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Subunit Structure and Dissociation of *Homarus americanus* Hemocyanin. Effects of Salts and Ureas on the Acetylated and Unmodified Hexamers†

Theodore T. Herskovits,* Susan E. Carberry, and Richard C. San George‡

ABSTRACT: At neutral pH and in the presence of divalent cations the hemocyanin of the lobster *Homarus americanus* exists largely as a dodecamer with a molecular weight of 940 000. Light-scattering investigation has shown that the dodecamers dissociate to hexamers followed by dissociation of the hexamers to monomers. In the absence of calcium ion, both the acetylated hemocyanin at pH 7.8 and the unmodified protein at pH 8.8 were found to be largely in the hexameric state, with molecular weights close to 450 000. The effects of various salts of the Hofmeister series and the urea series on the subunit organization and dissociation of the basic hexameric unit were investigated and analyzed. The salts as dissociating agents are found to be fairly effective, while the ureas are rather ineffective. The effects of these two groups of reagents on the dissociation of the hexameric structure to form monomers closely parallel their effects on the parent dodecamers, dissociating to hexamers [Herskovits, T. T., San George, R. C., & Erhunmwunsee, L. J. (1981) *Biochemistry 20*, 2580–2587]. This suggests that polar and ionic interactions, rather than hydrophobic forces, are the dominant forces that stabilize both the basic hexameric unit and the dodecamers in solution. The analysis of our data obtained with the acetylated hexamers gave apparent estimates of amino acids at the contact areas of the monomers that are nearly the same as the number of groups estimated previously for the contact areas of the hexamers. This suggests that the contact areas of the hexamers and monomers forming the dodecamer are comparable in size. Comparable surface areas of contact are also suggested by the recent models of arthropod hemocyanin dodecamers built by Lamy et al. [Lamy, J., Bijlholt, M. M. C., Sizaret, P.-Y., Lamy, J., & van Bruggen, E. F. J. (1981) *Biochemistry 20*, 1849–1856] and Markl et al. [Markl, J., Kempter, B., Linzen, B., Bijlholt, M. M. C., & van Bruggen, E. F. J. (1981) *Hoppe-Seyler's Z. Physiol. Chem. 362*, 1631–1641] on the basis of the X-ray crystallographic structure of the hexamer of *Panulirus interruptus* hemocyanin [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 Protens: Structure, Active Site and Function* (Lamy, J., & Lamy, J., Eds.) pp 353–362, Marcel Dekker, New York].

The hemocyanins found in the hemolymph of many invertebrates are copper-containing, multisubunit proteins of varying complexity charged with the transport of oxygen. The hemocyanins of the lobsters and other arthropods are assemblies consisting of one to eight basic hexameric units, ranging from approximately 0.45×10^6 to 3.3×10^6 daltons (Van Holde & van Bruggen, 1971; Bonaventura et al., 1977). The subunit structure and the interactions among the various subunits related to the stability and the oxygen binding of the various hemocyanins have been the topic of interest of a number of recent studies [for recent reviews, see Van Holde & van Bruggen (1971), Antonini & Chiancone (1977), and Bonaventura et al. (1977)]. Most hemocyanins exhibit subunit heterogeneity (Di Giamberardino, 1967; Konings et al., 1969; Van Holde et al., 1977), which alters their association–dissociation properties. This has been a complicating factor in the description and analysis of the fundamental monomer to hexamer step of assembly of the hemocyanins of the arthropod species. The few hemocyanins that have been found that appear to be homogeneous or show only moderate effects of subunit heterogeneity are those of the isopods *Ligia exotica* and *Bathynomus giganteus* (Terwilliger et al., 1979; Van Holde & Brenowitz, 1981) and the lobster *Homarus americanus* (Morimoto & Kegeles, 1971; Herskovits et al., 1981b). The association–dissociation behavior of the latter dodecameric

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protein to hexamers was found to be rapid and reversible, with the concentration dependence of the molecular weight correctly predicted by the law of mass action (Morimoto & Kegeles, 1971; Herskovits et al., 1981b).

We have initially investigated the effects of various neutral salts of the Hofmeister series and the urea series on the dodecamer to hexamer dissociation of this protein, using light scattering as a tool for probing the nature of the contact areas of the subunits. These studies have suggested that hydrophobic forces are not the dominant stabilizing interactions that hold the pair of hexamers of the protein together in solution. In order to investigate the nature of the contact areas be between the monomers forming the basic hexameric unit of the *Homarus* hemocyanin, we have extended these investigations to the dissociation behavior of the isolated hexamers (at pH 8.8 and 9.5) and the acetylated derivative of this protein.

