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Effect of Subunit Composition on Quaternary Structure of Isopod (*Ligia pallasii*) Hemocyanin†

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ABSTRACT: The quaternary structure and subunit composition of hemocyanin from the Pacific Coast isopod *Ligia pallasii* have been determined by using a variety of chromatographic and electrophoretic techniques. Two components, a 16S hexamer and a 24S two-hexamer or dodecamer, are present in a constant ratio of 40–60% in the hemolymph. The native hexamer appears to be composed of only one kind of polypeptide chain, M_1, whereas the two-hexamer is made up of two polypeptide chains, M_1 and M_2. The two-hexamer dissociates at neutral pH in the absence of calcium to two identical hexamers, each of which is composed of one M_2 and five M_1 subunits. Thus the native hexamers differ from the two-hexamers in their subunit compositions; the presence of an M_2 subunit in a hexamer enables it to assemble into a two-hexamer molecule. Dissociation studies of the 16S and 24S aggregates suggest that the M_1 and M_2 polypeptide chains have different intermolecular binding properties. The 16S polymer reversibly dissociates to monomer. Dissociation of the 24S polymer at alkaline pH results in a monomer fraction as well as the time-dependent formation of a very stable hexamer which contains an unusually high proportion of M_2 subunits. Formation of this anomalous hexamer may explain the difficulty of reassembling 24S polymers from alkaline pH dissociated subunits of isopod hemocyanin. The formation of anomalous hexamers may also be the reason for incomplete reassembly of many other crustacean 24S hemocyanins. The simplicity of subunit composition and complexity of polymer formation make *Ligia* hemocyanin a good model system for understanding the role of multiple subunits in hemocyanin structure.

Hemocyanins are blue, copper-containing respiratory proteins found dissolved in the hemolymph of many arthropods and molluscs. The structure and function of hemocyanins have been reviewed in a number of recent publications (Van Holde & Van Bruggen, 1971; Lontie & Witters, 1973; Bonaventura et al., 1977; Wood, 1980; Mangum, 1982). Arthropod hemocyanins are high molecular weight polymers assembled from subunits with molecular weights around 75 000 into multiples of six (M_r 450 000). Each subunit contains two copper atoms per one molecule of bound oxygen. Aggregates of one (16 S), two (24 S), four (36 S), and eight (60 S) hexamers can occur. In general, crustacean arthropods have hexamer or two-hexamer aggregates in their hemolymph, thalassinid shrimps and arachnids, four-hexamer aggregates, and horseshoe crabs, eight-hexamer aggregates.

Most arthropod hemocyanins exhibit a marked degree of subunit heterogeneity (Sullivan et al., 1974; Robinson & Ellerton, 1977; Markl et al., 1979a,b; Lamy et al., 1979; Larson et al., 1981) ranging from 3 to 12 subunits. The amount of subunit heterogeneity often parallels the level of aggregation. A major question concerning arthropod hemocyanin is whether, in those hemocyanins with multiple subunits, each different subunit has a specific role in determining the structure of the polymers. Several studies indicate that certain subunits are required in the assembly process, with some subunits apparently participating in the formation of hexamers and others linking the hexamers into higher aggregates (Schutter et al., 1977; Lamy et al., 1977; Jeffrey et al., 1978; Bijlholt et al., 1979; Van Bruggen et al., 1980). In some cases, the linking subunits appear to be dimers connected by disulfide bonds (Jeffrey et al., 1978; Markl, 1980; Pilz et al., 1980; Markl et al., 1981).

Previous studies have shown that hemocyanins from the isopods *Ligia exotica* (Terwilliger et al., 1979) and *Bathynomus giganteus* (Van Holde & Brenowitz, 1981) appear to have little subunit heterogeneity. However, *Bathynomus* hemocyanin assembles only to the hexamer state, whereas *Ligia exotica* hemocyanin forms both one-hexamer and two-hexamer aggregates, similar to crustacean hemocyanins with more extensive subunit heterogeneity. The relative simplicity of the subunit structure of *Ligia* hemocyanin together with its complex 16S and 24S quaternary structures suggested that a detailed study of isopod hemocyanin would be helpful in understanding the way in which specific subunits participate in the structure and function of hemocyanin.

This paper describes in detail the structure of hemocyanin from the Pacific Coast isopod, *Ligia pallasii*. The species is larger than *L. exotica*, and thus ample hemocyanin is available. The purpose of this study is to evaluate the roles of the subunits

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in formation of the 16S and 24S polymers.

Materials and Methods

Ligia pallasii (Brandt) was collected at night from the sea cliffs near the mouth of Coos Bay, OR. The animals were identified according to Richardson (1905). To avoid possible complications due to sex differences, molt cycle, or laboratory storage, only adult male intermolt isopods ranging in size from 3 to 5 cm in length were routinely used, and they were bled within a few days of collection. For specified experiments, hemocyanin was also obtained from small males as well as ovigerous and nonovigerous females. The hemolymph was obtained by inserting a capillary pipet between the fourth and fifth thoracic segments. About 100 μL of hemolymph could be obtained from one large male. Hemolymph from 50–75 animals was mixed with a small volume of ice-cold 0.05 ionic strength Tris-HCl[1] buffer, pH 7.5, that was made 0.1 M in NaCl, 10 mM in $MgCl_2$, and 10 mM in $CaCl_2$. The serine protease inhibitor, phenylmethanesulfonyl fluoride (1 mM), was included in the buffer to minimize the possibility of proteolysis. After centrifugation of the hemolymph at 12000*g* for 10 min, the supernatant was immediately applied to a 1.8 × 100 cm column of Bio-Gel A-5M (200–400 mesh) at 4 °C equilibrated with the same buffer minus phenylmethanesulfonyl fluoride. The column was calibrated with *Eudistylia vancouveri* chlorocruorin (M_r 2.8 × 10^6) (Terwilliger et al., 1975), *Cancer magister* 25S (M_r 940 000) and 16S (M_r 450 000) hemocyanin (Ellerton et al., 1970), and lactate dehydrogenase (M_r 150 000) (Sigma Chemical Co.).

Electrophoresis. The $NaDodSO_4$ electrophoresis procedure is based on the discontinuous system of Laemmli (1970) and has been described previously (Terwilliger & Terwilliger, 1982). Polyacrylamide slab gels (7.5%) were routinely electrophoresed at 100 V for about 3 h and stained with Coomassie brilliant blue R (Fairbanks et al., 1971). Molecular weight determinations larger than 100 000 were obtained on 5% gels.

