column of the table were calculated as outlined in Table III on the basis of no heat of interaction between the enzyme and the inhibitor. Although these heats differ from the observed values on average only by the estimated uncertainty in the measured values, the fact that all the differences are in the same direction suggests that there is a significant heat of interaction between the two proteins which is more positive in the native state than in the denatured state. Uehara et al. (1978) reported an enthalpy of interaction of 4.18 kcal mol^{-1} at 25 °C, based on fluorescence titrations over a range of temperatures. This corresponds to only 0.4 mcal at a mole ratio of 1.0 in the experiments under consideration here. The DSC results indicate a considerably larger interaction enthalpy. Whether the apparent difference between the calorimetric and fluorometric results can be accounted for on the basis of a heat capacity change in the denaturation of the complex is difficult to decide on the basis of the complex DSC curves. The fluorometric data gave no indication of a nonvanishing change in heat capacity accompanying the enzyme–inhibitor interaction.

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Erythrocruorin from the Crustacean *Caenestheria inopinata*. Quaternary Structure and Arrangement of Subunits†

Ehud Ilan, Melvyn M. David, and Ezra Daniel*

ABSTRACT: The subunit structure of erythrocruorin from the crustacean *Caenestheria inopinata* was studied. The native protein was found to have a sedimentation coefficient of 12.0 S and a molecular weight, as determined by sedimentation equilibrium, of 302 000. Iron and heme determinations gave 0.346 and 3.98% corresponding to minimal molecular weights of 16 100 and 15 500, respectively. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis gave one band with mobility corresponding to a molecular weight of 30 000. The molecular weight of the polypeptide chain was determined to be 30 500 by sedimentation equilibrium in 6 M guanidine hydrochloride and 0.1 M 2-mercaptoethanol. Dissociation of the 12S molecule was observed at acidic and alkaline pH. A dissociation species of 2.7 S was isolated and its molecular weight determined to be 28 000 by sedimentation equilibrium. On a molecular weight basis, the native molecule is composed of ten 2.7S subunits, each of which consists of a single polypeptide chain carrying two hemes. We propose a model for the molecule composed of ten spheres, each representing a 2.7S subunit, arranged in two layers stacked in an eclipsed orientation, the five spheres of each layer occupying the vertices of a regular pentagon. Support for this arrangement is provided by a comparison of projections of the model with molecular profiles seen in the electron microscope.

In Crustacea, erythrocruorin occurs mainly among the four orders of the subclass branchiopoda: anostraca, notostraca, cladocera, and conchostraca (Fox, 1957; Redmond, 1971). In a recent study (Ilan & Daniel, 1979a), it was found that erythrocruorins from species belonging to these four orders differ from each other with respect to the size of the native molecule, the size of the constituent polypeptide chains, or both. This means that different structures of erythrocruorin exist in the branchiopod crustaceans. This paper describes a detailed study of one of these structures, the structure of erythrocruorin from a conchostracan, the clam shrimp *Caenestheria inopinata*.

Materials and Methods

Preparation of Erythrocruorin. Caenestheria inopinata specimens were gathered during spring and early summer from a seasonal pond at Migdal Sedek, Israel. For the preparation of erythrocruorin, about 100 live animals were washed with cold distilled water, dried in air, and then placed in 10-mL plastic syringes (about 15 animals in each). Crude hemolymph was squeezed out by gentle application of pressure, diluted with an equal volume of 0.1 M sodium phosphate buffer, pH 6.8, and centrifuged for 20 min at low speed to remove particulate

† From the Department of Biochemistry, Tel-Aviv University, Tel-Aviv, Israel. *Received March 10, 1981.*

0006-2960/81/0420-6190$01.25/0

matter. The supernatant was then centrifuged for 5.5 h at 360000*g*. The precipitate was dissolved in the same buffer and centrifuged again. The resulting red pellet was redissolved in about 0.2 mL of the buffer and the concentrated erythrocruorin solution (50–150 mg/mL) stored in the cold until use. All work was done at 4 °C. The purified oxyerythrocruorin exhibited an absorbance ratio A_{416}/A_{280} of 2.9. Erythrocruorin that had been subjected to a third high-speed centrifugation showed the same A_{416}/A_{280} ratio. Erythrocruorin prepared in this manner could be stored for 1 week with no discernible changes in its spectral properties.