Materials and Methods

Homarus hemocyanin was isolated from the hemolymph of live Maine lobsters, as previously described (Herskovits et al., 1981a,b). Most of our work was done on the hemocyanin of male lobsters, since these were found to have the least contaminated crude protein. The hemolymph of female lobsters prepared during the summer months contained various amounts of reddish yellow pigment, which seems to be associated with their reproductive cycle. This pigment appeared as a trailing fraction in our chromatographic step of purification on Bio-Gel A-5m columns, resulting in significant loss of protein. Acetyated protein was prepared essentially according to the procedure of Gunther et al. (1973), who showed that concanavalin A tetramers are dissociated to half-molecules or dimers by this modification. *Homarus* hemocyanin in the hexameric state (at pH 7.8) was prepared by a similar acetylation procedure. Typically 53 cm^3 of 0.55% calcium-free protein was mixed with the appropriate amount of solid Tris[1] buffer (Trizma 8.3) to give 1.0 M, pH 8.3 buffered solutions, followed by the slow adition of 0.14 cm^3 of acetic anhydride. Magnetic stirring in an ice bath was employed to facilitate the reaction. After a 30-min stirring, the protein solutions were dialized against 2–3 changes of 0.1 M NaCl–0.05 M, pH 7.8 Tris in the cold. Eight of our preparations gave an average molecular weight of 449 500 ± 8600, close to the accepted value of 470 000 for the hemocyanin hexamer. The mean residue ellipticity at 218 nm of acetylated hemocyanin (at pH 7.8) was found to be −7240 deg·cm^2·$dmol^{-1}$, which is close to the value of −7050 ± 200 deg·cm^2·$dmol^{-1}$ obtained for the unmodified, parent protein. Hemocyanin concentrations were determined spectrophotometrically in a Cary 14 instrument by using the percent extinction coefficient $E^{1\%}_{278nm}$ = 13.4 (Morimoto & Kegeles, 1971; Herskovits et al., 1981b).

All the reagents and salts employed were analytical- or reagent-grade materials. The urea and guanidinium chloride was ultrapure grade purchased from Schwarz/Mann and used without further purification. The alkylureas were purified by recrystallization from hot ethanol and dried under vacuum.

Light-scattering measurements were made in a Wood instrument of Brice's design, at 436 nm and an angle of 90°, as previously described (Harrington et al., 1973; Elbaum & Herskovits, 1974). All the solutions used for light-scattering measurement were clarified by filtration in 0.2-μm Gelman metricell filters mounted in 25-mm diameter Millipore filter holders (Harrington et al., 1973; Elbaum & Herskovits, 1974).

[1] Abbreviations: GdmCl, guanidinium chloride; Tris, tris(hydroxymethyl)aminomethane; CD, circular dichroism.

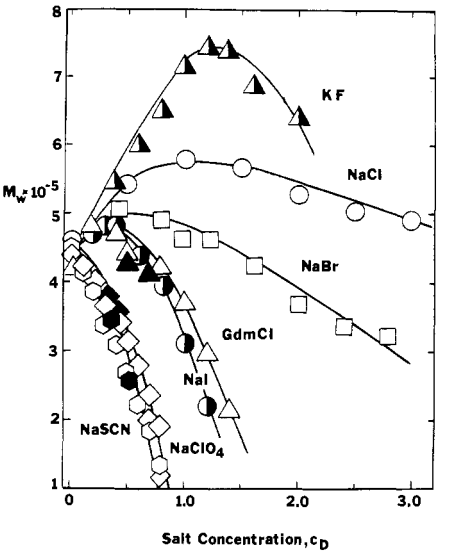

FIGURE 1: Effects of various salts on molecular weight of acetylhemocyanin hexamers at pH 7.8 plotted as a function of salt concentration. The NaSCN and $NaClO_4$ data were fitted by using eq 4 and 5, with $K_{w,app} = 2 \times 10^{-33}$ M^5 and $N_{app} = 25$ for NaSCN and $K_{w,app} = 1 \times 10^{-33}$ M^5 and $N_{app} = 21$ for $NaClO_4$. Data represented by filled symbols were obtained on the protein initially exposed to 0.8 M $NaClO_4$, 1.0 M NaSCN, and 1.4 M GdmCl. Protein concentration 0.4 g·L^{-1}; solvent 0.1 M NaCl–0.05 M Tris buffer.

