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Light-Scattering Investigation of the Subunit Structure and Sequential Dissociation of *Homarus americanus* Hemocyanin†

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ABSTRACT: The hemocyanin of the North American lobster *Homarus americanus* has a dodecameric subunit structure organized from two basic hexameric units characteristic of arthopod species. Various neutral salts and ureas have been found to dissociate this hemocyanin. The molecular weight profiles studied as a function of reagent concentration and constant hemocyanin concentration, using light-scattering intensity methods, suggest a two-step reaction of dissociation and assembly of the functional dodecameric protein. The dissociation data obtained with urea, NaBr, and some of the other salts of this study show an initial decrease in the molecular weights to values close to 4.7×10^5, the molecular weight of the basic hexameric unit. This intermediate plateau region of the molecular weight transitions is followed by further dissociation of the hexamers to monomers with a corresponding decrease in molecular weights approaching 0.8×10^5. The equations developed for analyzing the light-scattering dissociation data in previous studies were applied to the sequential steps of dodecamer to hexamer and hexamer to monomer dissociation–association reactions. The apparent number (N_{app}) of amino acid groups at the contact areas of the hexamers in the functional dodecamers and the contact areas of the monomers in the half-molecules or hexamers affected by the dissociating reagent were found to be about the same. Similar N_{app} estimates were obtained previously from the dissociation behavior of the *Homarus* hemocyanin dodecamers and the isolated hexamers, modified by acetylation. These findings suggest that the contact areas of the hexamers and the monomers forming the dodecamers of arthropod hemocyanins must be comparable in size.

The hemocyanin of the North American lobster *Homarus americanus* is a dodecameric subunit protein built from two basic hexameric units held together by noncovalent interactions in solution (Morimoto & Kegeles, 1971; Herskovits et al., 1981b). The hemocyanins of the lobsters *Panulirus interruptus* (Van Schaick et al., 1981) and *Homarus* (the subject of the present investigation) consist of one or two basic hexameric units, whereas the hemocyanins of some of the other arthropods, represented by the spider *Eurypelma californicum* (Markl et al., 1981) or the scorpion *Androctonus australis* (Lamy et al., 1981) and the horseshoe crab *Limulus poly-*

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phemus (Bijlholt et al., 1979), are more complex assemblies comprising four and eight hexameric units. The study of the subunit organization and the dissociation behavior of the simpler lobster protein is thus clearly of fundamental interest to our understanding of the processes of assembly of the arthropod hemocyanins and the forces that govern the self-assembly of the functional proteins in solution (Van Schaick et al., 1981; Herskovits et al., 1981b).

The ultracentrifugation studies of Morimoto & Kegeles (1971) and the light-scattering work from our laboratory (Herskovits et al., 1981b) have shown that the *Homarus* hemocyanin dodecamers can be dissociated to hexamers and reassociated by changes in pH and divalent ion concentration, and also by the use of various salts and ureas as dissociating reagents. The dissociation behavior of the hexameric species of this protein has also been investigated and characterized regarding the nature of the contact areas of the subunits by using the same salt of the Hofmeister series and ureas as probes. These studies have suggested that polar and ionic interactions, rather than hydrophobic forces, are the dominant interactions that stabilize the dodecamers and hold the subunits of the hexamers together in solution (Herskovits et al., 1981b, 1983b).

The molecular weight changes accompanying the dissociation of the *Homarus* hemocyanin have also suggested that the dissociation of the subunits is a sequential process. The light-scattering data of this study obtained with urea and various salts as dissociating agents show that the initial dissociation of the hemocyanin dodecamers to hexamers is followed sequentially by the dissociation of the hexamers to monomers at high reagent concentration. A relatively good account of the observed molecular weight data was obtained by using two sets of dissociation parameters, representing the dissociation of the dodecamers to hexamers followed by the dissociation of the hexamers to monomers. The two sets of estimates of the amino acid groups at the contact areas of the hexamers forming the dodecamer and the number of groups at the contact areas of the monomers forming the basic hexameric units were found to be relatively close to the previous estimates of these groups (Herskovits et al., 1981b, 1983b).

Materials and Methods

Hemocyanin was isolated from the hemolymph of live Maine lobsters as previously described, using Bio-Gel A-5m as chromatographic material and 0.1 M NaCl, 0.01 M $CaCl_2$, and 0.05 M tris(hydroxymethyl)aminomethane (Tris),[1] pH 7.8, as the eluent and solvent (Herskovits et al., 1981b, 1983b). The Ca^{2+}-free protein used (Figure 3) was prepared by dialysis in the cold against 0.01 M EDTA, 0.1 M NaCl, and 0.05 M Tris, pH 7.8, buffer followed by 0.1 M NaCl and 0.01 M Tris, pH 7.8. Protein concentration was determined spectrophotometrically on a Cary 14 spectrophotometer by using the percent extinction coefficient, $E^{1\%}_{278nm} = 13.4$ (Morimoto & Kegeles, 1971; Herskovits et al., 1981b).

All the salts and reagents used were analytical or reagent-grade materials. The urea and GdmCl were ultrapure grade purchased from Schwarz/Mann and used without further purification.

