Light-Scattering Investigation of the Subunit Structure and Dissociation of Octopoda Hemocyanins†

Theodore T. Herskovits*
Department of Chemistry, Fordham University, Bronx, New York 10458
German B. Villanueva
Department of Biochemistry, New York Medical College, Valhalla, New York 10595
Received August 6, 1985

ABSTRACT: The molecular weights, subunit dissociation, and conformation in solution of the hemocyanins of three species of octopi were investigated by light-scattering, ultracentrifugation, absorbance, and circular dichroism methods. The molecular weights of the hemocyanins of *Octopus bimaculoides*, *Octopus bimaculatus*, and *Octopus rubescens* obtained by light scattering were 3.3×10^6, 3.4×10^6, and $3.5 (\pm 0.3) \times 10^6$, respectively. The average molecular weights of the fully dissociated hemocyanins of the same octopi, investigated at alkaline pH and in the presence of 8 M urea and 6 M guanidinium chloride (GdmCl), were found to be close to one-tenth of those of the parent proteins, with average molecular masses of 3.4×10^5, 3.3×10^5, and $3.3 (\pm 0.3) \times 10^5$. These findings confirm the earlier observations of van Holde and co-workers with other cephalopod hemocyanins that the basic cylindrical assembly of molluscan hemocyanins consists of 10 subunits. Circular dichroism and absorbance measurements suggest that the dissociated subunits at alkaline pH and in concentrated urea solutions retain their native, multidomain folding. Fairly concentrated GdmCl above 3–4 M is necessary to unfold fully the dissociated hemocyanin chains. Molecular weight measurements studied as a function of reagent concentration with the urea and Hofmeister salt series as dissociating agents show that the ureas are very effective dissociating agents, while the salts are ineffective to moderately effective reagents for octopus hemocyanin. With the ureas the increasing order of effectiveness with greater hydrophobicity of the reagent, in going from urea to methyl-, ethyl-, propyl-, and butylurea, suggests that the basic decameric unit of molluscan hemocyanins in solution is hydrophobically stabilized. The more quantitative interpretation of the dissociation behavior of octopi hemocyanins is made simpler by the absence of stabilizing interdecamer contacts encountered with the land and marine snail hemocyanins, which are largely nonhydrophobic in character [Herskovits, T. T., Mazzella, L. J., & Villanueva, G. B. (1985) *Biochemistry 24*, 3862–3870]. The fit of the urea dissociation data obtained with *O. bimaculoides* hemocyanin could be best accommodated with a model of interacting dimers with about 40–50 apparent amino acid residues (N_{app}) at the contact areas of each dimer forming the parent decamer, with each dimer stabilized by a larger number of amino acid contacts represented by N_{app} values of 110–150 amino acid groups per monomer.

Since the earlier pioneering investigations of Svedberg and co-workers (Svedberg & Hedenius, 1934; Eriksson-Quensel & Svedberg, 1936) on the ultracentrifugal behavior of the hemoglobins and hemocyanins found in the hemolymph of various land and marine invertebrates, the elucidation of their structure and oxygen binding and carrying capacity has gained increasing attention [for recent reviews, see Antonini & Chiancone (1977), Mangum (1980), Van Holde & Miller (1982), and Ellerton et al. (1983)]. The hemocyanins of the arthropods and molluscs are large, copper-containing subunit proteins, with the latter usually consisting of one or two decameric assemblies of multidomained protein chains, each organized in the form of right circular cylindrical particles approximately 300 Å in diameter and 140–180 Å in height (Van Bruggen et al., 1962a,b). Exposure to alkaline pH or the use of salts and ureas as dissociating agents (Van Holde & Cohen, 1964; Konings et al., 1969a,b; Engelborghs & Lontie, 1973; Herskovits et al., 1984, 1985) is known to dissociate the hemocyanins either to the so-called half-molecules or decamers or to their constituent monomeric subunits. Ghiretti and co-workers (Tamburro et al., 1976; Salvato et al., 1979) have found that urea at relatively low concentrations effectively dissociates the hemocyanin decamers of *Octopus vulgaris* without any pronounced alterations in the secondary and tertiary structure of the subunits. The retention of the low intrinsic viscosity and the essentially unaltered circular dichroism and copper absorbance spectra suggest that the folded segments or domains of the subunits housing the active sites have suffered only minor modification. Similar results were obtained with the larger didecameric hemocyanin assemblies of the marine snails *Busycon canaliculatum*, *Littorina littorea*, and *Lunatia heros* studied in our laboratory (Herskovits et al., 1985a,b), which could be denatured only in 4–6 M GdmCl[1] solutions. The detailed interpretation of the association–dissociation behavior of the latter, more complex, hemocyanin assemblies is complicated by several factors, including partial irreversibility perhaps due to the less intact character of the subunit chains known to be very susceptible to proteolysis during the course of isolation (Gielens et al.,

† Supported in part by Grant RR-0715 from the National Institutes of Health, U.S. Public Health Service.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; GdmCl, guanidinium chloride; CD, circular dichroism; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-0931$01.50/0

Table I: Effects of pH, Urea, and GdmCl Dissociation and Unfolding on the Molecular Weight, Absorbance, and CD Parameters of Octopi Hemocyanins

solvent	M_w	ϵ_{278} (dL g^{-1} cm^{-1})	ϵ_{346} (dL g^{-1} cm^{-1})	$[\theta]_{222}$ (deg cm^2 dmol^{-1})	$[\theta]_{M,346}$ (deg cm^2 dmol^{-1})
Octopus bimaculoides					
pH 7.4, μ = 0.1 M Tris, 0.05 M Mg^{2+}, 0.01 M Ca^{2+}	3.3×10^6	15.7 ± 0.1	4.07 ± 0.10	−7100 ± 200	−28 000 ± 4000
pH 8.5, μ = 0.1 M Tris, 0.01 M EDTA	3.0×10^5	15.0	3.36	−6900	−32 100
pH 9.8, μ = 0.1 M bicarbonate–NaOH, 0.01 M Mg^{2+}	3.2×10^5	14.5	3.72	−6800	−28 500
2.0 M urea, μ = 0.1 M Tris, pH 8.5, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}	3.7×10^5		3.93	−5850[a]	−34 000
8.0 M urea, μ = 0.1 M acetate, pH 5.7, 0.05 M Mg^{2+}, 0.01 M Ca^{2+}	3.5×10^5	14.4	2.97	−4200	−29 300
6.0 M GdmCl, μ = 0.1 M acetate, pH 5.7, 0.05 M Mg^{2+}, 0.01 M Ca^{2+}	3.5×10^5	15.7	~0	−600	~0
Octopus bimaculatus					
pH 5.7, μ = 0.1 M acetate, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}	3.4×10^6	15.4 ± 0.3	2.0 ± 0.4	−7300 ± 300	−14 000 ± 2000
pH 10, μ = 0.1 M bicarbonate–NaOH	3.0×10^5	14.5	3.17	−6300	−31 000
8.0 M urea, μ = 0.1 M acetate, pH 5.7, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}	3.7×10^5	14.6	2.28	−4900	−21 600
6.0 M GdmCl, μ = 0.1 M acetate, pH 5.7, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}	3.3×10^5	15.1	~0.2	~−200	−3 500
Octopus rubescens					
pH 5.7, μ = 0.1 M acetate, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}	3.4×10^6	15.3 ± 0.1	1.7 ± 0.3	−6700 ± 300	
pH 7.4, μ = 0.1 M Tris, 0.05 M Mg^{2+}, 0.01 M Ca^{2+}	3.5×10^6			−7400	−26 800
pH 10, μ = 0.1 bicarbonate–NaOH	2.7×10^5	14.2	3.4	−6500	−31 700
8.0 M urea, μ = 0.1 M Tris, pH 7.5, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}	3.8×10^5[b]	14.1	2.45	−5100	−24 900
6.0 M GdmCl, μ = 0.1 M acetate, pH 5.7	3.4×10^5	14.8	0.2	−900	−2 000

[a] Interpolated values from smoothed concentration-dependence curve. [b] pH 5.7, μ = 0.1 M acetate.