The same Wood instrument was also employed for refractive index increment determination. The specific refractive index increment $(\partial n/\partial c)_\mu$ for *Homarus americanus* hemocyanin at pH 7.8 and at 436 nm was found to be 0.188 ± 0.003 g^{-1}·cm^3. In 1.0 and 2.0 M KF, the values were found to be 0.191 and 0.194 g^{-1}·cm^3, giving a molar increment of 0.003 g^{-1}·cm^3 for this salt. For the various other salts and ureas of this study, the previously determined molar increments or decrements obtained at 630 nm (Herskovits et al., 1981b) were used. The effects of dispersion due to differences in wavelength on these corrections are small (Herskovits et al., 1981b, 1983) and thus have been ignored.

Optical rotatory dispersion and circular dichroism measurements were made on a Cary 60 recording spectropolarimeter equipped with a CD attachment. The mean residue molecular weight M_0 of 124 was used on the basis of the amino acid data of related crustacean species (Ghiretti-Magaldi et al., 1966).

Results

The effects of various salts of the Hofmeister series and the ureas on molecular weight of the acetylated *Homarus* hemocyanin, investigated at pH 7.8, are shown in Figures 1 and 2. Salts such as KF, NaCl, and NaBr show an initial increase in molecular weight, M_w, signifying initial reassociation of the hemocyanin hexamers to dodecamers, followed by dissociation of the dodecamers to hexamers and monomers at higher reagent concentration. The more effective salts, $NaClO_4$, NaSCN, and NaI, and the urea series show little or no initial increase in molecular weight with increasing reagent concentration. We have attributed this initial association of *Homarus* hemocyanin in the presence of salts to electrostatic screening of some of the charges at the contact areas of the hexamers forming the dodecamer (Herskovits et al., 1981b). As shown in Figures 3 and 4, the behavior of unmodified *Homarus* hemocyanin at pH 8.8 and 9.5 in the presence of 2×10^{-3} M Ca^{2+} is closely analogous to that of the acetylated protein. The main difference is in the greater initial upswing in the molecular weights, which is also aparent for NaSCN

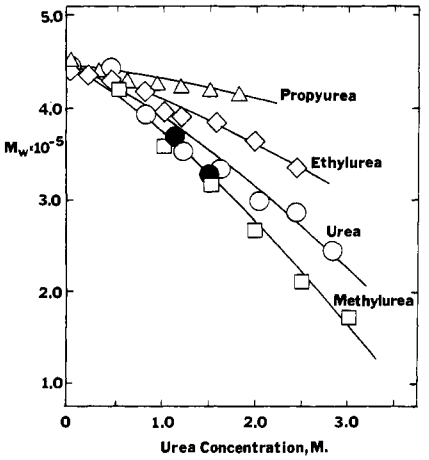

FIGURE 2: Effects of various ureas on molecular weight of acetylhemocyanin hexamers at pH 7.8 plotted as a function of urea concentration. Data represented by filled circles were obtained on protein solutions initially exposed to 2.8 M urea. Protein concentration and solvent conditions are the same as in Figure 1.

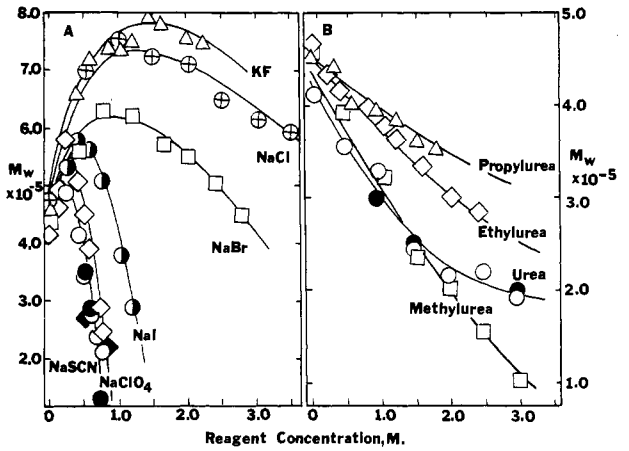

FIGURE 3: Effects of various salts (A) and ureas (B) on molecular weight of *Homarus* hemocyanin hexamers at pH 8.8 plotted as a function of reagent concentration. Filled symbols represent data obtained on hemocyanin initially exposed to 0.8 M $NaClO_4$, 0.8 M NaSCN, and 3.0 M urea. Protein concentration 0.4 g·L^{-1}; solvent 0.1 M NaCl–0.05 M Tris buffer.