Concentration Determinations. Erythrocruorin concentrations were measured by absorption spectroscopy with a Cary 118 spectrophotometer. Absorption coefficients were determined as described elsewhere (Ilan & Daniel, 1979b). At 280 nm, values of $E^{1\%}_{1cm}$ = 20.3 in 0.1 M phosphate buffer, pH 6.8, and of $E^{1\%}_{1cm}$ = 16.9 in 6 M guanidine hydrochloride, 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 as described by Weber et al. (1972). Gels (0.5 cm × 10 cm) contained 7.5% acrylamide, 0.20% methylenebis(acrylamide), and 0.15% *N,N,N′,N′*-tetramethylenediamine. Erythrocruorin samples were made 1% (w/v) in sodium dodecyl sulfate, 1% (v/v) in 2-mercaptoethanol, and 0.01 M in sodium phosphate buffer, pH 7.0. The samples were heated for 7 min at 90 °C before their application to the gels. A constant current, 4 mA per gel for the first 30 min and 8 mA for the following 5 h, was applied. Molecular weights were determined from band mobilities with a calibration curve obtained with seven protein markers. The markers were *E. coli* RNA polymerase (160 000, 150 000), rabbit muscle phosphorylase *b* (94 000), bovine serum albumin (67 000), egg white ovalbumin (43 000), bovine erythrocyte carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and bovine milk α-lactalbumin (14 400 daltons).

Ultracentrifugation was performed in a Beckman Model E analytical ultracentifuge. Sedimentation-velocity experiments were carried out at 20 °C or at a temperature close to it by using schlieren phase-plate or absorption scanning optics. Sedimentation coefficients, calculated from the rate of movement of the boundaries, were corrected to $s_{20,w}$ values in the usual way (Svedberg & Pedersen, 1940). Buffer densities were determined by means of a 25-mL pyknometer and buffer viscosities by means of an Ostwald viscometer.

Sedimentation equilibrium was carried out by using the short-column meniscus depletion technique (Yphantis, 1964). Interference optics were used. Protein solution (0.9–1.1 mg/mL) and solvent were placed in separate channels of a 12-mm double-sector cell. Photographs were taken immediately upon reaching speed and after attainment of equilibrium. The concentration distribution at equilibrium was obtained from base-line corrected readings of the interference fringe pattern. Local slopes of the ln c vs. r^2 plot, where c is the concentration and r is the distance from the axis of rotation, were determined at each concentration by least-squares straight line fitting of five points, two points on either side of the central point with which the slope is associated. The weight-average molecular weight at r, $\bar{M}_{w,r}$, was calculated from the equation $\bar{M}_{w,r} = [2RT/\omega^2(1 - \bar{v}\rho)]$ d ln c/dr^2, where R is the gas constant, T is the absolute temperature, ω is the angular velocity, $\bar{v}$ is the partial specific volume, ρ is the density of the solution, and d ln c/dr^2 is the local slope of the ln c vs. r^2 plot. Calculation of the molecular weight in concentrated guanidine hydrochloride solution was carried out by replacing $\bar{v}$ in the expression for $\bar{M}_{w,r}$ with the apparent specific volume $\phi' = (1/\rho_0)(1 - \Delta\rho/c)$, where $\Delta\rho = \rho - \rho_0$ is the difference in density between the solution and the solvent with which it is in dialysis equilibrium (Casassa & Eisenberg, 1964). The specific volumes in water and concentrated guanidine hydrochloride were obtained from density measurements. These were performed with a DMA-02 Digital densimeter (Anton Paar K.G., Austria). Details of the procedure are given elsewhere (Ilan & Daniel, 1979b).