1975; Brouwer et al., 1978) and also the presence of a further step in the assembly of the pair of decamers forming larger didecameric particles. The reversibility and the correct reassembly of *O. vulgaris* hemocyanin after exposure to urea observed by Salvato et al. (1976), together with some of our present findings that the hemocyanins of octopi escape degradation by proteolysis during isolation, make the study of the interactions of the subunits in the basic hemocyanin decamers an attractive subject of investigation using the simpler hemocyanins of this class of molluscs.

In the present investigation we have examined the effects of the hydrophobic reagents of the urea class and the Hofmeister series of salts on the subunit structure and dissociation of octopi hemocyanins. Due to the paucity of molecular weight and structural information on the hemocyanins of octopi (Svedberg & Pedersen, 1940; van Holde & Miller, 1982), we have also obtained the light-scattering molecular weights and circular dichroism and absorbance properties of both native and denatured forms of the hemocyanins of three octopi species, *Octopus bimaculoides*, *Octopus bimaculatus*, and *Octopus rebescens*.

Materials and Methods

The hemocyanins of three octopi species, *O. bimaculoides*, *O. bimaculatus*, and *O. rubescens*, were isolated by gel filtration in the cold (~4 °C) on Bio-Gel A-5m columns (ca. 35 × 2.5 cm) as previously described (Herskovits et al., 1981, 1985). The hemolymph of the octopi employed was secured through the efforts of Dr. R. C. Fay of Pacific Bio-Marine Laboratories, Inc., Venice, CA. A few milligrams of crystalline phenylmethanesulfonyl fluoride was included with all shipments of hemolymph, sent overnight to New York by Federal Express. The concentration of hemocyanin in the hemolymph of various preparations was found to be about 7–10%, and the measured pH ranged from 6.6 to 7.1. The eluent used for chromatographic purification was 0.1 ionic strength pH 5.7 acetate buffer or pH 7.4 Tris buffer, containing 0.01–0.05 M Mg^{2+} and 0.01 M Ca^{2+}. Where required, the divalent ions were removed by exhaustive dialysis in the cold against the appropriate buffers, initially containing 0.01 M EDTA.

Hemocyanin concentrations were determined spectrophotometrically in a Cary 14 recording instrument by using the extinction coefficients $E^{1\%}_{278}$ of 15.7, 15.4, and 15.3 for the hemocyanins of *O. bimaculoides*, *O. bimaculatus*, and *O. rubescens*, respectively. These extinction coefficients were based on refractive index increment determinations on dialyzed solutions coupled with absorbance measurements; the refractive index increment ($\partial n/\partial c$) of 0.194 g^{-1} cm^3 obtained with several other hemocyanins was used (Herskovits et al., 1985a,b). The concentration of hemocyanin in various dissociating and denaturing solvents was based on extinction coefficients given in Table I, determined by absorbance measurements on solutions diluted from stock solutions of known protein concentrations.

Light-scattering and refractive index increment measurements were made at 436 nm in an instrument of Brice's design made by Wood Manufacturing Co. The solutions and solvents used for light scattering were filtered directly into the cells, through 0.2-μm Gelman membrane filters secured in 25-mm diameter Millipore filter holders. Angular measurements were made between 35 and 145° angles in cylindrical cells requiring back-reflection corrections described by Tomimatsu and Palmer (1963). Most of our measurements were made at 90° in 2.4-cm square cells as previously described (Herskovits et al., 1981, 1985) with the same molar decrements of the specific refractive index increment for various urea solutions. The refractive index increment data were obtained on solutions dialyzed in the cold (Table II).

Circular dichroism measurements were made on a Cary 60 recording spectropolarimeter equipped with a CD attachment using 0.10- and 1.0-cm cylindrical cells. The mean residue molecular weight M_0 = 120 was used for our calculations of the mean reside ellipticities together with the minimum molecular weight of 2.5×10^4 per copper atom for calculating the molar ellipticities at 346 nm.

Ultracentrifugation measurements were made in a Beckman Model E analytical instrument. Corrections for standard conditions for water at 20 °C were made with the viscosity and density data for urea listed in the *Handbook of Chemistry and Physics* (1979).

Results

Concentration and Angular Dependence of the Light-Scattering Data. The light-scattering behavior of all three octopus hemocyanins was investigated in both their native, associated states in the presence of 0.01–0.05 M Ca^{2+} and in

Table II: Light-Scattering Molecular Weight Data of *Octopus bimaculoides*, *Octopus rubescens*, and *Octopus bimaculatus* Hemocyanins

solvent	protein concn range (g L^{-1})	$(\partial n/\partial c)_\mu$ (g^{-1} cm^3)	M_w	B' (L mol g^{-2})
Octopus bimaculoides				
pH 7.4, μ = 0.1 M Tris, 0.05 M Mg^{2+}, 0.01 M Ca^{2+}	0.1–6.2	0.194	(3.3 ± 0.30) × 10^{6a}	8.7 × 10^{-10a}
pH 9.8, μ = 0.1 M bicarbonate–NaOH	0.03–0.6	0.194	3.19 × 10^5	−8 × 10^{-8}
8.0 M urea, μ = 0.1 M acetate, pH 5.7, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}	0.17–1.1	0.147	(3.50 ± 0.1) × 10^5	2 × 10^{-7}
6.0 M GdmCl, μ = 0.1 M acetate, pH 5.7, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}	0.17–1.1	0.134	(3.47 ± 0.10) × 10^5	(3 ± 1) × 10^{-7}
Octopus rubescens				
pH 7.4, μ = 0.1 M Tris, 0.05 M Mg^{2+}, 0.01 M Ca^{2+}	1.2–3.9	0.194	(3.5 ± 0.30) × 10^{6a}	8.2 × 10^{-10a}
pH 10, μ = 0.1 M bicarbonate–NaOH	0.24–1.3	0.201	2.69 × 10^5	4 × 10^{-8}
8.0 M urea, μ = 0.1 M Tris, pH 7.4, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}	0.10–0.4	0.150	3.77 × 10^5	−1 × 10^{-7}
6.0 M GdmCl, μ = 0.1 M acetate, pH 5.7	0.16–1.0	0.132	3.37 × 10^5	−1 × 10^{-8}
Octopus bimaculatus				
pH 5.7, μ = 0.1 M acetate, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}	0.8–7.0[b]	0.194	(3.4 ± 0.30) × 10^{6b}	
pH 9.0, μ = 0.1 M Tris	0.11–1.2	0.204	(2.92 ± 0.23) × 10^5	2 × 10^{-7}
8.0 M urea, μ = 0.1 M acetate, pH 5.7, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}	0.17–1.2	0.153	3.68 × 10^5	7 × 10^{-8}
6.0 M GdmCl, μ = 0.1 M acetate, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}	0.13–1.3	0.139	(3.30 ± 0.28) × 10^5	2 × 10^{-7}

[a] Based on data fit with eq 2 assuming a hard sphere value for B' given in column 5. [b] Based on the average of three independent determination of different hemocyanin preparation with M_w = (3.00 ± 0.10) × 10^6 obtained in the concentration range specified in column 2 and corrected for the presence of 15% dissociation product assumed to be dimers with M_2 = 6.8 × 10^5.

their dissociated and denatured forms at alkaline pHs and the presence of 8 M urea and 6 M GdmCl. Figure 1 presents some of our data obtained with the hemocyanins of *O. bimaculoides*, and *O. rebescens*, studied as a function of both scattering angle and protein concentration. Loefler et al. (1978) have observed a slight angular dependence in the light-scattering behavior of *Busycon canaliculatum* hemocyanin using 632.8-nm laser light as the scattering source. A barely detectable angular dependence is also observable with the Wood instrument with the octopi hemocyanins of somewhat smaller particle dimensions and molecular mass, shown in Figure 1A. The light-scattering data are represented and plotted on the basis of the Zimm (1948) equation:

$$K'c/R_\theta = M_w^{-1}P(\theta)^{-1} + 2B'c \qquad (1)$$

$$P(\theta)^{-1} = 1 + [16\pi^2n^2/(3\lambda^2)]R_G^2 \sin^2(\theta/2) + \ldots$$

In these expressions, K' represents the light-scattering constant, R_θ is the excess Rayleigh relation representing the scattering per unit volume of protein solution at an angle θ over that of solvent, M_w is the weight-average molecular weight, B' is the second virial coefficient, and $P(\theta)^{-1}$ is the reciprocal particle scattering factor related to the radius of gyration of the particle R_G and the geometric and optical terms, given in the latter part of the scattering expression, having their usual meaning (Doty & Edsall, 1951). Experimentally, the angular dependence data shown in part in Figure 1A gave average values for the intercepts and slope corresponding to $P(\theta)^{-1}$ = 1.022 ± 0.008 and 1.035 ± 0.009 at a 90° angle and radii of gyration, R_G, equal to 93 ± 33 and 120 ± 15 Å for *O. bimaculoides* and *O. rubescens* hemocyanin, respectively. The molecular weight data based on 90° measurements and 0° extrapolations were found to be virtually the same. For example, the data represented by curves a and b in Figure 1A gave apparent molecular weights of 3.008 × 10^6 and 3.017 × 10^6 and 3.213 × 10^6 and 3.239 × 10^6, at 90° and 0°, respectively. The more accurate 90° determinations of molecular weights obtained for most of our work with various dissociating agents are thus clearly justified and require no angular dependence or dissymetry corrections.