Light-scattering and refractive index increment measurements were made at 436 nm in a Wood instrument of Brice's design, as previously described (Harrington et al., 1973; Elbaum & Herskovits, 1974). The solutions used for light-scattering measurements were clarified by filtration directly into the light-scattering cells through 0.2-μm Gelman membrane filters secured in 25-mm diameter Millipore filter holders. The specific refractive index increment obtained from *Homarus* hemocyanin at 436 nm in aqueous solutions, $(\partial n/\partial \bar{c})_\mu = 0.188$ g^{-1} cm^3, was employed for all our molecular weight calculations. For urea and salt solutions, the $(\partial n/\partial \bar{c})_\mu$ values were calculated by using the aqueous value and values of the molar increment or decrement ranging from 0 to about 0.010 g^{-1} cm^3 per mol of reagent. The latter values were obtained at 630 nm. However, since the wavelength dispersion effects were found to be rather small (Herskovits et al., 1981b), these effects on the molar increments or decrements at 436 nm could be ignored. The small Canannes depolarization correction of 1.01 was also applied to all our molecular weight calculations. The light-scattering data measured at a 90° angle were analyzed by using eq 1 where R_θ is the Rayleigh ratio, representing

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

the light scattering of any given protein solution minus that of solvent, M_w is the weight-average molecular weight, B' is the second virial coefficient, and K' is the light-scattering constant defined in our earlier publications (Harrington et al., 1971; Elbaum & Herskovits, 1974) which contains both the small depolarization correction due to the Cabannes factor, $(6 + 6\rho)/(6 - 7\rho)$, and the square of the specific refractive increment at constant chemical potential of the diffusible components, $(\partial n/\partial \bar{c})_\mu^2$.

Optical rotatory dispersion and circular dichroism measurements were made in a Cary 60 recording spectropolarimeter. The mean residue molecular weight, $M_0 = 124$, based on the amino acid data of Ghiretti et al. (1966), was used for all our calculations.

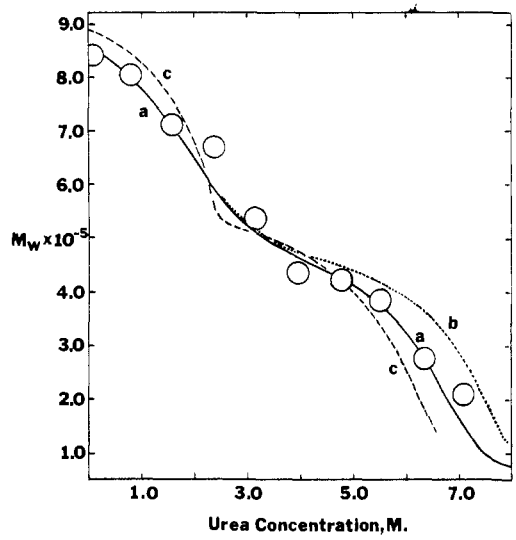

FIGURE 1: Effects of the dissociation reagent, urea, on the subunit dissociation and weight-average molecular weight (M_w) changes of *Homarus* hemocyanin at pH 7.8 predicted by eq 6–8. The following fitting parameters were employed: (a) $N_{12,6} = 28$, $N_{6,1}^{app} = 30$, $K_{12,6} = 5 \times 10^{-8}$ M, $K_{6,1}^{app} = 1 \times 10^{-43}$ M^5, $c = 0.4$ g·L^{-1}, and $K_B = 0.032$ M^{-1}; (b) $N_{6,1}^{app} = 27$; the rest of the parameters are the same as those for curve a; (c) $N_{12,6}^{app} = 30$, $K_{12,6} = 2 \times 10^{-8}$ M, $K_{6,1}^{app} = 1 \times 10^{-42}$ M^{-5}; the rest of the parameters are the same as those for curve a. Solvent, 0.1 M NaCl, 0.05 M Tris buffer, pH 7.8, and 0.01 M Ca^{2+}.

Results

Effects of Ureas and Neutral Salts on the Molecular Weight of Homarus Hemocyanin. The molecular weight profile of *Homarus* hemocyanin studied as a function of urea concentration at pH 7.8 is shown in Figure 1. The dissociation

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; GdmCl, guanidium chloride; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid.

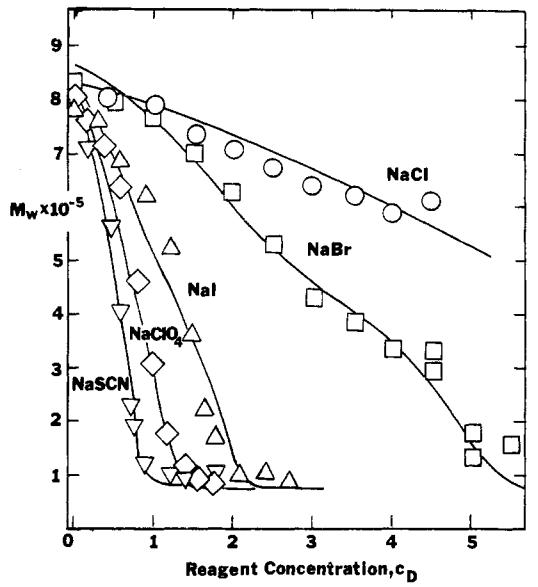

FIGURE 2: Effects of various Hofmeister salts on the weight-average molecular weight (M_w) of *Homarus* hemocyanin at pH 7.8 plotted as a function of the dissociating reagent concentration (c_D). The solid curves drawn through all the data were fitted by using eq 6–8 and the parameters listed in Table I. Protein concentration (0.4 g/L) and solvent conditions were the same as those in Figure 1.

