to minimal molecular weights of 20 700 and 19 200, respectively. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence and absence of 2-mercaptoethanol gave single bands with mobilities corresponding to molecular weights of 1.9×10^5 and $\sim 4 \times 10^5$, respectively. Sedimentation equilibrium in 6 M guanidine hydrochloride in the presence and absence of 2-mercaptoethanol gave molecular weights of 1.87×10^5 and 3.82×10^5, respectively. We conclude that a *Helisoma* erythrocruorin molecule is composed of 12 single polypeptide chain subunits, each carrying 10 hemes. In the molecule, the subunits are grouped in pairs, the members of each pair being held together by disulfide bonds. We propose a model for the molecule composed of 12 spherical subunits arranged in a shell structure with tetrahedral symmetry. Projections of the model are consistent with 10-membered ring and rhombic profiles observed in the electron microscopy of negatively stained *H. trivolvis* erythrocruorin [Terwilliger, N. B., Terwilliger, R. C., & Schabtach, E. (1976) *Biochim. Biophys. Acta 453*, 101–110] and with hexagonal ring structures seen in the electron micrographs of *Planorbis corneus* erythrocruorin [Wood, E. J., & Mosby, L. J. (1975) *Biochem. J. 149*, 437–445].

Erythrocruorin is the name given to extracellular hemoglobins that occur mainly among species from the phyla Annelida, Arthropoda, and Mollusca (Chung & Ellerton, 1979; Wood, 1980; Terwilliger, 1980; Vinogradov, 1985). In the molluscs, erythrocruorin is found in some marine bivalves and in a family of pulmonate snails, the Planorbidae (Read, 1966; Bonaventura & Bonaventura, 1983; Terwilliger & Terwilliger, 1985). The first structural study of a planorbid erythrocruorin was carried out by Svedberg and Eriksson-Quensel (1934). Recent studies include those on erythrocruorin from *Biomphalaria glabrata* (Figueiredo et al., 1973; Almeida & Neves, 1974), *Planorbis* sp. (Waxman, 1975), *Planorbis corneus* (Wood & Mosby, 1975), and *Helisoma trivolvis* (Terwilliger et al., 1976). The results show similarity in the sedimentation coefficient (33.5–35.2 S) and the molecular weight of the native molecule [$(1.65–1.75) \times 10^6$], the molecular weight of the polypeptide chain [$(1.75–2.20) \times 10^5$], and the minimal weight per heme (18 100–22 300) [see the review by Chung and Ellerton (1979)].

Planorbid erythrocruorin has also been studied by electron microscopy. Electron micrographs of *P. corneus* erythrocruorin reveal hexagonal ring structures (Wood & Mosby, 1975). Projections of negatively stained *H. trivolvis* erythrocruorin show 10-membered rings with a central structure (Terwilliger et al., 1976). This apparent difference in the electron micrographs may suggest, as pointed out by Chung and Ellerton (1979), differences in the quaternary structures of *Planorbis* and *Helisoma* erythrocruorins. In view of the taxonomical closeness of *Planorbis* and *Helisoma*, and the similarities in their erythrocruorins mentioned above, this idea seems rather improbable.

The present paper is concerned with a structural study of erythrocruorin from *H. trivolvis*. Our results lead us to propose a model for the quaternary structure of planorbid erythrocruorin. This model provides an explanation for the differences in the electron micrographs of erythrocruorins from *Planorbis* and *Helisoma*.

Materials and Methods

Preparation of Erythrocruorin. Snails identified as *H. trivolvis* were collected from a freshwater pond, located at the botanical gardens of Tel-Aviv University. Erythrocruorin was extracted in the following manner. About 50 animals were washed and dried and then placed in 5-mL syringes. They were then crushed and the resulting hemolymph collected in a small quantity of 0.1 M phosphate buffer, pH 6.8. The hemolymph was twice centrifuged at 15000*g* for 20 min in order to remove particulate matter. The resulting supernatant

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was then centrifuged for 2 h at 232000*g*. The precipitate was dissolved in the same buffer and centrifuged again. Dissolution of the red pellet in about 0.5 mL of the buffer gave a concentrated (50–150 mg/mL) solution of purified erythrocruorin (A_{415}/A_{280} = 2.8). All work was done at 4 °C.