Sedimentation equilibrium of the 2.7S isolated subunit was conducted according to the conventional low-speed method using an absorption scanning system. Recorder pen deflections from absorbance scans were corrected for nonlinearity by use of the stair-step calibration pattern. The weight-average molecular weight, $\bar{M}_w$, was calculated from the slope of the ln c vs. r^2 plot.

Electron microscopy was carried out by using the technique of negative staining (1% uranyl acetate). Observations were made with a Jeol-Jem-7 electron microscope. Image analysis was performed by Markham rotation (Markham et al., 1963).

Results

Native 12S Erythrocruorin. 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 = 0.4–5.0 mg/mL. The results were found to fit the relation $s_{20,w} = s^0_{20,w}(1 - Kc)$, where K = 0.0126 mL/mg and $s^0_{20,w}$ = 12.0 S. *Caenestheria* erythrocruorin showed a typical oxyhemoglobin spectrum with a Soret band at 416 nm. Determination of the iron and heme content gave 0.346 and 3.98% (w/w), values corresponding to minimal molecular weights of 16 100 and 15 500, respectively.

The molecular weight was determined by meniscus-depletion sedimentation equilibrium. Point-by-point weight-average molecular weights were found to be practically independent of concentration. Three experiments gave $\bar{M}_w$ = 311 000, 294 000, and 300 000, taking the experimentally determined value $\bar{v}$ = 0.747 mL/g, measured in water. The molecular weight of *Caenestheria* erythrocruorin will hereafter be taken as 302 000.

Dependence of Sedimentation Coefficient on pH. The effect of pH on the sedimentation coefficient is shown in Figure 1. It is seen that the 12S native molecule is stable between pH 3.0 and 8.2. In alkaline (>8.2) or acidic (<3.0) pH, dissociation into ~3S material takes place. The alkaline 12S → ~3S transition occurs over a significant pH range. Here, as the pH is increased, there is a decrease in the amount of the 12S species and a concomitant increase in that of the ~3S species, until at pH >10.7 the ~3S species becomes the preponderant one (>95% of the sedimenting material). The acidic 12S → ~3S transition is, in contrast, sharp. At the pH of acidic dissociation and in a narrow pH region where the alkaline dissociation begins, the presence of 5 mM ethylenediaminetetraacetic acid (EDTA) (EDTA was added to concentrated erythrocruorin solution at neutral pH, and then the pH was lowered or raised by dilution with the appropriate buffer) brought about a dissociation of the 12S molecule into material with a sedimentation coefficient of 3.9 S. At other pH values, the presence of EDTA did not cause any detectable changes in the sedimentation pattern.