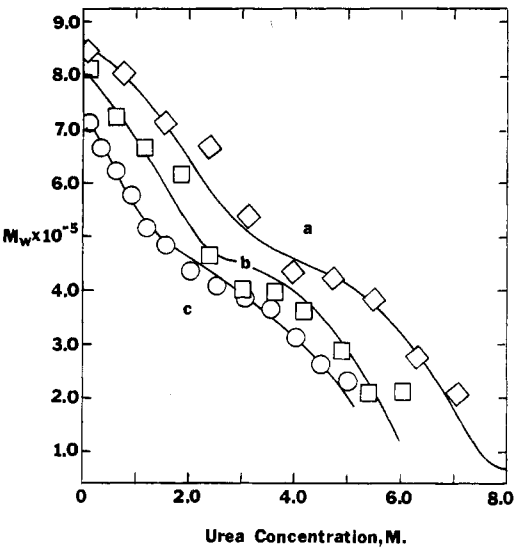

FIGURE 3: Stabilizing effects of calcium ion on the subunit dissociation of *Homarus* hemocyanin at pH 7.8 produced by urea: (a) 1×10^{-2} M Ca^{2+}; (b) 1×10^{-3} M Ca^{2+}; (c) no Ca^{2+}. Solid curves drawn through the data were fitted by using eq 6–8 and the fitting parameters listed in Table I. Protein concentration and solvent conditions were the same as those in Figure 1, except for the varying concentration of Ca^{2+}.

reaction is clearly a biphasic process, representing the initial dissociation of hemocyanin dodecamers going to hexamers followed by the dissociation of the hexamers to monomers. The observed plateau region, from about 3.2 to 5.0 M urea, corresponds to molecular weights that are close to 4.7×10^5, the accepted molecular weight of the half-molecule or hexamer of the lobster hemocyanin (Morimoto & Kegeles, 1971; Herskovits et al., 1981b). Similar data have been obtained with NaBr and some of the other dissociation agents of this study, including the effects of calcium ion concentration on the urea transition shown in Figures 2 and 3. Analysis of the molecular weight data, including the derived distribution of the species of hemocyanin dodecamers, hexamers, and monomers, as a function of dissociating reagent concentration is described in the following section.

Formulation and Analysis of the Sequential Dissociation of Homarus Hemocyanin Dodecamers. The dissociation behavior of hemocyanin which describes our light-scattering data adequately can be represented by the sequential reaction

$$\underset{c(1-\alpha_1)}{Hc_{12}} \overset{K_1}{\rightleftharpoons} \underset{c\alpha_1(1-\alpha_2)}{2Hc_6} \overset{K_2}{\underset{?}{\rightleftharpoons}} \underset{c(\alpha_1\alpha_2)}{12Hc_1} \qquad (2)$$

and the species concentrations of the hemocyanin dodecamers (Hc_{12}), hexamers (Hc_6), and monomers (Hc_1) present, with α_1 representing the weight fraction of the dodecamers that dissociate to hexamers and monomers and α_2 the weight fraction of monomers formed (Herskovits & Harring, 1975). The two equilibrium constants that describe the dissociation process have been expressed (Herskovits & Harrington, 1975) in the form

$$K_{12,6} = K_1 = \frac{4c[\alpha_1(1-\alpha_2)]^2}{(1-\alpha_1)M_{12}} \qquad (3)$$

and

$$K_{6,1}{}^{app} = K_2{}^{1/2} = \frac{(4.67 \times 10^4)c^5(\alpha_1\alpha_2)^6}{\alpha_1(1-\alpha_2)M_6{}^5} \qquad (4)$$

Because of the problems of subunit heterogeneity and its influence on the hexamer to monomer equilibrium, discussed in the following two sections, it is important to note that the latter constant ($K_{6,1}{}^{app}$) is an empirically important but apparent (app) quantity.

The effects of dissociating reagents and salts on protein equilibria as well as the changes in molecular weights of subunit proteins have been formulated and interpreted (Herskovits & Ibanez, 1976; Herskovits et al., 1977, 1978) by using the expression

$$K_D = \frac{m^m c^{m-1}\alpha^m}{(1-\alpha)(M_m)^{m-1}} \simeq K_w \exp(mN_{app}K_B c_D) \qquad (5)$$

where K_D and K_w represent the dissocation constant of the subunit protein in the presence and absence, respectively, of dissociating reagent having the concentration c_D, m is the number of subunits or fragments formed upon dissociation, K_B is the binding or interaction constant of the dissociating reagent with the average amino acid located at the contact areas of the subunits, and N_{app} is the apparent number of these amino acids. Combining eq 3, 5 and 4, 5 leads to eq 6 and 7, which are expressed in the form required to give estimates for the two sets of α values used to fit the light-scattering molecular weight data:

$$\frac{\alpha_1{}^2}{1-\alpha_1} = \left[\frac{K_{12,6}M_{12}}{4c(1-\alpha_2)^2}\right] \exp(2N_{12,6}K_B c_D) \qquad (6)$$

and

$$\frac{\alpha_2{}^6}{1-\alpha_2} = \left[\frac{(2.143 \times 10^{-5})K_{6,1}{}^{app}M_6{}^5}{c^5\alpha_1{}^5}\right] \exp(6N_{6,1}{}^{app}K_B c_D) \qquad (7)$$