Urea tube gel electrophoresis was performed at pH 2.3 in the presence of 6.25 M urea (Panyim & Chalkley, 1969; Poole et al., 1974). The urea solutions were deionized with Amberlite MB-1 resin immediately prior to use. Hemocyanin samples were dissolved in a solution of 10 M urea (deionized), 5% glacial acetic acid, and 1% 2-mercaptoethanol at a concentration of approximately 75 μg of protein/mL of solution. Samples were incubated at room temperature for at least 4 h. Preelectrophoresis of the gels and electrophoresis of the samples were carried out at 2 mA per tube. Hemocyanin was also incubated in 6 M guanidine hydrochloride and 2% 2-mercaptoethanol at pH 7.5 in a boiling water bath for 1 min, followed by 4 h at 22 °C. It was then dialyzed against the urea/acetic acid/mercaptoethanol incubation buffer before analysis by urea gel electrophoresis.

Regular gel electrophoresis in the absence of detergent or reducing agent (Davis, 1964) was modified for 5% and 7.5% polyacrylamide slab gels. The gels were preelectrophoresed in the appropriate gel buffer for at least 2 h and protein samples run under the same conditions. Calibrants included 25S, 16S, and 5S components of *Cancer magister* hemocyanin (Ellerton et al., 1970; Terwilliger & Terwilliger, 1982) and bovine serum albumin. Electrophoresis discontinuous buffer systems included 0.05 M Tris-HCl/Tris-maleate (pH 7.0 and 7.5), 0.05 M Tris–glycine/Tris-HCl, 10 mM in EDTA (pH 8.9), and 0.05 M sodium bicarbonate/sodium glycinate, 10 mM in EDTA (pH 10.0 and 10.5). For dissociation experiments at pH 7.0 and 7.5, the upper and lower buffers and the gel buffer were 10 mM in $CaCl_2$ or $MgCl_2$.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

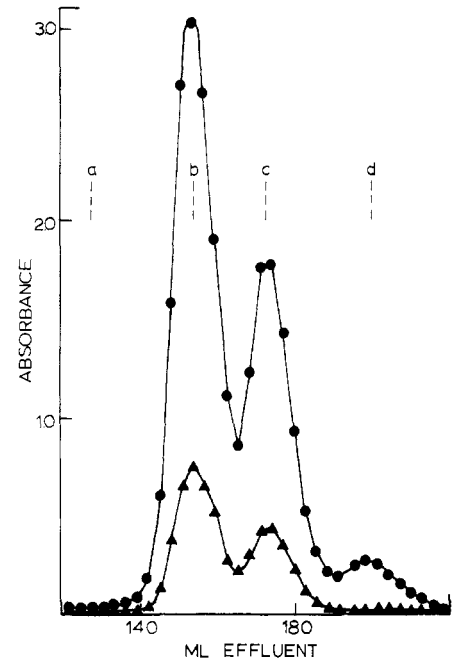

FIGURE 1: Elution pattern of *Ligia pallasii* hemocyanin chromatographed on Bio-Gel A-5 M (200–400 mesh), pH 7.5, 0.05 ionic strength Tris-HCl, 10 mM in $MgCl_2$, 10 mM in $CaCl_2$, and 0.1 M in NaCl. Column volume 1.8 × 100 cm. Calibration proteins: (a) *Eudistylia* chlorocruorin, (b) 25S and (c) 16S *Cancer magister* hemocyanin, and (d) lactate dehydrogenase. Absorbance at 280 (●) and 340 (▲) nm.

Destained gels were scanned at 550 nm with a Zeiss PMQ II spectrophotometer. Relative concentrations of protein bands were determined by calculating the area under the absorbance peaks.

Peptide Mapping. The procedures for obtaining peptide maps of protein bands isolated by gel electrophoresis are described by Cleveland et al. (1977). The preparative 7.5% $NaDodSO_4$ slab gels were loaded with 30–50 μg of hemocyanin and were electrophoresed for 5.5–6 h in order to have enough protein to visualize the peptide maps and also maintain sufficient band separation. The same peptide mapping procedure was also followed with protein bands obtained from urea gel electrophoresis.

Amino Acid Analysis. The amino acid composition of purified M_1 hemocyanin was determined according to Spackman et al. (1958). Cysteine and cystine were determined as cysteic acid on performic acid oxidized hemocyanin (Hirs, 1967).

Dissociation Experiments. Dissociation at high pH was carried out by dialyzing the purified hemocyanin fractions overnight at 4 °C against either 0.05 M sodium glycinate buffer, pH 10.0, or 0.05 M sodium glycinate buffer, pH 10.5, 10 mM in EDTA. The samples were then chromatographed on a 1.8 × 97 cm Bio-Gel A-1.5 M column equilibrated against the same buffer.

Detection of Copper on Gels. The localization of copper on polyacrylamide gels is based on the quenching of fluorescence of bathocuproinesulfonate as described by Bruyninckx et al. (1978).

Results

Purification and Quaternary Structure. The hemocyanin of *L. pallasii* can be separated on a Bio-Gel A-5 M column

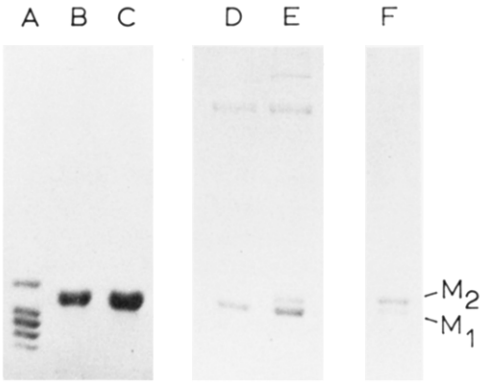

FIGURE 2: $NaDodSO_4$ gel electrophoresis of *L. pallasii* hemocyanin on 7.5% slab gel. (A–C) samples incubated in buffer containing 5% 2-mercaptoethanol: (A) control, *Cancer magister* 25S hemocyanin; (B) *Ligia* 16 S; (C) *Ligia* 24 S. (D–F) 2-Mercaptoethanol omitted from incubation buffer: (D) *Ligia* 16 S; (E) *Ligia* 24 S; (F) anomalous hexamer from Bio-Gel A-1.5 M fractionation, pH 10, of *Ligia* 24S fraction.

into two peaks (Figure 1). The first peak has an apparent molecular weight of approximately 900 000 and corresponds to a two-hexamer or 24S fraction. It will be designated 24S hemocyanin. The second peak has an apparent molecular weight of 450 000 and corresponds to a hexamer or 16S fraction. The 24S material comprises about 60% and the 16S material makes up 40% of the *L. pallasii* hemocyanin at pH 7.5 in the presence of 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.1 M NaCl. When a sample of either the 24S or the 16S material one-sixth the concentration of the original solution is rechromatographed on the same Bio-Gel column, each sample elutes as a single peak in the same position as before. The absorbance ratios at 280 nm/340 nm for both the 24S and the 16S hemocyanin peaks are 4.1. A trailing peak of yellow material with an apparent molecular weight of 150 000 is also present in *L. pallasii* hemolymph (Figure 1). Analysis by electrophoresis indicated it was not hemocyanin; thus it was not included as a hemocyanin component in the following experiments.