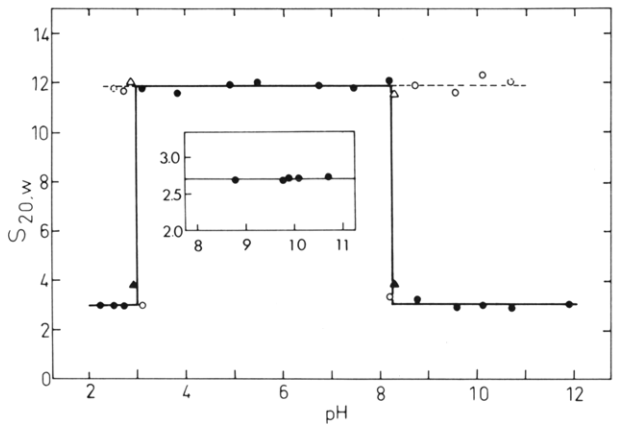

FIGURE 1: Dependence of sedimentation coefficient of *Caenestheria* erythrocruorin on pH. Protein concentration was 1 mg/mL except at pH 2.23, 2.92, and 8.30, where the concentration was 3 mg/mL. Buffers, about 0.1 M, were used in the pH ranges indicated: glycine–HCl (2.2–3.6); acetate (3.6–5.7); phosphate (5.7–7.3); Tris-HCl (7.3–9.0); glycine–NaOH (9.0–12.0). Different symbols were used to show the fraction x of protein sedimenting with the indicated sedimentation coefficient: (●) $1.00 \geq x > 0.50$; (○) $0.50 \geq x > 0.05$; (broken circle) $0.05 \geq x$. The presence of 5 mM EDTA is indicated by a triangular symbol. (Inset) Sedimentation coefficients of the alkaline dissociation product attained at very low protein concentration (~0.1 mg/mL). Optics: Absorption for experiments shown in inset, schlieren for all other experiments.

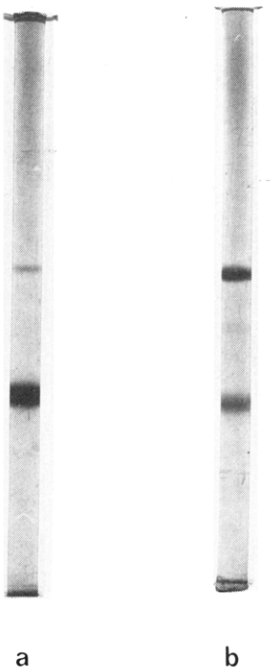

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (a) Sample, prepared as described under Materials and Methods, contained 10 μg of erythrocruorin; (b) same as (a) except for omission of 2-mercaptoethanol.

The alkaline dissociation of *Caenestheria* erythrocruorin can be reversed by lowering the pH back to neutrality. A solution of erythrocruorin in 0.1 M phosphate buffer, pH 6.8, was brought to pH 10.0 by dilution with 0.1 M glycine–NaOH buffer. After 3 h at the high pH, 1 mL of the solution was dialyzed twice in the cold against 1 L of 0.1 M phosphate buffer. The final pH was 6.8. A sedimentation-velocity experiment on the dialyzed erythrocruorin showed that about 70% of the sedimenting material reassociated back to 12S molecules.

The 2.7S Subunit. The sedimentation coefficient of the slow sedimenting material obtained upon alkaline dissociation of *Caenestheria* erythrocruorin was found to depend on the concentration of protein solution. At any given pH, decrease in the total protein concentration brought about a decrease in the sedimentation coefficient: 3.20 S at 1.0 mg/mL vs. 2.69 S at 0.12 mg/mL (pH 8.8), 2.99 S at 1.0 mg/mL vs. 2.72 S at 0.085 mg/mL (pH 10.1), and 2.84 S at 1.1 mg/mL vs. 2.73 S at 0.085 mg/mL (pH 10.7). The independence on pH of the limiting sedimentation coefficient attained at low (~0.1 mg/mL) protein concentration suggests the existence of a subunit with this sedimentation coefficient, the 2.7S subunit (Figure 1, inset). The next step was to determine the molecular weight of this subunit. Two possibilities were considered: to carry out sedimentation-equilibrium experiments at extreme pH values where the dissociation of the 12S molecule is virtually complete or to perform the experiment on the 2.7S subunit that had been isolated from the undissociated 12S component. Work at extreme pH did not seem attractive, since labile bonds in the polypeptide chain are liable to undergo hydrolysis. An attempt was therefore made to isolate the 2.7S subunit. A sample of native erythrocruorin at neutral pH was loaded on a Sephadex G75 column and immediately eluted with 0.1 M carbonate buffer, pH 9.9, containing 10^{-3} M EDTA. The eluted material was then examined by sedimentation velocity using absorption optics. Scans taken at 416 and 280 nm both showed a single sedimenting boundary, $s_{20,w}$ = 2.70 S at 0.10 mg/mL. Repeated examination by sedimentation velocity of a sample stored in an ultracentrifuge cell for 24 h gave the same sedimentation pattern, showing that the isolated 2.7S component did not reassociate back to the 12S molecule.