On the basis of the definition of the weight-average molecular weight, M_w, measured by light scattering, we have the equation

$$M_w = \frac{\sum c_m M_m}{\sum c_m} = \frac{1}{c}(c_{12}M_{12} + c_6M_6 + c_1M_1) = \frac{1}{c}[c(1-\alpha_1)M_{12} + c\alpha_1(1-\alpha_2)M_6 + c(\alpha_1\alpha_2)M_1] = M_{12}\left(1 - \frac{1}{2}\alpha_1 - \frac{5}{12}\alpha_1\alpha_2\right) \qquad (8)$$

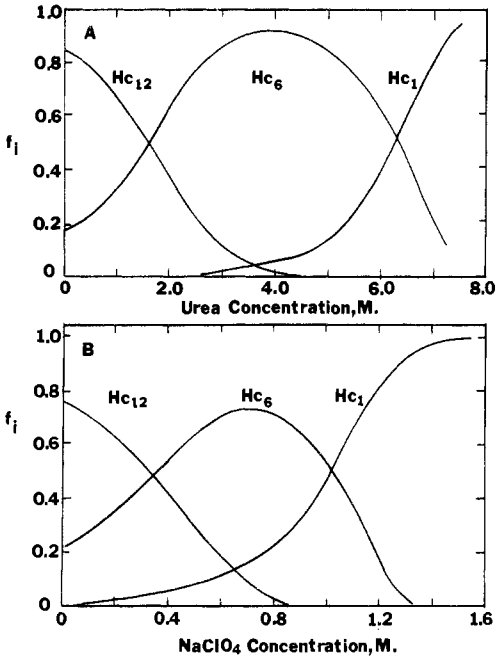

FIGURE 4: Distribution of *Homarus* hemocyanin dodecamers (Hc_{12}), hexamers (Hc_6), and monomers (Hc_1) plotted as a function of urea (A) and $NaClO_4$ (B) concentration. Protein concentration and solvent conditions were the same as those in Figure 1.

where c_{12}, c_6, and c_1 and M_{12}, M_6, and M_1 represent, respectively, the concentrations (grams per liter) and molecular weights of the dodecameric, hexameric, and monomeric species present in solution at a given set of solvent conditions. For all our calculations, we have used the accepted molecular weights, $M_{12} = 9.4 \times 10^5$ and $M_6 = 4.7 \times 10^5$ (Morimoto & Kegeles, 1971; Herskovits, et al., 1981b).

In order to be able to fit the molecular weight data, such as those of Figure 1, initial estimates of the two sets of dissociation constants and the amino acid contacts ($N_{i,j}$) required by eq 6 and 7 have to be obtained. Such estimates are provided from earlier studies of the dissociation of the dodecamers and hexamers of *Homarus* hemocyanin (Herskovits et al., 1981a, 1983b) or the analysis of the dissociation data at low and high reagent concentrations discussed below. Estimates of α_1 and α_2 as a function of the dissociating reagent concentration, c_D, have been obtained by successive approximations using eq 6 and 7 and applied to the molecular weight data through eq 8. Refinement of the data, on the basis of eq 6 and 7, has been found to be relatively straightforward by assuming initially that $\alpha_2 = 0$ at low reagent concentration and $\alpha_1 = 1.0$ at high reagent concentration.

The initial estimates of the two sets of K_w and N_{app} were obtained from the intercept and slope of the linearized logarithmic plot of K_D (eq 5), expressed on the free-energy or RT scale

$$\Delta G^\circ_D = \Delta G^\circ_w - mRTN_{app}K_Bc_D = -RT \ln K_D \quad (9)$$

which is usually plotted as function of regent concentration, c_D (Herskovits et al., 1977, 1981a,b) At low reagent concentration where monomeric species of hemocyanin are essentially absent (see Figure 4), such plots lead to the first set of the required $K_{12,6}$ and $N_{12,6}$ parameters. In principle, the second set of $K_{6,1}^{app}$ and $N_{6,1}^{app}$ estimates can be obtained by using the data at high reagent concentration, where the observed molecular weight falls below that of the hexamer[2] or

Table I: Summary of the Dissociation Parameters of *Homarus* Hemocyanin at pH 7.8 Based on Equations 6-8

reagent	Ca^{2+} concn (M)	K_B[a] (M^{-1})	$m = 2, c = 0.4$ g/L $K_{12,6}$ (M)	$N_{12,6}$	$m = 6, c = 0.4$ g/L $K_{6,1}^{app}$ (M^5)	$N_{6,1}^{app}$
urea	1×10^{-2}	0.032	5×10^{-8}	28 ± 3	1×10^{-43}	30 ± 3
urea	1×10^{-3}	0.032	2×10^{-7}	30	1×10^{-40}	30
urea	0	0.032	8×10^{-7}	30	1×10^{-37}	27
NaBr	1×10^{-2}	0.058	5×10^{-8}	18	1×10^{-40}	20
NaBr	1×10^{-3}	0.058	1×10^{-7}	16	1×10^{-38}	18
NaCl	1×10^{-2}	0.014	1×10^{-7}	30	1×10^{-40}	30
NaSCN	1×10^{-2}	0.160	1×10^{-7}	20	1×10^{-34}	20
$NaClO_4$	1×10^{-2}	0.175	1×10^{-7}	18	1×10^{-37}	21
NaI	1×10^{-2}	0.180	5×10^{-8}	15	1×10^{-40}	16

[a] Binding constants taken from Herskovits et al. (1977, 1978).