Subunit Composition. The purified 16S and 24S fractions each electrophorese on a 7.5% polyacrylamide–$NaDodSO_4$ slab gel in the presence of 2-mercaptoethanol as a single component with an apparent molecular weight of 80 000 ● 1500 (SD of six determinations) (Figure 2B,C). The six polypeptide chains of *Cancer magister* hemocyanin, which range in molecular weight from 81 800 to 67 300 (Larson et al., 1981), can easily be resolved on this same gel system (Figure 2A). In the presence of $NaDodSO_4$ but in the absence of 2-mercaptoethanol, the 16S fraction electrophoreses as a single band corresponding to a molecular weight of 80 000 (Figure 2D) whereas the 24S fraction can be resolved into two bands with apparent molecular weights of 80 000 and 82 000 (Figure 2E). These two putative monomer bands will be referred to as M_1 and M_2, respectively. The staining intensity of M_1 in Figure 2E is about 5 times greater than that of M_2. The 80 000 molecular weight monomer of the 16S fraction is electrophoretically identical with M_1 obtained from the 24S fraction. In the absence of 2-mercaptoethanol several bands corresponding to higher molecular weight components are also present; the 16S fraction shows one band (M_r 140 000) (Figure 2D) and the 24S fraction two bands (M_r 140 000 and 157 000) (Figure 2E). The relative concentration of the high molecular weight material increases with storage time in $NaDodSO_4$. When the bands obtained by $NaDodSO_4$ gel electrophoresis in the absence of reducing agent are excised from the gel and

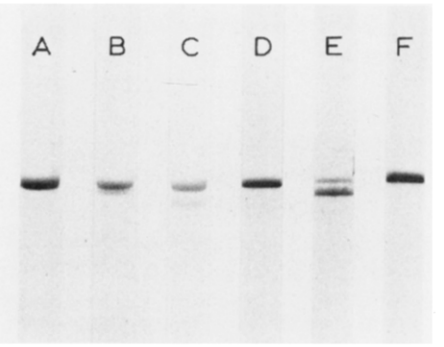

FIGURE 3: Urea gel electrophoresis at pH 2.3 of *Ligia pallasii* hemocyanin. Samples incubated with 2-mercaptoethanol except where noted. (A) 16 S; (B) 24 S; (C) 24 S minus mercaptoethanol; (D) monomer fraction from 16S sample, Bio-Gel A-1.5 M, pH 10; (E) anomalous hexamer fraction from 24S sample, Bio-Gel A-1.5 M, pH 10; (F) monomer fraction from 24S sample, Bio-Gel A-1.5 M, pH 10.

reelectrophoresed in $NaDodSO_4$ with 2-mercaptoethanol, they each electrophorese as a single band with a molecular weight of 80 000.

Gel electrophoresis of the two hemocyanin fractions in 6 M urea and 2-mercaptoethanol at pH 2.3 gives the following results. The 16S fraction contains only one component (Figure 3A). The 24S fraction consists of two closely migrating bands (Figure 3B). The upper band is present in approximately 5 times higher concentration than the lower band. Urea gel electrophoresis of hemocyanin fractions prepared without reducing agent give similar results except that the lower band in the 24S fraction migrates slightly faster and therefore is separated farther from the upper band (Figure 3C). The single band of the 16S fraction coelectrophoreses with the upper band of the 24S fraction. Both of these bands are equivalent to the M_1 band identified by $NaDodSO_4$ gel electrophoresis; the lower band of the 24S fraction is equivalent to M_2 from $NaDodSO_4$. These identities were confirmed by cutting out the bands from the urea gels and reelectrophoresing them in $NaDodSO_4$ in both the presence and absence of reducing agent. Further evidence that the urea gel bands and the $NaDodSO_4$ gel bands are equivalent is provided by the fact that the relative concentrations as determined by gel scans of M_1 and M_2 in the 24S fraction are similar in urea gels and $NaDodSO_4$ gels. The possibility existed that incubation of the samples in urea with 2-mercaptoethanol at low pH might not result in complete cleavage of disulfide bonds or unfolding of the polypeptide chain. Thus, hemocyanin fractions were incubated in guanidine hydrochloride and 2-mercaptoethanol at neutral pH and then dialyzed against urea and 2-mercaptoethanol at pH 2.3. Subsequent analysis of the fractions by urea gel electrophoresis showed the same results as described above.

Further characterization of M_1 subunits in the 16S fraction and M_1 and M_2 subunits in the 24S fraction was carried out by the modified peptide mapping technique of Cleveland et al. (1977). The M_1 and M_2 subunits were first separated by $NaDodSO_4$ preparative gel electrophoresis in the absence of reducing agents. Purified 16S and 24S proteins in $NaDodSO_4$ incubation buffer containing 2-mercaptoethanol were run on the analytical gel as controls. $NaDodSO_4$ band M_1 from the 24S fraction (Figure 4C) and $NaDodSO_4$ band M_1 from the 16S fraction (Figure 4E) show indistinguishable peptide patterns after limited digestion by *S. aureus* V8 protease. The control 16S protein (Figure 4A,F) shows the same digestion pattern as band M_1, although the control is present in higher concentrations. The peptide pattern of band M_2 from the 24S

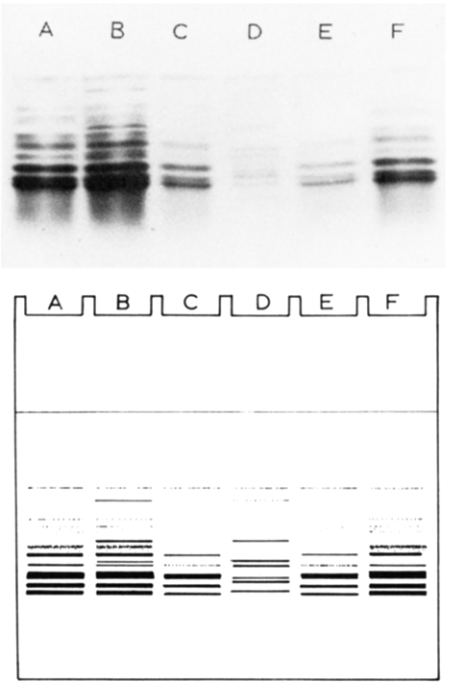

FIGURE 4: (Upper) Modified peptide maps on 15% gel of *Ligia pallasii* hemocyanin subunits. Each band was cut from an $NaDodSO_4$ gel (run in the absence of 2-mercaptoethanol) and treated with 0.6 μg of *Staphylococcus aureus* V8 protease as described under Materials and Methods. (A) 16S fraction; (B) 24S fraction; (C) M_1 from 24S fraction; (D) M_2 from 24S fraction; (E) M_1 from 16S fraction; (F) 16S fraction. (Lower) Diagram of upper part.

