Light-Scattering Investigation of the Subunit Structure and Dissociation of *Helix pomatia* Hemocyanin. Effects of Salts and Ureas†

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ABSTRACT: The effects of various salts of the Hofmeister and aliphatic acid salt series and hydrophobic reagents of the urea series on the subunit structure and the dissociation of *Helix pomatia* α-hemocyanin were investigated by employing light-scattering molecular weight methods. In moderate ranges of salt concentrations [0–1.0 M $NaClO_4$, NaSCN, NaI, and guanidinium chloride (GdmCl) and 0–2.0 M NaBr], the dissociation reaction is essentially a two-step process characterized by the dissociation of whole hemocyanin molecules dissociating to half-molecules of decamers followed by the dissociation of the half-molecules to five dimeric fragments. The effectiveness of the salts and relative ineffectiveness of the ureas and GdmCl as dissociating agents in the first step of the dissociation reaction suggest that the stabilization of the contact areas between half-molecules in solution is largely a nonhydrophobic energy process involving polar and ionic interactions. Hydrophobic forces appear to be important, however, for stabilization of the half-molecules through side to side contacts of the five dimeric units that make up each half-molecule. The analysis of our dissociation data by use of equations derived in our previous studies [Herskovits, T. T., & Harrington, J. P. (1975) *Biochemistry 14*, 4964–4971] gave apparent estimates of amino acid groups of about 60–150 for each of the contact areas between the cylindrically shaped half-molecules and 30–60 for each of the dimers in the half-molecules themselves. Light-scattering measurements in an instrument of Brice's design that gives absolute turbidities, together with refractive index increment data measured on dialyzed solutions at constant chemical potential of the diffusible components, gave molecular weights of $(7.55 \pm 0.50) \times 10^6$ for whole hemocyanin at pH 5.7, $(3.76 \pm 0.12) \times 10^6$ for the half-molecules of hemocyanin in 1.0 M NaCl, and $(3.72 \pm 0.042) \times 10^5$ for the fully dissociated subunits at pH 10.6.

The hemocyanins are copper-containing multisubunit proteins found in the hemolymph of many species of arthropods and molluscs. While charged with the same function of the delivery of oxygen, the hemocyanins of these two different phyla have entirely different subunit organization, molecular weights, and chain folding of the monomeric subunits (Van Holde & van Bruggen, 1971; Bonaventura et al., 1977; Siezen & van Bruggen, 1974). The arthropod hemocyanins are multiple aggregates of hexamers, consisting of one, two, four, or eight basic hexameric units, with molecular weights ranging from approximately 0.45×10^6 to 3.3×10^6 (Van Holde & van Bruggen, 1971; Bonaventura et al., 1977). The basic hexameric unit is organized of roughly kidney-shaped monomers, which occupy the six corners of a trigonal antiprism having a point group symmetry of 32 (Van Schaick et al., 1981). The molluscan hemocyanins, on the other hand, have cylindrical organization and symmetry. Depending on the species, the functional protein assemblies that exist at neutral pH have the form of hollow cylinders, organized of one or two decamers, each approximately 170–180 Å high and 300 Å in diameter (Van Holde & van Bruggen, 1971; Mellema & Klug, 1972; Siezen & van Bruggen, 1974). The decamers which have reported molecular weights of about $(3.6–4.5) \times 10^6$ dissociate by longitudinal splitting of the cylindrical particles into five dimeric fragments at slightly alkaline pH, followed by further dissociation to monomers at high pH (Van Holde & Cohen, 1964; Konings et al., 1969a,b; Siezen & Van Driel, 1973; Brouwer et al., 1976). Unlike the smaller monomeric units of the arthropods, which are folded into kidney-shaped single-domain structures, the much larger, $(3.6 \pm 0.3) \times 10^5$ dalton, molluscan monomers are multidomain chains consisting of seven or eight interconnected globular substructures (Brouwer et al., 1976).

The hemocyanin of the garden snail, *Helix pomatia*, has the two-decameric assembly common to the gastropod species (Svedberg & Pedersen, 1940; Brohult, 1947; Van Holde & van Bruggen, 1971; Siezen & van Bruggen, 1974). It can be readily dissociated to half-molecules, followed by further dissociation of the basic cylindrical unit to one-fifth and one-tenth size dimeric and monomeric fragments by an increase in pH above the physiological ranges (Svedberg & Pedersen, 1940; Brohult, 1947; Van Holde & van Bruggen, 1971; Siezen & van Bruggen, 1974) and by the dissociating effects of salts (Brohult, 1947; Engelborghs & Lontie, 1973). The efficacy of simple salts as dissociating agents has significant structural implications regarding the nature of the contact areas of the subunits, as well as the stabilizing forces that hold the functional subunit assemblies of the molluscan hemocyanins together in solution. In the past several years, we have investigated the latter phenomena as they relate to the subunit organization and stability of the one- and two-hexameric assemblies of two species of arthropod hemocyanins, the proteins of the blue crab and the lobster, *Callinectes sapidus* and *Homarus americanus*, respectively (Herskovits et al., 1981a,b, 1983). In the present work, we have extended these investigations to the major, α component of hemocyanin of *Helix pomatia* by using both salt and hydrophobic reagents of the urea and guanidinium chloride (GdmCl) classes as probes of the contact areas of the subunits. The light-scattering molecular weight technique employed in this work has also afforded us the opportunity to reinvestigate the problem of molecular weights of the parent α-hemocyanin of *Helix pomatia* as well as the half-molecules and the fully dissociated monomeric subunits (Brouwer et al., 1976; Van Holde & Miller, 1982).

Materials and Methods

The *Helix pomatia* α-hemocyanin employed in this study was a generous gift of Dr. Roel Van Driel, prepared by a slight modification (Konings et al., 1969; Siezen & Van Driel, 1973)

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of the method of Heirwegh et al. (1961). Stock solutions of the protein were prepared by dissolving the desired amount of protein in 0.1 M pH 5.7 acetate buffer, followed by dialysis in the cold against two to three changes of buffer in order to remove the sucrose employed in the lyophilization step of the preparation of the protein. Hemocyanin concentrations were based on optical density measurements, using the percent extinction coefficients, $E^{1\%}_{278nm} = 16.78$ at pH 5.7 and 14.16 at pH 9.2 (Heirwegh et al., 1961). The extinction coefficient for the half-molecule in 1.0 M NaCl and 0.5 M GdmCl was found to be 15.0.