Concentration Determinations. Erythrocruorin concentrations were measured by absorption spectroscopy with a Cary 118 spectrophotometer. Absorption coefficients were determined as described elsewhere (Ilan & Daniel, 1979). Values of $A^{1\%}_{1cm}$ = 20.6 at 280 nm in 0.1 M phosphate buffer, pH 6.8, and of $A^{1\%}_{1cm}$ = 19.2 at 278 nm in 6 M guanidine hydrochloride and 0.02 M Tris, pH 8.0, were obtained.

Heme and Iron Determinations. Heme determination was performed by the pyridine hemochromogen difference spectrum method (Falk, 1964). Iron determination was carried out by atomic absorption spectroscopy at 248.3 nm with a Varian Techtron Model AA-5 spectrophotometer.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed on 5% polyacrylamide gels as described by Weber et al. (1972) in the presence of 1% (w/v) sodium dodecyl sulfate and 1% (v/v) 2-mercaptoethanol. The samples were heated for 7 min at 90 °C before their application to the gels. Samples containing ferritin were heated for 15 min at 60 °C. Molecular weights were estimated from band mobilities with a calibration curve obtained with five protein markers. The markers (Pharmacia, Uppsala) were hog thyroid thyroglobulin (330000), horse spleen ferritin (220000), bovine serum albumin (67000), beef liver catalase (60000), and beef heart lactate dehydrogenase (36000).

Ultracentrifugation. Ultracentrifugation was performed with a Beckman Model E analytical ultracentrifuge. Sedimentation velocity was carried out with Schlieren phase plate optics. Sedimentation equilibrium studies were performed by using the short-column meniscus depletion technique (Yphantis, 1964), with interference optics. The fringe pattern was read at 0.1-mm intervals on photographs taken immediately on reaching speed and after attainment of equilibrium. Local slopes of the plot of ln c vs. r^2, d(ln c)/dr^2, c being the concentration and r the distance from the axis of rotation, were determined by least-squares fitting of the base-line-corrected concentration distribution at equilibrium and used to calculate the weight-average molecular weight at r, $\bar{M}_{w,r}$, according to the relation

$$\bar{M}_{w,r} = [2RT/\omega^2(1 - \bar{v}\rho)]\mathrm{d}(\ln c)/\mathrm{d}r^2$$

where R is the gas constant, T is the absolute temperature, ω is the angular velocity, $\bar{v}$ is the partial specific volume, and ρ is the density of the solution. For the calculation of the molecular weight in 6 M guanidine hydrochloride solution, in both the presence and absence of 2-mercaptoethanol, $\bar{v}$ in the expression for $\bar{M}_{w,r}$ has to be replaced by ϕ', the apparent specific volume of the protein in dialysis equilibrium with the solvent (Casassa & Eisenberg, 1964). The values of $\bar{v}$ and ϕ' were experimentally determined from density measurements made with a Digital Densimeter DMA-02 (Anton Paar K. G., Graz, Austria) [for details, see Ilan and Daniel (1979)].

Electron microscopy was carried out using the technique of negative staining (2% uranyl acetate). Observations were made with a Jeol Jem 100B electron microscope.

RESULTS

Native 35S Molecule. Erythrocruorin prepared by the method described gave a single symmetrical peak upon sedimentation in the ultracentrifuge. The sedimentation coefficient was determined over the concentration range c = 0.25–14 mg/mL. The results were found to fit the relation $s_{20,w} = s^0_{20,w}(1 - Kc)$, where K = 0.0129 mL/mg and $s^0_{20,w}$ = 34.7 S. *Helisoma* erythrocruorin showed a typical oxyhemoglobin spectrum with a Soret band at 415 nm. Determination of the iron and heme content gave 0.270 and 3.21% (w/w), values corresponding to minimal molecular weights of 20700 and 19200, respectively.