Table I: Amino Acid Composition of M_1 Subunits of *Ligia pallasii* Hemocyanin

amino acid	residues/ 100 residues	amino acid	residues/ 100 residues
Lys	5.2	Ala	6.5
His	6.8	Cys	0.7[a]
Arg	4.8	Val	8.0
Asp	13.4	Met	2.2
Thr	5.7	Ile	3.9
Ser	5.4	Leu	7.8
Glu	10.6	Tyr	3.8
Pro	5.5	Phe	4.9
Gly	7.3	Trp	*b*

[a] Determined as cysteic acid. [b] Not measured.

fraction (Figure 4D) is clearly different from that of band M_1. The control 24S hemocyanin peptide pattern (Figure 4B) is a composite of the peptides from both M_1 and M_2. The peptide mapping procedure was also carried out on M_1 and M_2 bands obtained by urea gel electrophoresis. The results were indistinguishable from the peptide patterns of bands obtained by $NaDodSO_4$ gel electrophoresis.

The experiments described above were carried out on samples of hemolymph which had been pooled from a large number of animals. For determination of whether the M_1 and M_2 subunit pattern seen in the pooled 24S fraction was simply a reflection of polymorphism in the *Ligia* population, hemolymph samples were removed from individual animals and run separately on $NaDodSO_4$ gels in the absence of reducing agent. Hemolymph from animals of different sizes and sexes and females in both ovigerous and nonovigerous stages of their reproductive cycle all showed the pattern of a major M_1 and a minor M_2 band on $NaDodSO_4$ gels.

The amino acid composition of the M_1 subunits from the 16S fraction is shown in Table I. Purified M_2 subunits were not available in adequate concentration for an accurate amino acid analysis.

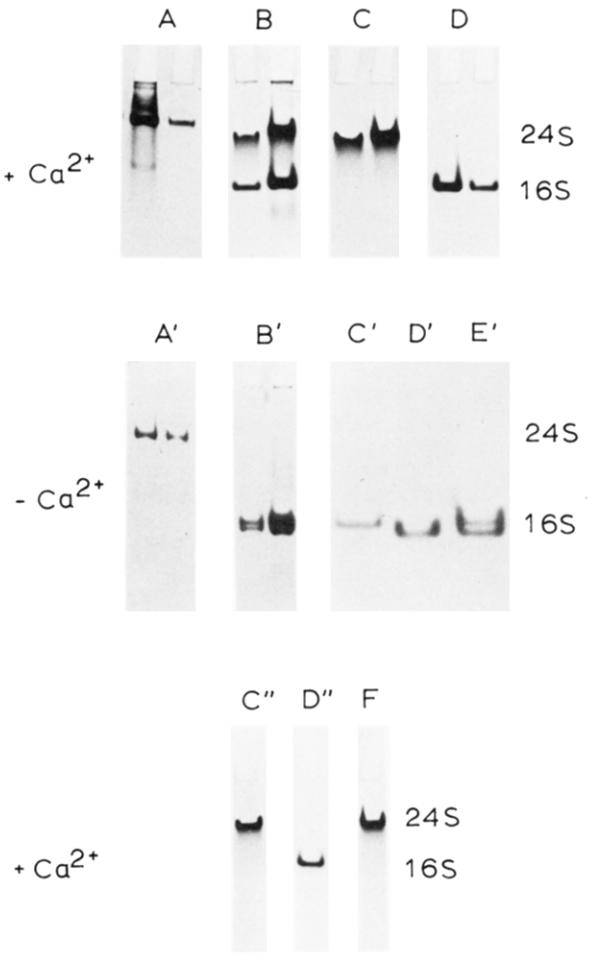

FIGURE 5: Regular slab gel electrophoresis, pH 7.0, on 5% acylamide gels of *Ligia pallasii* 16S and 24S fractions in the presence and absence of Ca^{2+}. (A–D) Gel buffers 10 mM in $CaCl_2$. (A) *Cancer magister* hemocyanin, two concentrations; (B) unfractionated *Ligia* hemolymph; (C) *Ligia* 24S fraction; (D) *Ligia* 16S fraction. (A′–E′) No calcium added: (A′–D′) same as above; (E′) *Ligia* 24S fraction plus 16S fraction. (C″, *D″*, *F*) Gel buffers 10 mM in $CaCl_2$: (C″) *Liqia* 24S fraction; (D″) *Ligia* 16S fraction; (F) *Ligia* 24S fraction which had been dissociated to 16 S by dialysis against Ca^{2+}-free buffer prior to reassembly in this electrophoresis. The *s* values reflect accepted terminology for aggregation states of crustacean hemocyanin rather than specifically determined $s_{20,w}$ values.

Ca^{2+}-Sensitive Reversible Dissociation to Hexamers. *L. pallasii* unpurified whole blood electrophoreses as two major components on a regular 5% polyacrylamide gel at pH 7.0–7.5 in the presence of $CaCl_2$ (Figure 5B). Copper analysis showed positive fluorescence quenching of both bands, indicating the presence of copper in each. The upper band corresponds to a two-hexamer aggregate (24S fraction) and the lower band, a hexamer (16S fraction) based on comparisons with *Cancer magister* hemocyanin (Figure 5A). However, when whole hemolymph is electrophoresed in the absence of divalent cations or when an equimolar concentration of $MgCl_2$ is substituted for $CaCl_2$, the 24S band is no longer present (Figure 5B′). Instead, two distinct hexamer bands are seen. Reelectrophoresis of each of these bands on $NaDodSO_4$ indicated that the upper hexamer band (Figure 5B′) consists of both M_1 and M_2 subunits in a ratio of about 5:1 and results from cleavage of the 24S polymer into hexamers. The lower hexamer band is composed solely of M_1 subunits and is derived from the original 16S material.