The slight upswing of the $K'c/R_\theta$ vs. c data of the octopi hemocyanins, observed at low protein concentrations and shown in Figure 1B, suggests dissociation of the hemocyanin particle to its constituent subunits. We have analyzed the

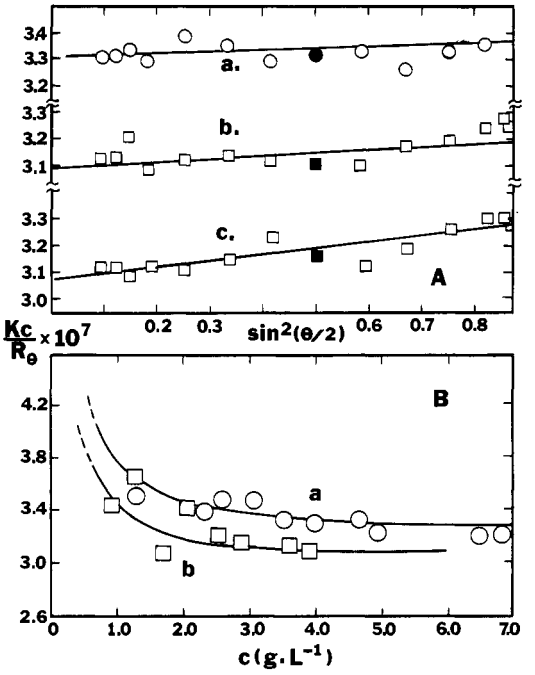

FIGURE 1: Angular dependence (A) and concentration dependence (B) of the light scattering of *Octopus bimaculoides* and *Octopus rubescens* hemocyanins plotted according to eq 1–3. (A) (Curve a) *O. bimaculoides* hemocyanin, c = 4.97 g L^{-1}; (curve b) *O. rubescens* hemocyanin, c = 3.92 g L^{-1}; (curve c) *O. rubescens* hemocyanin, c = 1.69 g L^{-1}. (B) (Curve a) *O. bimaculoides* hemocyanin, data fitted with the parameters $K_{app}^{10,2}$ = 3 × 10^{-26} M^4, B' = 8.7 × 10^{-10} L mol g^{-2}, and M_{10} = 3.3 × 10^6; (curve b) *O. rubescens* hemocyanin, data fitted with $K_{app}^{10,2}$ = 1 × 10^{-26} M^4, B' = 8.2 × 10^{-10} L mol g^{-2}, and M_{10} = 3.5 × 10^6. Solvent was 0.1 M Tris, pH 7.4, 0.05 M Mg^{2+}, and 0.01 M Ca^{2+}.

dissociation behavior of hemocyanin using the light-scattering expression

$$K'c/R_\theta = (M_{10}[1 - (m - 1/m)\alpha_i])^{-1} + 2B'c \qquad (2)$$

and the related apparent equilibrium constant

$$K_{app} = \frac{m^m c^{m-1}\alpha_i^m}{M_{10}^{m-1}(1 - \alpha_i)} \qquad (3)$$

appropriate for dissociation of hemocyanin decamers (Kerskovits et al., 1977, 1978), where α_i represents the weight fraction of the protein that undergoes dissociation forming

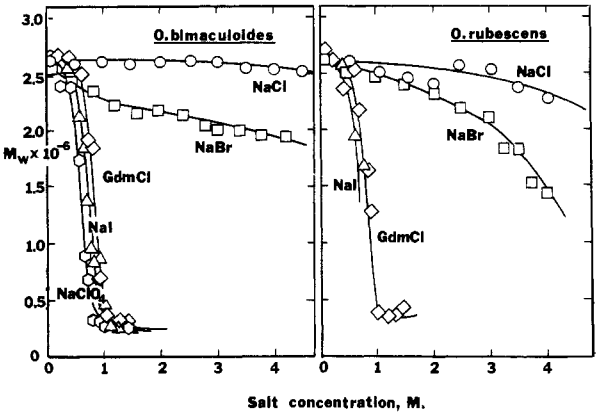

FIGURE 2: Effects of various salts on the molecular weight (M_w) of *O. bimaculoides* and *O. rubescens* hemocyanins plotted as a function of salt concentration. Hemocyanin concentrations employed were 0.1 g L^{-1}; all solutions were buffered with 0.1 M Tris, pH 8.5, containing 0.01 M Mg^{2+} and 0.01 M Ca^{2+}.

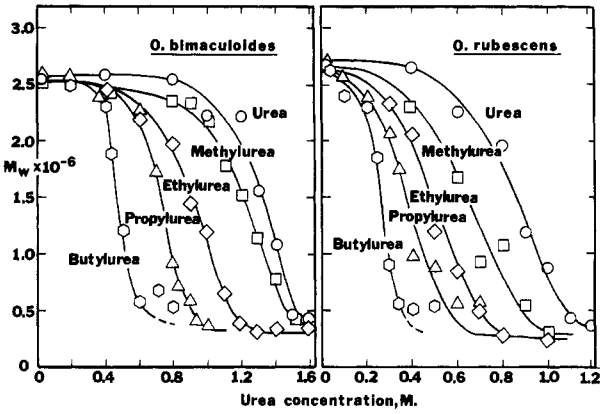

FIGURE 3: Effects of the urea series of dissociating reagents on the molecular weights of *O. bimaculoides* and *O. rubescens* hemocyanin (pH 8.5). Hemocyanin concentration and solvent conditions the same as in Figure 2.

respectively 5 and 10 fragments (=m). Since the dissociation of the octopi hemocyanins at pH 5.7 and 7.4 is not very extensive, the dissociation data obtained could be fitted equally well with a decamer to monomer or a decamer to dimer scheme of dissociation. The best fit of the *O. bimaculoides* data investigated at pH 7.4 in the presence of 0.05 M Mg^{2+} and 0.01 M Ca^{2+} and shown in Figure 1B were obtained with $K_{app}^{10,2} = 3 \times 10^{-26}$ M^4, $K_{app}^{10,1} = 1 \times 10^{-77}$ M^9, $B' = 8.7 \times 10^{-10}$ L mol g^{-2}, and $M_{10} = 3.3 \times 10^6$. Similar results were obtained with *O. rubescens* hemocyanin studied at the same pH and ionic conditions, giving the parameters $K_{app}^{10,2} = 1 \times 10^{-26}$ M^4, $B' = 8.2 \times 10^{-10}$ L mol g^{-2}, and $M_{10} = 3.5 \times 10^6$.

Table II presents a summary of our molecular weight data obtained on both the native octopi hemocyanins and the fully dissociated and denatured proteins obtained at alkaline pH, 8 M urea, and 6 M GdmCl. It is significant that in the latter solvents the molecular weights extrapolated to infinite dilutions have values close to one-tenth of the molecular weights of the parent decameric protein assemblies.