All the salts, ureas, and reagents were analytical or reagent-grade materials. The urea and GdmCl were ultrapure grade purchased from Schwarz/Mann and used without further purification. Where required, the alkylureas and the acid salts were purified further by recrystallization from hot ethanol. Since the carboxylate acid salts have strong buffering action, the pHs of the 1–2 M stock solutions employed were adjusted to 5.7 by use of concentrated HCl or NaOH, and the volume was made up with 0.1 M acetate buffer.

Light-scattering and refractive index measurements were made at 436 nm in a Wood Mfg. Co. photometer of Brice's design, equipped with a differential refractometer. This instrument measures turbidities on an absolute scale (Brice et al., 1950). The protein solutions used for light scattering were prepared by serial dilution from common dialyzed stock solutions using the appropriate amounts of buffer and concentrated salt or urea solutions made up with the same buffer. The solutions were clarified by filtration directly into the light-scattering cells using single 0.2-μm Gelman metricell filters secured in Millipore filter holders as previously described (Harrington et al., 1973; Elbaum & Herskovits, 1974). The light-scattering data, measured at an angle of 90°, were interpreted by use of the familiar expression (Doty & Edsall, 1951)

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

where R_θ is the excess Rayleigh ratio, related to the measured turbidity of the protein solution minus that of the solvent, M_w is the weight-average molecular weight, B' is the second virial coefficient, c is the protein concentration, and K' is the light-scattering constant, which among other factors contains the square of the specific refractive index increment at constant chemical potential, $(\partial n/\partial c)_\mu$ obtained on dialyzed solutions (Doty & Edsall, 1951; Casassa & Eisenberg, 1964).

The specific refractive index increment, $(\partial n/\partial c)_\mu$, obtained on dialyzed α-hemocyanin solutions in 0.1 M acetate (pH 5.7) was found to be 0.191 ± 0.003 $g^{-1}\cdot cm^3$ and in 1.0 M NaCl and 0.1 M acetate was 0.183 $g^{-1}\cdot cm^3$. Since these $(\partial n/\partial c)_\mu$ values are virtually the same as those obtained for *Callinectes sapidus* and *Homarus americanus* hemocyanin (Herskovits et al., 1981a,b), we have assumed that the values of the molar increments or decrements due to the secondary effects of salts and ureas are also the same. Consequently, we have used the published data of the latter hemocyanin in order to make these corrections. For the aliphatic acid salts, the molar decrements of $(\partial n/\partial c)_\mu$ used appear to be the same as the values obtained for NaCl and the other Hofmeister salts, or about 0.008–0.012 $g^{-1}\cdot cm^3$ (Herskovits & Ibanez, 1976).

Circular dichroism (CD) measurements were made on a Cary 60 recording spectropolarimeter equipped with a CD attachment. The mean residue molecular weight (M_0) of 122, estimated on the basis of the amino acid data of Dijk et al. (1970), was used for all our calculations of the reported mean residue ellipticities $[\theta]_\lambda$.

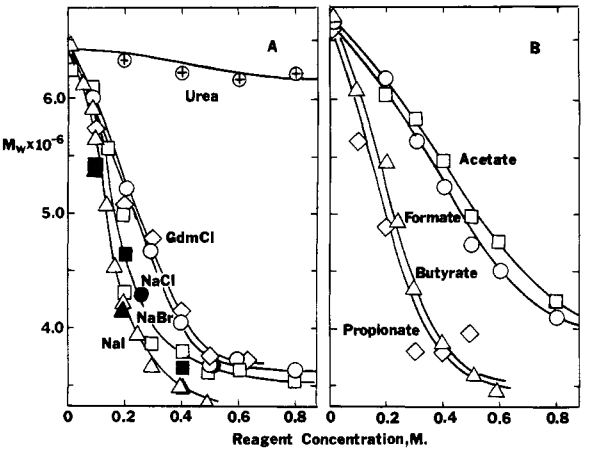

FIGURE 1: Comparative effects of halide salts, aliphatic acid salts, GdmCl, and urea on the eicosamer–decamer equilibrium of *Helix pomatia* α-hemocyanin at pH 5.7, plotted as molecular weight (M_w) vs. reagent concentration. Data represented by filled symbols were obtained on protein initially exposed to 0.5 M NaCl and 0.4 M NaBr and NaI. Protein concentrations employed were 5×10^{-2} g·L^{-1}, and the solvent was 0.1 M acetate, pH 5.7.

Results

Dissociating Effects of Neutral Salts, GdmCl, and Various Ureas. The Hofmeister salts are known to be effective dissociating agents of *Helix pomatia* α-hemocyanin (Brohult, 1947; Engelborghs & Lontie, 1973). With most salts, the relative molecular weights in 0.3–0.5 M salt solutions were found to decrease to about half of the initial values, suggesting dissociation of the parent protein to half-molecules (Engelborghs & Lontie, 1973). Our light-scattering data in Figure 1 show similar changes for the aliphatic acid salts and GdmCl. Significantly, however, the latter group of reagents is less effective than the neutral halide salts. The urea data obtained in the 0–1.0 M concentration range have also been included in Figure 1A. It shows essentially no dissociation in this reagent concentration region. The dissociation effects of GdmCl, urea, and methyl-, ethyl-, and propylurea investigated also at higher reagent concentrations are shown in Figure 2. The effects of the ureas on the molecular weights of α-hemocyanin have also been studied in the presence of 0.5 M NaCl, where the protein is largely in the dissociated decameric state. The latter results are shown in panel B of Figure 2. The molecular weight data shown in these figures have been obtained at a protein concentration close to 5×10^{-2} g·L^{-1}, where nonideality effects are less than the experimental uncertainties of the light-scattering measurements and could thus be ignored.

Analysis of the Stepwise or Sequential Dissociation of α-Hemocyanin Eicosamers to Decamers and Dimers. The initial dissociation measurements with the Hofmeister salts shown in Figure 1 were extended to higher salt concentrations beyond the initial plateau region of approximately 3.6×10^6 daltons. The molecular weight data of Figures 3 and 4 obtained with $NaClO_4$ and NaBr, for example, show a second plateau region corresponding to the molecular weights of about one-tenth of the parent hemocyanin. In this limited region of reagent concentration, we find that our molecular weight data can be adequately represented by the sequential dissociation scheme

$$\underset{c(1-\alpha_1)}{(1/2)Hc_{20}} \overset{K_1}{\rightleftharpoons} \underset{c\alpha_1(1-\alpha_2)}{Hc_{10}} \overset{K_2}{\rightleftharpoons} \underset{c(\alpha_1\alpha_2)}{5Hc_2} \qquad (2)$$

where Hc_{20}, Hc_{10}, and Hc_2 represent the eicosamer, decamer, and dimer species of *Helix pomatia* α-hemocyanin present at equilibrium in solution and α_1 and α_2 represent the weight