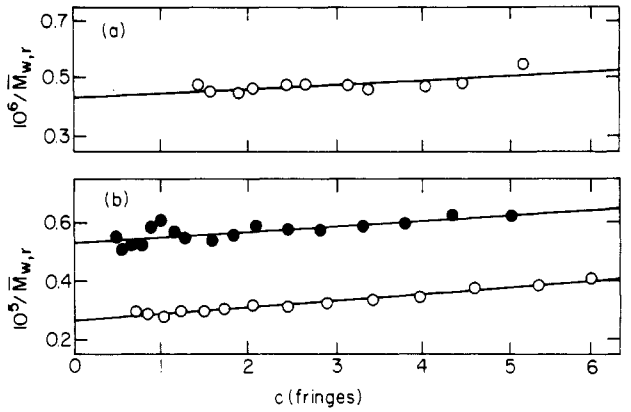

FIGURE 1: Sedimentation equilibrium of *H. trivolvis* erythrocruorin. (a) Reciprocal weight-average molecular weight of native 35S erythrocruorin as a function of protein concentration. Protein concentration was initially 0.7 mg/mL in 0.1 M phosphate buffer, pH 6.8, containing 0.01 M $MgCl_2$. Conditions: speed, 5593 rpm; temperature, 14.0 °C. (b) Reciprocal weight-average molecular weight of erythrocruorin in 6 M guanidine hydrochloride as a function of protein concentration. Protein concentration was initially ~1 mg/mL. (○) In the absence of 2-mercaptoethanol [conditions: speed, 14091 rpm; temperature, 18.0 °C]; (●) in the presence of 0.1 M 2-mercaptoethanol and 0.02 M Tris, pH 7.7 [conditions: speed, 17872 rpm; temperature, 16.7 °C].

The molecular weight of the 35S native molecule was determined by meniscus-depletion sedimentation equilibrium. Point-by-point weight-average molecular weights were found to be somewhat dependent on protein concentration. The molecular weight was therefore obtained by extrapolation to zero concentration of a plot of the reciprocal weight-average molecular weight against concentration. Three experiments gave $\bar{M}_w = 2.29 \times 10^6$ (Figure 1), 2.12×10^6, and 2.33×10^6. Taken together, the data give for the molecular weight of *Helisoma* erythrocruorin $\bar{M}_w = 2.25 \times 10^6$. In this calculation a value of $\bar{v}$ = 0.740 mL/g, measured in water at 20.1 °C, was used. The latter value represents an average of three independent determinations that gave 0.739, 0.740, and 0.741 mL/g.

Polypeptide Chain. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of *Helisoma* erythrocruorin in the presence of 2-mercaptoethanol resulted in one band with a mobility corresponding to a molecular weight of 1.9×10^5 (Figure 2). At relatively high protein loading (~50 μg/gel), an additional faint band of mobility corresponding to about twice the molecular weight of the major band, can be discerned. The same pattern was obtained with hemolymph drawn from a live animal. The molecular weight of the polypeptide chain was determined by sedimentation equilibrium in solution containing 6 M guanidine hydrochloride, 0.1 M 2-mercaptoethanol, and 0.02 M Tris. A linear extrapolation to zero concentration of a plot of the reciprocal of the weight-average molecular weight against concentration was carried out. Two experiments gave $\bar{M}_w = 1.87 \times 10^5$ (Figure 1) and $\bar{M}_w = 1.86 \times 10^5$. The molecular weight of *Helisoma* erythrocruorin polypeptide chain will hereafter be taken as 1.87×10^5. For this calculation, we used a value for the apparent specific volume ϕ' of 0.720 mL/g, experimentally determined by us in 6 M guan-

