Forsén, S., & Hoffman, R. A. (1963) *J. Chem. Phys. 39*, 2892.
Gelin, B. R., & Karplus, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2002.
Hyde, E. I., Birdsall, B., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1980a) *Biochemistry 19*, 3738.
Hyde, E. I., Birdsall, B., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1980b) *Biochemistry 19*, 3746.
Lunazzi, L., Magagnoli, C., & Macciantelli, D. (1980) *J. Chem. Soc., Perkin Trans. 2*, 1704.
Matsubayashi, G. E., Takaya, Y., & Tanaka, T. (1970) *Spectrochim. Acta, Part A 26A*, 1851.
Matthews, D. A., Alden, R. A., Bolin, J. T., Filman, D. J., Freer, S. T., Hamlin, R., Hol, W. G. J., Kisliuk, R. L., Pastore, E. J., Plante, L. T., Xuong, N., & Kraut, J. (1978) *J. Biol. Chem. 253*, 6946.
Porath, J. (1957) *Ark. Kemi 11*, 97.
Roberts, G. C. K. (1978) *Ciba Found. Symp. 60*.
Roberts, G. C. K., Feeney, J., Birdsall, B., Kimber, B., Griffiths, D. V., & Burgen, A. S. V. (1977) in *NMR in Biology* (Dwek, R. A., Campbell, I. D., Richards, R. E., & Williams, R. J. P., Eds.) p 95, Academic Press, London.
Snyder, G. H., Rowan, R., Karplus, S., & Sykes, B. D. (1975) *Biochemistry 14*, 3765.
Uyeda, K., & Rabinowitz, J. C. (1965) *J. Biol. Chem. 240*, 1701.
Wagner, G., De Marco, A., & Wüthrich, K. (1975) *J. Magn. Reson. 20*, 565.
Wagner, G., De Marco, A., & Wüthrich, K. (1976) *Biophys. Struct. Mech. 2*, 29.
Wyeth, P., Gronenborn, A., Birdsall, B., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1980) *Biochemistry 19*, 2608.

Effect of Oxygen and Allosteric Effectors on Structural Stability of Oligomeric Hemocyanins of the Arthropod, *Limulus polyphemus*, and the Mollusc, *Helix pomatia*†

Marius Brouwer,* Celia Bonaventura, and Joseph Bonaventura

ABSTRACT: The hemocyanins of arthropods and molluscs are allosteric proteins whose reactivity toward oxygen is influenced by the ionic composition and pH of the buffering medium. In general, the oxygen dissociation curves exhibited by these proteins can be described by models which invoke conformational transitions between high- and low-reactivity states. Since the degree of ligation and the concentration of allosteric effectors are both influential in determining the conformational state which the molecule will assume, it would seem likely that the intersubunit contacts which determine the structural stability of the oligomers might also be affected by these conditions. We have used a stopped-flow light-scattering technique and parallel sedimentation velocity experiments in order to probe the stability of the quaternary structure of *Helix pomatia* α-hemocyanin and *Limulus polyphemus* hemocyanin. We find that there are substantial oxygen- and NaCl-dependent differences in the time courses of dissociation of both hemocyanins when divalent cations are removed at high pH. Notably, *Limulus* oxy- and deoxyhemocyanin can exist in a 48-subunit aggregation state, but the oxygenated form is much more quickly dissociated into monomeric subunits when calcium ions are removed at high pH. A similar difference between the stability of oxy and deoxy forms of *Helix* α-hemocyanin was found. These results and an analysis of oxygen binding curves of the aggregated and dissociated forms lead us to the conclusion that although the allosteric units of these high molecular weight proteins appear to be restricted structural units, the overall stability and quaternary state of the high molecular weight aggregate are substantially affected by the conformational changes which accompany oxygenation. The rates of dissociation of *Limulus* oxyhemocyanin and *Helix* α-oxyhemocyanin were found to be approximately equal to the rates of dissociation when the deoxygenated molecules were rapidly mixed with air-equilibrated dissociation buffer. We conclude that the changes in conformational state which accompany oxygenation occur within the dead time of the stopped-flow apparatus and thus have first-order rate constants with a minimum value of 2000 s^{-1}.

The blue, copper-containing hemocyanins of the horseshoe crab *Limulus polyphemus* and the vineyard snail *Helix pomatia* are among the largest of respiratory proteins. *Limulus* hemocyanin, which has a sedimentation coefficient of 60 S and a molecular weight of $\sim 3.2 \times 10^6$, contains 48 oxygen binding sites (Johnson & Yphantis, 1978). *Helix* hemocyanin, which has a sedimentation value of 100 S and a molecular weight of $\sim 9 \times 10^6$, contains 180 oxygen binding sites (Van Holde & van Bruggen, 1971). Both hemocyanins show cooperative interactions between their subunits in the process of oxygen binding. In both cases, their functional properties can basically be described by the two-state model for allosteric transitions (Colosimo et al., 1974; Brouwer et al., 1977; van Driel et al., 1978; Zolla et al., 1978). This paper concerns the stability of these high molecular weight oligomers and the changes in quaternary constraints that accompany the interaction of these molecules with oxygen and with allosteric effectors.