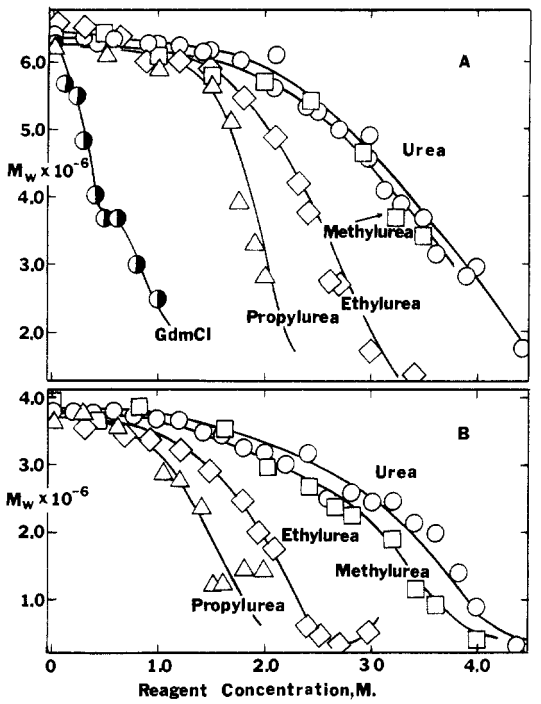

FIGURE 2: Effects of the hydrophobic urea and GdmCl series of reagents on the molecular weight (M_w) of *Helix pomatia* α-hemocyanin (A) and its dissociated half-molecules (B) in the presence of 0.5 M NaCl. Protein concentration and solvent conditions are the same as those in Figure 1.

fractions of whole molecules that dissociate to decamers and dimers (Herskovits & Harrington, 1975). The two apparent equilibrium constants that describe the dissociation reaction can be expressed as

$$K_1^2 = K_{app}^{20,10} = \frac{4c[\alpha_1(1-\alpha_2)]^2}{(1-\alpha_1)M_{20}} \quad (3)$$

and

$$K_2 = K_{app}^{10,2} = \frac{(5.0 \times 10^4)c^4(\alpha_1\alpha_2)^5}{\alpha_1(1-\alpha_2)M_{20}^4} \quad (4)$$

With the effects of dissociating reagents such as salts and ureas on protein equilibria given by the general expressions shown in eq 5 and 6 (Herskovits et al., 1977, 1978) eq 3 and 4 can

$$K_{D,app} = \frac{m^m c^{m-1}\alpha^m}{(1-\alpha)M_n^{m-1}} \quad (5)$$

$$K_{D,app} = K_{w,app}\exp(mN_{app}K_B c_D) \quad (6)$$

be rearranged in the form convenient for calculating the α_1 and α_2 values necessary to fit the molecular weight data. The latter equations are

$$\frac{\alpha_1^2}{1-\alpha_1} = \left[\frac{K_{w,app}^{20,10}M_{20}}{4c(1-\alpha_2)^2}\right]\exp(2N_{app}^{20,10}K_B c_D) \quad (7)$$

and

$$\frac{\alpha_2^5}{1-\alpha_2} = \left[\frac{(2.0 \times 10^{-5})M_{20}^4 K_{w,app}^{10,2}}{c^4\alpha_1^4}\right]\exp(5N_{app}^{10,2}K_B c_D) \quad (8)$$

In these equations, $K_{D,app}$ and $K_{w,app}$ represent the apparent dissociation constants of the protein in the presence and absence of a dissociating reagent, respectively, N_{app} is the apparent number of amino acids at the contact areas of the subunits, respectively specified by the superscripts for the two stages of dissociation, K_B is the binding or interaction constant of the dissociating reagent with the average amino acid at the contact areas of the subunits, M_{20} is the molecular weight of the undissociated hemocyanin (consisting of 20 polypeptide chains designated by the subscript), c is the concentration of the hemocyanin expressed in grams per liter, and c_D is the molar concentration of the dissociating reagent.

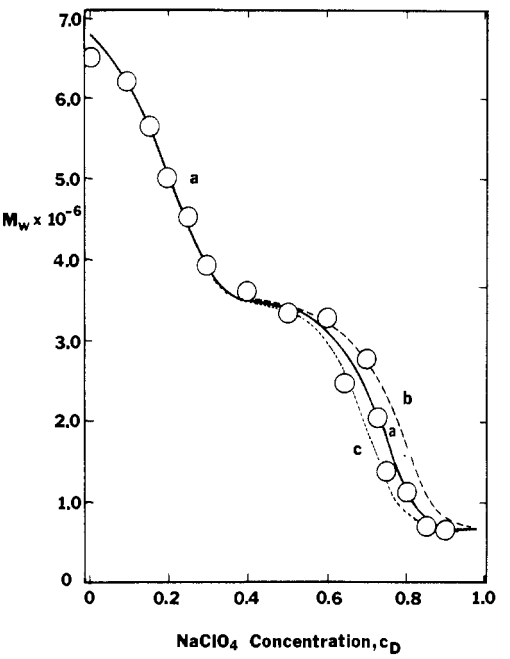

FIGURE 3: Fit of the molecular weight data (M_w) of *Helix pomatia* α-hemocyanin obtained with 0–0.9 M $NaClO_4$ as dissociating agent. Fitting parameters with eq 7–9 were as follows: curve a, $N_{app}^{20,10} = 60$, $K_{w,app}^{20,10} = 4 \times 10^{-10}$ M, $N_{app}^{10,2} = 56$, $K_{w,app}^{10,2} = 3 \times 10^{-45}$ M^4, $K_B = 0.175$ M^{-1}, and $c = 5 \times 10^{-2}$ g·L^{-1}; curves b and c, $N_{w,app}^{10,2} = 53$ and 59, respectively; the rest of the parameters are the same as those for curve a.

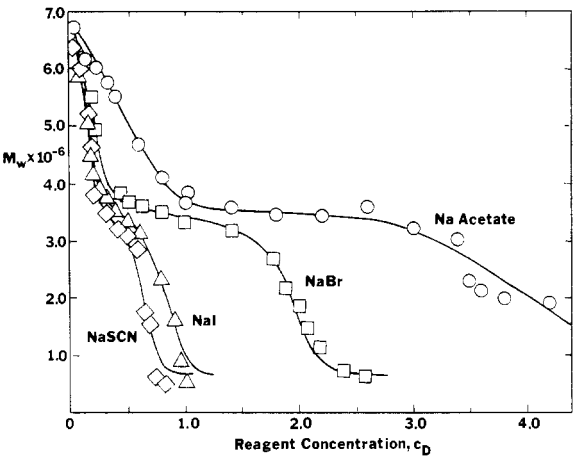

FIGURE 4: Effects of several salts on the molecular weight (M_w) of *Helix pomatia* α-hemocyanin fitted by means of eq 7–9 and the parameters listed in Table I. Protein concentration and solvent conditions are the same as those in Figure 1.