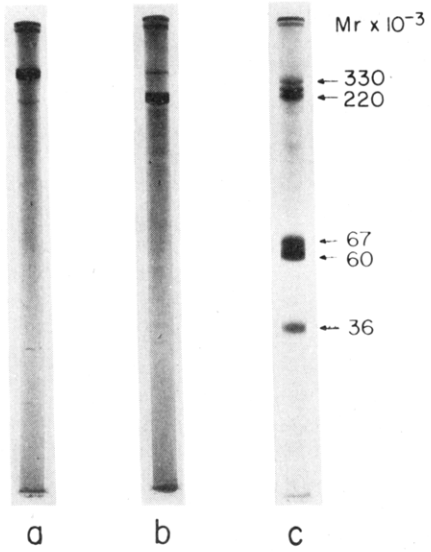

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of *H. trivolvis* erythrocruorin: (a) purified erythrocruorin in the absence of 2-mercaptoethanol; (b) purified erythrocruorin in the presence of 2-mercaptoethanol; (c) mixture of protein markers.

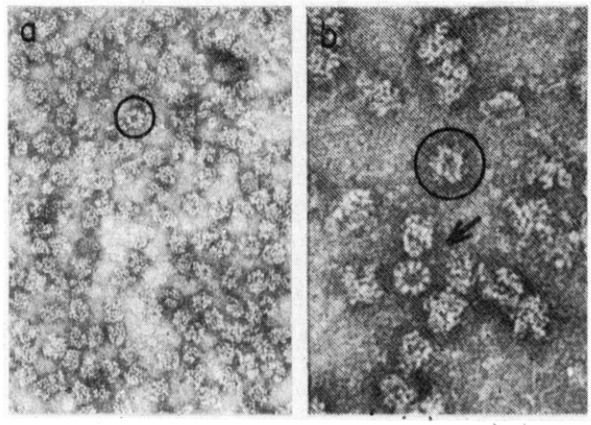

FIGURE 3: Electron micrographs of negatively stained *H. trivolvis* erythrocruorin: (a) present study (120 000×) [circled is a 10-membered ring projection]; (b) reprint from Terwilliger et al. (1976) by courtesy of Elsevier Biomedical Press [pointed by an arrow is a 10-membered ring projection; circled is a rhombic projection].

idine hydrochloride and 0.02 M Tris at 20.1 °C.

Smallest Covalently Linked Submultiple. The effect of omission of 2-mercaptoethanol on the sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of *Helisoma* erythrocruorin is shown in Figure 2. Virtually all the protein migrates in a single band, with a mobility corresponding to a molecular weight of $\sim 4.0 \times 10^5$ (value obtained by extrapolation of the molecular weight calibration curve). A faint band with a mobility corresponding to about half the molecular weight of the major band can be observed.

The molecular weight of the smallest covalently linked submultiple was determined by sedimentation equilibrium in solution containing 6 M guanidine hydrochloride, in the absence of 2-mercaptoethanol. Two experiments gave $\bar{M}_w = 3.78 \times 10^5$ (Figure 1) and 3.85×10^5. Taken together, the data from the two experiments give $\bar{M}_w = 3.82 \times 10^5$. As in the determination of the molecular weight of the polypeptide chain, a value $\phi' = 0.720$ mL/g was used.

Electron Microscopy. Difficulties were encountered in the electron microscopy of *Helisoma* erythrocruorin. Figure 3a is an electron micrograph of a negatively stained preparation. Most of the projections seen in the field have a globular shape with a granular-like appearance. Very few projections, however, reveal a symmetrical shape about 200 Å in diameter. These projections consist of 10 spots arranged in a ring with an additional spot in the center.