For confirmation of this interpretation, the same experi-

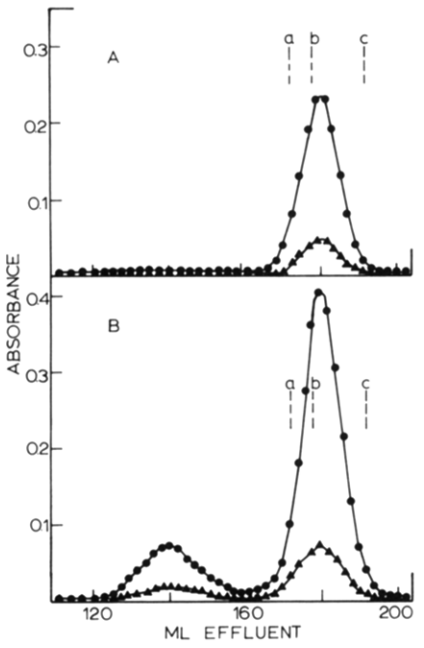

FIGURE 6: Elution patterns of *L. pallasii* purified 16S (A) and 24S (B) hemocyanin chromatographed on Bio-Gel A-1.5 M after dialysis vs. 0.05 M sodium glycinate buffer, pH 10.0. Column volume 1.8 × 97 cm; column buffer same as dialyzate. Calibration proteins: (a) phosphorylase A; (b) bovine serum albumin; and (c) ovalbumin. Absorbance at 280 (●) and 340 (▲) nm.

ments were carried out on purified 24S and 16S fractions. In the presence of Ca^{2+}, the 24S fraction electrophoreses as a single band corresponding to intact 24S molecules (Figure 5C). In the absence of Ca^{2+}, the 24S polymer electrophoreses as a single band corresponding to a hexamer (Figure 5C′). The purified 16S fraction electrophoreses as a single hexamer band of slightly faster mobility than the dissociated 24S polymer regardless of whether or not Ca^{2+} is present (Figure 5D,D′). Coelectrophoresis of purified 24S and 16S fractions in the absence of Ca^{2+} resolves both hexamer bands (Figure 5E′).

The Ca^{2+}-sensitive dissociation of *L. pallasii* 24S hemocyanin into hexamers is a reversible process. Purified 24S polymers were dialyzed overnight against the pH 7.0 Tris–maleate electrophoresis buffer made 10 mM in EDTA to ensure removal of divalent cations and dissociation into hexamers. Next, EDTA was removed by dialysis against the same buffer minus EDTA. The samples were then electrophoresed on a pH 7.0 regular gel in the presence of Ca^{2+}. The 24S molecules which had been dissociated to hexamers now electrophoresed as two-hexamer molecules (Figure 5F).

Dissociation to Monomers. Dissociation of the native hexamers and two-hexamers into monomers was studied by dialyzing the two purified fractions against high pH, low ionic strength buffer as described under Materials and Methods. Each fraction was then chromatographed on a Bio-Gel A-1.5 M column equilibrated against the same buffer. The 16S hexamers dissociate completely into monomers with an apparent molecular weight of 70 000 under these conditions (Figure 6A). $NaDodSO_4$ gel electrophoresis indicates that this monomeric fraction is composed solely of M_1 subunits. The 24S two-hexamers dissociate into two fractions. About 80% of the 24S protein is dissociated into monomers (M_r 70 000); the monomeric fraction from the native 24S two-hexamers (Figure 6B) is composed solely of M_1 subunits. The remaining 20% chromatographs as putative hexamers (Figure 6B); these putative hexamers contain M_2 plus M_1 subunits (Figure 2F). The M_2 subunits are present in a ratio of four M_2 to two M_1 subunits, compared to the one M_2 to five M_1

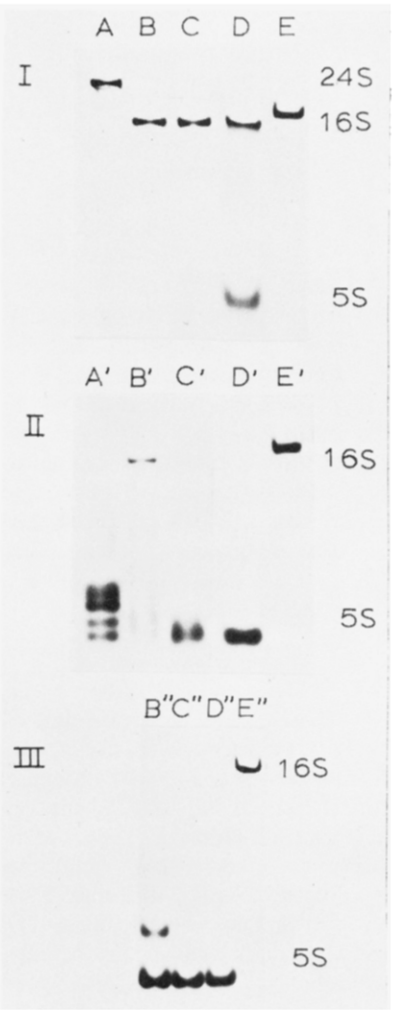

FIGURE 7: Regular gel electrophoresis of *L. pallasii* hemocyanin. (I) pH 7.5 gel, 5% acrylamide; (II) pH 8.9 gel, 10 mM EDTA, 7.5% acrylamide; (III) pH 10.5 gel, 10 mM EDTA, 7.5% acrylamide. (A) *Cancer magister* hemocyanin; (B) *Ligia* 24 S; (C) *Ligia* 16 S; (D) monomers from 16S fraction, Bio-Gel A-1.5 M, pH 10.0; (E) anomalous hexamers from 24S fraction, Bio-Gel A-1.5 M, pH 10.0. (A′–E′) (B″–E″) Same as above.

ratio found in the native 24S molecules. The same distribution of subunits is found when the Bio-Gel A-1.5 M fractions are analyzed by urea gel electrophoresis (Figure 3D–F).