The molecular weight data can be readily fitted as previously described (Herskovits & Harrington, 1975; Herskovits et al., 1984) using eq 9

$$M_w = \frac{\sum c_i M_i}{\sum c_i} = \frac{1}{c}[c(1-\alpha_1)M_{20} + c\alpha_1(1-\alpha_2)M_{10} + c(\alpha_1\alpha_2)M_2] = M_{20}[1 - (1/2)\alpha_1 - (4/10)\alpha_1\alpha_2] \quad (9)$$

Table I: Summary of the Dissociation Parameters of *Helix pomatia* α-Hemocyanin at pH 5.7 Based on Equations 7–10

dissociating reagent	K_B[a]	slope (kcal·mol^{-1}·M^{-1})	$\Delta G°_{w,app}$ (kcal·mol^{-1})	$K_{w,app}$	r[b]	N_{app}
		Eicosamer–Decamer (m = 2, c = 5 × 10^{-2} g·L^{-1})				
NaBr	0.053	−9.21	12.13	1.0 × 10^{-9}	0.966	147
NaSCN	0.160	−15.03	12.83	8.0 × 10^{-10}	0.930	80
$NaClO_4$	0.175	−12.47	12.83	4.0 × 10^{-10}	0.997	60
NaI	0.180	−12.92	12.24	1.0 × 10^{-9}	0.993	60
sodium acetate	0.021	−3.83	12.33	8.8 × 10^{-10}	0.969	(155)
sodium butyrate	0.086	−9.77	12.68	5.0 × 10^{-10}	0.997	96
						89 ± 36[d]
		Decamer–Dimer (m = 5, c = 5 × 10^{-2} g·L^{-})				
NaBr		−8.37	55.99	9.0 × 10^{-42}	0.942	53
NaSCN		−19.30	51.94	8.0 × 10^{-39}	0.976	42
$NaClO_4$		−29.03	60.77	3.0 × 10^{-45}	0.958	56
NaI		−16.48	52.77	2.0 × 10^{-39}	0.952	30
sodium acetate		−3.92	54.87	5.6 × 10^{-41}	0.922	63
urea[c]	0.032	−3.32	50.68	1.0 × 10^{-37}	0.961	35
ethylurea[c]	0.061	−7.34	54.05	2.2 × 10^{-40}	0.994	41
						46 ± 12[d]

[a] K_B parameters taken from Herskovits et al. (1977, 1978) and Herskovits & Ibanez (1976). [b] Correlation coefficients based on least-squares treatment of the data based on eq 10 (Figure 5). [c] Based on the data of Figure 2B contained in the presence of 0.5 M NaCl. [d] Average value.

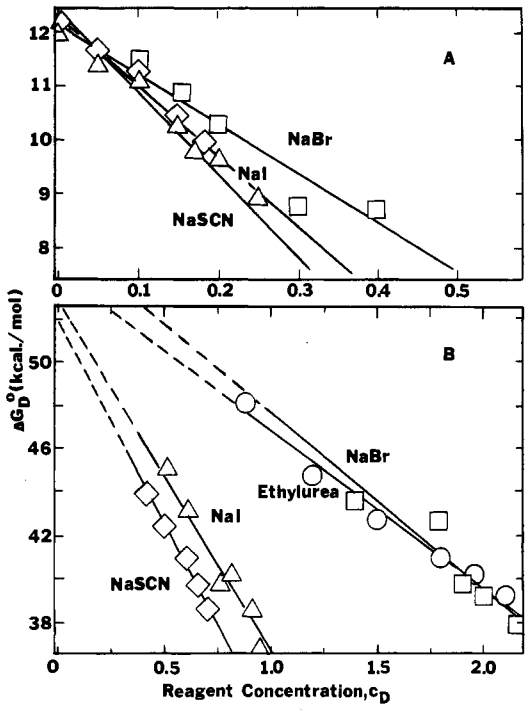

FIGURE 5: Plots of $\Delta G°_{D,app}$ vs. c_D based on eq 10 for eicosamer to decamer dissociation (A) and decamer to dimer (B) dissociation of *Helix pomatia* α-hemocyanin, obtained with several dissociating agents. The derived slope, intercept ($\Delta G_{w,app}$), and N_{app} parameters are listed in Table I.

This equation is based on the definition of the weight-average molecular weight and the concentrations and molecular weights of eicosameric, decameric, and dimeric species of hemocyanin specified in eq 2. The required two sets of α values were generated by using eq 7 and 8 and the "best-fit" $K_{w,app}$ and N_{app} parameters obtained from the refinement of the molecular weight data as shown in Figure 3. Since the two observed transitions are fairly well separated, fairly good initial estimates of these parameters could be obtained from the slope and intercept of the free-energy plots ($\Delta G°_{w,app}$) based on eq 5 and 6:

$$\Delta G°_{D,app} = \Delta G°_{w,app} - mRTN_{app}K_Bc_D = -RT \ln K_{D,app} \quad (10)$$

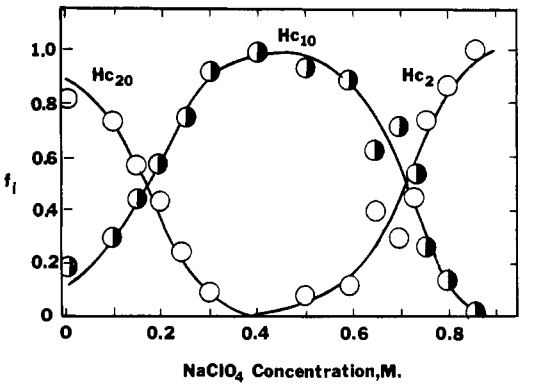

FIGURE 6: Effects of $NaClO_4$ concentration on the species distribution of the eicosamer (Hc_{20}), decamer (Hc_{10}), and dimer (Hc_2) components of *Helix pomatia* α-hemocyanin plotted as the weight fraction, f_i, vs. $NaClO_4$ concentration. The solid lines were computed by using eq 7, 8, and 12 and the $NaClO_4$ parameters of Table I. The data points were based on the molecular weights shown in Figure 3, calculated by use of eq 11.