Table I: Summary of Molecular Weight Data for *H. trivolvis* Erythrocruorin

mol wt of native molecule	2.25×10^6
mol wt of smallest covalently linked submultiple	3.82×10^5
mol wt of polypeptide chain	1.87×10^5
min mol wt calcd from the heme content	1.92×10^4
no. of heme groups per chain	10
no. of polypeptide chains per submultiple	2
no. of polypeptide chains per molecule	12

Discussion

The values found in the present study for the sedimentation coefficient, the molecular weight of the polypeptide chain, and minimal weight per heme for *H. trivolvis* erythrocruorin (Table I) are in agreement with the corresponding values reported for planorbid erythrocruorins in general (see the introduction) and those reported by Terwilliger et al. (1976) for erythrocruorin from the same species [33.8 S, $(1.75–2.00) \times 10^5$, 18 100], in particular. The fact that a single band is obtained in sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol indicates that the native molecule is composed of identical, or very similar, polypeptide chains. A similarity in size of the constituent polypeptide chains was also demonstrated for *Planorbis* sp. erythrocruorin (Waxman, 1975).

The results from sodium dodecyl sulfate–polyacrylamide gel electrophoresis and sedimentation equilibrium in 6 M guanidine hydrochloride, both carried out in the absence of 2-mercaptoethanol, indicate that the smallest covalently linked submultiple is not identical with a single polypeptide chain. From the molecular weight data (Table I), we conclude that, in the native molecule, the polypeptide chains are grouped in pairs, each chain carrying 10 heme groups. The two chains in each pair are held together by disulfide bonds. These conclusions are in agreement with those reported by Terwilliger et al. (1976).

The molecular weight determined by us for native *Helisoma* erythrocruorin, 2.25×10^6, is appreciably higher than the values reported for erythrocruorin from the same and other planorbid species. Part of the discrepancy is undoubtedly due to the values of $\bar{v}$ used in the calculation of the molecular weight. We used a value $\bar{v} = 0.740$ mL/g while others used $\bar{v} = 0.722$ [*Biomphalaria glabrata*; Almeida & Neves (1974)], 0.730 [*H. trivolvis*; Terwilliger et al. (1976)], and 0.733 mL/g [*P. corneus*; Wood & Mosby (1975)]. It should be noted that our $\bar{v}$ value for *Helisoma* erythrocruorin is close to the value $\bar{v} = 0.745$ mL/g experimentally determined for *P. corneus* erythrocruorin by Svedberg and Eriksson-Quensel (1934). At this point, we draw attention to a problem connected specifically with the determination of the molecular weight of native planorbid erythrocruorins. The 35S molecule is stable within a narrow pH range. For *Helisoma*, this range was found to be 4.5–7.5 (Hammel, 1976). For *P. corneus*, a range of 3.0–7.8 was reported (Svedberg & Eriksson-Quensel, 1934). It is obvious that in order to obtain the molecular weight of the 35S intact molecule one has to work within the pH stability range. Experiments carried out in 0.1 M phosphate buffer, pH 6.8, showed, however, evidence for partial dissociation at concentrations less than about three fringes (0.75 mg/mL) (Shnit, 1977). This finding has to do, in our opinion, with the fact that the dissociation of the intact molecule at alkaline pH occurs gradually (Svedberg & Eriksson-Quensel, 1934; Hammel, 1976). The problem was overcome either by addition of