Electrophoretic analyses of these high pH dissociation products on regular gels in the absence of denaturants or reducing agents are shown in Figure 7. The monomeric dissociation product of the native 16S hemocyanin electrophoreses as a single band corresponding to a 5S subunit at alkaline pH (Figure 7D′,D″) and shows partial reassociation to a 16S hexamer when electrophoresed at pH 7.5 (Figure 7D). The monomeric dissociation product of the 24S fraction behaves in the same manner. In contrast, the putative hexamer dissociation product of the 24S fraction electrophoreses as a single hexamer component under all conditions studied (Figure 7E–E″). Its mobility at pH 7.5 in the absence of Ca^{2+} is slower than either the hexamer band of a dissociated 24S molecule or that of a native undissociated 16S molecule. Even at high pH, this unusual hexamer does not dissociate to monomer.

What is the origin of the putative hexamer component with the high M_2 subunit ratio? Initial experiments on *L. exotica* pooled 16S and 24S hemocyanins suggested that a similar component was a dissociation-resistant hexamer (Terwilliger et al., 1979). The following results indicate that the putative hexamer is a reassembly of dissociated subunits into an ar-

tificial or anomalous hexamer. When the native 24S hemocyanin at pH 7.5 is electrophoresed directly into a pH 10.5 gel without prior dialysis, no "resistant" hexamer band is observed; all the protein dissociates into two monomeric bands (Figure 7B″). It is only after the 24S fraction is dissociated into subunits at high pH and the subunits remain in proximity with one another during the overnight dialysis that the resulting putative resistant hexamer is observed (Figure 6B and 7E–E″).

Reassembly of Monomers. Dissociation of the native 16S molecules to M_1 monomers is a reversible process. When the monomers obtained by dialysis at high pH in EDTA of either the 16S or 24S fraction are dialyzed back to neutral pH in the presence or absence of divalent cations, they reassemble to hexamers as determined by column chromatography. No other molecular weight aggregates are observed. When the native 24S fraction is dialyzed against high pH in EDTA and the resulting mixture of artificial M_2:M_1 hexamers and M_1 monomers then returned to neutral pH in the presence of divalent cations, reassembly occurs, but only to hexamers. The dissociated 24S fraction does not reassemble into two-hexamer aggregates under these conditions.

Discussion

Ligia hemocyanin is unusual in that appreciable amounts of both 16S and 24S aggregates are present in the hemolymph, whereas in many other crustaceans, either the 16S or 24S polymer predominates. Only a few crustaceans, such as *Astacus fluviatilis* (Eriksson-Quensel & Svedberg, 1936), *Homarus americanus* (Pickett et al., 1966), and *Cherax destructor* (Murray & Jeffrey, 1974), contain roughly equal concentrations of both 16S and 24S forms. Even among the isopod hemocyanins that have been studied, the structure of *Ligia* hemocyanin is noteworthy since the hemolymph of other isopod species is reported to contain only 16S components (Berthet et al., 1964; Van Holde & Brenowitz, 1981). However, sedimentation constants determined by Berthet et al. (1964) were performed in the absence of divalent cations. As discussed below, these buffer conditions may account for the apparent absence of any 24S components in their study.

The molecular weights of *L. pallasii* hemocyanin subunits, 80 000–82 000, and the amino acid composition of the M_1 subunits from the 16S fraction are similar to those of other arthropod hemocyanins (Van Holde & Van Bruggen, 1971; Sevilla, 1978). However, a unique feature of *L. pallasii* hemocyanin is that it apparently is composed of only two monomers, M_1 and M_2. This is in contrast to the more extensive subunit heterogeneity observed in most crustacean hemocyanins. Both subunits migrate on $NaDodSO_4$ gels with indistinguishable apparent molecular weights when the disulfide bonds are reduced. Only in the absence of reducing agent can M_1 and M_2 be resolved on $NaDodSO_4$ gels. This behavior suggests that M_1 and M_2 are two distinct polypeptide chains which have very similar molecular weights but which may have different conformations due to a variation in internal disulfide bonds. The subunit heterogeneity in *Ligia* hemocyanin does not appear to be due to genetic polymorphism or degradation during sample preparation. The differences in subunit composition between the 16S and 24S polymers provide an explanation for the coexistence of both polymers in apparently nonequilibrium conditions in the hemolymph.

The high molecular weight bands observed when purified *Ligia* hemocyanin is electrophoresed in $NaDodSO_4$ in the absence of reducing agent most likely correspond to partially denatured or reaggregated M_1 and M_2 subunits rather than to "native" disulfide-linked dimeric assemblages. The formation of spurious high molecular weight aggregates during molecular weight determinations in the absence of reducing agents has been documented (Poole et al., 1974). While it is conceivable that future refinements in protein chemistry may reveal a heretofore unrecognized subunit heterogeneity in *L. pallasii*, as has been found for other hemocyanins (cf. Markl et al., 1979b; Larson et al., 1981; Marlborough et al., 1981), it is unlikely that these high molecular weight bands represent additional subunits.

The reversible dissociation of *L. pallasii* hemocyanin two-hexamer to hexamer shows a calcium specificity. While the calcium requirement can be obviated by a sufficiently high ionic strength (0.1 M NaCl), an equimolar concentration of Mg^{2+} instead of Ca^{2+} is not sufficient to either maintain or reassociate the two-hexamer component. Neither the native 16S hexamers nor the hexamers resulting from cleavage of the 24S molecule when Ca^{2+} is removed dissociate further to 5S monomers under the Ca^{2+} free, low ionic strength conditions. It appears that the interhexamer affinities in the 24S aggregate are more sensitive to ionic perturbations, especially Ca^{2+} concentration, than are the intrahexamer bonds. Consequently, the monomer–hexamer assembly process must differ from the assembly of hexamers to higher aggregation states, and discretion must be used in extrapolating from one process to the next. The direct dissociation of *L. pallasii* 24S hemocyanin into hexamers simply by removing Ca^{2+} at neutral pH is strong evidence that the two hexamers of the *Ligia* 24S hemocyanin aggregate are not linked by a disulfide bond.

The 5:1 ratio of M_1 and M_2 subunits present in the 24S fraction suggests several possible subunit arrangements. The most probable pattern in *L. pallasii* hemocyanin is a 24S polymer composed of two identical hexamers, each consisting of one M_2 and five M_1 subunits, based on the following reasons. First, the hexamer population resulting from the dissociation of the 24S fraction electrophoreses at neutral pH in the absence of Ca^{2+} as a single component on regular gel electrophoresis. Futhermore, this hexamer band is clearly resolvable from the band corresponding to the native M_1 hexamer. It can also be distinguished from the putative artificial hexamers (2 M_1:4 M_2) which migrate as a third hexamer band in this electrophoresis system. If the 24S fraction were composed of hexamers containing variable ratios of M_1 and M_2 subunits as seen in *Cherax destructor* hemocyanin (Jeffrey et al., 1976), it is expected that they too could be resolved on this gel system. Similarly, two separate populations of homogeneous M_1 and homogeneous M_2 24S aggregates could also be resolved at the hexamer level. Instead, only one band is present.

