Subunit Structure and Oxygen Binding by Hemocyanin of the Isopod *Ligia exotica*†

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ABSTRACT: Isopods are small crustacean arthropods that are generally found in damp or humid areas. The "rock louse", "sow bug", "pill bug", and "wood louse" are members of this group. While the hemocyanins of crabs and shrimps have been the object of numerous investigations, very little work has been done on isopod hemocyanins. This is undoubtedly due, in part, to the small size of the isopods which makes it difficult to obtain hemocyanin samples in appreciable quantity. We have examined in detail the hemocyanin of the marine isopod *Ligia exotica*. The molecular architecture of the protein resembles that of decapod crustaceans in that dodecamers and hexamers are found in the hemolymph. Only the hexameric form persists after removal of divalent cations by treatment with EDTA at pH 9.0. Monomeric forms appear at elevated pH and molecular sieve chromatography can be used to separate hexamers and monomers. At a given pH the isolated fractions are stable populations of either monomers or hexamers, implying the existence of multiple pK values for hexamer–monomer equilibria. At pH 9.5 the isolated monomeric subunits have lower oxygen affinities than the hexamers, indicating that in this hemocyanin system the aggregation of subunits introduces constraints which lead to increased oxygen affinity. The monomers which are isolated by molecular sieve chromatography give single bands on regular and $NaDodSO_4$ gels and are thus considered to be homogeneous with respect to charge and molecular weight. They reaggregate at neutral pH and the reassembled hexamers show cooperative interactions in oxygen binding. The high degree of cooperativity and positive Bohr effect exhibited by hexameric aggregates made up of apparently identical subunits make *Ligia* hemocyanin a good model system for investigation of homo- and heterotropic interactions in a high molecular weight system.

Hemocyanins, the high molecular weight copper proteins which serve as oxygen carriers in many arthropods and molluscs, have been the subject of many investigations (Eriksson-Quensel & Svedberg, 1936; Van Holde & Van Bruggen, 1971; Bonaventura et al., 1977). In most cases, the crustacean hemocyanins are found in aggregation states having sedimentation coefficients of 5 S, 16 S, and 24 S. The relative proportions of these states have been observed to be dependent upon pH, ionic strength, and cation concentration. The 5S state corresponds to an approximately 70 000-dalton polypeptide chain with a single oxygen binding site. The 16S and 24S states correspond to 450 000-dalton hexamers and 900 000-dalton dodecamers. This view of the assembly of crustacean hemocyanins is supported by electron microscope studies of the various components (Van Holde & Van Bruggen, 1971; Schepman, 1975). A number of the arthropod hemocyanins have been studied in detail, with attention directed primarily toward the relationship between the aggregation state of the hemocyanin and the homotropic and heterotropic interactions which influence oxygen binding. However, the interpretation of the results obtained is complicated by the presence of electrophoretically distinct subunits. Subunit diversity has now been described for the hemocyanins of a large number of arthropods and appears to be a fairly general property (Busselen, 1970; Murray & Jeffrey, 1974; Bonaventura et al., 1975; Schepman, 1975; Sullivan et al., 1976; Hamlin & Fish, 1977; Miller et al., 1977). It is of interest in this regard that the isolated subunits of *Ligia exotica* hemocyanin appear to be homogeneous with respect to charge and molecular weight.

The marine isopod *Ligia exotica* is a small, active crustacean which lives in the damp, aerial climate of the high intertidal zone. Hemocyanin occurs in relatively high concentration in its hemolymph. In this paper, we document the highly cooperative oxygen binding behavior and the pronounced Bohr effect of *Ligia* hemocyanin and present data on various ionic interactions which influence its state of aggregation. In addition, the absence of conspicuous charge heterogeneity among the 5S subunits of *Ligia* hemocyanin, which is in contrast to most arthropod hemocyanins, makes the relationship between its structure and function accessible to theoretical interpretation.

Materials and Methods

Ligia exotica (Roux) was collected from the sea wall on Pivers Island, NC. The animals ranged in size from ~1.5 to 3 cm in length. Hemolymph was obtained from the live animal by inserting a capillary pipet between the fifth and sixth thoracic segments. The hemolymph from 100 to 150 animals was mixed with a small volume of ice-cold 0.1 ionic strength Tris-HCl buffer, pH 8.0, that was made 0.01 M in $MgCl_2$ and 0.1 M in NaCl. Phenylmethanesulfonyl fluoride (PMSF,[1] 1 mM) was included in the extraction buffer to inhibit proteolytic degradation by serine proteases. The pooled hemolymph was allowed to coagulate during gentle stirring. After centrifugation at 12000g for 10 min, the supernatant was immediately applied to a 1.8 × 70 cm column of Sepharose 4B at 4 °C equilibrated with the same buffer (without PMSF). Absorbance of the eluted fractions was measured at both 280 and 340 nm. All further experiments, except the electron microscopy, were performed on the Sepharose 4B purified hemocyanin. Samples were dialyzed overnight at 4 °C against the appropriate buffer prior to centrifugation or oxygen binding experiments.

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[1] Abbreviations used: PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; $p_{1/2}$, oxygen pressure in mmHg necessary for half-saturation; $n_{1/2}$, slope of the Hill plot of oxygen binding at half-saturation.