and $NaClO_4$ dissociation. The molecular weight data shown in these figures were obtained at a fixed protein concentration of 0.4 g/L, where nonideality effects are close to minimal, altering the observed apparent molecular weight by less than 1%. We have calculated the molecular weights using the expression

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

where R_θ is the measured Rayleigh ratio, K' is the light-scattering constant, and B' is the nonideality or second virial coefficient taken as 1×10^{-8} L·mol·g^{-2} (Herskovits et al., 1981b).

The effects of dissociating agents and salts on the molecular weight of subunit proteins have been interpreted from the general expressions (Herskovits & Ibanez, 1976; Herskovits et al., 1977, 1978)

$$K_{D,app} = \frac{m^m c^{m-1} \alpha^m}{(1-\alpha) M_m^{m-1}} \simeq K_{w,app} \exp(m N_{app} K_B c_D) \quad (2)$$

and

$$M_w = M_m(1 - [(m-1)/m]\alpha) \quad (3)$$

where $K_{D,app}$ and $K_{w,app}$ represent the apparent dissociation constant of the subunit protein in the presence and absence

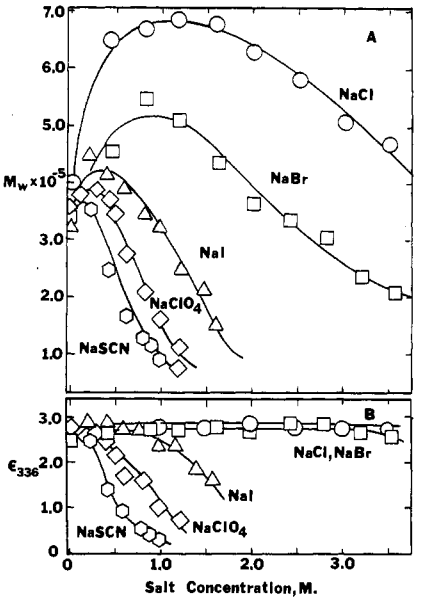

FIGURE 4: Effects of various salts on molecular weight, M_w (A), and absorbance of the copper band at 336 nm, ϵ_{336} (B), of *Homarus* hemocyanin at pH 9.5 in the presence of 2×10^{-3} M Ca^{2+}. Protein concentration 0.4 g·L^{-1}; solvent 0.2 M pH 9.5 glycine–2×10^{-3} M Ca^{2+}.

of dissociating reagent, respectively, M_m is the molecular weight of the parent, undissociated protein, m is the number of subunits or fragments of the protein formed as a result of dissociation, α is the weight fraction of the protein that dissociates at any reagent concentration c_D, K_B is the binding or interaction constant of the dissociating reagent with the average amino acid found at the contact areas of the subunits, and N_{app} is the apparent number of these amino acids. For the dissociation of hemocyanin hexamers to monomers, investigated in this study, N_{app} refers to the apparent number amino acids at the contact areas of the monomers that become exposed as a result of dissociation. For the dissociation of hexamers to monomers ($m = 6$), we obtain the working eq 4

$$M_w = M_6[1 - (5/6)\alpha] \quad (4)$$

and 5 required to fit the molecular weight data. The $NaClO_4$

$$\frac{\alpha^6}{1-\alpha} = \frac{(2.14 \times 10^{-5}) K_{w,app} M_6^5}{c^5} \exp(6 N_{app} K_B c_D) \quad (5)$$

and NaSCN data of Figure 1 were fitted by using these equations with the required N_{app} and $K_{w,app}$ parameters extracted from the slope and the intercept of the linearized, logarithmic form of eq 2, expressed as

$$\Delta G_D^\circ = \Delta G_w^\circ - 6RTN_{app}K_B c_D \quad (6)$$

$$\Delta G_D^\circ = -RT \ln K_{D,app} \quad (7)$$

Figure 5 shows some of data obtained on acetylated hemocyanin studied at pH 7.8 and plotted according to eq 6 and 7. The α-values required for calculating $K_{D,app}$ (eq 2, with $m = 6$) were based on eq 4, written as

$$\alpha = (6/5)(1 - M_w/M_6) \quad (8)$$

with the molecular weight of the hemocyanin hexamer, M_6, taken as 470 000. Table I gives a summary of some of the derived dissociation parameters for the acetylated hemocyanin hexamers, on the basis of eq 6–8 and the molecular weight data of Figures 1 and 2.