Effects of Ureas and Salts on Subunit Dissociation. Both ureas and salts are known to dissociate the α- and β-type hemocyanins of land and marine snails (Englborghs & Lontie, 1973; Herskovits et al., 1984, 1985). The effects of these two groups of reagents on the subunit structure of octopi hemocyanins were also investigated in regard to the mode of association–dissociation of the subunits and the nature of the forces that hold the subunits together in solutions. Figures 2 and 3 present some of our results obtained with *O. bimaculoides* and *O. rubescens* hemocyanin, studied at the low protein concentration of 0.10 g L^{-1}, which usually require no nonideality corrections. Dissociation by the urea series is interesting in the sense that it follows the order of effectiveness with an increasing hydrocarbon content expected of hydrophobicity stabilized subunit systems. In fact the effectiveness of the higher members of the series, propyl- and butylurea, is comparable to, if not greater than, GdmCl, one of the more effective salts usually employed in protein denaturation work.

We have consequently analyzed the urea dissociation of both *O. bimaculoides* and *O. rubescens* hemocyanins in significant detail using the decamer to monomer, decamer to dimer, and decamer to dimer to monomer schemes of subunit dissociation. The general expression (eq 4) describing the influence of

$$K_{D,app} \simeq K_{w,app} \exp(mN_{app}K_B c_D) \quad (4)$$

dissociation reagents such as salts and ureas on protein equilibria (Herskovits & Ibanez, 1976; Herskovits et al., 1977, 1978) has been combined with eq 3, giving eq 5 and 7, required to generate the weight fractions α_1 and α_2 for monomer and dimer formation used to fit the molecular weight data based on eq 6 and 8:

$$\frac{\alpha_2^5}{1-\alpha_2} = \frac{(3.2 \times 10^{-4}) M_{10}^4 K_{w,app}^{10,2}}{c^4} \exp(5N_{app}^{10,2} K_B c_D) \quad (5)$$

$$M_w = M_{10}(1 - 0.8\alpha_2) \quad (6)$$

and

$$\frac{\alpha_1^{10}}{1-\alpha_1} = \frac{(1 \times 10^{-10}) M_{10}^9 K_{w,app}^{10,1}}{c^9} \exp(10N_{app}^{10,1} K_B c_D) \quad (7)$$

$$M_w = M_{10}(1 - 0.9\alpha_1) \quad (8)$$

In eq 4–7, $K_{w,app}^{10,1}$ and $K_{w,app}^{10,2}$ are the apparent dissociation constants of the hemocyanin decamers dissociating to monomers and dimers, $N_{app}^{10,1}$ and $N_{app}^{10,2}$ are the apparent numbers of amino acids at the contact areas of monomer and dimer in the assembled hemocyanin decamer, c_D is the urea concentration, and K_B is the binding or interaction constant of the average amino acid with urea taken as 0.032 M^{-1} (Herskovits et al., 1977, 1978). For the decamer–dimer–monomer scheme of dissociation, we have the pair of related expressions:

$$\frac{\alpha_2^5}{1-\alpha_2} = \frac{(3.2 \times 10^{-4}) M_{10}^4 K_{w,app}^{10,2}}{c^4(1-\alpha_3)^5} \exp(5N_{app}^{10,2} K_B c_D) \quad (9)$$

and

$$\frac{\alpha_3^2}{1-\alpha_3} = \frac{K_{w,app}^{2,1} M_{10}}{20c\alpha_2} \exp(2N_{app}^{2,1} K_B c_D) \quad (10)$$

where α_3 represents the weight fraction of dimeric intermediates formed intially from the hemocyanin decamers, which dissociate to the final monomeric species, and $N_{app}^{2,1}$ represents the apparent number of amino acids at the contact area of each monomer. The weight-average molecular weight obtained for this model can be written in the form

$$M_w = M_{10}(1.0 - 0.8\alpha_2 - 0.1\alpha_2\alpha_3) \quad (11)$$

Equations 9–11 have been obtained from the sequential scheme of three species of subunits present, described in greater detail in previous papers (Herskovits et al., 1984; Herskovits & Russell, 1984). Figure 4 presents the urea dissociation data obtained at pH 8.5 on *O. bimaculoides* hemocyanin, in the presence of 0.01 M Mg^{2+} and 0.01 M Ca^{2+}, analyzed according to eq 5–11. Curve a of the data fit shows clearly that the dissociation proceeds beyond the dimeric stage. The

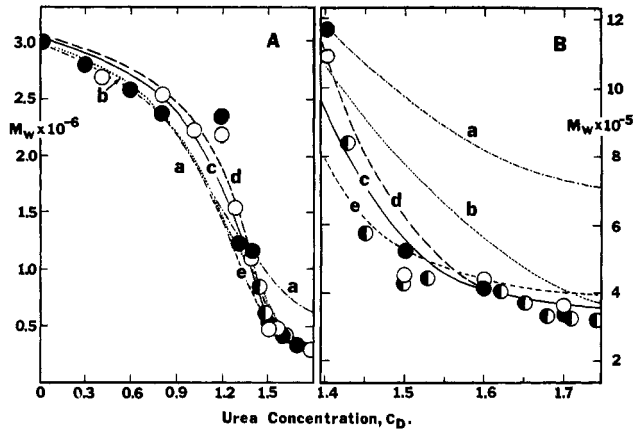

FIGURE 4: Analysis of the urea dissociation data of *O. bimaculoides* hemocyanin on the basis of eq 5–8 (curves a and b) and eq 9–11 (curves c–e) plotted as M_w vs. urea concentration, c_D. (Curve a) $N_{app}^{10,2} = 55$, $K_{w,app}^{10,2} = 2 \times 10^{-28}$ M^4; (curve b) $N_{app}^{10,1} = 50$, $K_{w,app}^{10,1} = 2 \times 10^{-59}$ M^9; (curve c) $N_{app}^{10,2} = 40$, $K_{w,app}^{10,2} = 5 \times 10^{-28}$ M^4, $N_{app}^{2,1} = 150$, $K_{w,app}^{2,1} = 3 \times 10^{-12}$ M; (curve d) $N_{app}^{10,2} = 40$, $K_{w,app}^{10,2} = 2 \times 10^{-28}$ M^4, $N_{app}^{2,1} = 150$, $K_{w,app}^{2,1} = 3 \times 10^{-12}$ M; (curve e) $N_{app}^{10,2} = 40$, $K_{w,app}^{10,2} = 1 \times 10^{-27}$ M^4, $N_{app}^{2,1} = 110$, $K_{w,app}^{2,1} = 1 \times 10^{-10}$ M. With all calculations, $M_{10} = 3.3 \times 10^6$, $K_B = 0.032$ M^{-1}, and $c = 1.0$ g L^{-1} were used. Hemocyanin concentrations employed were 1.0 (●), 0.2 (◐), and 0.1 g L^{-1} (○); solvent and buffer (pH 8.5) were the same as in Figure 2.

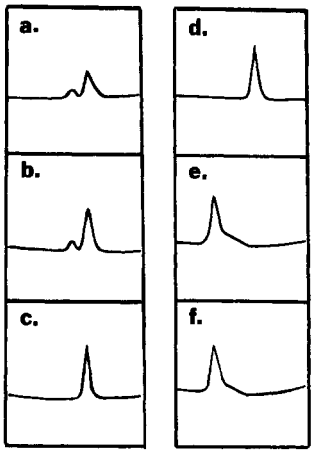

FIGURE 5: Tracings of ultracentrifugation patterns of octopus hemocyanins: (a) *O. bimaculatus* hemocyanin, pH 5.7, μ = 0.1 M acetate, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}; (b) *O. bimaculoides* hemocyanin, pH 5.7, μ = 0.1 M acetate, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}; (c) *O. rubescens* hemocyanin, pH 7.4, μ = 0.1 M Tris, 0.05 M Mg^{2+}, 0.01 M Ca^{2+}; (d) *O. bimaculoides* hemocyanin, pH 8.5, μ = 0.1 M Tris, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}; (e) *O. bimaculoides* hemocyanin, 1.35 M urea, pH 8.5, μ = 0.1 M Tris, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}; (f) *O. bimaculoides* hemocyanin, 1.6 M urea, pH 8.5, μ = 0.1 M Tris, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}. Rotor speed 24 630 and 35 600 rpm, temperature 20 °C, protein concentration 3–5 g L^{-1}.

dissociation to monomers, represented by curve b, seems to be somewhat too gradual in the important 1.4–1.7 M transition region shown in panel B of the figure. Despite the scattering of some of our data points obtained at three different protein concentrations of 0.1, 0.2, and 1.0 g L^{-1}, the decamer–dimer–monomer scheme of subunit dissociation gives clearly the best account for the observed molecular weight data. Essentially the same dissociation behavior was observed for both *O. bimaculoides* and *O. rubescens* hemocyanin with the urea dissociation data giving essentially the same apparent number of amino acid group estimates of $N_{app}^{10,2} = 40$ and $N_{app}^{2,1} = 110$–150.