The molecular architectures of the hemocyanins of the mollusc *H. pomatia* and the arthropod *L. polyphemus* are very different (Hendrickson, 1977; Bonaventura et al., 1977a). The hemocyanins of molluscs are constructed like hollow cylindrical drums partially closed at the ends by "collars" (Mellema & Klug, 1972; Siezen & van Bruggen, 1974). The cylinders are composed of 20 very long polypeptide chains, each of which

† From the Marine Biomedical Center, Duke University Marine Laboratory, Beaufort, North Carolina 28516. *Received July 8, 1980; revised manuscript received November 24, 1980.* This work was supported by grants from the National Science Foundation (BMS-7S-15246) and the National Institutes of Health (HL 15450 and ESO-1908). M.B. was supported by the Netherlands Organization for the Advancement of Pure Research. J.B. is an Established Investigator for the American Heart Association.

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contains between seven and nine domains that are capable of binding a single oxygen molecule (Brouwer & Kuiper, 1973; Siezen & van Bruggen, 1974; Brouwer et al., 1976; Gielens et al., 1975, 1977; Brouwer et al., 1979). The domains within a polypeptide chain have been shown to be functionally nonequivalent (Bonaventura et al., 1977a,b; Brouwer et al., 1978; Gielens et al., 1977).

Hemocyanins of arthropods have a less complex molecular structure. The fundamental functional unit in this case is a 70 000 molecular weight polypeptide chain, which contains a single oxygen binding site. Subunit heterogeneity seems to be quite common (Bonaventura et al., 1975, 1977a). Arthropod hemocyanins often occur as 16S hexamers, 25S dodecamers, 35S 24-mers, and 60S 48-mers (Van Holde & van Bruggen, 1971; Hendrickson, 1977; Bonaventura et al., 1977a).

The stability of both classes of hemocyanins is influenced by allosteric effectors such as protons and divalent cations (Van Holde & van Bruggen, 1971; Siezen & van Driel, 1974; Zolla et al., 1978). The subunit aggregation of some mollusc hemocyanins has also been shown to be affected by oxygenation (DePhillips et al., 1969, 1970). The studies cited above strongly suggest that some subunit contacts are altered by the conformational changes which accompany ligation of the active site or binding of allosteric effectors. We consequently undertook an investigation of the kinetic basis for these observations. Our premise is that the transient kinetics of subunit dissociation can provide information about the stability of various conformational states and about the rates of the conformational changes induced by copper and noncopper ligands. A comparison of the spectral characteristics of a mixture of *Limulus* subunits and the 48-mer of *Limulus* hemocyanin was also undertaken to further clarify the constraints imposed on the subunits by virtue of their associations within the giant molecule. Some aspects of this study were presented at a recent workshop in Tours, France (Brouwer et al., 1981).

Materials and Methods

Limulus hemocyanin was prepared and purified as described previously (Brouwer et al., 1977). Lyophilized α-hemocyanin from *Helix pomatia*, prepared as described by Siezen & van Driel (1973, 1974), was a gift from Drs. R. Torensma and E. F. J. van Bruggen, University of Groningen, The Netherlands.

Sedimentation analysis and tonometric oxygen equilibrium experiments were performed as described elsewhere (Brouwer et al., 1977; Riggs & Wolbach, 1956). Samples were in Tris[1]-HCl, made up to the desired ionic strength with NaCl (Bates, 1973).

Fluorescence excitation and emission spectra of *Limulus* 60S hemocyanin (in 50 mM Tris, pH 8.9, ionic strength 0.05, buffer to which 10 mM $CaCl_2$ was added, giving a total ionic strength of 0.08) and of 5S *Limulus* hemocyanin (in 50 mM Tris and 10 mM EDTA, pH 8.9) were measured by using a Turner Model 430 fluorometer. Excitation wavelengths ranged from 270 to 300 nm. Fluorescence emission was monitored at 340 nm. The absorbance of the protein samples used for fluorescence studies was 0.45 at 280 nm.

Difference spectra in the ultraviolet region were measured for *Limulus* 60S oxy- vs. *Limulus* 60S deoxyhemocyanin (in 50 mM Tris, pH 8.9, ionic strength 0.08 buffer containing 10 mM $CaCl_2$) and for *Limulus* 5S oxy- vs. 5S deoxyhemocyanin (in 50 mM Tris and 10 mM EDTA, pH 8.9). Measurements were made with an Aminco double-beam DW-2A spectrophotometer at sensitivity 0.1. Prior to recording of difference spectra, samples were filtered through 1-μm nucleopore filters. The following procedure was used in order to have identical protein concentrations of the oxy and deoxy samples: a 6-mL sample with an absorbance at 280 nm of 1.4 was pipetted into a tonometer equipped with a quartz cuvette and rubber cap. The sample tonometer and a second empty tonometer were deoxygenated by repeatedly evacuating and flushing with water vapor saturated nitrogen. Both tonometers were then filled with this nitrogen. Subsequently, a syringe equipped with a needle and a three-way stopcock was flushed with nitrogen and inserted through the rubber cap into the sample tonometer. After the syringe was flushed a few times with the nitrogen atmosphere in the tonometer, 3 mL of the deoxyhemocyanin solution was withdrawn and transferred to the second tonometer. Spectra were taken of both samples to make sure they were deoxygenated. With one tonometer in the sample position and the other in the reference position of the spectrophotometer, the base line was corrected for variation over the selected wavelength range by adjusting the trim potentiometers until the recorder pen was positioned at the selected base line level at all wavelengths. The tonometer in the sample position was then opened and flushed slowly for $\sim$3 min with oxygen that was saturated with water vapor. After 10 min of rotation and equilibration of the oxygenated sample at 20 °C the oxy–deoxy difference spectrum was recorded over the range from 250 to 320 nm.