An important assumption made in our work is that the dissociating reagent does not alter significantly the nature of the contact areas of the subunits by unfolding or denaturation.[2]

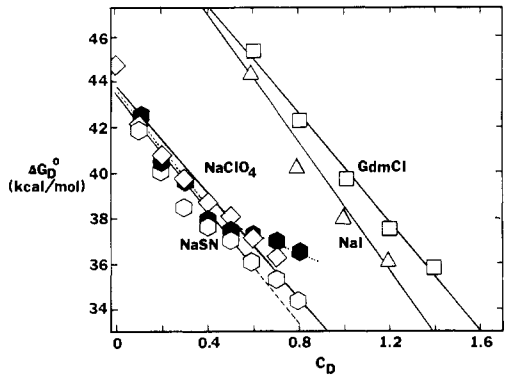

FIGURE 5: Plot of ΔG_D° vs. c_D for hexamer to monomer dissociation of acetylhemocyanin (pH 7.8) on the basis of eq 6. The derived ΔG_D°, slope, and N_{app} parameters are listed in Table I. Open symbols for the NaSCN data represent measurements after 15–20 min following mixing of the solutions, while the filled symbols represent an independent set of measurements after 1 h of equilibration of solutions at 25 °C.

Table I: Dissociation Parameters of Acetylhemocyanin Hexamers Forming Monomers at pH 7.8, on the Basis of Equations 6 and 7

reagent	K_B[a]	K_s[a]	slope (kcal·mol^{-1}·M^{-1})	ΔG_D° (kcal·M^{-1})	r[b]	N_{app}
urea	0.032		−3.86	45.7	0.921	33
NaSCN	0.160	−0.070	−14.2[c]	44.2[c]	0.98[c]	25 ± 3[c]
$NaClO_4$	0.175	−0.076	−12.9[c]	44.3[c]	0.97[c]	21 ± 2[c]
NaI	0.180	−0.078	−14.0[d]		0.992	22
GdmCl	0.200	−0.088	−12.0[d]		0.993	17

[a] Values of K_B and K_s taken from Herskovits et al. (1977, 1978). [b] Correlation coefficients based on least-squares treatment of the data. [c] Parameters represent average of two independent determinations. [d] Parameters based on data above 0.5 M NaI and GdmCl shown in Figure 5. Below these concentrations the molecular weight data is above 470 000, suggesting little or no dissociation and the presence of a significant amount of dodecamer (see Figure 1).

The circular dichroism (CD) and the absorbance data of Figure 6 suggest that there is little or no denaturation of the modified hemocyanin below about 0.8–1.2 M $NaClO_4$, GdmCl, and NaI and also below 3 M urea. These are the ranges of reagent concentration that have been used for quantitative interpretation of our data (Table I) and also the ranges where most of the dissociation of the hexamers to monomers is observed. There may be some ambiguities with the NaSCN data above 0.5–0.6 M, since significant absorbance changes are seen with this reagent above these concentrations (Figure 6B). Circular dichroism measurements would have been informative in the conformationally sensitive 218–20-nm region. Unfortunately, such measurements could not be made with NaSCN and most of the other salts of this study because of their strong absorbance in this wavelength

[2] We would expect that the destabilization of the dodecameric structure and also the monomer to monomer contacts due to acetylation of hemocyanin should result in changes in the dissociation constant, $K_{w,app}$, but not in the N_{app} parameters. The latter reflect the size of the contact areas between the subunits (eq 2). Acetylation should not change the protein conformation to any significant degree since both the mean residue ellipticity and the copper–oxygen extinction coefficient, at 218 and 336 nm, are found to be largely unaltered (see Figures 4 and 6 and Materials and Methods). With regard to the sort of interactions that may be weakened by acetylation, the X-ray crystallographic and solution data of concanavalin A show that the blocking of a few salt bridges by acetylation or succinylation (two lysine residues in case of concanavalin A) results also in the dissociation of the parent protein to half-molecules (Gunther et al., 1973).