Ultracentrifugal Behavior. The hemocyanins of octopi species exist primarily as decameric assemblies having sedimentation constants of 48–51 S (Eriksson-Quensel & Svedberg, 1936; Salvato et al., 1979; Miller & van Holde, 1982). Depending on pH, dimeric and monomeric fragments with sedimentation constants of 15–21 and 11–12 S have also been noted with some octopi species. Our limited data obtained with the three hemocyanin species of this study suggest essentially the same behavior (Figure 5). At pH 7.5 and 8.5 in the presence of stabilizing Mg^{2+} and Ca^{2+} ions, the sedimentation patterns show a single boundary of the fully associated 50S particle. At slightly acidic pH 5.7, however, we find that approximately 15% of both *O. bimaculatus* and *O. bimaculoides* hemocyanins are dissociated, forming dimeric fragments with sedimentation constants of 21–27 S, while *O. rubescens* hemocyanin studied at the same pH and ionic conditions shows little or no dissociation (Figure 5, tracings a and b). The sedimentation patterns of *O. rubescens* hemocyanin were found to be essentially the same at pH 5.7 (0.01 M Mg^{2+}, 0.01 M Ca^{2+}) and pH 7.4 (0.05 M Mg^{2+}, 0.01 M Ca^{2+}), both showing a single sedimentation boundary (Figure 5, tracing c). In the presence of urea the dissociation patterns are found to be somewhat more complex. Here the final dissociation products appear to be monomers rather than dimers, with sedimentation values in the neighborhood of 12 S. In the dissociation transition region above 1.2 M urea, the sedimentation patterns are not resolved into clearly identifiable components, the slowest boundary having sedimentation values of 12–15 S. At 1.35 M urea, the observed sedimentation constant associated with this boundary was 12.5 S, while at 1.6 M urea it was found to be 15 S (Figure 5, tracings e and f). The more rapidly sedimenting broad shoulder observed with the latter centrifuge patterns suggests the presence of all three species of hemocyanin in rapid equilibrium, relative to the sedimentation rates necessary for radial separation of the individual components. Rapid dimer to monomer equilibria producing single broad boundaries, with changing sedimentation values of about 19–11 S, having first been noted by van Holde & Cohen (1964), who studied the subunit structure and dissociation of the hemocyanin of the squid *Loligo pealei*.

pH Dependence of the Dissociation Data. The increase in pH above the physiological ranges of about 6.6–7.1 noted with different hemocyanin preparations produces dissociation of the parent decameric assemblies. The observed changes in light-scattering molecular weights shown in Figure 6A suggest dissociation of the subunits to dimers and monomers over a relatively narrow range of pH. The presence of divalent ions such as 0.01 M Mg^{2+} seems to stabilize the decameric assemblies and tends to shift the dissociation transitions above pH 9.0. It is significant that the dissociation of the hemocyanin subunits seems to have no pronounced effect on the folded conformation of the polypeptide chains since the optical activity at 222 nm remains essentially unaltered in the entire pH region from 5.7 to about 10. The copper absorbance at 346 nm is actually intensified as the decameric structure is abolished, above pH 7 (Figure 6B). The increased oxygen binding with pH is also reflected in the increased amplitude of the related CD band centering at the latter wavelength (Table I). Even 8 M urea appears to have only marginal effect on the CD spectra of the three octopi hemocyanins studied. As with the other molluscan hemocyanins investigated in the past 2 years in our laboratory (Herskovits et al., 1984, 1985a,b) by the latter techniques, only in fairly concentrated GdmCl solutions of 4–6 M do we see significant changes in the mean residue ellipticity at 222 nm and corresponding changes in the absorbance and CD spectra of the copper–oxygen bands centered at 346 nm (Figure 7).

Reversibility and Subunit Heterogeneity. The octopi hemocyanins exposed to dissociating but largely nondenaturing

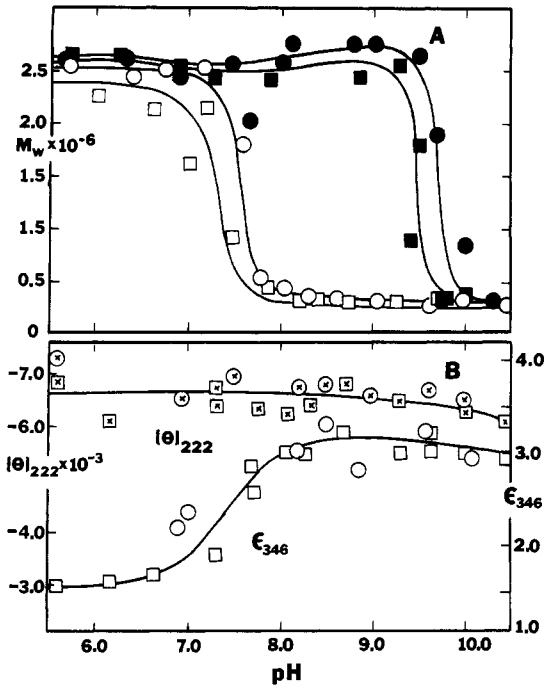

FIGURE 6: Effects of pH and Mg^{2+} ions on the molecular weights (A), the CD spectra at 222 nm and the copper absorbance spectra at 346 nm (B) of *O. bimaculoides* (circles) and *O. bimaculatus* (squares) hemocyanins. Data represented by open symbols were obtained on solutions containing no divalent ions while the data represented by filled symbols were obtained on solutions containing 0.01 M Mg^{2+}.

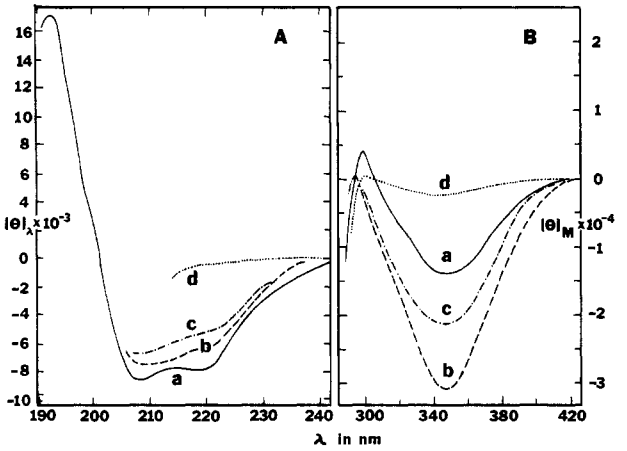

FIGURE 7: Circular dichroism spectra of *O. bimaculatus* hemocyanin in the peptide absorbing (A) and the copper absorbing (B) spectral regions: (curve a) pH 5.7; (curve b) pH 10; (curve c) 8.0 M urea, pH 5.7; (curve d) 6.0 M GdmCl, pH 5.7. Ionic conditions and buffers used are given in Table I. For spectra in the far-ultraviolet region, the buffer and divalent ion concentrations were reduced to half or one-third of their initial values.

conditions such as alkaline pH or 8.0 M urea have exhibited a high degree of reassociation, approaching in some instances nearly complete reassociation. For example, *O. bimaculoides* hemocyanin exposed to pH 8.5 in the presence of 0.01 M EDTA gave initial molecular weights of about 2.9×10^5 and 2.1×10^6 and 2.82×10^6 (at protein concentrations of 0.24 and 0.59 g L^{-1}) following the removal of EDTA and the introduction of 0.05 M Mg^{2+} and 0.01 M Ca^{2+} into the supporting pH 8.5 solvent, by dialysis. Exposure to 8.0 M urea (pH 5.7, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}) where the molecular weight was initially found to be 3.5×10^5 (Table II) gave a molecular weight of 1.8×10^6 ($c = 0.21$ g L^{-1}) upon removal of the urea by dialysis in the cold. The reassociation was found