The kinetics of the dissociation of 60S *Limulus* hemocyanin into 5S subunits and of 100S *Helix* α-hemocyanin into 18S units were measured by a stopped-flow light-scattering technique. The samples were rapidly mixed with EDTA-containing buffers at high pH to bring about the change in aggregation state. Experiments were performed with a Durrum stopped-flow spectrophotometer, with the photomultiplier at a right angle to the observation beam. The decrease in light-scattering intensity was monitored at 425 nm, with a slit width of 2.5 mm and a photomultiplier voltage of 600 V. The photomultiplier output was processed by an Aminco analog to digital converter and transient recorder (DASAR) and analyzed with a PDP-11/34 computer (Digital Equipment Corp.).

Results

Subunit Interactions in Oxygen Binding. Oxygen-carrying proteins typically contain multiple subunits. This provides an opportunity for changes in reactivity toward a ligand as ligation proceeds. The high molecular weight hemocyanins of *L. polyphemus* and *H. pomatia* show cooperative interactions in oxygen binding. Under the conditions used in this study the maximum Hill coefficient for oxygen binding by *Limulus* hemocyanin is 4.2, and that of *Helix* α-hemocyanin is 2.1. The Hill plots for 60S *Limulus* hemocyanin and 100S *Helix* α-hemocyanin shown in Figure 1 correspond to the experimental conditions of the stopped-flow light-scattering experiments and the spectral analysis presented in the following sections. The binding curve shown for *Limulus* hemocyanin in Figure 1 can be described by the two-state model for allosteric transitions (Monod et al., 1965) with the dodecamer as the allosteric unit and using values of 7.2×10^{12} and 0.0397 for the allosteric equilibrium constant, L, and the nonexclusive binding coefficient, c, respectively.

Limulus hemocyanin contains functionally and structurally distinct subunits. The isolated subunits bind oxygen noncooperatively and differ significantly in their oxygen affinities (Sullivan et al., 1974). The 48-mer of *Limulus* hemocyanin can be dissociated into a mixture of these subunits by removal of calcium. As is shown in Figure 1, the binding curve for

[1] Abbreviations used: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid.

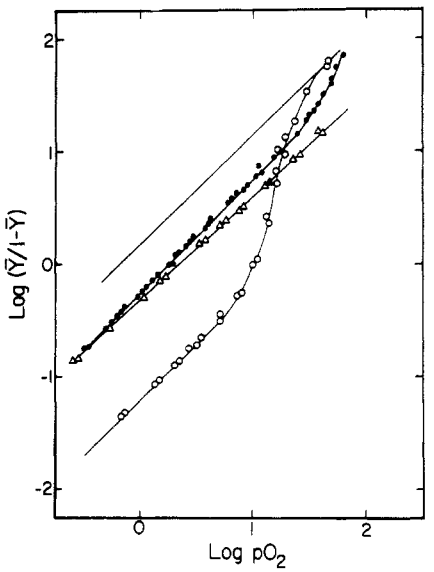

FIGURE 1: Hill plots of oxygen binding by *L. polyphemus* and *H. pomatia* α-hemocyanin at 20 °C, with protein concentration of 4 mg/mL. (○) 60S *Limulus* hemocyanin in a 50 mM Tris buffer, ionic strength 0.05, pH 8.9, to which 10 mM $CaCl_2$ was added, giving a total ionic strength of 0.08; (△) 5S *Limulus* hemocyanin in 50 mM Tris and 10 mM EDTA, pH 8.9; (●) 100S *Helix* α-hemocyanin in 50 mM Tris, ionic strength 0.05, pH 8.9, containing 35 mM $CaCl_2$ (total ionic strength 0.155).

the subunit mixture is a straight line with a slope of 0.93. It is located between the hypothetical binding curves of the "T" and "R" states of the 48-mer, indicating that the subunits in the R state of the 48-mer have higher affinities than do the free subunits.

The binding curve shown in Figure 1 for *Helix* α-hemocyanin does not contain enough information for the calculations of the parameters of the two-state model. *Helix* contains α and β types of hemocyanin with differing pH and ion sensitivities. Detailed studies on oxygen binding by both types of *Helix* hemocyanin are reported elsewhere (Colosimo et al., 1974; Zolla et al., 1978). Both α and β forms of *Helix* hemocyanin have been reported to bind oxygen cooperatively and in a manner compatible with the two-state concerted model. As in the case of *Limulus* hemocyanin, the subunits of *Helix* hemocyanin show Hill coefficients <1, and the binding function for the subunits lies between the T and R state binding functions of the oligomer (Van Driel et al., 1974).