The $K_{D,app}$ and α_1 and α_2 values were estimated by using eq 5 and 11:

$$\alpha = [m/(m-1)](1 - M_w/M_n) \quad (11)$$

Figure 5 shows some of the latter results obtained at low and high dissociating reagent concentrations. In the molecular weight region above 3.6 × 10^6, the dissociation is to half-molecules, requiring a dimer–monomer type of analysis with m = 2 and M_n = 7.2 × 10^6; below the plateau region, the half-molecules or decamers dissociate to dimers; thus, pentamer to monomer parameters are applicable, with m = 5 and M_n = 3.6 × 10^6. Table I presents a summary of the derived slope, $\Delta G°_{w,app}$, and related N_{app} and $K_{w,app}$ parameters. The latter parameters were used to calculate molecular weight curves as a function of dissociating reagent concentration as shown in Figures 3 and 4.

The distribution of hemocyanin species obtained with $NaClO_4$ as a dissociating reagent is shown in Figure 6. The weight fractions of species (f_i) were evaluated by using the species concentrations given with eq 2.

$$f_{20} = c_{20}/c = 1 - \alpha_1$$
$$f_{10} = \alpha_1(1 - \alpha_2) \quad (12)$$
$$f_2 = \alpha_1\alpha_2$$

Table II: Light-Scattering Molecular Weight Data of *Helix pomatia* α-Hemocyanin

solvent	protein concn range ($g \cdot L^{-1}$)	$\partial n/\partial c$ ($g^{-1} \cdot cm^3$)	M_w[a]	B'[a] ($L \cdot mol \cdot g^{-2}$)
0.1 M acetate, pH 5.7	0.5–2.3	0.191 ± 0.003	(7.55 ± 0.50) × 10^6	-7×10^{-9}
1.0 M NaCl–0.1 M acetate, pH 5.7	0.4–1.5	0.181	(3.76 ± 0.12) × 10^6	-5×10^{-10}
0.1 M bicarbonate–NaOH, pH 10.6	0.2–1.3	0.191[b]	(3.72 ± 0.042) × 10^5	$(-6 \pm 2) \times 10^{-9}$

[a] The pH 5.7 value given in the absence of NaCl is the average of the values obtained in the concentration range of hemocyanin specified in column 2, assuming $B' = 0$ (see text). The rest of the molecular weight values given are extrapolated values based on eq 1, with least-squares treatment of the data specified in column 2. Computed error estimates are standard deviations from the mean. [b] Values used assuming independence of pH.

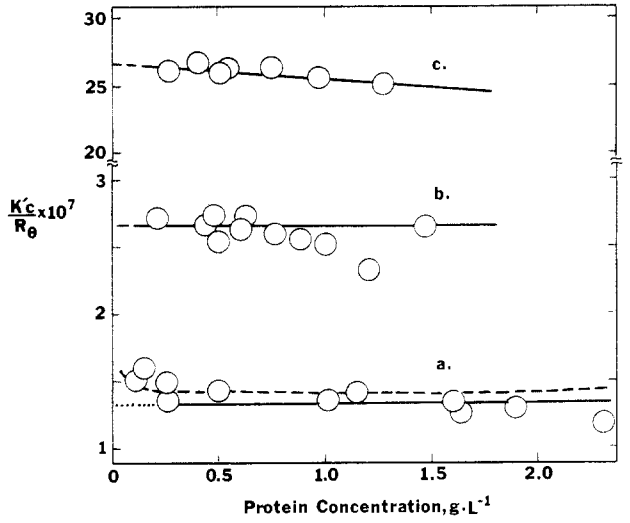

FIGURE 7: Concentration dependence of the light-scattering data of *Helix pomatia* α-hemocyanin plotted according to eq 1 and 13. Solid lines: (a) 0.1 M sodium acetate, pH 5.7; (b) 1.0 M NaCl–0.1 M sodium acetate, pH 5.7; (c) 0.1 M sodium bicarbonate–NaOH, pH 10.6. The dashed lines represent the fit of the 0.1 M acetate data based on eq 13 and the $K_{w,app}$ data of Table I with $K_{w,app}^{20,10} = 1 \times 10^{-9}$ M, $K_{w,app}^{10,2} = 1 \times 10^{-40}$ M^4, $M_{20} = 7.2 \times 10^6$, and $B' = 4 \times 10^{-10}$ $L \cdot mol \cdot g^{-2}$.

The curves based on these equations are represented as solid lines, while the fractions of the three components calculated by using the actual molecular weight data of Figure 3 are represented as open and half-filled circles. It is apparent from the distribution of species as a function of $NaClO_4$ concentration that the *Helix pomatia* α-hemocyanin dissociation is essentially a stepwise reaction with relatively little overlap between the dissociation of the whole molecules represented by the first step of the reaction and with the appearance of dimers signifying the initiation of the second step of the dissociation reaction.

Concentration Dependence of Light-Scattering Data. The light-scattering behavior of *Helix pomatia* α-hemocyanin whole molecules, half-molecules, and dissociated monomers at pH 10.6 is shown in Figure 7. The data of both the parent protein and the dissociated subunits show slightly negative slopes and negative second virial coefficients (B'), observed also by Engelborghs & Lontie (1973). This suggests slight dissociation of both the parent protein and the half-molecules at low protein concentration and the presence of a small fraction of dimers at pH 10.6 at protein concentrations above 1 $g \cdot L^{-1}$. The average molecular weight of the parent protein in the 0.5–2.3 $g \cdot L^{-1}$ concentration range was found to be (7.55 ± 0.5) × 10^6, which is not significantly different from the value of 7.2 × 10^6 suggested by the studies of Seizen & van Bruggen (1974) and Brouwer et al. (1976). The molecular weights and second virial coefficients are given in Table II. The molecular weights in 1.0 M NaCl and 0.1 M bicarbonate–NaOH at pH 10.6 based on the usual intercepts of the light-scattering plots are only slightly lower than the average values of (3.79 ± 0.20) × 10^6 and (3.83 ± 0.072) × 10^5 daltons observed at the concentration ranges indicated in Table II.

In order to see what effects subunit dissociation will have on the light-scattering plots at low protein concentrations where our data show a slight upswing in the $K'c/R_\theta$ plots, we have calculated the curve represented with the dashed line by using assumed values of the apparent dissociation constants, $K_{w,app}$ of Table I, and the hard-sphere value of the second virial coefficient ($4\bar{V}/M_{20}$). The light-scattering expression used for this calculation is based on the combined eq 1 and 9, written in the form

$$\frac{K'c}{R_\theta} = \{M_{20}[1 - (1/2)\alpha_1 - (4/10)\alpha_1\alpha_2)\}^{-1} + 2B'c \qquad (13)$$

The parameters used to construct this curve are given in the caption of Figure 7. The calculated curve shows the expected upswing in the $K'c/R_\theta$ curve of a hemocyanin, characteristic of dissociating subunit systems at low protein concentrations (Noren et al., 1974; Elbaum & Herskovits, 1974).