The absorption spectrum of the 2.7S species was very similar to that of the native 12S oxy derivative as regards wavelengths of absorption maxima and ratio of Soret band to protein absorption. The molecular weight was determined by low-speed sedimentation equilibrium using scanner optics. A molecular weight of 28 000 was calculated from the slope of the linear ln c vs. r^2 plot. For this calculation $\bar{v}$ = 0.747 mL/g, determined for the native 12S molecule, was used.

The Polypeptide Chain. Figure 2 presents typical results of sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The electrophoretic pattern of *Caenestheria* erythrocruorin shows a single band with a mobility corresponding to a molecular weight of 30 000. At relatively high-protein loading, an additional faint band corresponding to a molecular weight twice that of the major monomeric band component can be observed. Omission of 2-mercaptoethanol caused an increase in the relative intensity of the dimeric band.

The molecular weight of the polypeptide chain was determined by sedimentation equilibrium in solutions containing 6 M guanidine hydrochloride and 0.1 M 2-mercaptoethanol. Linear extrapolation to zero concentration of a plot of reciprocal weight-average molecular weight against concentration gave $\bar{M}_w$ = 30 500. For this calculation, a value for the apparent specific volume ϕ' of 0.737 mL/g determined in 6 M guanidine hydrochloride was used. For verification that the molecular-weight determination had been carried out on intact polypeptide chains, the solution from the ultracentrifuge cell was subjected, after the sedimentation-equilibrium experiment was completed, to sodium dodecyl sulfate gel electrophoresis in the presence of 2-mercaptoethanol. No band with a mobility greater than that of the monomeric chain could be detected.

Electron Microscopy. Figure 3a is an electron micrograph of *Caenestheria* erythrocruorin. Two main projections are seen: a polygon with a central hole and a rectangle subdivided into halves by a longitudinal gap. A micrograph taken

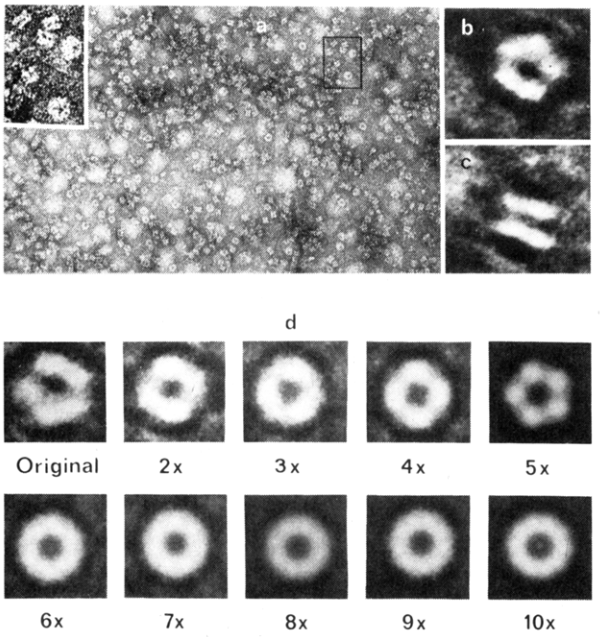

FIGURE 3: Electron microscopy of *Caenestheria* erythrocruorin. (a) Molecules negatively stained with uranyl acetate (66000×); a magnified view of part of the field (inside rectangular frame) is shown in inset; (b, c) molecules seen in pentagonal and rectangular projections, respectively; (d) rotation photograph of a molecule in pentagonal projection. Rotation steps: 2 × 180°, 3 × 120°, 4 × 90°, 5 × 72°, 6 × 60°, 7 × 51.4°, 8 × 45°, 9 × 40°, and 10 × 36°.

Table I: Summary of Molecular Weight Data for *Caenestheria* Erythrocruorin

mol wt of native molecule	302 000
mol wt of polypeptide chain	30 500
mol wt of 2.7S subunit	28 000
min mol wt calcd from heme content	15 500
min mol wt calcd from iron content	16 100
no. of heme groups per chain	2
no. of chains per molecule	10

somewhat underfocus (Figure 3b,c) clearly shows that the polygonal projection is five-sided. Further evidence for the fivefold symmetry is provided by application of the technique of rotational photography (Figure 3d).