Spectral Changes Associated with Aggregation. As mentioned above, the oxygen-binding experiments performed with *Limulus* and *Helix* hemocyanins suggest that there is an increase in the reactivity of the oxygen-binding sites when subunits are assembled into high molecular weight oligomers (see Discussion). To further explore this effect, we monitored the changes in fluorescence emission which accompany aggregation of *Limulus* hemocyanin. Fluorescence spectra were measured for the oxygenated forms of 60S and 5S *Limulus* hemocyanin molecules. Fluorescence excitation and emission spectra showed broad maxima for whole (60S) and dissociated (5S) *Limulus* hemocyanin. These excitation and emission maxima were found to be at about 288 and 340 nm, respectively. Fluorescence excitation spectra of 60S and 5S oxyhemocyanin, monitored at 340 nm, are shown in Figure 2. The observed emission intensities were corrected for trivial reabsorption due to the overlap of the emission and the 340-nm absorption band according to

$$F_{cor} = F_{obsd} \text{ antilog } (A/2)$$

where F_{obsd} and F_{cor} are respectively the observed and corrected

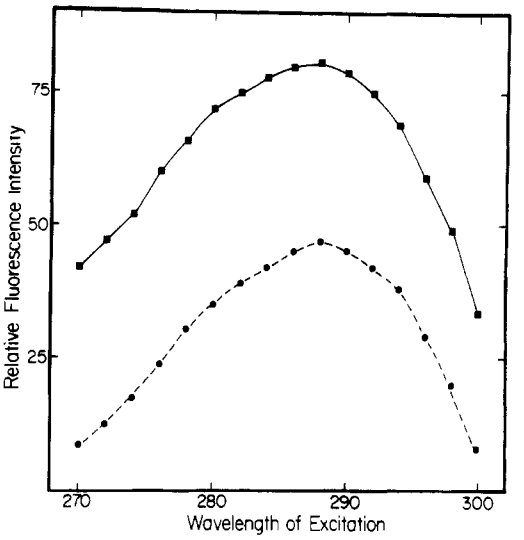

FIGURE 2: Fluorescence excitation spectra of 60S and 5S *Limulus* oxyhemocyanin at 0.34 mg/mL, monitored at 340 nm. Protein concentrations were determined by using $E^{1\%,1cm} = 13.9$ at 280 nm (Nickerson & Van Holde, 1971). (●) 60S oxyhemocyanin in 50 mM Tris, pH 8.9, ionic strength 0.05, with 10 mM $CaCl_2$ (total ionic strength 0.08). (■) 5S oxyhemocyanin in 50 mM Tris and 10 mM EDTA, pH 8.9.

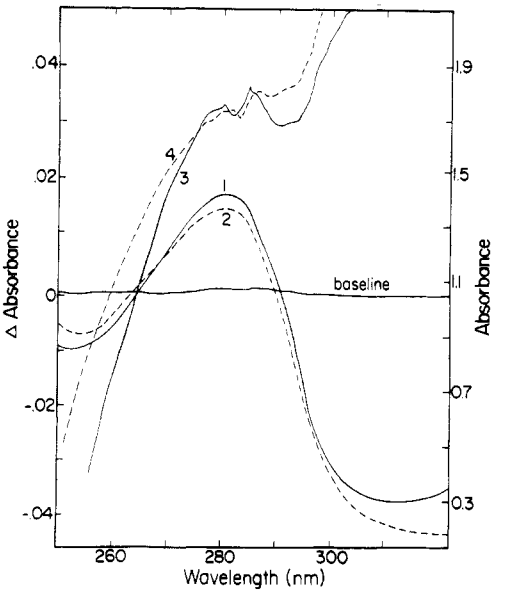

FIGURE 3: Absorption spectra of 60S *Limulus* hemocyanin in 50 mM Tris, ionic strength 0.05, pH 8.9, with 10 mM $CaCl_2$ (total ionic strength 0.08). (Curve 1) Oxyhemocyanin, (curve 2) deoxyhemocyanin, (curve 3) 60S hemocyanin (oxy vs. deoxy), and (curve 4) 5S hemocyanin (oxy vs. deoxy) in 50 mM Tris and 10 mM EDTA, pH 8.9.

fluorescence intensities and A is the absorbance at the emission wavelength (Er-El et al., 1972). Figure 2 shows that the relative fluorescence intensity of the free oxygenated subunits is about twice as high as that of the subunits in the oxygenated 60S molecule. This is a further indication that the subunits in the R state of 60S *Limulus* hemocyanin are in a significantly different conformation than when they are free in solution.

Oxy–deoxy difference spectra were measured for 60S and 5S *Limulus* hemocyanin in looking for other spectral indications of conformational changes which accompany aggregation. The results are shown in Figure 3. Oxygenation of hemocyanin is accompanied by the well-known spectral change at 340 nm that is associated with the formation of the copper–oxygen complex and by spectral changes in the 250–280-nm region where absorption is dominated by aromatic amino acid