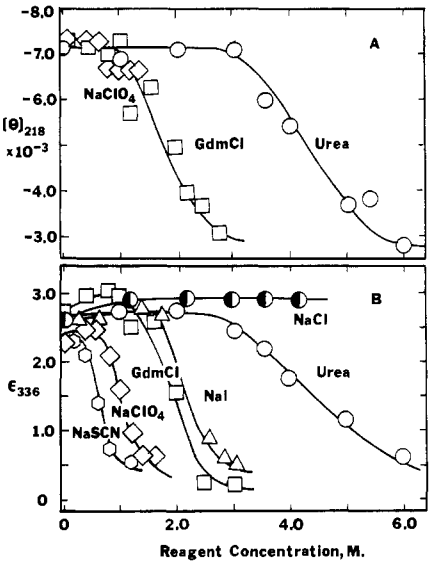

FIGURE 6: Effects of various dissociating reagents on mean residue ellipticity, $[\theta]_{218}$, at 218 nm (A) and the copper extinction coefficient, ϵ_{336}, at 336 nm (B) of acetylhemocyanin at pH 7.8 (0.1 M NaCl–0.05 M Tris buffer).

region. The light-scattering data obtained with 0.5–0.8 M NaSCN show also time-dependent changes in molecular weight, signifying gradual aggregation of the unfolded protein. This has resulted in a definite break or upswing in the ΔG_D° vs. c_D curves represented by the filled symbols in Figure 5.

The data obtained on hemocyanin hexamers shown in Figure 4 suggest that the unmodified protein at alkaline pHs is somewhat less stable than the acetylated protein at pH 7.8. In addition, we find that the greater initial stabilization of the dodecameric structure by the salts and the resultant increase in molecular weights tend to shift the hexamer to monomer transition region to higher reagent concentration (by about 0.3–0.5 mol). The result is that the dissociation and unfolding transitions observed at high pH almost coincide. The latter findings preclude any quantitative interpretation of the molecular weight data obtained, in terms of the number of amino acids groups at the contact areas of the subunits.

The structural organization of the hemocyanin hexamer (Van Schaick et al., 1981) suggests the possibility that the dissociation to monomers may involve the accumulation of a significant fraction of trimer intermediates. That such intermediates are in fact totally absent is fairly difficult to prove. However, what seems to argue strongly against the presence of a significant contribution of intermediates to our description of the monomer to hexamer dissociation reaction are both the close fit of our $NaClO_4$ and NaSCN data with the theoretical curves shown in Figure 1 and the very satisfactory correlation coefficients of all our data analyzed by means of eq 6–8, giving r values that are mostly within the ranges of 0.97–0.99 (Table I). Also, such intermediates have not been observed directly in any ultracentrifugation studies[3] on *Homarus* and other arthropod hemocyanins (Morimoto & Kegeles, 1971; Ellerton et al., 1970; Herskovits et al., 1981a).

The dissociation of *Homarus* hemocyanin at the protein concentration of 0.4 g/L and below employed for most of our work was found to be relatively rapid and reversible, provided

[3] Morimoto & Kegeles (1971) have observed only 25S, 17S, and 5S species of dodecamers, hexamers, and monomers in the case of *Homarus* hemocyanin dissociation at alkaline pHs. Again, only these three species have been reported in the case of *Cancer magister* (Ellerton et al., 1970) and *Callinectes sapdisu* hemocyanin (Herskovits et al., 1981a).

the reagent concentration was kept below the level where there is significant unfolding of the subunits suggested by the changes in absorbance at the 336-nm copper band and the CD spectra at 218 nm (Figures 4 and 6). The observed changes in the turbidity and molecular weight were usually complete within 5–15 min following mixing of the protein solutions and the dissociating reagent or salt. The reversibility data obtained on solutions exposed to $NaClO_4$, NaSCN, GdmCl, and urea are shown as filled symbols in Figures 1–3. The equilibrium values of the molecular weights seem to fall on the smoothed forward curves or the theoretical curves calculated by use of eq 4 and 5, suggesting that the subunit dissociation is fully reversible.