Reversibility and Denaturation. The dissociation of *Helix pomatia* α-hemocyanin to half-molecules is found to be a nearly reversible and fairly rapid process. This is suggested both by the studies of Engelborghs & Lontie (1973) using α-hemocyanin exposed to 1.0 M NaCl and by our data of Figure 1 represented by the filled symbols. The protein solutions employed in these experiments were exposed initially to 0.4 M NaI and NaBr and 0.5 M NaCl, respectively, followed by 2–4-fold dilution with the pH 5.7 acetate buffer free of any dissociating reagent. There appears to be a slow step in the reassociation process, at least with 0.4 M $NaClO_4$ as a dissociating reagent, suggested by the molecular weight changes studied as a function of time. The data of Table III present a summary of the work obtained with hemocyanin initially exposed to both 0.4 and 0.8 M $NaClO_4$. In 0.8 M $NaClO_4$, *Helix pomatia* α-hemocyanin is largely in the dimeric state, having a molecular weight of (1.1 ± 0.3) × 10^6 (see Figure 6). Reassociation from the latter stages of dissociation is found to be relatively slow and somewhat incomplete even after 5–14 days of equilibration at room temperature. However, since the molecular weights obtained with the reassociated hemocyanin initially exposed to 0.8 M $NaClO_4$ are higher than 3.6 × 10^6, the reassociation does not seem to be blocked at the half-molecular or decameric stage. This was more clearly demonstrated by the ultracentrifugation studies of Konings et al. (1969a), who have found that the hemocyanin initially exposed to pH 9.5 can be nearly completely reassociated to whole molecules by adjustment of the pH to neutrality and prolonged standing at 20 °C (260 h or 11 days), or by the addition of stabilizing divalent ions such as Ca^{2+}.

Helix pomatia α-hemocyanin seems to be remarkably time stable with regard to denaturation, and also fairly resistant to urea and GdmCl denaturation, as suggested by the circular dichroism data of Figure 8A. Changes in the mean residue

Table III: Reversibility and Reassociation of *Helix pomatia* α-Hemocyanin Exposed to 0.4 and 0.8 M $NaClO_4$ at pH 5.7[a]

final $NaClO_4$ concn (M)	equilibration period (h) at 25 ± 2 °C	M_w 0 M Ca^{2+}	M_w 0.01 M Ca^{2+}	expected equilibrium values[b]
		Reassociation from 0.4 M $NaClO_4$[a]		
0.4 (control)		3.2×10^6		
0.2	1	4.3×10^6		
0.2	22	4.6×10^6		$(5.0 \pm 0.3) \times 10^6$
0.1	1	5.3×10^6		
0.1	22	5.5×10^6		$(6.0 \pm 0.3) \times 10^6$
		Reassociation from 0.8 M $NaClO_4$[a]		
0.8 (control)		$(1.1 \pm 0.3) \times 10^6$		
0.4	0.5	1.9×10^6	1.4×10^6	
0.4	22	2.0×10^6	1.9×10^6	
0.4	120	2.3×10^6	1.9×10^6	
0.4	330	2.1×10^6	2.1×10^6	$(3.2 \pm 0.3) \times 10^6$
0.2	0.5	2.2×10^6	2.3×10^6	
0.2	22	3.0×10^6	3.3×10^6	
0.2	120	3.3×10^6	3.0×10^6	
0.2	330	4.1×10^6	3.5×10^6	$(5.0 \pm 0.3) \times 10^6$
0	90	4.0×10^6		$(6.5 \pm 0.3) \times 10^6$

[a] Hemocyanin ($c = 5 \times 10^{-2}$ g·L^{-1}) initially exposed to 0.4 or 0.8 M $NaClO_4$ (pH 5.7) for 0.5 h at 25 °C was diluted with 0.1 M (pH 5.7) acetate to 0.4, 0.2, or 0.1 M $NaClO_4$ concentration and equilibrated at room temperature for the time period specified in column 2. The 0 M $NaClO_4$ solution was obtained by dialysis in the cold against several changes of buffer. [b] Based on the molecular weight data of Figure 3.

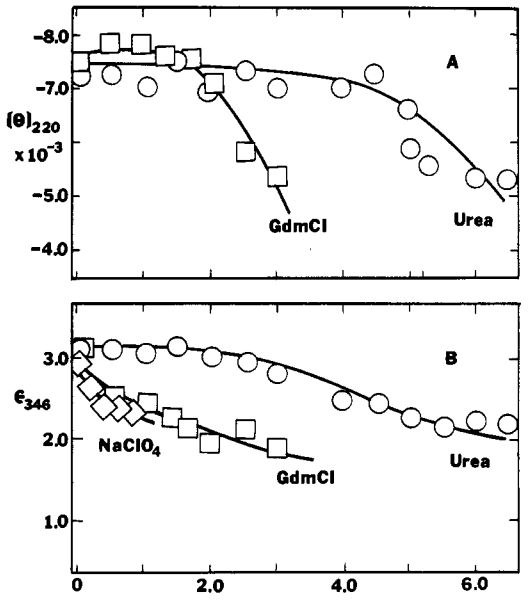

FIGURE 8: Effects of urea, GdmCl, and $NaClO_4$ on the mean residue ellipticity at 220 nm (A) and the copper absorbance at 346 nm (B).

ellipticity at the conformationally sensitive 220-nm region are only apparent above 4 M urea and above 1.8 M GdmCl. This suggests that the observed dissociation with most of the reagents is not complicated by the effects of unfolding of the hemocyanin subunits. GdmCl is known to be the most strongly denaturing reagent from among the various compounds used in this study. Some time effects have been noted with propylurea and ethylurea above the 1.5 and 2.0 M concentrations ranges, respectively, suggesting slow denaturation and unfolding of the polypeptide chains in these solvents (see Figure 2B). The absorbance changes at the 346-nm copper band have also been studied as a function of dissociating reagent concentration, with the data obtained shown in Figure 8B. These are somewhat less informative than the corresponding changes in the CD spectra. This is because of the coupling of the turbidity and absorbance changes at this wavelength, associated with the dissociation of the subunits of hemocyanin (Heirwegh et al., 1961). The sharp drop in the extinction coefficient at 346 nm seen with 0–0.5 M GdmCl and also with $NaClO_4$ is due to these changes in turbidity. No corresponding changes are seen, however, in the mean residue ellipticity that would signify unfolding of the hemocyanin chains. The absorbance changes observed with urea above 2.0 M are the result of dissociation of the whole molecules and also the half-molecules of hemocyanin suggested by the molecular weight data of Figure 2.