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The Sepharose 4B column was calibrated with the following proteins: *Eudistylia vancouverii* chlorocruorin (M_r = 2.8 × 10^6) (Terwilliger et al., 1975), *Lumbricus terrestris* hemoglobin (M_r = 2.6 × 10^6) (Wiechelman & Parkhurst, 1972), *Helisoma trivolvis* hemoglobin (M_r = 1.7 × 10^6) (Terwilliger et al., 1976), *Callinectes sapidus* hemocyanin (M_r = 9.4 × 10^5) (Hamlin & Fish, 1977), *Glycera dibranchiata* polymeric hemoglobin (M_r = 1.25 × 10^5) (Seamonds et al., 1971), and bovine serum albumin.

Electron microscopy was performed with hemocyanin which had been collected in filtered seawater containing sodium tetrathionate (Eastman) and α-toluenesulfonyl fluoride (Eastman) as proteolytic inhibitors. The hemocyanin sample was millipore filtered before shipment to Holland. Prior to electron microscopy, 10 μL of the pigment was diluted with 1 mL of 0.1 ionic strength Tris-HCl buffer, pH 7.1. The diluted blood was sprayed onto a specimen grid covered with a thick carbon film. The molecules were negatively stained with a few droplets of unbuffered 1% uranyl acetate. Pictures were taken with a Philips EM-200 electron microscope (80 kV) at a magnification of 26 500× on Kodak FRP 35-mm roll film. Prints of the negatives were made at 6× magnification.

Sedimentation velocity experiments were carried out at 20 °C by use of a Beckman Model E analytical ultracentrifuge equipped with a mechanical speed control and Schlieren optics. Sedimentation coefficients were corrected to standard conditions of the viscosity and density of water according to the method of Svedberg & Pedersen (1940). Protein concentrations were 5 mg/mL, obtained from absorption measurements at 340 nm, assuming $\epsilon(1\%)$ = 2.23 for crustacean hemocyanin (Sullivan et al., 1974).

Regular disc gel electrophoresis of *Ligia* hemocyanin at pH 8.9 followed the procedures of Ornstein (1964) and Davis (1964). Electrophoresis at pH 7.4 was run in a 0.05 M Tris-HCl/Tris-maleate buffer system, while at pH 10.5 a 0.05 M sodium bicarbonate/sodium glycinate buffer system was used. All buffers included 0.01 M EDTA. Similar results were obtained both with and without the use of a spacer gel on 7.5% gels.

$NaDodSO_4$ disc gel electrophoresis was performed using 5, 6, and 10% acrylamide gels, with a constant ratio of acrylamide to methylenebis(acrylamide) of 37:1 (Weber & Osborn, 1975). Thin-slab $NaDodSO_4$ gel electrophoresis on 7.5 and 10% gels was also done (Laemmli, 1970; Studier, 1973; Ames, 1974). Prior to $NaDodSO_4$ gel electrophoresis, the proteins were heated to 100 °C for 1.5 min in boiling $NaDodSO_4$ incubation buffer which contained 1 mM PMSF. Calibrants for the 5 and 6% gels were myosin, phosphorylase A, β-galactosidase, and bovine serum albumin, while the 7.5 and 10% gel markers included phosphorylase A, transferrin, bovine serum albumin, ovalbumin, α-chymotrypsinogen A, sperm whale metmyoglobin, and lysozyme (Sigma Chemicals). The gels were stained with Coomassie blue according to Fairbanks et al. (1971).

Oxygen equilibrium studies were carried out by the spectrophotometric method of Riggs & Wolbach (1956). Spectral changes were measured with a Cary 14 recording spectrophotometer. Ligand binding kinetics were determined by rapid mixing methods which have been previously described (Bonaventura et al., 1974).

Results

Structural Properties. *Ligia* hemocyanin chromatographs as a single asymmetric peak on Sepharose 4B as shown in Figure 1. As calculated from a plot of log molecular weight vs. elution volume, the main peak has an apparent molecular weight of approximately 900 000 and the trailing shoulder an apparent molecular weight of 450 000. A yellow nonhemocyanin fraction elutes in the 140 000–170 000 molecular weight range. The 900 000 and 450 000 molecular weight components are present in a ratio of about 65 to 35%, respectively. All subsequent studies were done on the pooled fractions shown under the bar in Figure 1.

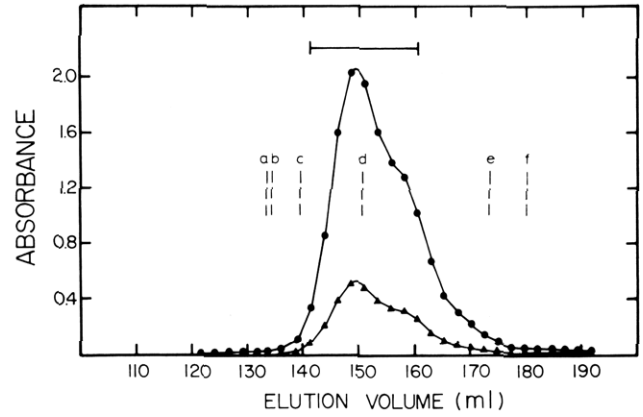

FIGURE 1: Elution pattern of *Ligia* hemocyanin chromatographed on Sepharose 4B, 0.1 ionic strength Tris-HCl, pH 8.0, made 0.01 M in $MgCl_2$ and 0.1 M in NaCl. Column volume 1.8 × 70 cm. Calibrants: (a) *Eudistylia* chlorocruorin, (b) *Lumbricus* erythrocruorin, (c) *Helisoma* hemoglobin, (d) *Callinectes* hemocyanin, (e) *Glycera dibranchiata* polymeric hemoglobin, and (f) bovine serum albumin. Absorbance at 280 (—●—) and 340 nm (—▲—).