4.7×10^5. The required K_D values for this treatment are calculated with eq 5, with the α_1 and α_2 values obtained by using the observed molecular weights, M_w, and the equations

$$\alpha_1 = 2(1 - M_w/940\,000) \quad (10)$$

and

$$\alpha_2 = (6/5)(1 - M_w/470\,000) \quad (11)$$

The data in Figure 1 represented by the solid curve and the dashed and dotted curves were calculated with slightly different fitting parameters, illustrating the relative sensitivity of these parameters as applied to the description of the molecular weight data. The stabilizing effects of calcium ion on the molecular weight transitions were also studied and are shown in Figure 3. Table I presents a summary of the derived dissociation parameters characterizing the hexamer–hexamer and monomer–monomer contacts of *Homarus* hemocyanin. The K_B constants used for these calculations were taken from the literature (Herskovits et al., 1977, 1978).

The distribution of the hemocyanin species as a function of reagent concentration is shown in Figure 4. The weight fractions of species, f_m, were estimated by using eq 12–14 on the basis of the stoichiometry and species concentrations given by eq 2. It is apparent from the distribution of species as a

$$f_{12} = 1 - \alpha_1 \quad (12)$$

$$f_6 = \alpha_1(1 - \alpha_2) \quad (13)$$

$$f_1 = \alpha_1\alpha_2 \quad (14)$$

function of urea or $NaClO_4$ concentration that the dissociation reaction is a sequential process, with essentially only dodecamers and hexamers present at low reagent concentrations. Subsequent depletion of dodecamers and dissociation of the dodecamers to monomers are observed at medium to high reagent concentrations. The addition of calcium ion was found to shift the equilibrium and the dissociation of species to higher reagent concentrations. Thus, with urea, for example, the maximum of the hexameric fraction seen in Figure 4 shifted from approximately 2 M urea to 3 and 4 M urea as the calcium ion concentration was increased from 0 M to 1×10^{-3} and 1×10^{-2} M.

[2] In practice, we found it simpler to assume previous values of the dissociation constants taken from the literature (Herskovits et al., 1981a, 1983b) for the initial fit of the data or to assume that $N_{12,6}$ and $N_{6,1}^{app}$ have the same initial values, followed by further refinement of the data (see, for example, curve c of Figure 1). The reason for this is the relatively poor quality and the narrow range of available data as a function of reagent concentration (see Figure 6B) which are required for the evaluation of $K_{6,1}^{app}$.

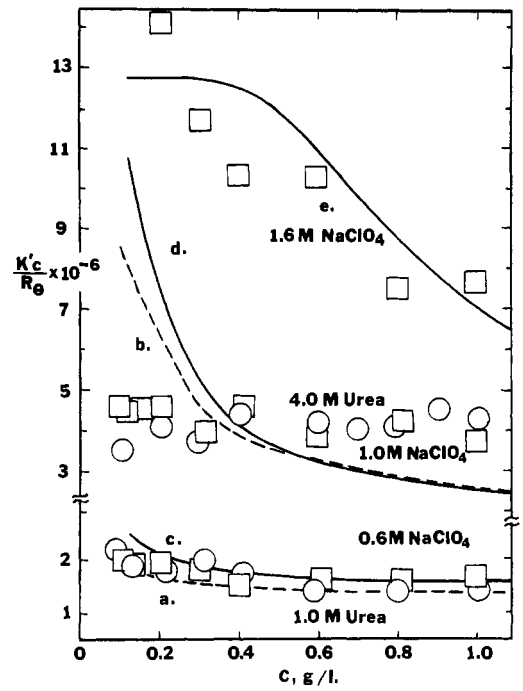

FIGURE 5: Concentration dependence of the light scattering of *Homarus* hemocyanin in 1.0 and 4.0 M urea (○) and in 0.6, 1.0, and 1.6 M $NaClO_4$ (□) plotted according to eq 15. The fitting of the curves represented by dashed and solid lines was based on eq 6 and 7, required to generate the necessary α_1 and α_2 data and the following parameters: (a and b) 1.0 and 4.0 M urea, $N_{12,6} = 35$, $N_{6,1}^{app} = 30$, $K_{12,6} = 1 \times 10^{-7}$ M, $K_{6,1}^{app} = 1 \times 10^{-37}$ M^5, and $K_B = 0.032$ M^{-1}; (c) 0.6 M $NaClO_4$, $N_{12,6} = 18$, $N_{6,1}^{app} = 21$, $K_{12,6} = 5 \times 10^{-8}$ M; $K_{6,1}^{app} = 1 \times 10^{-37}$ M^5, and $K_B = 0.175$ M^{-1}; (d) 1.0 M $NaClO_4$, same parameters as curve c except for $K_{6,1}^{app} = 5 \times 10^{-37}$ M^5; (e) 1.6 M $NaClO_4$, same parameters as curve c except for $K_{6,1}^{app} = 2 \times 10^{-39}$ M^5. Solvent, 0.1 M NaCl–0.05 M pH 7.8 Tris buffer.

Concentration Dependence and the Effects of Subunit Heterogeneity. The data in Figure 5 represent the light-scattering behavior of *Homarus* hemocyanin in the presence of 1.0 and 2.0 M urea and 0.6, 1.0, and 1.6 M $NaClO_4$ (at pH 7.8) studied as a function of protein concentration. For protein dissociation, the light-scattering data are usually represented by equations combining the light-scattering expression (eq 1) and the reciprocal of the weight-average molecular weight (Noren et al., 1971; Elbaum & Herskovits, 1974; Harrington & Herskovits, 1975) such as eq 8. Thus, we obtain the expression

$$K'\bar{c}/R_\theta = \left[M_{12}\left(1 - \frac{1}{2}\alpha_1 - \frac{5}{12}\alpha_1\alpha_2\right)\right]^{-1} + B'c \quad (15)$$

which we have used in conjunction with eq 6 and 7 to generate the required α_1 and α_2 estimates needed to fit the experimental data. We have used the literature value for $B' = 1 \times 10^{-8}$ L·mol·g^{-2} (Herskovits et al., 1981b) together with the N_{app} and K_B parameters given in the legend of Figure 5 to fit the data of this figure.