The dissociation behavior of the two-hexamer is more complex than that of the native M_1 hexamer. When alkaline pH dissociation of the two-hexamer to monomers takes place during electrophoresis, with no prior dialysis, the M_1 and M_2 subunits are physically separated from one another and no reassembly takes place. However, a time-dependent reassembly of all M_2 and some M_1 subunits into the unusually stable anomalous hexamer occurs during dialysis at alkaline pH. It is possible that the M_2 subunits undergo a change in charge distribution or conformation at alkaline pH which alters their affinity for one another. The conformational change would then be maintained upon aggregation into the anomalous hexamer such that it is stable over the pH range 7.0–10.5, unlike the native molecules. The "dissociation-resistant" hexamer of *L. exotica* (Terwilliger et al., 1979) is more likely a rearrangement of subunits into an anomalous hexamer, as in *L. pallasii*.

The formation of these anomalous hexamers helps explain the inability of the 24S component, dissociated to 5S, to reassemble beyond the hexamer state. Since the M_2 subunits have all become incorporated into the stable anomalous hexamers, they are not available to participate in the formation of the 24S two-hexamers. However, reassembly of the M_1 monomer pool proceeds easily to the level of the M_1 homohexamer as do *L. exotica* monomers (Terwilliger et al., 1979) and *Bathynomus* monomers (Van Holde & Brenowitz, 1981). The difficulties reported in the literature by numerous researchers of reassembling other crustacean hemocyanin subunits to a polymerization level beyond the hexamer (for example, Morimoto & Kegeles, 1971; Jeffrey, 1979) compared to the relative ease of reassembly of chelicerate polymers may well be related to the tendency of certain crustacean hemocyanin subunits to form anomalous aggregates at high pH, as illustrated by *Ligia* hemocyanin.

In a comparative study of crustacean hemocyanins (Markl & Kempter, 1981), the number of electrophoretically distinguishable polypeptide chains was always greater than the same hemocyanin examined by immunological methods. Markl & Kempter (1981) have suggested that the electrophoretically heterogeneous subunits in arthropod hemocyanin can be grouped into two general categories, the antigenically "variable" subunits, which form homohexamers, and the antigenically more "conservative" subunits, which appear to link hexamers into higher aggregates. Immunological studies have not yet been done on *L. pallasii* or *Bathynomus* hemocyanins. It is likely, however, that *L. pallasii* subunit M_1 belongs to the variable group and subunit M_2 to the conservative group. While M_2 has not been demonstrated to dimerize, its presence is necessary for the formation of the two-hexamer. The three subunits of *Bathynomus* hemocyanin may all be variable subunits, since no polymerization to two-hexamer occurs (Van Holde & Brenowitz, 1981). *L. pallasii* hemocyanin subunits provide an example of the minimal number of subunit types necessary for crustacean hemocyanin polymer assembly. The *Ligia* 16S polymer can be formed from M_1 alone; the 24S polymer requires the additional M_2 subunit, a possible "two-hexamer linker". Differences in kinds of linker subunits are supported by the variations observed in interhexamer affinity from hemocyanins of different arthropods, ranging from the calcium-sensitive dissociation at neutral pH in *Ligia* and lobster (*Homarus americanus*) 25S hemocyanins (Morimoto & Kegeles, 1971) and the alkaline pH dissociation of the brachyuran crab (*Cancer pagurus*) 25S hemocyanin into hexamers (Markl et al., 1979a) to the multihexameric aggregates of chelicerate hemocyanins which contain dimers sensitive to high concentrations of EDTA, alkaline pH, and 4 M urea (Schneider et al., 1977; Lamy et al., 1979). The strongest interhexamer bonds are found in those hemocyanins which appear linked by covalent disulfide bonded dimers. Thus, the variations in interhexamer affinities may be a reflection of species-specific differences in amino acid side chains available for subunit binding sites.

In summary, it is likely that subunits M_1 and M_2 of *L. pallasii* hemocyanin represent the two basic subunit types of arthropod hemocyanin. Both M_1 and M_2 play a unique role in the level of polymerization. It appears that these two subunits represent the minimal number of subunit types necessary for assembly to the two-hexamer state of crustacean hemocyanin.

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References

Berthet, J., Baudhuin, P., & Wibo, M. (1964) *Arch. Int. Physiol. Biochim. 72*, 676–677.

Bijlholt, M. C., Van Bruggen, E. F. J., & Bonaventura, J. (1979) *Eur. J. Biochem. 95*, 399–405.

Bonaventura, J., Bonaventura, C., & Sullivan, B. (1977) in *Oxygen and Physiological Function* (Jobsis, F., Ed.) pp 177–220, Professional Information Library, Dallas, TX.

Bruyninckx, W. J., Gutteridge, S., & Mason, H. S. (1978) *Anal. Biochem. 89*, 174–177.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Ellerton, H. D., Carpenter, D. E., & Van Holde, K. E. (1970) *Biochemistry 9*, 2225–2232.

Erikkson-Quensel, I. B., & Svedberg, T. (1936) *Biol. Bull. (Woods Hole, Mass.) 71*, 498–547.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2616.

Hirs, C. H. W. (1967) *Methods Enzymol. 2*, 199–203.

Jeffrey, P. D. (1979) *Biochemistry 18*, 2508–2512.

Jeffrey, P. D., Shaw, D. C., & Treacy, G. B. (1976) *Biochemistry 15*, 5527–5533.

Jeffrey, P. D., Shaw, D. C., & Treacy, G. B. (1978) *Biochemistry 17*, 3078–3084.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lamy, J., Lamy, J., Baglin, M. C., & Weill, J. (1977) in *Structure and Function of Haemocyanin* (Bannister, J. V., Ed.) pp 37–49, Springer-Verlag, West Berlin.

Lamy, J., Lamy, J., & Weill, J. (1979) *Arch. Biochem. Biophys. 193*, 140–149.

Larson, B. A., Terwilliger, N. B., & Terwilliger, R. C. (1981) *Biochim. Biophys. Acta 667*, 294–302.

Lontie, R., & Witters, R. (1973) in *Inorganic Biochemistry* (Eichorn, G. L., Ed.) pp 344–358, Elsevier, Amsterdam.

Mangum, C. P. (1982) in *Biology of Crustacea* (Mantel, L. H., Ed.) Vol. II, Academic Press, New York (in press).