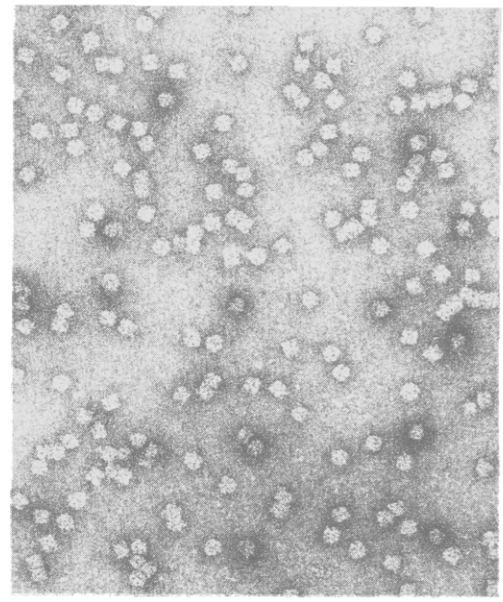

FIGURE 2: Electron micrograph of *Ligia* hemocyanin, pH 7.1, negatively stained with 1% uranyl acetate, 159 000×.

Sedimentation velocity experiments from pH 7 to 9 with purified *Ligia* hemocyanin in the presence of 0.01 M $MgCl_2$ or $CaCl_2$ show two molecular weight components, a major peak with $s^0_{20,w}$ of approximately 24 S and a minor peak with $s^0_{20,w}$ equal to 16 S. Only one component, corresponding to 16S material, is present at pH 9.0 if divalent cations are removed by dialysis against 0.01 ionic strength Tris-HCl, 0.01 M in EDTA.

An electron micrograph of *Ligia* hemocyanin is shown in Figure 2. Single hexagonal and square profiles are present as well as dimeric structures composed of two squares, or a hexagon and a square, joined side by side. The dimeric structures correspond to the 900 000 molecular weight material (24 S) and the single structures to the 450 000 molecular

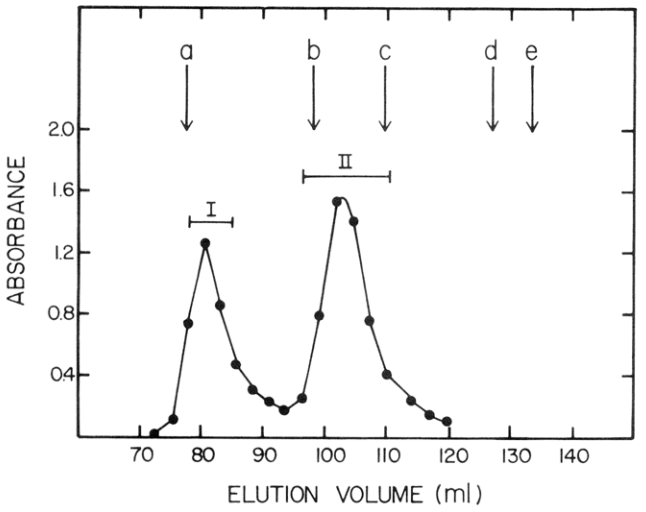

FIGURE 3: Elution pattern for purified *Ligia* hemocyanin chromatographed on Sephacryl S-200 after dialysis vs. 0.05 *I* Tris-HCl, 0.01 M in EDTA, pH 9.5. Column volume 1.4 × 110 cm; column buffer same as dialysate. Calibrants: (a) blue dextran, (b) bovine serum albumin, (c) ovalbumin, (d) α-chymotrypsinogen A, and (e) sperm whale metmyoglobin.

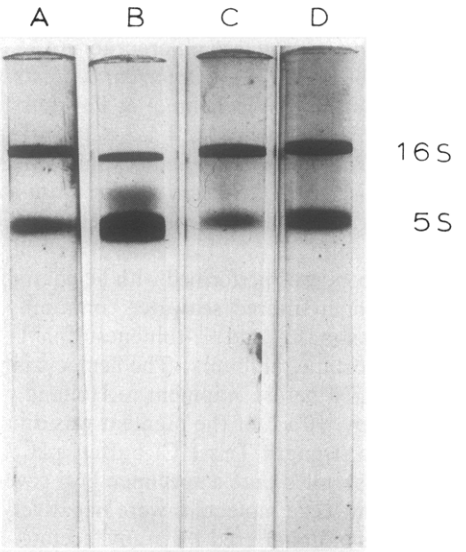

FIGURE 4: Disc gel electrophoresis, pH 8.9, on 7.5% acrylamide gels of fractions I and II from Sephacryl S-200. (A) Fraction I, major component is 16 S; (B) fraction II, major component is 5 S; (C) fraction II, after dialysis at pH 8.0, major component is reassociated 16 S; and (D) fractions I and II (after dialysis at pH 8.0) combined, major component is 16 S. The *s* values indicate the approximate sedimentation velocity of the samples prior to electrophoresis.