It is apparent from the fit of the data obtained with 1.0 M urea and 0.6 M $NaClO_4$ represented by dashed and solid curves that the dissociation behavior of *Homarus* hemocyanin is initially correctly predicted by the apparent stoichiometry given by eq 2. From the data of Figure 4, it is clear that at these reagent concentrations the predominant species in solution are dodecamers and half-molecules or hexamers. The studies of Morimoto & Kegeles (1971) and our earlier studies (Herskovits et al., 1981b) have clearly established that these two hemocyanin species are in rapid and reversible equilibrium. However, this is clearly not the case at intermediate reagent

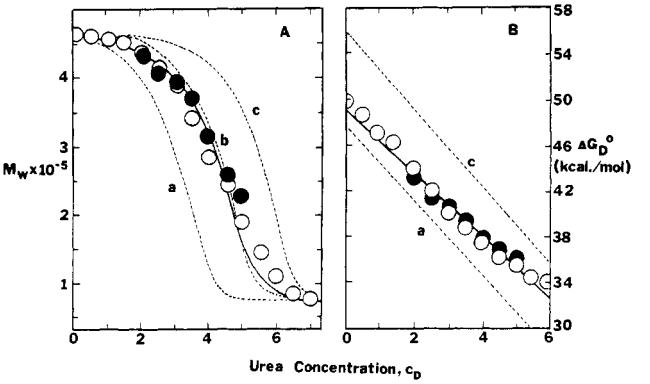

FIGURE 6: Simulated effects of subunit heterogeneity on the molecular weight and $\Delta G°_D$ plots obtained with three equal mixtures of hexamers having different stabilization energies. (A) Molecular weight curves calculated by eq 7 and 8 with the following parameters: $K_{6,1}^{app} = 1 \times 10^{-35}$, 1×10^{-38}, and 1×10^{-41} M^5 (dashed curves a, b, and c, respectively), $N_{6,1}^{app} = 30$, $c = 0.4$ g/L, $K_B = 0.032$ M^{-1}, and $\alpha_1 = 1.0$. Open circles, weight-average molecular weights computed for a mixture consisting of equal weight fractions of hexamers represented by curves a–c. Filled circles, 2.0–5.0 M urea data of Figure 3, curve b. (B) Computed $\Delta G°_D$ plots based on eq 5, 9, and 11 and the calculated molecular weight data from (A). Derived parameters for a simulated three-component mixture represented by open circles are $K_{6,1}^{app} = 4 \times 10^{-37}$ M^5 and $N_{6,1}^{app} = 26$. Filled circles represent urea data from Figure 3.

concentrations, such as 4.0 M urea and 1.0 M $NaClO_4$. Again, the data of Figure 4 suggest that at these concentrations of dissociating reagent we deal nearly exclusively with hexamers and monomers in solution. The divergence between the experimental and calculated curves, represented by the dashed and solid curves b and d in Figure 5, indicates that the concentration dependence of the light-scattering data at these solvent conditions is not predicted correctly by the stoichiometry describing the solution behavior of homogeneous subunit systems in equilibrium. Similar effects have been noted with other hemocyanins of both molluscan and arthropod origin (Konings et al., 1969; Engelborghs & Lontie, 1973; Herskovits et al., 1981a) and attributed to heterogeneity of the hemocyanin subunits. We hope to study these effects of the subunit dissociation parameters in the future, using isolated subunits or perhaps hemocyanins from species such as *Bathynomus giganteus*, which seem to exhibit only a single association–dissociation equilibrium (Van Holde & Brenowitz, 1981). In this regard, it is interesting that the dissociation behavior of *Homarus* hemocyanin at high reagent concentration (1.6 M $NaClO_4$), represented by curve e in Figure 5, seems to be correctly predicted by the light-scattering expression eq 15 and the associated eq 6 and 7. Unfortunately, at these reagent concentrations, there is a significant degree of denaturation suggested by the absorption of the copper band of hemocyanin at 335 nm and the circular dichroism discussed in the next section.

In order to gain some further insight into the effects of subunit heterogeneity on the most relevant parameter, $N_{6,1}^{app}$, derived from the analysis of subunit dissociation data, we have calculated and analyzed dissociation curves by assuming a moderate degree of subunit heterogeneity. Mixtures of three equal weight fractions of hemocyanin hexamers were considered having differences in stabilization energies of 2.7–4.1 kcal/mol. Figure 6 represents some of our results based on calculations on hypothetical mixtures of three equal fractions of hemocyanin hexamers having stabilization energies that differ by ±4.1 kcal/mol, corresponding to a change in dissociation constants by a factor of 1000. The calculated weight-average molecular weight, and $\Delta G°_D$ as a function of