While the dodecamers and hexamers have been found to be in rapid equilibrium (Morimoto & Kegeles, 1971) and the concentration dependence of the light-scattering curves was correctly predicted by the law of mass action describing the stoichiometry of the dissociation reaction, this does not appear to be the case with the dissociation of the hexamers to monomers. In several experiments with urea and $NaClO_4$ as dissociation agents, the $K'c/R_\theta$ vs. protein concentration curves investigated in the region of 0.05–1.0 g/L were found to be fairly flat and invariant with concentration, suggesting complications due to the effects of subunit heterogeneity observed also with other hemocyanins (Di Giamberardino, 1967; Konings et al., 1969, Herskovits et al., 1981a). Consequently, our N_{app} estimates obtained in this study (Table I) must be viewed as apparent quantities having only comparative or empirical value. The effects of subunit heterogeneity on the derived estimates of amino acids at the contact areas of the monomers will require further investigation using a homogeneous preparation of the subunits isolated by chromatographic procedures developed by Sullivan and Lamy and their coworkers (Sullivan et al., 1974; Schutter et al., 1977; Lamy et al., 1981) or the use of hemocyanins such as the protein of the isopod *Bathynomus giganteus*, which exhibit only a single association–dissociation equilibrium (Van Holde & Brenowitz, 1981). It is, nevertheless, significant that our present N_{app} estimates obtained for the monomer contacts, examined more fully under Discussion, are comparable to the N_{app} estimates for the contact areas of the hexamers, where the problem of subunit heterogeneity poses fewer experimental uncertainties (Herskovits et al., 1981b).

Discussion

At physiological pH and ionic conditions, the hemocyanin of the North American lobster *H. americanus* exists predominantly as a dodecamer with a molecular weight of 940 000 (Herskovits et al., 1981a,b). Light-scattering studies in the presence of various salts and ureas have suggested that the dodecamers dissociate to half-molecules of hexamers, followed by further dissociation to monomers. The dodecamers and hexamers are found to be in rapid equilibrium that can be shifted by increasing pH and reduced divalent cation concentration to give nearly exclusively hexamers. This is apparent from both the ultracentrifugation studies of Morimoto & Kegeles (1971) at alkaline pHs in the presence of reduced amounts of calcium ion and our present light-scattering investigations on hemocyanin at pH 8.8 and 9.5, as well as the acetylated protein at pH 7.8.

The molecular weight data obtained with the various salts of the Hofmeister series and the urea series shown in Figures 1–4 suggest that the *Homarus* hemocyanin hexamers can be readily dissociated to monomers by a number of these reagents. The higher members of the Hofmeister series such as NaSCN, $NaClO_4$, and NaI are found to be the most effective dissociating agents while the ureas appear to be relatively ineffective. Qualitatively, the effects of these two groups of reagents on the dissociation of the hexamers to monomers closely parallel their effects on the parent dodecameric protein, dissociating to hexamers (Herskovits et al., 1981b). This suggests that polar and ionic interactions, rather than hydrophobic forces, represent the dominant interactions that stabilize both the monomer–monomer contacts of the basic hexameric unit and the hexamer–hexamer contacts in the dodecameric structure of this hemocyanin. The ineffectiveness of ethyl- and propylurea relative to urea and methylurea (Figures 2 and 3) and the inverse trend in effectiveness with increasing hydrophobicity or hydrocarbon content were also observed with the molecular weight behavior of the hemocyanin of the blue crab *Callinectes sapidus* (Herskovits et al., 1981a). This would tend to support the generality of our findings with regard to the stabilizing forces that hold together the subunits of the hemocyanins of the arthropod species. Engelborghs & Lontie (1973) have investigated the dissociation behavior of the α-component of the hemocyanin of *Helix pomatia* using light-scattering and ultracentrifugation methods. This molluscan hemocyanin has a molecular weight of approximately 9×10^6 and is found to dissociate to half-molecules by the various Hofmeister salts (Engelborghs & Lontie, 1973), with closely similar order of effectiveness found for the hemocyanins of the two arthorpod species studied in our laboratory (Herskovits et al., 1981a,b). However, with regard to the question of stabilization of the subunit structure of the molluscan hemocyanins, so far no dissociation studies have been reported with any of the alkylurea series or other hydrophobic reagents probing the nature of the contact areas of the subunits. The molluscan hemocyanins have different structural organization from the arthropod hemocyanins, with basic subunits having molecular weights of 360 000 ± 30 000 (Brouwer et al., 1976; Van Holde & Miller, 1982), arranged to form a rodlike cylindrical structure in their native state (Mellema & Klug, 1972).