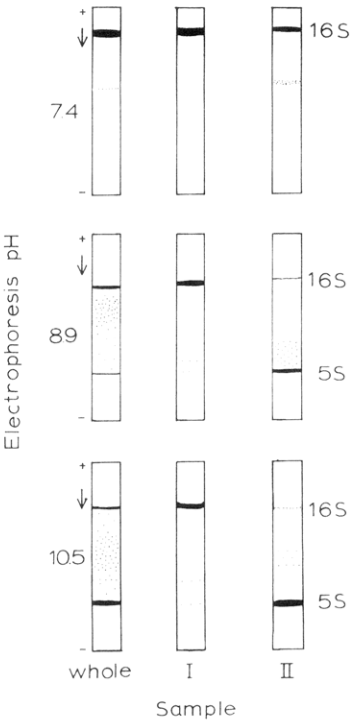

FIGURE 5: Disc gel electrophoresis of purified *Ligia* hemocyanin (pH 8.0, Mg^{2+}), fraction I (pH 10.5, EDTA), and fraction II (pH 10.5, EDTA). Electrophoresis buffers, pH 7.4, 8.9, and 10.5; 7.5% acrylamide gels stained with Coomassie blue.

weight species (16 S). This interpretation is based on the results of column chromatography and ultracentrifugation and by analogy with the aggregation states of other crustacean hemocyanins (Van Holde & Van Bruggen, 1971; Schepman, 1975).

Dissociation of *Ligia* hemocyanin into lower aggregation states occurs under conditions of low ionic strength, of high pH, and in the presence of a chelating agent such as EDTA. At pH 9.0 only 16S material is observed. At pH 9.5, there is substantial dissociation into 5S monomers. When purified hemocyanin is dialyzed against 0.05 *I* Tris-HCl, 0.01 M in EDTA, pH 9.5, and chromatographed on Sephacryl S-200, the elution profile illustrated in Figure 3 is obtained. Fraction I, about 44% of the hemocyanin, elutes with the void volume of the column. Sedimentation velocity measurements show that fraction I is predominantly 16 S with a slight trace of 5S material. Fraction II, about 56% of the protein, sediments with a major $s^0_{20,w}$ peak of 5 S and a very small peak of 16 S. Fraction II therefore corresponds to the 5S monomers reported for other crustacean hemocyanins. The relative percentage of fraction II can be increased to 70% by dialysis and chromatography at pH 10.5.

When fraction I hexamers from a pH 10.5 chromatography are rechromatographed on the same column, they elute as hexamers in exactly the same position as before. Thus the proportions of the 16S and 5S populations at pH 10.5 are not established by a simple pH-dependent equilibrium. Neither 16S nor 5S fractions lose copper at pH 10.5 as judged by 280/340-nm absorbance ratios.

The 5S subunits in fraction II can be reassociated to 14–16S hexamers by simply lowering the pH of the Tris-EDTA dialysis buffer to 8.0. Attempts to reconstitute the 24S dodecamers by dialysis against 0.1 ionic strength Tris-HCl, pH 8.0, made 0.01 M in Mg^{2+} and Ca^{2+} and 0.1 M in NaCl, were unsuccessful.

Disc gel electrophoresis can be used to study the distribution between 16S and 5S populations as a function of pH. Figure 4A shows that at pH 8.9 fraction I gives a single sharp band corresponding to the 16S material and a single, less intense band of 5S material. Figure 4B shows that fraction II gives one sharp major band corresponding to the 5S material, a thin single band corresponding to 16S molecules, and a diffuse stained area between the 5S and 16S material. Figure 4C shows that after dialysis at pH 8.0 fraction II gives an electrophoretic pattern just like that of fraction I hexamers. Finally, Figure 4D shows that when fractions I and II (after dialysis at pH 8.0) are coelectrophoresed, they give a single sharp band corresponding to 16S material (major band) and a single faint band corresponding to 5S material.

Figure 5 is a further illustration of how pH influences the aggregation state of *Ligia* hemocyanin. Samples of purified, unfractionated hemocyanin and fractions I and II from a pH 10.5 chromatography were electrophoresed at pH 7.4, 8.9, and

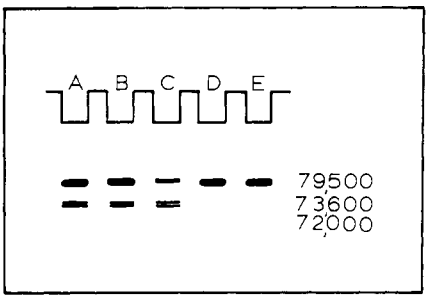

FIGURE 6: $NaDodSO_4$ electrophoresis of *Ligia* hemocyanin on 7.5% slab gel. (A) Purified pigment, 24 S and 16 S; (B) fraction I hexamers from Sephacryl S-200, pH 9.5; (C) fraction I hexamers from Sephacryl S-200, pH 10.5; (D) fraction II monomers from Sephacryl S-200; and (E) fraction II reaggregated hexamers from Sephacryl S-200 monomers.

Table 1: Effect of pH and Cofactors on O_2 Binding Characteristics of Purified *Ligia* Hemocyanin at 20 °C

molecular species	treatment of hemocyanin	co-factor	pH	log $p_{1/2}$	$n_{1/2}$	k^a (s^{-1})
dodecamers and hexamers	Sepharose 4B	Mg^{2+}	7.1	1.44	3.6	400
		EDTA	7.1	1.53	2.9	>400
		Mg^{2+}	8.0	0.95	4.0	350
		EDTA	8.0	1.15	3.4	
		Mg^{2+}	9.2	0.93	4.7	170
hexamers	Sepharose 4B	EDTA	9.0	1.02	3.2	325

[a] The estimates of oxygen dissociation rates (k) are averages from multiple experiments. These rates represent the final stages of oxygen dissociation since much of the reaction was lost in the instrumental dead-time.