Discussion

Caenestheria erythrocruorin is a 12S molecule of molecular weight 302 000. Sodium dodecyl sulfate gel electrophoresis results in a single band, indicating that the native molecule is composed of chains of the same molecular weight. The exact molecular weight of the polypeptide chain was obtained by sedimentation equilibrium in 6 M guanidine hydrochloride. A minimal molecular weight of 15 500 was obtained from the heme content and a slightly higher vaue from the iron content. Comparison of the molecular weight data (Table I) indicates that the molecule of *Caenestheria* erythrocruorin is composed of ten identical, or nearly identical, polypeptide chains carrying two hemes each.

At acidic and alkaline pH, dissociation of the 12S native molecule occurs. The smallest species obtained in both acidic and alkaline dissociation is a 2.7S subunit. On the basis of its molecular weight, one may conclude that this subunit consists of a single polypeptide chain. The 2.7S subunit is spectrally identical with the native 12S oxy derivative, implying that it retains its oxygen-binding function. It possesses the ability, once the solution is brought back to neutrality, to reassociate to the 12S molecule. The 2.7S subunit can therefore be envisaged as the structural building unit of

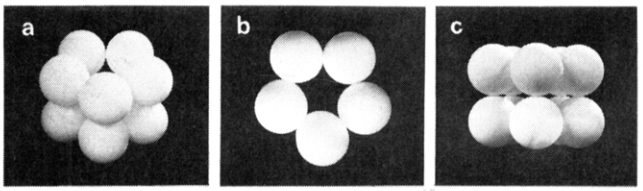

FIGURE 4: Proposed model for *Caenestheria* erythrocruorin. (a) Photograph of a model made up of ten identical spheres arranged in two layers stacked in an eclipsed orientation, the five subunits of each layer occupying the vertices of a regular pentagon; (b) top view; (c) face view.

Caenestheria erythrocruorin. The increase in the sedimentation coefficient of the 2.7S subunit brought about by increase of the protein concentration suggests that one is dealing with a system in association–dissociation equilibrium. The sedimentation coefficient attained in the presence of EDTA at the acidic and alkaline transition pHs, 3.9 S, indicates that the association–dissociation is of the "monomer–dimer" type. Indeed, a value of 2.7 × 1.50 = 4.05 S is expected for the dimer, if one uses the theoretical value of 1:1.50 calculated for the ratio of the sedimentation coefficients of a spherical particle and its dimer (Van Holde, 1975). It is noteworthy that EDTA is effective in halting the dissociation of *Caenestheria* erythrocruorin at the dimer stage. This finding is not surprising in view of the well-known effect of EDTA in inhibiting dimer to monomer dissociation in human hemoglobin (Kellett & Schachman, 1971).

On the basis of the molecular weight relationships determined from the ultracentrifuge and the findings from the electron microscope, we propose a model for *Caenestheria* erythrocruorin composed of ten spheres, each representing a 2.7S subunit, arranged in two layers stacked in an eclipsed orientation, the five subunits of each layer occupying the vertices of a regular pentagon (Figure 4). The projections of the model bring forward the characteristic features seen in the electron micrographs. The top view clearly shows the pentagonal symmetry. The face view shows the rectangular profile and the central longitudinal gap. Further support for the proposed arrangement of the subunits is provided by a consideration of the hydrodynamic data of the native molecule and the isolated subunit. From the model, the sedimentation coefficient predicted for a spherical subunit by the Kirkwood treatment is 2.76 S, a value close to the 2.70 S actually determined.[1]