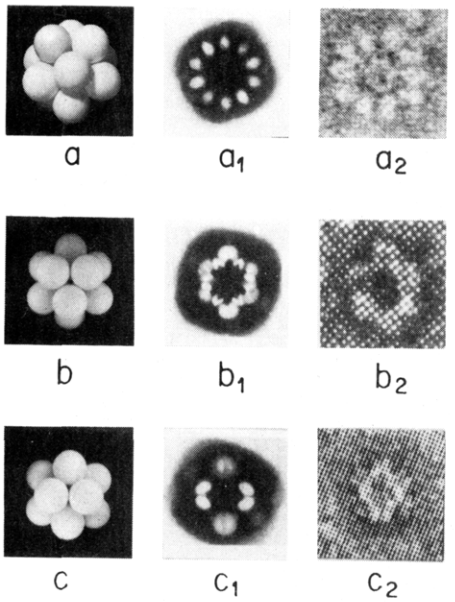

FIGURE 4: Proposed model for planorbid erythrocruorin: (a–c) projections of the model along the pseudo-fivefold, -threefold, and -twofold axes; (a_1–c_1) predicted electron microscopic profiles along the three axes obtained by the X-ray analogue technique [reprinted from Haschemeyer (1970), copyright by Wiley]; (a_2) 10-membered ring projection of *H. trivolvis* erythrocruorin observed in electron microscopy [reprinted from Terwilliger et al. (1976) by courtesy of Elsevier Biomedical Press]; (b_2) hexagonal ring projection of negatively stained *P. corneus* erythrocruorin [reprinted from Wood and Mosby (1975) by permission from *The Biochemical Journal*, copyright 1975, The Biochemical Society, London]; (c_2) rhombic projection of *H. trivolvis* erythrocruorin observed in electron microscopy [reprinted from Terwilliger et al. (1976) by courtesy of Elsevier Biomedical Press].

0.01 M $MgCl_2$ to the buffer used for the equilibrium experiment (0.1 M phosphate, pH 6.8) or by working with 0.1 M acetate buffer, pH 6.0. From the molecular weight data (Table I), we conclude that the 35S molecule of native *Helisoma* erythrocruorin is composed of 12 single polypeptide chain subunits.

On the basis of the number of subunits and the evidence from electron microscopy, we propose a model for planorbid erythrocruorin. The model is composed of 12 spherical subunits arranged in a shell structure with tetrahedral symmetry. The proposed model is consistent with the grouping of the 12 identical single polypeptide chain subunits into six dimeric submultiples (Haschemeyer, 1970). Figure 4a–c shows three projections of the model along the pseudo-fivefold, -threefold, and -twofold axes. Predicted electron microscopic profiles along the three axes obtained by the X-ray analogue technique (Haschemeyer, 1970) are presented in Figure 4a_1–c_1. The 10-membered ring projection of *Helisoma* erythrocruorin actually observed in electron microscopy (Figure 4a_2) is in good agreement with the one predicted (Figure 4a_1), except for the presence of a central spot in the electron microscopic projection. It should be realized, however, that the presence or absence of a central spot within the 10-membered ring depends on the ability of the staining material to penetrate the central cavity of the molecule. The predicted electron microscopic profile for the projection of the model along the threefold axis (Figure 4b_1) is composed of six major spots situated at the vertices of a regular hexagon, adjacent spots being connected to one another by spots of smaller size. Such profiles were not reported for *Helisoma* erythrocruorin. Hexagonal ring projections closely resembling the predicted ones were, however, observed in electron miscroscopy of *P. corneus* erythrocruorin by Wood and Mosby (1975) (Figure 4b_2). The predicted profile for the third projection of the model, the one along the twofold axis (Figure 4c_1) is a rhombus. Such profiles were not reported for planorbid erythrocruorin. Inspection of electron micrographs of negatively stained *Helisoma* erythrocruorin published by Terwilliger et at. (1976) reveals, however, the presence of such a profile (Figures 3b and 4c_2). Finally, the globular-shaped profiles with the granular-like appearance seen in electron micrographs of *Helisoma* erythrocruorin (Terwilliger et al., 1976; this study) may be attributed, according to our model, to projections of the molecule along directions not coincident with one of the three axes referred to above.

The model proposed here for planorbid erythrocruorins explains the seemingly conflicting findings in the electron microscopy of *Planorbis* and *Helisoma* erythrocruorins as different view of the molecule. The fact that a single model can do so is in line with the taxonomical closeness of the planorbid gastropods and the similarity in the physicochemical properties of their erythrocruorins.

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