10.5. It can be seen that the proportions of bands corresponding to putative hexamer, monomer, and intermediate aggregation states vary as a function of pH. The 16S material generally predominates at pH 7.4 and the monomeric form predominates at pH 10.5. As previously noted, the hexamers isolated by chromatography at pH 10.5 do not dissociate into monomers and thus do not show a monomeric band. Although the pattern of staining due to the intermediate aggregation states varies from one electrophoresis pH to another, the 16S and 5S components consistently migrate as single, homogeneous bands.

$NaDodSO_4$ gel electrophoresis of the freshly purified pigment on 5, 6, and 10% acrylamide disc gels in tubes shows a single band of about 70 000 daltons, corresponding to the molecular weight of the smallest polypeptide chain. $NaDodSO_4$ electrophoresis of crude hemolymph removed from the animal and immediately put into boiling incubation buffer containing the proteolytic blocking agent PMSF also gives a major band of about 70 000 daltons, although several other minor bands are present. Other studies were carried out using $NaDodSO_4$ disc gels on slabs. The $NaDodSO_4$ slab gels have, in general, greater resolving capability than $NaDodSO_4$ tube gels. Figure 6 summarizes these extended studies. $NaDodSO_4$ slab gel electrophoresis of purified *Ligia* hemocyanin on a 7.5% gel shows three bands, a major component corresponding to 79 500 daltons and two minor components of 73 600 and 72 000 daltons (Figure 6A). Fraction I hexamers obtained from both the pH 9.5 and 10.5 Sephacryl separations also show the same three bands. However, while the relative proportions of the three bands from the 16S material obtained at pH 9.5 (Figure 6B) are similar to those of the whole hemocyanin, the 16S fraction isolated at pH 10.5 (Figure 6C) shows a decrease in staining intensity of the 79 500-dalton component. The fraction II samples, obtained from chromatographies at both pH 9.5 and 10.5, migrate on $NaDodSO_4$ slab gels as a single band

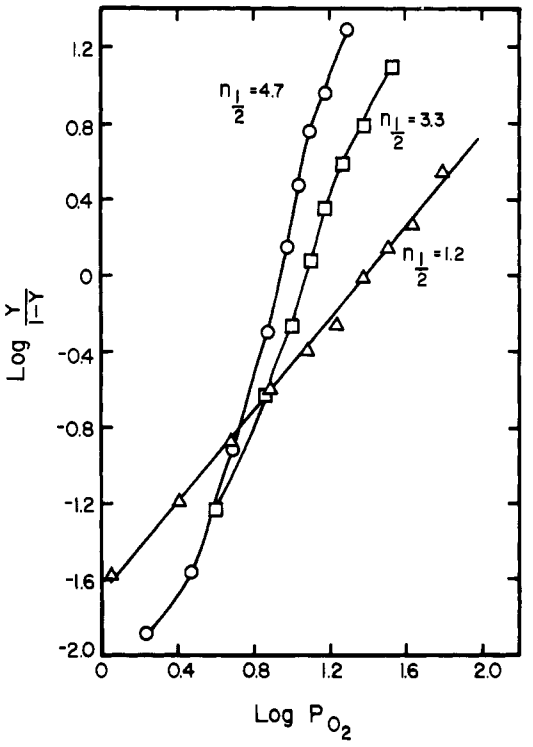

FIGURE 7: Hill plots of oxygen binding to various aggregation states of *Ligia* hemocyanin. Temperature, 20 °C; oxygen pressure is expressed in mmHg. Purified hemocyanin dialyzed vs. 0.1 *I* Tris-HCl, pH 9.2, made 0.01 M in $MgCl_2$ and 0.1 M in NaCl (—○—); fraction I from Sephacryl S-200, pH 9.3 (—□—); fraction II from Sephacryl S-200, pH 9.5 (—△—).

Table II: Oxygen Binding Characteristics of Dissociated and Reassociated *Ligia* Hemocyanin at 20 °C

molecular species	treatment of hemocyanin[a]	cofactor	pH	log $p_{1/2}$	$n_{1/2}$	k^b (s^{-1})
hexamer (from Table I)	Sepharose 4B	EDTA	9.0	1.02	3.2	325
hexamer	fraction I	EDTA	9.3	1.07	3.3	
hexamer	(a) reassociated from monomers of fraction II	EDTA	8.0	1.28	2.1	>400
	(b) reassociated from monomers of fraction II	EDTA + NaCl	8.0			100
monomer	(a) fraction II	EDTA	9.5	1.37	1.2	340
	(b) fraction II	EDTA + NaCl	9.5			120

[a] Fraction I (hexamers) and fraction II (monomers) are the two major zones from a Sephacryl column. [b] The estimates of oxygen dissociation rates (k) are averages from multiple experiments. These rates represent the final stages of oxygen dissociation since much of the reaction was lost in the instrumental dead-time.

with a molecular weight of 79 500, whether applied to the gel as 5S monomers or as reaggregated 16S hexamers (Figure 6D,E). Thus, the smaller molecular weight bands observed in $NaDodSO_4$ gels of the purified hemocyanin and the fraction I hexamers are not present in $NaDodSO_4$ gels of the fraction II monomers.