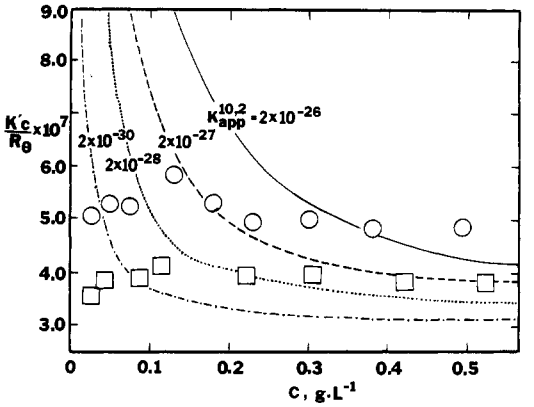

FIGURE 8: Concentration dependence of the light scattering of partially dissociated octopus hemocyanins testing their mass action properties, on the basis of eq 2 and 3. *Octopus bimaculatus* hemocyanin data in 3.0 M NaBr (pH 5.7, 0.1 M acetate, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}) are represented by circles; *O. bimaculoides* data in pH 9.6 0.1 M bicarbonate–NaOH buffer containing 0.01 M Mg^{2+} are represented by squares. The calculated four sets of curves were obtained with $K^{10,2}_{app} = 2 \times 10^{-26}$ to 2×10^{-30} M^4, $B' = 8.7 \times 10^{-10}$ L mol g^{-2}, and $M_{10} = 3.3 \times 10^6$.

to be generally protein concentration dependent, producing more extensive reassociation at higher protein concentrations, usually employed for molecular weight and ultracentrifugation work (Figures 1 and 5).

The concentration dependence of partially dissociated octopus hemocyanin was also investigated with regard to the question of the influence of subunit heterogeneity on the light-scattering behavior. Some of our data obtained on dialyzed solutions of *O. bimaculatus* hemocyanin in 3.0 M NaBr and *O. bimaculoides* hemocyanin at pH 9.6 bicarbonate/NaOH buffer, diluted with dialyzate, are shown in Figure 8. The absence of any significant upswing in the $K'c/R_\theta$ vs. c data at decreasing protein concentration indicates a lack of adherence to the law of mass action at these conditions (3.0 M NaBr and pH 9.6) dictated by eq 2 and 3. The curves representing four sets of different lines were calculated with $K^{10,2}_{app} = 2 \times 10^{-26}$ to 2×10^{-30} M^4, $M_{10} = 3.3 \times 10^6$, and $B' = 8.7 \times 10^{-10}$ L mol g^{-2}. Similar data were obtained in 1.3 and 2.3 M urea solutions corresponding to the dissociation transitions of Figures 4 and 9, curve b.

Discussion

The investigation of three octopi hemocyanin species in this study has addressed the problem of molecular weights, solutions conformation, and subunit dissociation of these hemocyanins by means of pH changes and the use of various dissociating reagents such as the ureas, GdmCl, and salts of the Hofmeister series. We shall discuss these two main topics of our work in turn.

The earlier estimates of the molecular weights reported by Svedberg and co-workers gave initial estimates of 2×10^6 for *Octopus vulgaris* hemocyanin (Svedberg & Eriksson, 1932) followed by a higher estimate of 2.785×10^6 and also a value of 2.791×10^6 for *Eledona moschata* hemocyanin (Eriksson-Quensel & Svedberg, 1936) on the basis of sedimentation–diffusion measurements. More recent estimates based on sedimentation–equilibrium determinations and light-scattering measurements place the molecular weights of octopi hemocyanins about 20–30% above the latter values. Thus, Miller and van Holde (1982) have obtained a value of 3.58×10^6 for *Octopus dofleini* hemocyanin, and our own values of this study give molecular weight estimates of 3.3×10^6, 3.4×10^6, and $3.5 (\pm 0.3) \times 10^6$ for the hemocyanins of *Octopus*

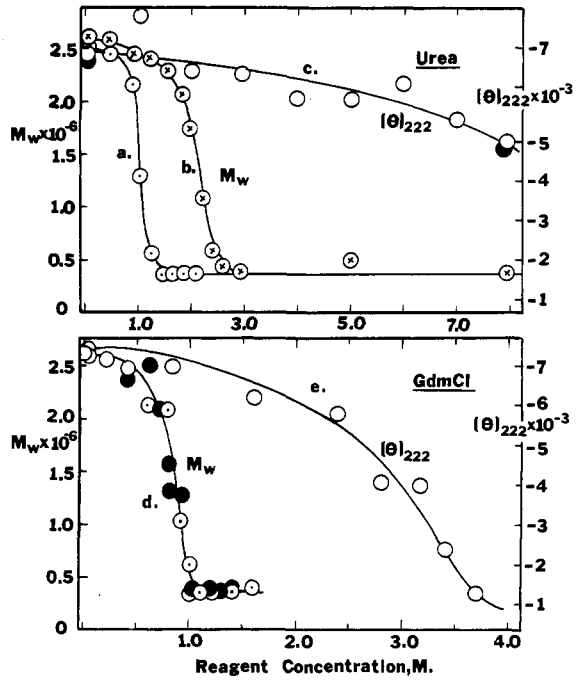

FIGURE 9: Effects of urea (A) and GdmCl concentration on the subunit dissociation and polypeptide chain conformation of octopus hemocyanins reflected by the changes in molecular weights (M_w) and the CD spectra at 222 nm ($[\theta]_{222}$). (Curves a and b) Effects of urea on M_w of *O. bimaculatus* hemocyanin at pH 8.0 and 5.7; (curve c) effects of urea on $[\theta]_{222}$ of *O. bimaculatus* (○) and *O. rubescens* (●) hemocyanin at pH 5.7; (curve d) effects of GdmCl on M_w of *O. bimaculatus* (○) and *O. rubescens* (●) hemocyanin at pH 5.7; (curve e) effects of GdmCl on $[\theta]_{222}$ of *O. bimaculatus* hemocyanin at pH 5.7. All the solutions contained 0.01 M Mg^{2+} and 0.01 M Ca^{2+}.

bimaculoides, *O. bimaculatus*, and *O. rubescens*, respectively (Table II). Moreover, these investigations also have firmly established the fact that the molecular weights of the undegraded monomeric subunits of molluscan hemocyanins are close to one-tenth the molecular masses of the parent hemocyanins. Miller and van Holde (1982) have obtained a molecular weight of 3.58×10^5 for the subunits of *O. dofleini*, and again our own estimates give average values of 3.4×10^5, 3.3×10^5, and $3.3 (\pm 0.3) \times 10^5$ for the high pH, urea, and GdmCl-dissociated hemocyanin subunits of the three species of octopi of this study (Table II). Some intrinsic chain heterogeneity and/or instability of the dissociated subunits has been noted, however, by both Miller and van Holde (1982) and Ryan et al. (1985). Both the SDS gel patterns of *O. dofleini* hemocyanin used by these workers and our light-scattering molecular weight data obtained at alkaline pHs with *O. rubescens* and *O. bimaculatus* hemocyanin (Table II) suggest the possibility of some chain degradation.[2] A slight decrease in molecular weight of the subunits was also observed with *O. bimaculoides* hemocyanin upon prolonged standing in 6 M GdmCl. The initial molecular weight of 3.3×10^5 obtained with one of our preparations was found to decrease to about 2.9×10^5 after 3 days of standing in this solvent at room temperature.

Circular dichroism measurements made on the three octopi species of hemocyanins (Table I and Figure 7) suggest a relatively low helix constant of about 12–15% of the chain conformation of these hemocyanins which is not significantly different from the estimate of 17% obtained by Tamburro et al. (1976) on *O. vulgaris* hemocyanin. With the reference parameters for α-helical, β-structured, and random chain conformations obtained by Yang and co-workers (Chen et al., 1972, 1974), our data also give estimates of about 50–65% β-structure and 20–40 unordered or random chain conformations. These estimates of polypeptide chain conformation are about the same as those obtained for several gastropod hemocyanins (Herskovits & Russell, 1984; Herskovits et al., 1985a,b). The CD data obtained with concentrated urea and GdmCl solutions indicate that the folded functional domains of the hemocyanin subunits are strongly resistant to denaturation (Figure 9). The latter characteristic of the octopod hemocyanins is also shared with other hemocyanins of the gastropods investigated in our laboratory. Salvato and co-workers (Salvato et al., 1974; Tamburro et al., 1976) have found that the fully dissociated hemocyanin of *O. vulgaris*, investigated in 3 M urea, retains its functional oxygen binding property. Moreover, the reassociated hemocyanin regains its characteristic cylindrical structure seen in electron microscopy, upon removal of the urea by dialysis.