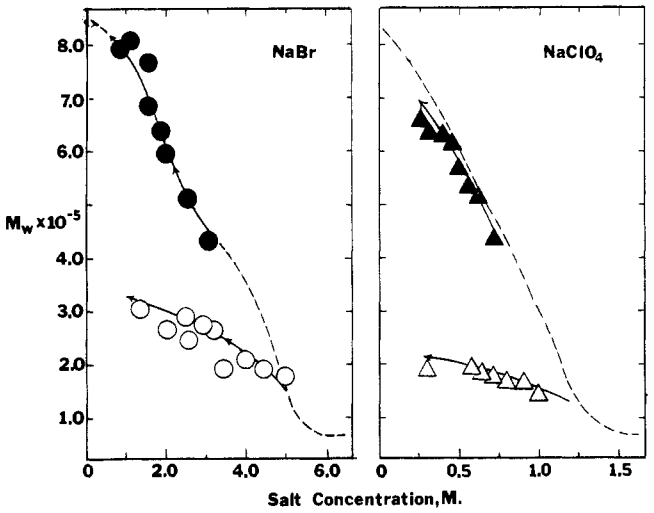

FIGURE 7: Test of the reversibility and reassociation of *Homarus* hemocyanin at pH 7.8 in 0.01 M Ca^{2+} exposed to 3.0 and 5.0 M NaBr and 0.7 and 1.2 M $NaClO_4$. The data represented by filled symbols represent reassociation from the hexameric state with a molecular weight close to 4.7×10^5, while the data represented by open symbols represent reassociation from the largely monomeric state. Protein solutions initially exposed to the above conditions were diluted by a factor of 2–4 with the appropraite buffer and reagent mixture. The curves represented by dashed lines are forward curves based on the data of Figure 2. Final protein concentrations were 0.1–0.3 $g \cdot L^{-1}$.

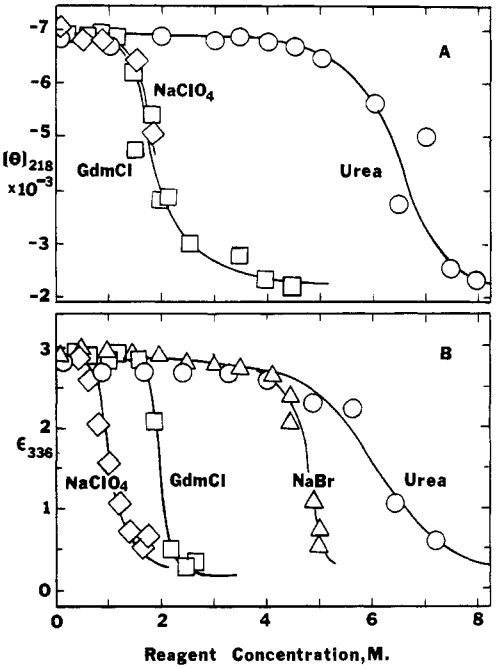

FIGURE 8: Effects of urea, GdmCl, $NaClO_4$, and NaBr on the circular dichroism (A) and absorbance spectra (B) of *Homarus* hemocyanin, expressed as the mean residue ellipticity at 218 nm, $[\theta]_{218}$, and the percent extinction coefficient at 336 nm, ϵ_{336}.

dissociating reagent concentration, is represented by the open circles.

Our most significant findings suggested by the data and the derived parameters are (a) that the curves simulating the effects of a moderate degree of microheterogeneity (data represented by solid lines) retain most of the characteristics of the average curves of the central homogeneous component and (b) that the derived $N_{6,1}^{app}$ parameters are only moderately lowered by the broadening effect of subunit heterogeneity. The actual $N_{6,1}^{app}$ values were changed from the initial value of 30 used for all three homogeneous components to 26–28, or a change of about 10–15%. It is not suggested that in the case of *Homarus* hemocyanin hexamers we deal with a simple three-component system of hexamers, used for these calculations, but rather that moderate effects of subunit heterogeneity should not alter very significantly the derived estimates of amino acid groups at the contact areas of the subunits.

Reversibility and Denaturation. The dissocation of *Homarus* hemocyanin dodecamers to hexamers has been found to be a rapid and reversible reaction (Morimoto & Kegeles, 1981; Herskovits et al., 1981b) provided the protein is not exposed to dissociating reagent concentrations that produce significant amounts of unfolded or denatured material. Closely similar behavior has been observed with the isolated hexamers dissociating to monomers studied at pH 8.8 in the absence of stabilizing calcium ions or prepared by acetylation (Herskovits et al., 1983b). The present data obtained at pH 7.8 in the presence of 0.01 M Ca^{2+} shown in Figure 7 show this clearly for the first step of the dissociation process. The data represented by filled symbols were obtained on protein solutions (c = 0.1–0.3 g/L) initially exposed to 3.0 M NaBr, 0.7 M $NaClO_4$, and 3–4 M urea (data not shown). The molecular weights at these reagent concentrations are close to 4.5–4.7 $\times 10^5$, the plateau region where the species distribution data show predominantly hexamers and dodecamers present and only about 5–8% monomers in solution (Figure 4). The monomer to hexamer reassociation data represented by open symbols in Figure 7 show only partial reassociation that seems to be blocked at the hexamer stage since the molecular weights of the hemocyanin initially exposed to high reagent concentration fall substantially below 4.7×10^6, the molecular weight of the hexameric species. Blocking of the reassociation of hemocyanin monomers at the hexamer stage has also been noted for other arthropod species (Ellerton et al., 1970; Terwilliger et al., 1979; Herskovits et al., 1981a). We ascribe this to the effects of subunit heterogeneity introduced by the reassociated hexamers that are different as far as subunit composition is concerned relative to the natural composition of the basic hexameric unit.