Oxygen Equilibria. The pH dependence of oxygen binding by Sepharose 4B purified hemocyanin is summarized in Table I. The purified material, containing 24S and 16S molecules, shows a positive Bohr effect and a high degree of cooperativity. Values of the Hill coefficient at half-saturation, $n_{1/2}$, varied from 3.6 to 4.7 in the presence of Mg^{2+}. Figure 7 and Table II summarize the oxygen binding characteristics of the monomeric and hexameric forms of *Ligia* hemocyanin. Figure 7

contrasts the Hill plots of oxygen binding by the Sepharose 4B purified hemocyanin (24 S and 16 S) with those of the hexameric forms (16 S) and the monomeric forms (5 S with a trace of 16 S) of *Ligia* hemocyanin. The hexamers obtained by chromatography at high pH and low ionic strength (fraction I of Figure 3) have approximately the same oxygen affinity and high degree of cooperative interactions as the unchromatographed hexamers that were prepared by dialysis against EDTA-containing buffer at pH 9.0. In contrast, hexamers prepared by reassociation of chromatographically isolated monomers (fraction II of Figure 3) show somewhat reduced cooperativity and a lower oxygen affinity. The 5S subunits of *Ligia* hemocyanin, prepared by chromatography at high pH and low ionic strength, show little or no cooperativity in oxygen binding. At high pH in EDTA-containing buffers the monomers have a much lower oxygen affinity than do the 16S hexamers.

Kinetics of Oxygen Dissociation. Rapid-mixing methods were used to study the kinetics of oxygen dissociation from the various *Ligia* hemocyanin preparations. The reaction was extremely fast under all conditions examined. Although much of the reaction was lost in the dead-time of the rapid-mixing apparatus, the observed portion shows pH-dependent changes that parallel the pH dependence seen in oxygen equilibrium studies. The observed portion of the oxygen dissociation process from the 24S–16S mixture at pH 7.0 in magnesium occurs at a rate of approximately 400 s^{-1}. The high reaction rate results in a large degree of uncertainty in this estimate. The observed rate is considerably slower at pH 9.0, approximately 170 s^{-1}. Although more of the reaction can then be observed, it was not possible to determine if more than a single exponential process was involved. Kinetic data on the hexamer–dodecamer and hexamer preparations of *Ligia* hemocyanin are presented in Table I. The apparent oxygen dissociation rates are increased in the presence of EDTA, which is consistent with the EDTA-induced decrease in oxygen affinity observed in oxygen equilibria determinations. Addition of NaCl to either the fraction II monomer or the hexamer reassembled from fraction II monomers results in a marked decrease in the rate of oxygen dissociation. This suggests that there may be an increased oxygen affinity in the presence of NaCl. These kinetic aspects of the oxygen binding properties of *Ligia* hemocyanin are summarized in Table II.

Discussion

Ligia hemocyanin exhibits many of the structural and functional characteristics common to crustacean hemocyanins. On the structural level, the data from analytical ultracentrifugation, column chromatography, and electron microscopy indicate that *Ligia* hemocyanin exists in the hemolymph as 24S dodecamers and 16S hexamers with molecular weights of about 900000 and 450000, respectively. A similar pattern of aggregation states is found in many other crustacean hemocyanins (Ellerton et al., 1970; Van Holde & Van Bruggen, 1971; Murray & Jeffrey, 1974; Kuiper et al., 1975; Hamlin & Fish, 1977). *Ligia* hemocyanin is set apart from most crustacean hemocyanins, however, in that very high pH is required to dissociate it into monomers, and the isolated monomers appear to be of a single electrophoretic type. A recent study on the electrophoretic patterns of hemocyanin from six different species of isopods (Oniscoidea) was suggestive of charge heterogeneity both within and between species (Sevilla, 1977). However, since the electrophoresis which indicated heterogeneity was performed at pH 9.1, the variations in banding patterns might well reflect intermediate association phenomena rather than heterogeneous populations of monomers. Although many crustacean hemocyanins dissociate into their monomeric subunits at high pH and low ionic strength in the presence of EDTA, this is not always the case. Electrophoresis at a pH lower than that required for complete dissociation into monomers may result in alterations in the banding pattern due to association phenomena during the electrophoresis. The existence of "resistant" hexameric forms thus makes it hazardous to make judgements on homogeneity or heterogeneity based on electrophoresis alone.