Discussion

With most of the salts employed as dissociating reagents, *Helix pomatia* α-hemocyanin is found to dissociate in a two-step reaction. In the initial step, at relatively low reagent concentrations, the whole molecule dissociates to half-molecules of decamers, followed by the dissociation of this intermediate assembly to one-tenth molecular weight fragments (see Figures 3 and 4). These have been identified as dimeric aggregates of the smallest subunits of approximately 3.6×10^5 daltons (Seizen & van Bruggen, 1974). As observed by Engelborghs & Lontie (1973), who were the first to study the effects of the Hofmeister salts on the initial stages of dissociation of α-hemocyanin, the salts are remarkably effective dissociating agents. On the other hand, we find that the hydrophobic reagents of the urea series and also GdmCl are rather ineffective. For example, GdmCl is only found to be about as effective as NaCl, and urea is nearly inert in the 0–1.0 M concentration range, where most salts nearly fully dissociate α-hemocyanin (see Figure 1A). This would suggest that hydrophobic forces are not the dominant interactions that stabilize the decamer to decamer contacts between the two halves of the α-hemocyanin molecule. There is, however, an ambiguity with the urea series of dissociating agents that requires further discussion. Dissociation of the whole molecule by this group of reagents is observed above 1.5–2.0 M urea. In addition, the higher ureas, represented by ethyl- and propylurea, appear to be more effective dissociating agents (see Figure 2A). There are two possibilities that could be used to explain the increased effectiveness of more hydrophobic compounds. Either the up and down contacts between the half-molecules are destabilized by partial unfolding or denaturation or, al-

ternatively, the side to side contacts of the subunits in the half-molecules are destabilized by favorable hydrophobic interactions with the ureas. We have ruled out denaturation as the possible cause of the observed effects on the basis of the circular dichroism spectra, studied at the conformationally sensitive 220-nm peptide band. The data of Figure 8A shows fairly clearly that the mean residue ellipticity of hemocyanin at 220 nm remains essentially unaltered in the 2.0–4.0 M concentration region of urea. Both the whole molecule and the half-molecules of α-hemocyanin are nearly fully dissociated in this concentration region as shown by the molecular weight data of Figure 2. Moreover, the data obtained on the (3.6–3.8) $\times 10^6$ dalton half-molecules, shown in panel B of this figure, exhibit the correct trend in dissociation expected of a hydrophobically stabilized subunit system, showing increasing dissociation with increasing hydrophobicity of the dissociating reagent (Elbaum & Herskovits, 1974; Herskovits et al., 1977, 1978). We interpret this to mean that the half-molecules of α-hemocyanin are predominantly stabilized by hydrophobic interactions, involving the side to side contacts of its five dimeric fragments. The relative instability of the whole molecule in fairly dilute salt solutions and the much greater stability of the half-molecules reflected by the biphasic character of the salt dissociation data (see Figures 3 and 4) suggest that polar and ionic interactions are more important for the stabilization of the up and down contacts between the two half-molecules of hemocyanin.

We have been able to account for the dissociation effects of several of the salts in terms of the two-step reaction scheme eq 2 and the appropriate equilibria used to describe the effects of dissociating agents on the subunit structure of proteins (Herskovits & Ibanez, 1976; Herskovits et al., 1977, 1978). The apparent number of amino acid groups (N_{app}) used to fit the salt dissociation data of Figures 3 and 4 suggests and average of about 60–150 groups for the contact areas of each half-molecule and a smaller number of 30–60 groups for each of the dimeric fragments representing the side to side interactions in the half-molecules of α-hemocyanin (Table I).

These two estimates of amino acid groups reflect the fairly large contact areas involved in the stabilization of the cylindrical hemocyanin assembly of 7.2×10^6 daltons and the constituent half-molecules in solution. The multidomain structure of the hemocyanin subunits suggests the involvement of one or two domain structures per monomer forming part of the contact areas between the two halves of the hemocyanin molecule, and six or perhaps seven domains forming the walls of the complete assembly (Van Bremen et al., 1977). Maximally there would be 20 domains per half-molecule in the up and down contact areas relative to about 12 in side to side contacts between neighboring dimers. Such a geometric arrangement of dimeric chains would be consistent with our N_{app} estimates, which are found to be appreciably larger for the former set of contact areas (Table I). It is important to note, however, that our N_{app} estimates are only apparent quantities, complicated by the uncertainties associated with the problem of subunit heterogeneity on protein equilibria (Siezen & Van Driel, 1973; Engelborghs & Lontie, 1973; Kegeles, 1977; Van Holde et al., 1977) discussed in our previous studies on hemocyanin (Herskovits et al., 1983, 1984).

In order to fit the molecular weight data as a function of dissociation reagent concentration by using eq 7–11, relatively accurate knowledge of the molecular weights of the undissociated protein and the intermediate subunit assembly is required. Our salt dissociation data of Figures 3 and 4 have suggested molecular weights of about 3.5×10^6 to 3.7×10^6 for the half-molecules of α-hemocyanin, on the basis of the plateau region of the observed molecular weights. Consequently, we have employed the value of 7.2×10^6 daltons suggested also by the studies of Brouwer et al. (1976) and Siezen & van Bruggen (1974) as the basis of all our data fitting. Actually, molecular weights of *Helix pomatia* hemocyanin reported in the earlier literature range from about 6.3×10^6 to 8.9×10^6 (Svedberg & Pedersen, 1940; Putzeys & Brosteaux, 1941; Brohult, 1947; Lontie & Witters, 1966; Konings et al., 1969a). Partly because of this fairly large range of values and also the desire for checking the molecular weight of α-hemocyanin on an instrument that gives absolute rather than relative molecular weight values (Brice et al., 1950), we have extended our work to higher protein concentrations necessary for molecular weight determination (Figure 7) together with the determination of the refractive index increments at constant chemical potential (Casassa & Eisenberg, 1964). Our molecular weights of $(7.55 \pm 0.50) \times 10^6$, $(3.76 \pm 0.12) \times 10^6$, and $(3.72 \pm 0.42) \times 10^5$ obtained for the whole molecules, the half-molecules, and the monomer chains of α-hemocyanin, respectively (Table II), are within 3–5% of the molecular weight value of $(3.6 \pm 0.3) \times 10^5$ for the monomer chains estimated by gel electrophoresis and ultracentrifugation methods (Brouwer et al., 1976; Siezen & van Bruggen, 1974). The molecular weight values of the half-molecule and monomer chain of *Helix pomatia* α-hemocyanin are very close to the values of 3.75×10^6 and 3.85×10^5 obtained by Van Holde & Cohen (1964) for the hemocyanin of the squid *Loligo pealei* and its fully dissociated one-tenth molecular weight subunits. The closeness in the molecular weights of undissociated squid hemocyanin and the molecular weights of the half-molecules of the snail *Helix pomatia* hemocyanin, as well as their fully dissociated subunits, supports the observation that the molecular architecture of the basic decameric unit of the two different classes of molluscs, the gastropods and cephalopods, is closely similar (van Bruggen et al., 1962a,b).