According to the model proposed for *Caenestheria* erythrocruorin, the molecule has D_5 symmetry—one fivefold and, in a plane perpenticular to it, five twofold rotation axes. It is well-known that in a molecule with this symmetry, the bonds holding the subunits together cannot all be of the same type (Klotz et al., 1970; Matthews & Bernhard, 1973). The 2.7S subunit has been shown to be a single-chain particle. It is therefore clear that the 12S → 2.7S dissociation involves the rupture of all intersubunit bonds. The 3.9S particle has been previously identified as a dimer of the 2.7S subunit. The fact that such a particle is observed indicates that, under certain

[1] This calculation was made by using the relation (Van Holde, 1975)

$$s_n^0/s_1^0 = 1 + (1/n)\sum_{i=1}^{n}\sum_{j=1}^{n}(1/\alpha_{ij}) \qquad i \neq j$$

where s_n^0 is the intrinsic sedimentation coefficent of an assembly of n spherical subunits of radius R and intrinsic sedimentation coefficient s_1^0 and $\alpha_{ij} = R_{ij}/R$, R_{ij} being the center-to-center distance between subunits i and j. Here, $n = 10$, $s_n^0 = 12.0$ S, and α_{ij} were calculated from the proposed model.

conditions, part of the intersubunit linkages (most likely the intralayer bonds holding together the five subunits of each layer) are ruptured, and part (interlayer bonds holding together subunits from different layers) are not. This means that at least two types of bonds hold the 2.7S subunits together in the 12S molecule, as predicted from the symmetry of the model.

At the time this work was undertaken, the results of two structural studies on erythrocruorin from another clam shrimp *Cyzicus hierosolymitanus* were already available in the literature (Ar & Schejter, 1970; David et al., 1977). Two values had been reported for the molecular weight of native *Cyzicus* erythrocruorin: 220 000 from sedimentation diffusion and a more reliable value of 280 000 from sedimentation equilibrium. For the polypeptide chain, molecular weights of 14 500 from sedimentation equilibrium and 15 500 from sodium dodecyl sulfate gel electrophoresis were determined. These findings led to the conclusion that *Cyzicus* erythrocruorin is composed of 20 polypeptide chains carrying one heme each. The subunit structure of *Caenestheria* erythrocruorin which was emerging from the present study was distinctly different. This was surprising in view of the similarity of the electron micrographs of the two erythrocruorins and the fact that *Cyzicus* and *Caenestheria* are very closely related species, both being conchostracan crustaceans. Recently it has been shown that multiheme chains of invertebrate erythrocruorins (*Helisoma*, *Lepidurus*) are sensitive to cleavage by proteolytic enzymes into single-heme fragments of similar size—"domains" (Terwilliger et al., 1977; Dangott & Terwilliger, 1979). We performed some experiments which indicated that such a cleavage was probably responsible for the low molecular weights reported for the polypeptide chain of *Cyzicus* erythrocruorin. Indeed, if proper precautions are taken, a polypeptide chain of the same size as that reported here for *Caenestheria* erythrocruorin can be demonstrated (Ilan & Daniel, 1979a). Moreover, use of the experimentally determined specific volume for *Caenestheria* erythrocruorin, $\bar{v}$ = 0.747 mL/g, instead of the value estimated from the amino acid composition, $\bar{v}$ = 0.729 mL/g, brings up the molecular weight of *Cyzicus* erythrocruorin from 280 000 to 301 000, almost exactly the value determined here for *Caenestheria* erythrocruorin. The two erythrocruorins are thus very similar both in subunit structure and molecular architecture.

The weight per heme of *Caenestheria* and *Cyzicus* erythrocruorins is very similar to the molecular weight of the single-heme chains in vertebrate and *Chironomus* larval hemoglobins. The molecular weight of the polypeptide chain is twice that value. This suggests that the polypeptide chains of *Caenestheria* and *Cyzicus* erythrocruorins, and probably of other multiheme chains of invertebrate erythrocruorins, are composed of repeating covalently linked sequences. The production of single-heme "domains" by mild proteolytic hydrolysis makes this suggestion all the more plausible. Definite confirmation of this idea must, however, await the results of amino acid sequence determinations.

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