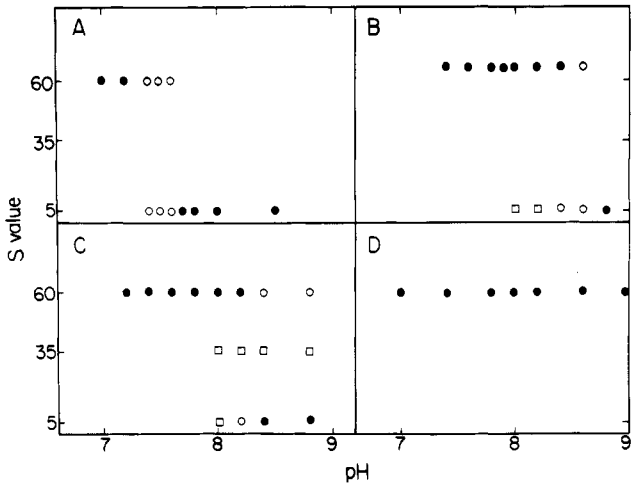

FIGURE 4: Dissociation diagrams showing effects of pH, NaCl, and $CaCl_2$ on aggregation of *L. polyphemus* hemocyanin at 20 °C. The protein concentration was 5 mg/mL. Changes in ionic strength were brought about by NaCl addition. (A) 20 mM Tris, ionic strength 0.1; (B) 20 mM Tris, ionic strength 0.5; (C) 20 mM Tris, ionic strength 0.1, with 1 mM $CaCl_2$ (total ionic strength 0.103); (D) 20 mM Tris, ionic strength 0.1, with 10 mM $CaCl_2$ (total ionic strength 0.13). (●) Main component; (○) intermediate component; (□) minor component.

residues. We find that the spectral change at 250 nm which accompanies oxygenation of *Limulus* hemocyanin is significantly larger for the 60S molecule ($\Delta OD_{250}/\Delta OD_{340} = -0.22$) than for the 5S monomers ($\Delta OD_{280}/\Delta OD_{340} = -0.15$). The change in the 280-nm region is nearly the same for both the 5S and 60S molecules: $\Delta OD_{280}/\Delta OD_{340} = 0.13$ and 0.14, respectively (based on average values calculated from five difference spectra). The similarity of difference spectra for 60S and 5S states indicates that these spectral differences are primarily due to localized changes in subunit conformation that accompany oxygenation.

Analysis of Dissociation Products by Ultracentrifugation. In the course of this investigation, analytical ultracentrifugation was used to monitor the state of aggregation of *Limulus* and *Helix* hemocyanins under specific experimental conditions. As with many other hemocyanins, the dissociation of these oligomers is favored by conditions of high pH, low ionic strength, and low concentrations of divalent cations. Figure 4 summarizes the results of our studies on *Limulus* hemocyanin. Sedimentation coefficients of 60S, 35S, and 5S are associated with the sedimentation behavior of whole molecules (48-mers), half-molecules (24-mers), and subunits (monomers), respectively, as described by Johnson & Yphantis (1978). Figure 4 illustrates the fact that decreasing the concentration of calcium or increasing the pH favors the dissociation of the whole molecules (48-mers) into monomeric subunits. The dissociation does not appear to involve 24-mers as a stable intermediate under the conditions examined. Varying concentrations of NaCl were added to investigate the dissociation behavior under varied ionic strength conditions. Both the NaCl concentration and the pH of the solution influence the extent of monomer formation when divalent cations are removed. Dissociation into monomers is complete at ionic strength 0.1 at pH 7.7, while at $I = 0.5$ the dissociation into monomers is complete only at pH 8.8 (Figure 4A,B). The effects of salts other than NaCl were not studied systematically, but ionic strength increases, even those brought about by increased EDTA concentration, were found to exert a stabilizing effect. As shown in Figure 4C,D, the presence of calcium greatly stabilizes the 48-subunit ensemble. The 48-mer is stable in 10 mM $CaCl_2$ from pH 7.0 to pH 9.5 at ionic strength 0.1

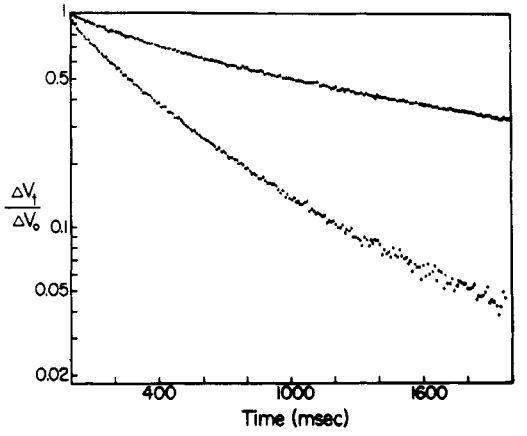

FIGURE 5: Stopped-flow light-scattering experiments with oxy and deoxy forms of 60S *Limulus* hemocyanin. Oxy- or deoxyhemocyanin in 50 mM Tris, ionic strength 0.05, pH 8.9, and 10 mM $CaCl_2$ (total ionic strength 0.08) at 20 °C and at a protein concentration of 5 mg/mL was rapidly mixed with oxy or deoxy Tris buffer containing 50 mM EDTA, pH 8.9. (Upper curve) Deoxyhemocyanin mixed with deoxy EDTA buffer; initial rate 1 s^{-1}, final rate 0.45 s^{-1}. (Lower curve) Oxyhemocyanin mixed with oxy EDTA buffer and deoxyhemocyanin with oxy EDTA buffer; initial rate 2.4 s^{-1}, final rate 1.5 s^{-1}. The dissociation into 5S particles was monitored by measuring the decrease of the light-scattering intensity signal at 425 nm.