Registry No. $NaClO_4$, 7601-89-0; NaSCN, 540-72-7; NaI, 7681-82-5; NaBr, 7647-15-6; GdmCl, 50-01-1; sodium acetate, 127-09-3; sodium butyrate, 156-54-7; sodium formate, 141-53-7; sodium propionate, 137-40-6; urea, 57-13-6; ethylurea, 625-52-5; methylurea, 598-50-5; propylurea, 627-06-5.

References

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

Brice, B. A., Halwer, M., & Spaiser, R. (1950) *J. Opt. Soc. Am. 40*, 768–778.

Brohult, S. (1947) *J. Phys. Colloid Chem. 51*, 206–217.

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

Casassa, E. F., & Eisenberg, H. (1964) *Adv. Protein Chem. 19*, 287–395.

Dijk, J., Brouwer, M., Coert, A., & Gruber, M. (1970) *Biochim. Biophys. Acta 221*, 467–479.

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

Elbaum, D., & Herskovits, T. T. (1974) *Biochemistry 13*, 1268–1278.

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

Harrington, J. P., Pandolfelli, E. R., & Herskovits, T. T. (1973) *Biochim. Biophys. Acta 328*, 61–73.

Heirwegh, K., Borginon, H., & Lontie, R. (1961) *Biochim. Biphys. Acta 48*, 517–526.

Herskovits, T. T., & Harrington, J. P. (1975) *Biochemistry 14*, 4964–4971.

Herskovits, T. T., & Ibanez, V. S. (1976) *Biochemistry 15*, 5715–5721.
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., Carberry, S. E., & San George, R. C. (1983) *Biochemistry 22*, 4107–4112.
Herskovits, T. T., Russell, M. W., & Carberry, S. E. (1984) *Biochemistry 23*, 1875–1881.
Kegeles, G. (1977) *Arch. Biochem. Biophys. 180*, 530–536.
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.
Lontie, R., & Witters, R. (1966) in *The Biochemistry of Copper* (Peisach, J., Aisen, P., & Blumberg, W. E., Eds.) Academic Press, New York.
Mellema, J. E., & Klug, A. (1972) *Nature (London) 239*, 146–150.
Noren, I. B. E., Bertoli, D. E., Ho, C., & Casassa, E. F. (1974) *Biochemistry 13*, 1683–1686.
Putzeys, P., & Brostreaux, J. (1941) *Meded. K. Vlaam. Acad. Wet., Lett. Schone Kunsten Belg., Kl Wet. 3*, 3–23.
Siezen, R. J., & Van Driel, R. (1973) *Biochim. Biophys. Acta 295*, 131–139.
Siezen, R. J., & van Bruggen, E. F. J. (1974) *J. Mol. Biol. 90*, 77–89.
Svedberg, T., & Pedersen, K. O. (1940) *The Ultracentrifuge*, Oxford University Press, Oxford, England.
van Breemen, J. F. L., Schuurhuis, G. J., & van Bruggen, E. F. J. (1977) in *Structure and Function of Hemocyanin* (Bannister, J. V., Ed.) pp 122–127, Spring-Verlag, New York.
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., & van Bruggen, E. F. J. (1971) *Biol. Macromol. 5*, 1–53.
Van Holde, K. E., & Miller, K. I. (1982) *Q. Rev. Biophys. 15*, 1–129.
Van Holde, K. E., Blair, D., Eldren, N., & Arisaka, F. (1977) in *Structure and Function of Hemocyanin* (Bannister, J. V., Ed.) pp 22–30, Springer-Verlag, West Berlin.
Van Schaick, E. J. M., Schutter, W. G., Gaykema, W. P. J., van Bruggen, E. F. J., & Holt, W. G. J. (1981) in *Invertebrate Oxygen-Binding Proteins. Structure, Active Site and Function* (Lamy, J., & Lamy, J., Eds.) pp 353–362, Marcel Dekker, New York.

Hydroxide Ion Binding to Methemerythrin. An Investigation by Resonance Raman and Difference Spectroscopy†

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ABSTRACT: The pH dependence for the interconversion of the acid and base forms of methemerythrin from *Themiste dyscritum* was investigated by difference spectroscopy. A new technique was designed to be able to study mixtures without knowledge of extinction coefficients or exact protein concentrations. The resultant pK_a value of 8.4 proved that *T. dyscritum* hemerythrin crystals used for previous X-ray crystallographic studies at pH ≤6.5 were in the acid form. Since this material contains a 5-coordinate iron atom with no evidence of a ligated water molecule, it is more appropriately referred to as methemerythrin than aquomethemerythrin. The presence of an iron-bound hydroxide in the base form of methemerythrin was verified by resonance Raman spectroscopy for both *T. dyscritum* and *Phascolopsis gouldii*. At pH >9, the protein from either species exhibited a new feature at 490 cm^{-1} that shifted to 518 cm^{-1} in D_2O and was assigned to a coupled Fe–OH stretching and O–H bending vibration. Thus, hydroxomethemerythrin is the correct designation for the base form of the protein. The other resonance-enhanced vibration, the Fe–O–Fe symmetric stretch, was observed at 506 cm^{-1} in hydroxomethemerythrin and at 511 cm^{-1} in methemerythrin and was unaffected by deuteration. Addition of perchlorate to methemerythrin had no effect on the Raman spectrum, despite its known role in stabilizing the met form relative to the hydroxomet form.

Hemerythrin is an oxygen transport protein found in several phyla of marine invertebrates. It contains a non-heme binuclear iron center that reversibly binds one molecule of oxygen for every two iron atoms (Boeri & Ghiretti-Magaldi, 1957). Upon oxygenation both iron atoms are oxidized from the ferrous to the ferric state while the oxygen is reduced to peroxide (Kurtz et al., 1977; Loehr & Loehr, 1979). The protein can also be oxidized by chemical reagents such as ferricyanide or hydrogen peroxide (Wilkins & Harrington, 1983) to produce methemerythrin, a form that no longer binds dioxygen but has the binuclear iron center fixed in the ferric state. Methemerythrin has been shown to bind small anions such as chloride, azide, and thiocyanate (Keresztes-Nagy & Klotz, 1965; Garbett et al., 1969). All anionic adducts of

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