Markl, J. (1980) *J. Comp. Physiol. 140*, 199–207.

Markl, J., & Kempter, B. (1981) in *Invertebrate Oxygen Binding Proteins, Structure, Active Site and Function* (Lamy, J., & Lamy, J., Eds.) pp 125–138, Marcel Dekker, New York.

Markl, J., Hofer, A., Bauer, G., Markl, A., Kempter, B., Brenzinger, M., & Linzen, B. (1979a) *J. Comp. Physiol. 133*, 167–175.

Markl, J., Markl, A., Schartau, W., & Linzen, B. (1979b) *J. Comp. Physiol. 130*, 283–292.

Markl, J., Decker, H., Stöcker, W., Savel, A., Linzen, B., Schutter, W. G., & Van Bruggen, E. F. J. (1981) *Hoppe-Zeyler's Z. Physiol. Chem. 362*, 185–188.

Marlborough, D. I., Jeffrey, P. D., & Treacy, G. B. (1981) *Biochemistry 20*, 4816–4821.

Morimoto, K., & Kegeles, G. (1971) *Arch. Biochem. Biophys. 142*, 247–257.

Murray, A. C., & Jeffrey, P. D. (1974) *Biochemistry 13*, 3667–3671.

Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337–346.

Pickett, S. M., Riggs, A. F., & Larimer, J. L. (1966) *Science (Washington, D.C.) 151*, 1005–1007.

Pilz, I., Goral, K., Hoylaerts, M., Witters, R., & Lontie, R. (1980) *Eur. J. Biochem. 105*, 539–543.

Poole, T., Leach, B. S., & Fish, W. W. (1974) *Anal. Biochem. 60*, 596–607.
Richardson, H. S. (1905) *A Monograph on the Isopods of North America*, pp 682–684, U.S. Government Printing Office, Washington, D.C.
Robinson, H. A., & Ellerton, H. D. (1977) in *Structure and Function of Haemocyanin* (Bannister, J. V., Ed.) pp 55–70, Springer-Verlag, West Berlin.
Schneider, H. J., Markl, J., Schartau, W., & Linzen, B. (1977) *Hoppe-Seyler's Z. Physiol. Chem. 358*, 1133–1141.
Schutter, W. G., Van Bruggen, E. F. J., Bonaventura, J., Bonaventura, C., & Sullivan, B. (1977) in *Structure and Function of Haemocyanin* (Bannister, J. V., Ed.) pp 13–21, Springer-Verlag, West Berlin.
Sevilla, C. (1978) *Comp. Biochem. Physiol. B 60B*, 467–471.
Spackman, H. D., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.
Sullivan, B., Bonaventura, J., & Bonaventura, C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2558–2562.
Terwilliger, N. B., & Terwilliger, R. C. (1982) *J. Exp. Zool.* (in press).
Terwilliger, N. B., Terwilliger, R. C., Applestein, M., Bonaventura, C., & Bonaventura, J. (1979) *Biochemistry 18*, 102–108.
Terwilliger, R. C., Garlick, R. L., Terwilliger, N. B., & Blair, D. P. (1975) *Biochim. Biophys. Acta 453*, 101–110.
Van Bruggen, E. F. J., Bijlholt, M. M. C., Schutter, W. G., Wichertjes, T., Bonaventura, J., Bonaventura, C., Lamy, J., Lamy, J., LeClerc, M., Schneider, H. J., Markl, J., & Linzen, B. (1980) *FEBS Lett. 116*, 207–210.
Van Holde, K. E., & Van Bruggen, E. F. J. (1971) in *Subunits in Biological Systems* (Timasheff, S. N., & Fasman, G. D., Eds.) pp 1–53, Marcel Dekker, New York.
Van Holde, K. E., & Brenowitz, M. (1981) *Biochemistry 20*, 5232–5239.
Wood, E. J. (1980) *Essays Biochem. 16*, 1–47.

Guanidine Hydrochloride Induced Unfolding of the α Subunit of Tryptophan Synthase and of the Two α Proteolytic Fragments: Evidence for Stepwise Unfolding of the Two α Domains†

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ABSTRACT: The relationship between the domain structure of the α subunit of *Escherichia coli* tryptophan synthase and the mechanism of unfolding of the α subunit is investigated. Previous studies of the unfolding of the α subunit by increasing concentrations of guanidine hydrochloride or urea detected a partially unfolded form of the α subunit at intermediate concentrations of either denaturant. The possibility that this partially unfolded form of the α subunit results from the preferential unfolding of one of the two domains of the α subunit is now investigated. This study utilizes two proteolytic fragments of the α subunit, α-1 and α-2, which have been shown to refold independently and to correspond to two domains of the α subunit. The effects of guanidine hydrochloride concentration on the separate α-1 and α-2 fragments, on the intact α subunit, and on the derivative nicked by trypsin (α') are compared by measuring ellipticity at 222 nm and by measuring the susceptibility of tyrosyl residues to chemical modification. The results show that guanidine hydrochloride induced unfolding of the α subunit results from the stepwise unfolding of the two domains: the α-2 fragment and the corresponding domain in the intact α subunit are unfolded by low concentrations of guanidine hydrochloride; the α-1 fragment and the corresponding domain in the intact α subunit are unfolded by higher concentrations of guanidine hydrochloride.

Although the mechanism of unfolding and refolding of proteins has been extensively studied and reviewed [see Anfinsen (1973), Baldwin (1975, 1978), Creighton (1978), and Wetlaufer (1981)], it is still incompletely understood. The classic approach to determining the pathway of protein unfolding has been to isolate intermediates, to characterize them, and to place them in correct order on the pathway (Baldwin, 1978). This unfolding pathway can be represented as a multistate process:

$$N \rightleftarrows I \rightleftarrows U$$

where N is the native state, U is the unfolded state, and I is one or more unfolding intermediates with a conformation different from N or U. However, in most equilibrium studies of globular proteins, the process is so highly cooperative that intermediates are not detected, and the pathway can be represented by a two-state process:

$$N \rightleftarrows U$$

Since a number of large, single-chain globular proteins appear to contain two or more largely independent regions of structure termed domains, it has been independently proposed by two groups that these domains represent the products of independent folding processes, which begin with independent nucleation steps (Goldberg, 1969; Wetlaufer, 1973). Since several of these multidomain proteins have been shown to unfold by multistate processes, these proteins have been useful for characterizing unfolding intermediates (see Results and Discussion).

One important approach to the study of protein folding has been to isolate protein fragments prepared by limited pro-

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