The interpretation of both the dissociation and the reassociation data assumes that we have little or no denaturation at the higher reagent concentrations used in our work (Figures 1–3). The circular dichroism data at the conformationally sensitive peptide band near 220 nm and the absorbance data at the 336-nm copper band of hemocyanin, shown in Figure 8, suggest that denaturation in most instances is not a complicating factor, except perhaps at the highest reagent concentrations. There may, however, be some ambiguities regarding the extent of reassociation of hemocyanin monomers from the higher $NaClO_4$ and NaBr concentrations shown in Figure 7, since there are significant changes in absorbance at 336 nm. Irreversible changes associated with the unfolding of the subunits may lower the extent of reassociation suggested by the molecular weight data.

Discussion

The hemocyanin of the lobster *Homarus americanus* investigated in the serum of the animal is found to be nearly completely in the dodecameric state, having a sedimentation coefficient of 25 S (Morimoto & Kegeles, 1971). The molecular weight study of this paper shows clearly that the dissociation of the dodecamers to hexamers and monomers produced by the ureas and various neutral salts is a sequential process. The distribution of hemocyanin species on the basis of the molecular weight data shows initially the conversion of dodecamers to hexamers with essentially no monomers present, followed by the dissociation of the hexamers to monomers at high reagent concentration (Figure 4). The plateau region

seen in the molecular weight data of Figure 1, obtained with urea as the dissociating reagent, corresponds to a molecular weight of 460 000, which is close to half the molecular weight of the parent dodecamer, 940 000 (Morimoto & Kegeles, 1971; Herskovits et al., 1981b). The calculated weight distribution of species represented in Figure 4A suggests that at this urea concentration more than 90% of the protein is in the intermediate hexameric state, with only about 5% of the initial dodecameric material having been converted to monomers. The effects of pH and the lowering of calcium ion concentration present in solution seem to have the same consequences on the distribution of hemocyanin species. Thus, Morimoto & Kegeles (1971) have noted that at pH 9.6 and 1×10^{-3} M Ca^{2+} essentially all the hemocyanin sediments as a single ultracentrifugal component with a sedimentation constant of 17 S, characteristic of the hexameric species.

The sequential dissociation process and the two sets of dissocation parameters based on our eq 6–8 seem to give a good account of most of our data shown in Figures 1–4, including the stabilizing effects of calcium ion on the observed molecular weight transitions. It is perhaps most significant that the derived estimates of the amino acids at both the hexamer and the monomer area of contacts exposed as a result of dissociation of the subunits seem to be comparable. This has also been suggested by our previous estimates of such groups based on our analysis of the dissociation of the dodecamers to hexamers and the isolated hexamers going to monomers (Herskovits et al., 1981b, 1983b). The $N_{12,6}$ and $N_{6,1}^{app}$ values of 28–35 and 16–20 groups obtained with urea and the salts NaI, NaSCN, $NaClO_4$, and GdmCl, respectively, should be compared to previous estimates of 28–31 and 16–28 with these two groups of compounds, respectively.

Subunit contacts of similar surface areas between the pair of hexamers in the dodecamer and the monomers in each hexamer are also suggested by the subunit models for arthropod hemocyanins proposed recently by Lamy, Linzen, and van Bruggen and their associates (Lamy et al., 1981; Markl et al., 1981). These models are based on the X-ray crystallographic structure of the prototype for the basic hexameric unit, the hemocyanin of the spiny lobster *Panulirus interruptus* (Van Schaick et al., 1981), which is organized of kidney-shaped subunits located at the six corners of a trigonal antiprism. Models of the dodecamer suggest that the two basic hexameric units are perpendicularly arranged with respect to their 3-fold axes of symmetry so as to maximize the surface areas of contact between the subunits (Lamy et al., 1981; Markl et al., 1981). In the basic hexameric unit, each subunit makes an average of four contacts with its neighbors, with about the same number of contacts being produced between the pair of hexamers. Thus, it is reasonable to assume that the size of the contact areas formed between the monomers and the hexamer pairs should be about the same, comprising approximately the same number of amino acids at the two different sets of contact sites.

In light of the latter observations, the closely similar estimates of amino acid groups at the two sets off contact areas of *Homarus* hemocyanin would tend to suggest that moderate effects of subunit heterogeneity should not alter significantly the estimates of contact groups, based on molecular weight data. Both our calculations described under Concentration Dependence and the Effects of Subunit Heterogeneity and the recent investigations on homogeneous and heterogeneous concanavalin A preparations (Senear & Teller, 1981; Herskovits et al., 1983a) tend to support this contention. In the case of concanavalin A, the estimates of the number of pH-linked histidine residues (Senear & Teller, 1981) as well as the apparent number of contact groups based on urea and salt dissociation measurements were found to be largely the same (Herskovits et al., 1983a). Of course, the demonstration that this is also true for the dissociation of the basic hexameric unit of arthropod hemocyanins will require further work with isolated homogeneous subunit preparations, described recently by Bijlholt and Lamy and their co-workers (Bijlholt et al., 1979; Lamy et al., 1981).

Registry No. NaBr, 7647-15-6; $NaClO_4$, 7601-89-0; NaCl, 7647-14-5; NaI, 7681-82-5; NaSCN, 540-72-7; calcium, 7440-70-2; urea, 57-13-6; guanidinium chloride, 50-01-1.

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