Are the electrophoretically homogeneous subunits of *Ligia* hemocyanin identical by other criteria? Molecular weight estimates for both denatured *Ligia* hemocyanin and denatured fraction I are suggestive of some degree of heterogeneity at the subunit level. These preparations show one major and two minor bands when electrophoresed on high-resolution 7.5% $NaDodSO_4$ slab gels. A doublet pattern on $NaDodSO_4$ gels has been reported for *Cancer magister* (Carpenter & Van Holde, 1973; Loehr & Mason, 1973) and *Callinectes sapidus* (Hamlin & Fish, 1977) hemocyanins and three bands within the same 70000–85000 molecular weight range have been found for *Cherax destructor* (Murray & Jeffrey, 1974) and *Panulirus* (Kuiper et al., 1975) hemocyanins. The multiple bands seen in these decapod hemocyanins, especially apparent on high-resolution $NaDodSO_4$ slab gels, have been cited as evidence for multiple polypeptide chains. It is of interest that when *Ligia* hemocyanin monomers (fraction II) are electrophoresed on $NaDodSO_4$ slab gels, a single sharp band appears with a calculated molecular weight of 79500. The same results are obtained with the 5S material isolated by chromatography at pH 9.5 (50% of the total protein) or at pH 10.5 (70% of the total protein). We cannot, at present, distinguish between a number of possible explanations for the differences in banding patterns between the isolated monomeric and hexameric forms of *Ligia* hemocyanin. The minor bands of lower molecular weight in the hexameric fraction could be either real or artifactual. The interpretation that these minor bands represent structurally distinct monomers that are preferentially held in hexameric aggregates cannot be excluded. However, this possibility would imply that the hexameric forms made up of diverse subunits show the same electrophoretic mobility as hexamers containing only identical subunits. As pointed out in the results, no charge heterogeneity has been observed under three different conditions of pH in either 16S or 5S material.

There are several ways minor bands could appear that do not involve intrinsic subunit heterogeneity. If proteolysis, enzymatic removal of carbohydrate bound to the hemocyanin, or carbohydrate contaminants are invoked, one must presume that the chromatographic separation of hexamers and monomers keeps the contaminating factor with the hexameric fraction. There is no evidence to either support or refute the possibility of a nonuniform binding of carbohydrate which results in heterogeneous behavior on $NaDodSO_4$ gels. Extensive precautions were taken to eliminate proteolysis during protein purification and in the incubation with $NaDodSO_4$. Furthermore, it is likely that a proteolytic or glycolytic contaminant would have been removed by the initial purification process. Alternatively, the differences in $NaDodSO_4$ patterns of hexamers (fraction I) and monomers (fraction II) may reflect conformational heterogeneity of the hexameric population. Fraction I hexamers of a particularly "tight" conformation may impose constraints upon some of the subunits so that they denature differently in $NaDodSO_4$–mercaptoethanol solutions. A likely result might be an unequal binding of $NaDodSO_4$ to the subunits, which would give rise

to spurious molecular weight heterogeneity.

The ratio of the 24S and 16S components of *Ligia* hemocyanin appears to be independent of protein concentration at pH 8.0, Mg^{2+}, 20 °C, since dilute hemocyanin rechromatographed on the same Sepharose 4B column gives an elution pattern similar to the more concentrated pigment. Furthermore, at pH 9.5 or 10.5 in EDTA, the 16S hexamers and the 5S monomers can be separated from one another by chromatography and remain as separate populations under these conditions. The presence of these unique populations of components under specific solvent conditions suggests heterogeneity. The results may be explained if the aggregated forms exist in distinct conformational states that have different p*K* values for dissociation. Microheterogeneity within purified protein solutions has been suggested previously to describe the pH-dependent dissociation transitions in other hemocyanins (Di Giamberardino, 1967; Konings et al., 1969; Siezen & Van Driel, 1973).

The oxygen binding properties of *Ligia* hemocyanin, as summarized in Tables I and II, are clearly dependent on pH and divalent cation concentration. The aggregated forms show a high degree of cooperative interaction and a strong positive Bohr effect. Both the cooperativity and the oxygen affinity of the purified hemocyanin are increased in the presence of Mg^{2+}. This allosteric effect of divalent cations has been shown to occur in many other crustacean hemocyanins such as *Callianassa* (Miller & Van Holde, 1974) and *Homarus* (Pickett et al., 1966), although in *Panulirus* (Kuiper et al., 1975) and *Cupiennius* (Loewe & Linzen, 1975) hemocyanins the effect of Mg^{2+} is to lower the oxygen affinity.

The cooperative binding behavior of both dissociation-resistant and nonresistant hexamers in the absence of Mg^{2+} contrasts sharply with the nearly noncooperative binding curves shown by the 17S forms of *Callianassa* hemocyanin in the absence of Mg^{2+} (Miller & Van Holde, 1974) and the hexameric form of *Cupiennius* hemocyanin (Loewe & Linzen, 1975). It is relevant that when purified *Ligia* monomers (fraction II) are brought to lower pH, the hexamers which form exhibit cooperative interactions in oxygen binding. The Hill coefficient, $n_{1/2} = 2.1$, is somewhat lower than that of the native hexamers, $n = 3.2$, but it is clear that cooperativity is maintained. This is important because the reassembled hexamers are known to be composed of subunits that are homogeneous with respect to charge and molecular weight.

Ligia monomers (fraction II) show almost no cooperativity in oxygen binding. The fact that $n_{1/2} = 1.2$ instead of 1.0 at pH 9.5 is probably due to the presence of a small percentage of hexamers in the preparation. Fraction II, composed largely of 5S molecules, clearly has a lower affinity than either 16S or 16S–24S preparations. Low-affinity monomers have also been reported in *Callianassa* hemocyanin (Miller & Van Holde, 1974) and *Panulirus* hemocyanin (Kuiper et al., 1975), although most arthropod hemocyanin monomers show the reverse phenomenon. It is now clear that the aggregation of subunits can introduce constraints which either raise or lower the oxygen affinity of the binding sites.