The studies of Salvato et al. (1979) have also shown that urea is a very effective dissociating reagent for *Octopus vulgaris* hemocyanin. Our molecular weight data of Figures 3 and 4 suggest that this reagent is an equally effective dissociating probe for other octopi hemocyanins. Significantly, we also find that the effectiveness of the ureas as a class of dissociating agents increases with increasing chain length or hydrophobicity of their substituent alkyl groups. The increasing potency of the higher members of the series and their correlation with greater hydrophobicity suggest hydrophobic stabilization of the basic decameric unit of hemocyanin in solution. The absence of decamer to decamer interactions encountered with the didecameric hemocyanin assemblies of the snail hemocyanins (Herskovits & Russell, 1984; Herskovits et al., 1985b) places this interpretation of the dissociation of the octopi decamers by the hydrophobic urea series of probes on a much firmer footing. The up and down contacts between the pair of decamers of the land and marine snail hemocyanins were found to be largely nonhydrophobic in character, and it was necessary to use 0.5 M NaCl and 1.2 M urea as partial dissociating reagents for *Helix pomatia* and *Littorina littorea* hemocyanins, in order to be able to probe the side to side contacts of the subunits in the half-molecules or decamers of these hemocyanins. It is interesting that salts such as NaCl and to some extent also NaBr have only marginal effects on the subunit stability of the octopi hemocyanins, placing these proteins in the β-type category of hemocyanin chain constituents. The higher members of the Hofmeister series, NaI and $NaClO_4$ are also found to be less potent dissociating reagents for octopus hemocyanin than for the α-component of *Helix pomatia* hemocyanin (Herskovits & Russell, 1984).

The analysis of the urea dissociation data shown in Figure 4 for *O. bimaculoides* is significant in that it suggests a fairly large number of amino acids, on the order of 40–50, at the contact areas of the subunits necessary to produce the fairly sharp initial decline in the observed molecular weight transition. A further interesting feature of our analysis is the finding that the observed dissociation transition is most probably a two-step reaction of decamers dissociating to dimers, followed by a splitting or untangling of the dimers to monomers of one-tenth of the molecular mass of the parent hemocyanin. It is fairly apparent from the data fit of the expanded Figure 4B that the very abrupt decline in molecular

[2] Preliminary SDS gel data obtained by Dr. Mary Hamilton on one of our *O. bimaculoides* preparations suggest also subunit heterogeneity, showing a major band with an approximate molecular weight of (3.2–3.4) $\times 10^5$ and closely spaced minor bands with molecular weights of about 3.6×10^5, 3.5×10^5, 3.1×10^5, and 2.9×10^5.

weights in the neighborhood of 1.5 M urea, investigated at three different protein concentrations ranging from 0.1 to 1.0 g L^{-1}, cannot be accommodated by a two-state dissociation transition of either decamers dissociating to dimers or decamers dissociating directly to monomers. The calculated cruves a and b drawn by dotted and dash-dotted lines represent to shallow transition profiles relative to the actual molecular weight data, and the more appropriate data fit is given by curves c–e, calculated for the three-state transition of decamer–dimer–monomer. The apparent values of amino acid groups (N_{app}) at the two areas of contact between dimers in the parent decamers of octopus hemocyanin and the monomer to monomer contacts are also significant. The former with $N_{app}^{10,2}$ = 40 groups is not very different from previous estimates of 46 ± 12 groups obtained for the decamer to dimer dissociation step of the land snail hemocyanin of *Helix pomatia*, obtained with different salts and urea (Herskovits & Russell, 1984), considering fairly large experimental uncertainties associated with the fit of such data. The much larger $N_{app}^{2,1}$ of about 110–150 groups is interesting in that it suggests a larger contact surface or area between the pair of monomeric units forming each dimer than the number of contacts between neighboring subunits in the decamers. An intertwining structure involving contact between both the folded domains of each monomeric chain and the connecting chain segments (Gielens et al., 1975; Brouwer et al., 1976) represents a reasonable description of the contact areas of the monomers forming the dimers. Again, the analysis of the urea dissociation data of *Busycon canaliculatum* hemocyanin gave $N_{app}^{2,1}$ of 110 (Herskovits et al., 1985a) for the monomer to monomer contacts, which also represents a very large apparent number of amino acid contacts between individual monomer chains.

The three-dimensional structure of *Panulirus interruptus* hemocyanin hexamers at 3.2-Å resolution has been recently determined by Gaykema and co-workers (1984). Amino acid sequence comparison with other arthropodan hemocyanins has shown that the subunits of the basic 450000-dalton hexamers are folded into three domain structures, with the central binuclear copper site located at the center of the second folded domain (Linzen et al., 1985). The studies of Gaykema et al. (1984) and other spectroscopic investigations (Himmelwright et al., 1980; Torensma & Phillips, 1981; van Holde & Miller, 1982) suggest that the basic 50000-dalton oxygen-binding center of the molluscan hemocyanins may have similar structural organization. The central folded domain together with the first or third domain in close contact may represent the basic molluscan globular unit, with eight such units forming each subunit of the hemocyanin (Brouwer et al., 1976; Siezen & Van Bruggen, 1974; Gielens et al., 1980). In the absence of any detailed information concerning the location of the amino acids at the contact areas of the molluscan subunits, it is reasonable to assume that each of the eight globular domains will form an area of contact with neighboring subunits comparable to about two-thirds of the area of contact of an arthropodan hemocyanin subunit, such as the American lobster hemocyanin or the $\alpha\beta$ contact area of the dimer of human hemoglobin, investigated in our laboratory (Herskovits et al., 1977, 1978, 1984). With the approximately 18–20 amino acid groups[3] per contact area in each domain, the eight molluscan domains represent 144–160 amino acid groups, not significantly different for our $N_{app}^{2,1}$ estimate of 110–150 groups obtained for the second dissociation step of *Octopus* and *Busycon* hemocyanins, discussed above.

The invariance of the molecular weight data of the three different protein concentrations examined in the urea transition region in Figure 4B (data represented by filled, half-filled, and open circles) represents one subtle indication of the influence of subunit heterogeneity on the protein equilibria of octopi hemocyanins, a phenomenon also observed with various hemocyanins of both orthropodan and molluscan origin (Di Giamberardino, 1967; Konings et al., 1969a; Engelborghs & Lontie, 1967; van Holde et al., 1977; Herskovits et al., 1981a, 1984b). As a consequence, our amino acid group estimates (N_{app}) based on our present analysis must be viewed as apparent quantities, in need of independent verification and further study.

[3] The later estimate of 18 residues is based on the X-ray crystallographic model of horse hemoglobin of Perutz et al. (1968).

Registry No. GdmCl, 50-01-1; Mg, 7439-95-4; NaCl, 7647-14-5; NaBr, 7647-15-6; NaI, 7681-82-5; $NaClO_4$, 7601-89-0; $(NH_2)_2CO$, 57-13-6; $MeNHC(O)NH_2$, 598-50-5; $EtNHC(O)NH_2$, 625-52-5; $PrNHC(O)NH_2$, 627-06-5; $BuNHC(O)NH_2$, 592-31-4.

REFERENCES

Antonini, E., & Chiancone, E. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 239–271.

Bonaventura, C., Bonaventura, J., Miller, K. I., & Van Holde, K. E. (1981) *Arch. Biochem. Biophys. 211*, 589–598.

Brouwer, M., Walters, M., & Van Bruggen, E. F. J. (1976) *Biochemistry 15*, 2618–2623.

Brouwer, M., Ryan, M., Bonaventura, J., & Bonaventura, C. (1978) *Biochemistry, 17*, 2810–2815.

Chen, Y. H., Yang, Y. T., & Martinez, H. M. (1972) *Biochemistry 11*, 4120–4131.

Chen, Y. H., Yang, Y. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350–3359.

Doty, P., & Edsall, J. T. (1951) *Adv. Protein Chem. 6*, 35–121.

Ellerton, H. D., Ellerton, N. F., & Robinson, H. A. (1983) *Prog. Biophys. Mol. Biol. 41*, 143–248.