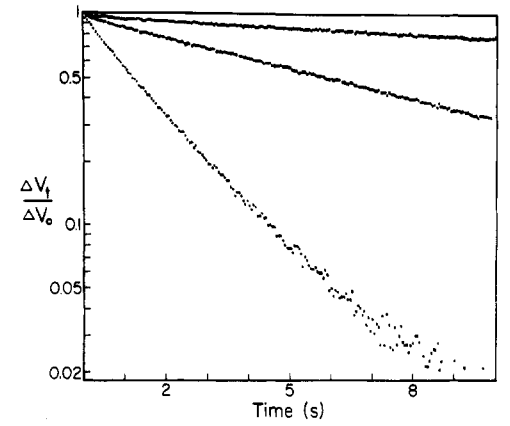

FIGURE 6: Experiments were as described in Figure 5 with 60S oxyhemocyanin and varied concentrations of NaCl in protein solution. (Upper curve) 0.3 M NaCl with hemocyanin; rate constant 0.035 s^{-1}. (Middle curve) 0.2 M NaCl; initial rate 0.15 s^{-1}, final rate 0.11 s^{-1}. (Lower curve) 0.1 M NaCl; initial rate 0.9 s^{-1}, final rate 0.5 s^{-1}.

(Figure 4D). At lower levels of calcium the 24-subunit ensemble appears as a minor component at ionic strength 0.1 at and above pH 8 (Figure 4C).

Stopped-Flow Experiments in Transmission Mode. Limulus and *Helix* deoxyhemocyanins, under conditions used in stopped-flow light-scattering experiments, were mixed with air-equilibrated buffers in a Gibson-Durrum stopped-flow spectrophotometer. The reaction with oxygen was monitored at 340 nm. No signal could be detected, showing that the combination with oxygen takes place within the 2.3-ms dead time of the stopped-flow apparatus. The same experiment was repeated with *Limulus* deoxyhemocyanin, but with 250 or 280 nm as the monitoring wavelength. The spectral changes at these wavelengths that accompany oxygenation (Figure 3) are also too fast to be measured with this technique.

Kinetics of Dissociation of Limulus and Helix Hemocyanins. Stopped-flow light-scattering measurements of hemocyanin dissociation were performed as described under Materials and Methods. The results of these experiments are shown in Figures 5–7. Here it is important to mention that the photomultiplier output, in the scattering mode, was determined to be a linear function of both the protein concen-

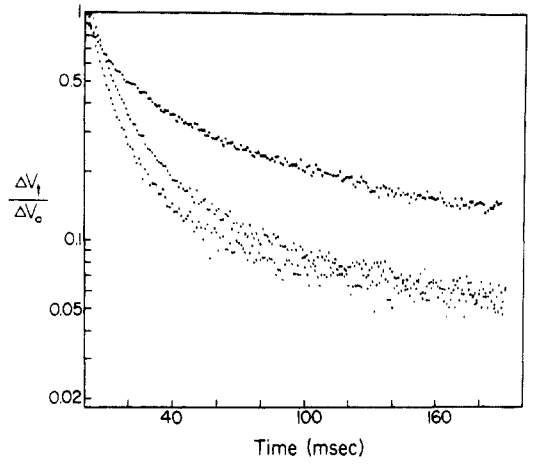

FIGURE 7: Stopped-flow light-scattering experiments with oxy and deoxy forms of 100S α-hemocyanin from *H. pomatia*. The protein concentration was 4 mg/mL at 20 °C. (Upper curve) Deoxyhemocyanin in 50 mM Tris, ionic strength 0.05, pH 8.9, containing 35 mM $CaCl_2$ (total ionic strength 0.155), mixed with deoxygenated 50 mM Tris, 50 mM EDTA, pH 8.9; initial rate 40 s^{-1}, final rate 4 s^{-1}. (Middle curve) Oxyhemocyanin mixed with oxy EDTA buffer; initial rate 70 s^{-1}, final rate 20 s^{-1}. (Lower curve) Deoxyhemocyanin mixed with oxy EDTA buffer; initial rate 77 s^{-1}, final rate 20 s^{-1}. The dissociation of whole 100S molecules into $^1/_{10}$ (18S) molecules was monitored at 425 nm.

tration (up to 3 mg/mL) and the molecular weight of the light-scattering species. This was checked by plotting the digitized voltage readings vs. the protein concentrations for 60S *Limulus* hemocyanin (pH 7, ionic strength 0.01, with 10 mM $CaCl_2$ giving a total ionic strength of 0.04) and 35S *Limulus* hemocyanin (pH 7, ionic strength 0.01, with no $CaCl_2$). The plots are straight lines with correlation coefficients of 0.97. The slope of the 35S plot is exactly half of the slope of the 60S plot. The deviation from linearity at protein concentrations above 3 mg/mL is possibly caused by residual absorption by the copper–oxygen complex at the scattering wavelength (425 nm) and perhaps by nonideality as well. All subsequent experiments were carried out with protein concentrations in the linear region.