Perhaps the most significant finding of this study, on the basis of analysis of our acetylhemocyanin data with eq 4–8, is that the apparent estimate of the number of amino acids (N_{app}) at the contact areas of the monomers is about the same as the estimates of groups for the hexamer to hexamer contacts. Our present estimates for the monomer to monomer contacts obtained with urea and the salts NaSCN, $NaClO_4$, NaI, and GdmCl are 33 and 17–25 groups, respectively (Table I). These stimates should be compared with the values of 28–31 and 16–28 obtained previously for the hexamer to hexamer contacts with the same two groups of reagents (Herskovits et al., 1981b). Unlike the molluscan hemocyanins, which have cylindrical organization and symmetry, the arthropod hemocyanins have compact globular structures with the basic hexameric unit organized in the form of trigonal antiprism, having point-group symmetry 32 (Schutter et al., 1977; Van Schaick et al., 1981). The X-ray crystallographic structure proposed by Van Schaick et al. (1981) for the spiny lobster *Panulirus interruptus* hemocyanin suggests that each kidney-shaped monomer is in contact with four neighboring subunits. In the hemocyanin dodecamer built on the basis of a pair of such hexamers (Lamy et al., 1981; Markl et al., 1981), the hexamers appear to make the same number of contacts as the monomers. This would suggest that these two different areas of contact should be comparable in size, comprising approximately the same surface area and the same number of amino acids. Our finding that the N_{app} values for the two sets of contact area are about the same is consistent

with such models of the hemocyanin hexamer and dodecamer of arthropod species.

It is important to note that much of our present work deals with the dissociation behavior of acetylated hemocyanin in the hexameric state. As such, the implications regarding the apparent estimates of amino acids at the contact areas of the monomers (N_{app}) are open to question and must be viewed with necessary caution.[2] Clearly, more work needs to be done on *Homarus* hemocyanin, including dissociation studies on the isolated homogeneous subunit assemblies. The closely similar N_{app} values associated with the monomer and hexamer contact areas poses a further challenging question about the effects of microheterogeneity of the subunits on the derived macromolecular parameters based on multiple equilibria formulations such as ours (Herskovits et al., 1977, 1978; Herskovits & Ibanez, 1976).

The effects of subunit heterogeneity would tend to broaden the dissociation transition and thus lead to a lower estimate of the corresponding N_{app} value. We have estimated that changes in the stabilization energy of the order of 1.4–2.8 kcal/mol of subunit or fragment formed should lead to lower N_{app} estimates by perhaps three to eight amino acid groups (Herskovits et al., 1981b). Thus the uncertainty introduced by a moderate degree of microheterogeneity would not alter greatly our conclusions pertaining to the nature and approximate number of amino acids at the contact areas of the subunits.[4] We have recently examined one aspect of the problem of microheterogeneity related to the present study by comparing the dissociation parameters of homogeneous with heterogeneous concanavalin A tetramers forming dimers (Herskovits et al., 1983). Heterogeneous concanavalin A preparations contain about 50% of the total number of subunits that are proteolytically cleved at a single region of the polypeptide chains, between amino acid residues 118 and 119 (Cunningham et al., 1972). Homogeneous intact chain protein preparations are readily obtained by NH_4HCO_3 fractionation and removal of the hydrolyzed polypeptide chains (Cunningham et al., 1972; Senear & Teller, 1981). Both of the concanavalin A preparations gave within experimental uncertainty the same N_{app} estimates (Herskovits et al., 1983) and also the same estimates of the number of protonating histidine residues, the latter extracted from the pH dependence of the dissociation of this protein (Senear & Teller, 1981).

Registry No. KF, 7789-23-3; NaCl, 7647-14-5; NaBr, 7647-15-6; GdmCl, 50-01-1; NaI, 7681-82-5; $NaClO_4$, 7601-89-0; NaSCN, 540-72-7; propylurea, 627-06-5; urea, 57-13-6; ethylurea, 625-52-5; methylurea, 598-50-5; Ca, 7440-70-2.

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[4] These estimates have been calculated for the dodecamer to hexamer dissociation, with an average $\Delta G_w°$ of about 10 kcal·mol^{-1}. For the hexamer to monomer dissociation examined in this study, the higher $\Delta G_w°$ of about 40 kcal·mol^{-1} for the reaction (Table I) would mean a 3–4 times lower N_{app} estimate, assuming the same 1.4–2.8 kcal·mol^{-1} lowering in $\Delta G_w°$.