Engelborghs, Y., & Lontie, R. (1973) *J. Mol. Biol. 77*, 577–587.

Eriksson-Quensel, I.-B., & Svedberg, T. (1936) *Biol. Bull. 71*, 498–547.

Gaykema, W. P. J., Hol, W. G. J., Vereijken, J. M., Soeter, N. M., Bak, H. J., & Beintema, J. J. (1984) *Nature (London) 309*, 23–29.

Gielens, C., Preaux, G., & Lontie, R. (1975) *Eur. J. Biochem. 60*, 271–280.

Gielens, C., Verschueren, L. J., Preaux, G., & Lontie, R. (1980) *Eur. J. Biochem. 103*, 463–470.

Handbook of Chemistry and Physics (1979) CRC Press, Boca Raton, FL.

Herskovits, T. T., & Ibanez, V. S. (1976) *Biochemistry 15*, 5715–5721.

Herskovits, T. T., & Russell, M. W. (1984) *Biochemistry 23*, 2812–2819.

Herskovits, T. T., Cavanagh, S. M., & San George, R. C. (1977) *Biochemistry 16*, 5795–5801.

Herskovits, T. T., San George, R. C., & Cavanagh, S. M. (1978) *J. Colloid Interface Sci. 63*, 226–234.

Herskovits, T. T., Erhunmwunsee, L. J., San George, R. C., & Herp, A. (1981a) *Biochim. Biophys. Acta 667*, 44–58.

Herskovits, T. T., San George, R. C., & Erhunmwunsee, L. J. (1981b) *Biochemistry 20*, 2580–2587.

Herskovits, T. T., Russell, M. W., & Carberry, S. E. (1984) *Biochemistry 23*, 1873–1881.

Herskovits, T. T., Carberry, S. E., & Villanueva, G. B. (1985a) *Biochim. Biophys. Acta 828*, 278–289.

Herskovits, T. T., Mazzella, L. J., & Villanueva, G. B. (1985b) *Biochemistry 24*, 3862–3870.

Himmelwright, R. S., Eickman, N. C., LuBien, C. D., & Solomon, E. I. (1980) *J. Am. Chem. Soc. 102*, 5378–5388.
Konings, W. N., Siezen, R. J., & Gruber, M. (1969a) *Biochim. Biophys. Acta 194*, 376–385.
Konings, W. N., Van Driel, R., van Bruggen, E. F. J., & Gruber, M. (1969b) *Biochim. Biophys. Acta 194*, 55–66.
Linzen, B., Soeter, N. M., Riggs, A. F., Schneider, H.-J., Schartau, W., Moore, M. D., Yokota, E., Behrens, P. Q., Nakashima, H., Takagi, T., Nemoto, T., Vereijken, J. M., Bak, H. J., Beintema, J. J., Volbeda, A., Gaykema, W. P. J., & Hol, W. G. J. (1985) *Science (Washington, D.C.) 229*, 519–524.
Loeffler, M. C., Su, S.-S., & Li, N. C. (1978) *Bioinorg. Chem. 8*, 133–138.
Mangum, C. P. (1980) *Am. Zool. 20*, 19–38.
Miller, K. I., & van Holde, K. E. (1982) *Comp. Biochem. Physiol., B: Comp. Biochem. 73B*, 1013–1018.
Perutz, M. F., Muirhead, H., Cox, J. M., & Goaman, L. C. G. (1968) *Nature (London) 219*, 131–139.
Ryan, M., Terwilliger, N. B., Terwilliger, R. C., & Schabtach, E. (1985) *Comp. Biochem. Physiol., B: Comp. Biochem. 80B*, 647–656.
Salvato, B., Ghiretti-Magaldi, A., & Ghiretti, F. (1974) *Biochemistry 13*, 4778–4783.
Salvato, B., Ghiretti-Magaldi, A., & Ghiretti, F. (1979) *Biochemistry 18*, 2731–2736.
Siezen, R. J., & Van Bruggen, E. F. J. (1974) *J. Mol. Biol. 90*, 77–89.
Svedberg, T., & Eriksson, I. B. (1932) *J. Am. Chem. Soc. 64*, 4730–4738.
Svedberg, T., & Hedenius, A. (1934) *Biol. Bull. 66*, 191–223.
Svedberg, T., & Pedersen, K. O. (1940) *The Ultracentrifuge*, Oxford University Press, Oxford, England.
Tamburro, A. M., Salvato, B., & Zatta, P. (1976) *Comp. Biochem. Physiol., B: Comp. Biochem. 55B*, 347–356.
Tomimatsu, Y., & Palmer, K. J. (1963) *J. Phys. Chem. 67*, 1720–1722.
Torensma, R., & Phillips, J. C. (1981) *FEBS Lett. 130*, 314–316.
van Bruggen, E. F. J., Wiebenga, E. H., & Gruber, M. (1962a) *J. Mol. Biol. 4*, 1–7.
van Bruggen, E. F. J., Wiebenga, E. H., & Gruber, M. (1962b) *J. Mol. Biol. 4*, 8–9.
van Holde, K. E., & Cohen, L. B. (1964) *Biochemistry 3*, 1803–1808.
van Holde, K. E., & Miller, K. I. (1982) *Q. Rev. Biophys. 15*, 1–129.
van Holde, K. E., Blair, D., Eldren, N., & Ariska, F. (1977) in *Structure and Function of Hemocyanin* (Bannister, J. V., Ed.) pp 22–30, Springer-Verlag, West Berlin.
Zimm, B. H. (1948) *J. Chem. Phys. 16*, 1099–1116.

Neoglycoproteins: Preparation and Properties of Complexes of Biotinylated Asparagine–Oligosaccharides with Avidin and Streptavidin†

Victor J. Chen‡ and Finn Wold*
Department of Biochemistry and Molecular Biology, The University of Texas Medical School, Houston, Texas 77225
Received August 12, 1985

ABSTRACT: Neoglycoproteins in which the oligosaccharide moieties are attached noncovalently to the protein through a high-affinity ligand have been prepared from biotinylated oligosaccharides and avidin or the nonglycosylated microbial analogue streptavidin. One of the asparagine–oligosaccharides purified from Pronase-digested ovalbumin (Man_6-$GlcNAc_2$-Asn) was reacted with an excess of the hydroxysuccinimide ester of biotin or, for the purpose of quantitation, [^{3}H]biotin. Derivatives were also prepared with an extension "arm", a 6-aminohexanoyl group, between biotin and asparagine. When the purified biotinyl-Asn–oligosaccharide was added to avidin or streptavidin, a complex was formed containing 3 mol of oligosaccharide/mol of protein. The complexes were stable at neutral pH in the absence of biotin and could be dialyzed for 2 weeks without any significant loss of ligand. In the presence of biotin, or under denaturing conditions, the oligosaccharide derivative was released and could be quantitatively recovered. To assess the influence of the protein matrix on the reactivity of the oligosaccharide units, free biotinyl-Asn–oligosaccharide and the corresponding avidin and streptavidin complexes were exposed to α-mannosidase in parallel experiments. The rate of hydrolysis of the free derivative was severalfold faster than that of the two protein complexes, and at the time when about 90% of the free derivative had all five α-mannosyl residues removed, the majority of the protein-bound derivatives contained two to four undigested α-mannosyl residues and also had a significant amount of undigested starting material. The ease of preparation and the properties of these neoglycoproteins suggest that they should be excellent models for the study of glycoprotein–receptor binding and glycoprotein processing.

A variety of biological recognition processes use glycoproteins as specificity determinants. Some examples of these processes include circulatory clearance of serum proteins (Neufeld & Ashwell, 1980; Ashwell & Morell, 1974), compartmentalization of lysosomal enzymes (Neufeld & Ashwell, 1980; Kaplan et al., 1977), developmental processes of slime mold and certain vertebrates (Barondes, 1981), and the specificity involved in legume–symbiont interaction (Schmidt, 1979). In order to fully evaluate how various oligosaccharide

†This work was supported by U.S. Public Health Service Grant GM31305 and Robert A. Welch Foundation Grant AU916.
‡Present address: Lilly Research Laboratories, Indianapolis, IN 46285.

0006-2960/86/0425-0939$01.50/0