For comparison of the kinetics of the dissociation processes under varied conditions, data are presented in Figures 5–7 as first-order plots. The rates of reaction (k) were estimated according to

$$\ln \frac{V_t - V_\infty}{V_0 - V_\infty} = \ln \frac{\Delta V_t}{\Delta V_0} = -kt$$

where V_0, V_t, and V_∞ are relative digitized readings of photomultiplier output observed at the beginning of the reaction, at time t, and at the end of the reaction, respectively. The maximum light-scattering level (V_{max}) was determined by mixing the protein with a buffer that did not affect its aggregation state. The fraction of the dissociation reaction observed after mixing the protein with a buffer that induced dissociation was then found from

$$\text{fraction of reaction obsd} = \frac{V_0 - V_\infty}{V_{max} - V_\infty} = \frac{\Delta V_0}{\Delta V_{max}}$$

Figure 5 shows the results obtained when 60S *Limulus* hemocyanin (pH 8.9, ionic strength 0.08, with 10 mM $CaCl_2$) was mixed with a pH 8.9 buffer containing 50 mM EDTA. The conditions established after mixing result in a complete dissociation of the 60S aggregate into its 5S subunits. This was established by analytical ultracentrifugation as discussed in a previous section. The dissociation of R-state *Limulus* oxyhemocyanin under these conditions has an initial rate of 2.4 s^{-1} and slows to a final rate of 1.5 s^{-1}. Slower dissociation of the oligomer occurs when the experiment is repeated with deoxy protein and deoxy buffer. The T-state *Limulus* deoxyhemocyanin shows an initial dissociation rate of 1 s^{-1} and a final rate of 0.45 s^{-1} (Figure 5). This indicates that there is a release of quaternary constraint upon the binding of oxygen. When the deoxy 60S *Limulus* hemocyanin was mixed with air-equilibrated EDTA buffer, the dissociation process observed was identical in all respects with that of the fully oxygenated R state. As previously noted, the oxygenation process under these conditions is complete within the dead time of the stopped-flow apparatus. This implies that the oxygen-linked T → R transition of *Limulus* hemocyanin is fast and complete within 2.3 ms, the dead time of the stopped-flow apparatus. After such an experiment, deoxygenated buffer containing EDTA was again introduced into one of the drive syringes of the stopped-flow apparatus. The observed dissociation behavior was again that of the T state, showing that no oxygen had leaked into the system.

It was mentioned in a previous section that NaCl stabilizes the 60S form of *Limulus* hemocyanin (Figure 4A,B). The effect of NaCl on the kinetics of the EDTA-induced dissociation of 60S *Limulus* oxyhemocyanin is shown in Figure 6. Increasing the concentration of NaCl dramatically slows the dissociation process. If the hemocyanin is equilibrated with 0.3 M NaCl the dissociation occurs at a rate constant of 0.035 s^{-1}! It is of interest that identical results are obtained in experiments in which NaCl is not with the hemocyanin solution but is, instead, a component of the EDTA buffer. This equivalence indicates that the stabilizing influence of NaCl is established within the 2.3-ms dead time of the rapid-mixing apparatus.

Similar stopped-flow light-scattering experiments were carried out with the more complex 100S α-hemocyanin of *H. pomatia*. When *Helix* oxyhemocyanin (pH 8.9, ionic strength 0.08, with 10 mM $CaCl_2$) was mixed with a pH 8.9 buffer containing 50 mM EDTA, the dissociation was complete within the dead time of the stopped-flow apparatus. The final dissociation products under these conditions are 18S ($^1/_{10}$) molecules as revealed by analytical ultracentrifugation. An extremely rapid dissociation process also occurred when deoxyhemocyanin was mixed with deoxy buffer containing EDTA. When the $CaCl_2$ concentration in the hemocyanin solution was raised to 35 mM, ~50% of the dissociation reaction could be observed. The results of dissociation studies with *Helix* oxy- and deoxyhemocyanin are shown in Figure 7. As in the case of *Limulus* hemocyanin, the deoxygenated form dissociates more slowly (40 → 4 s^{-1}) than the oxy form (70 → 20 s^{-1}). Deoxyhemocyanin from *H. pomatia*, when mixed with oxy EDTA buffer, shows dissociation kinetics which closely resemble those of the oxy protein (Figure 7).

The effect of NaCl on the dissociation kinetics of *H. pomatia* α-hemocyanin is very different from the NaCl effect obsered with *Limulus* hemocyanin. When 100S *Helix* α-hemocyanin with 10 mM $CaCl_2$ is rapidly mixed with a pH 8.9 buffer containing 2 M NaCl, there is a very rapid decrease of the light-scattering intensity (within 2.3 ms) followed by a very slow decrease of the light-scattering signal. The very rapid decrease in the light-scattering signal corresponds to ~50% of that observed in the absence of EDTA. As judged by sedimentation analysis, the rapid signal change is due to dissociation of whole molecules into half-molecules, and the very slow signal change is due to an incomplete dissociation of the half-molecules into $^1/_{10}$ molecules. When 100S *Helix* α-hemocyanin with 10 mM $CaCl_2$ is rapidly mixed with a pH

8.9 buffer containing 2 M $CaCl_2$ and 50 mM EDTA, we observe again a very rapid decrease of the light-scattering intensity (within 2.3 ms). The dissociation of the half-molecules into $^1/_{10}$ molecules proceeds now via a heterogeneous dissociation process with initial and final rates of 57 and 15 s^{-1}. At this $CaCl_2$ concentration (10 mM) the complete dissociation into $^1/_{10}$ molecules, induced by mixing with 50 mM EDTA, occurs too quickly to be observed if the NaCl is omitted. We conclude, therefore, that NaCl has two different effects on the stability of *Helix* α-hemocyanin. It disrupts the bonds which hold the two-tiered cylinder together as a 100S species, and it hinders the fast dissociation of the 60S half-molecules.