The oxygen affinity of whole *Ligia* hemocyanin is relatively low, as one would expect for a fast-moving animal living at the air–water interface of the high intertidal where, presumably, there is no problem in obtaining oxygen. As determined by oxygen equilibrium experiments and oxygen dissociation kinetics, the process of oxygen binding is sensitive to changes in pH, $MgCl_2$, and NaCl. With steeply changing oxygen binding curves, very slight changes in the pO_2 could significantly facilitate unloading of the oxygen at the tissues. Since H^+ lowers the oxygen affinity and both Mg^{2+} and NaCl increase the affinity, perhaps there is "enantiostatic" regulation of oxygen binding in *Ligia* as described by Mangum & Towle (1977) for *Callinectes*. It has been reported that the chloride ion is an allosteric regulator of *Limulus* hemocyanin function although its effect in that system is to lower the oxygen affinity rather than to raise it (Brouwer et al., 1977). An allosteric effect of NaCl similar to that observed for *Ligia* hemocyanin has been observed in experiments with *Callinectes* hemocyanin (M. Brouwer, J. Bonaventura, and C. Bonaventura, unpublished observations) and with *Penaus* hemocyanin (Brouwer et al., 1978).

The apparent lack of equilibrium between populations of hexamers and dodecamers, the pH dependence of the hexamer–monomer equilibria, and the minor bands seen in $NaDodSO_4$ banding patterns for the purified hemocyanin suggest caution in concluding that *Ligia* hemocyanin is entirely composed of homogeneous subunits. However, it is clear that at least 70% of the hexamers are composed of a homogeneous pool of polypeptide chains. Since these homohexamers are pH dependent and are capable of binding oxygen cooperatively, *Ligia exotica* hemocyanin presents us with an intriguing model for a prototype of a high molecular weight respiratory protein with highly developed homo- and heterotropic interactions in the absence of conspicuous subunit heterogeneity.

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Inhibition of the (Ca^{2+})ATPase from Sarcoplasmic Reticulum by Dicyclohexylcarbodiimide: Evidence for Location of the Ca^{2+} Binding Site in a Hydrophobic Region†

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ABSTRACT: Dicyclohexylcarbodiimide (DCCD) inhibits the (Ca^{2+})ATPase, Ca^{2+} uptake by sarcoplasmic reticulum vesicles and Ca^{2+} binding to the (Ca^{2+})ATPase from sarcoplasmic reticulum. Ca^{2+} (at μM concentrations) specifically protects against DCCD inhibition. The inhibition can, therefore, be readily demonstrated only in the presence of Ca^{2+} chelating agents such as EGTA. In the presence of EGTA, the ionophore A-23187 increased the sensitivity to DCCD. The ionophore also increased the phosphorylation of the enzyme by inorganic phosphate in the presence of Mg^{2+}. These results indicate that tightly bound Ca^{2+} is located in a hydrophobic region of the enzyme which is not accessible to EGTA. Complete inhibition of the (Ca^{2+})ATPase is accompanied by binding of 4–5 nmol of [^{14}C]DCCD per mg of ATPase protein in the absence of Ca^{2+} compared with 2 nmol bound per mg in the presence of Ca^{2+} with no ATPase inhibition. Assuming a molecular weight of 100 000 for the ATPase monomer, about 1 nmol of DCCD inhibits 4 nmol of ATPase. This result suggests that the minimal functional unit of the enzyme is a tetramer. Following trypsin digestion of the [^{14}C]DCCD-labeled ATPase most of the radioactivity appears in the 20 000-dalton fragment. We propose that DCCD reacts with the Ca^{2+}-binding site of the ATPase.

The (Ca^{2+})ATPase from sarcoplasmic reticulum is a membrane-bound enzyme which catalyzes Ca^{2+} transport into the vesicles coupled to the hydrolysis of ATP (Hasselbach & Makinose, 1961). Ca^{2+} uptake is accompanied by a reversible sequence of phosphorylation and dephosphorylation of the ATPase protein. The involvement of Ca^{2+} in the different partial reactions can be summarized as follows: Ca^{2+} binding to sarcoplasmic reticulum vesicles (Meissner, 1973) and to the purified (Ca^{2+})ATPase protein (Ikemoto, 1975) indicates the existence of two high-affinity binding sites and one to three low-affinity binding sites. The high-affinity Ca^{2+} binding sites are probably located at the outer surface of the sarcoplasmic reticulum membrane since the apparent K_m for Ca^{2+} uptake (Hasselbach & Makinose, 1961; Mermier & Hasselbach, 1976), for Ca^{2+}-dependent ATPase (Hasselbach & Makinose, 1961; Shigekawa et al., 1976), and for phosphorylation of the ATPase protein by ATP (Martonosi, 1969) is similar to the dissociation constant of the high-affinity Ca^{2+} binding site (1–3 μM).

de Meis & Carvalho (1974) suggested that the low-affinity Ca^{2+} binding site is at the inner surface of the sarcoplasmic reticulum membrane. This conclusion was based mainly on the observation that in leaky vesicles the P_i–ATP exchange reaction requires high Ca^{2+} concentrations (ca. 2 mM), whereas in intact vesicles, which accumulate Ca^{2+}, low external concentrations sufficed. In line with this conclusion is the fact that high internal Ca^{2+} concentrations are required for the reversal of the pump (Barlogie et al., 1971; Panet & Selinger, 1972).

Knowles & Racker (1975) demonstrated that the purified (Ca^{2+})ATPase catalyzes ATP formation from P_i and ADP without involving an ion gradient in a two-step reaction:

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