Discussion

By virtue of their high molecular weight and subunit diversity, the hemocyanins can be regarded as good model systems for the study of assembly processes in high molecular weight heteropolymers. The degree of aggregation of hemocyanin oligomers is often found to be affected by the interaction with allosteric effectors.

Stopped-flow light scattering as used in the present study can be a useful tool for analysis of the stability of various aggregation states. We find that differences in the structural stability of hemocyanin oligomers in response to allosteric effectors give rise to dramatic differences in the time courses of dissociation when divalent cations are removed. In the present study, our analysis of dissociation rates as influenced by oxygen and allosteric effectors was performed with the 60S hemocyanin of the arthropod, *L. polyphemus*, and with the 100S α-hemocyanin of the mollusc, *H. pomatia*.

We find that the 60S structure of the hemocyanin of the arthropod *Limulus* can be stabilized by NaCl with respect to increasing pH (Figure 4) and with respect to EDTA-induced dissociation (Figure 6). Previous experiments have shown that chloride ions can act as allosteric effectors of the 60S molecule of *Limulus* hemocyanin by stabilizing the T state (Brouwer et al., 1977). Thus, for *Limulus* hemocyanin, the addition of NaCl promotes the T state and also stabilizes the 60S oligomer.

Hemocyanin of the mollusc, *H. pomatia*, is quite differently affected by NaCl. In this molluscan hemocyanin, dissociation is promoted by high ionic strength conditions brought about by NaCl addition (Siezen & van Driel, 1974; Zolla et al., 1978). The light-scattering experiments presented here lead us to the conclusion that NaCl rapidly disrupts the bonds which hold together the two-tiered cylinder as a 100S species. In contrast to this, the subsequent dissociation of the 60S half-molecules is slowed by NaCl. The structural stability of various aggregation states is thereby illustrated to be affected differently. This is of importance in a general consideration of assembly of high molecular weight proteins where metabolic effectors may selectively promote specific steps in the assembly process.

The data presented on the effect of NaCl on the structural stability of hemocyanin oligomers (this paper) and on their oxygen binding behavior (Brouwer et al., 1977; Zolla et al., 1978) do not allow us to infer whether or not these two phenomena are linked. In order to obtain direct information about the linkage between changes in intersubunit contacts and the binding of oxygen, we performed a number of experiments (a) to assess the influence of aggregation on the oxygen affinity of the active sites and (b) to further probe the effect of oxygenation on the structural stability of the high molecular weight oligomers. There are differences between the oxygen affinities of free subunits and subunits within the R states of oligomers of both *Helix* and *Limulus* hemocyanin. The differences are indications that the conformation of the subunits is modified by assembly into the high molecular weight structures. For *Limulus* hemocyanin, this conclusion is further supported by the observation that the relative fluorescence intensity of a mixture of fully oxygenated subunits is about twice as high as that of the oxygenated 60S molecule (Figure 2). We must keep in mind, however, that the extinction coefficients used for the determination of the 60S and 5S species were uncorrected for light scattering (Nickerson & Van Holde, 1971). Consequently, the protein concentration of a solution of 5S particles will be $\sim$10% higher than the concentration of a solution of 60S particles of the same absorbance at 280 nm [see, e.g., Van Holde & Cohen (1964)]. The effect of this difference in protein concentration on the fluorescence spectra is small compared to the observed difference. The fluorescence yield of *Limulus* deoxyhemocyanin has been reported to be $\sim$9 times as high as that of the oxy derivative (Ma et al., 1978). The fluorescence yield of the subunits is then inferred to lie between that of the R and T states of the 60S molecule of *Limulus* hemocyanin. The quenching of the fluorescence of hemocyanins upon the binding of oxygen is thought to be a result of radiationless electronic energy transfer from an excited tryptophanyl residue to the copper–oxygen complex (Shaklai & Daniel, 1970). This energy transfer is apparently more effective in the oxygenated 60S structure of *Limulus* hemocyanin than in the oxygenated *Limulus* hemocyanin subunits that are free in solution. We should point out that the structural differences which give rise to a difference in fluorescence yield for the oligomer and the free subunits are not necessarily the same as the structural differences that result in differences in ligand affinity.

Direct indications that the quaternary changes induced by oxygenation affect the structural stability of hemocyanin oligomers are presented in Figures 5 and 7. The oxygenated forms of both *Limulus* and *Helix* hemocyanin dissociate faster than their deoxy counterparts when calcium is removed at high pH. Since oxygen binding by both hemocyanins can be described by the two-state model for allosteric transitions (Brouwer et al., 1977; Zolla et al., 1978; Colosimo et al., 1974), the observed dissociation behavior constitutes direct evidence that oxygen binding by both hemocyanins is accompanied by release of quaternary constraint in the high molecular weight aggregates. Oxygen linked changes in structural stability have also been reported for the hemocyanins of *Loligo* and *Busycon* (DePhillips et al., 1969, 1970). For these two proteins, however, oxygenation was reported to favor association.

It is readily apparent that the dissociation of *H. pomatia* α-hemocyanin that is induced by removal of calcium at high pH proceeds much more quickly than that of *L. polyphemus* hemocyanin. Under identical measuring conditions, in the presence of 10 mM $CaCl_2$, the apparent first-order rate constant for the dissociation of oxygenated *Limulus* hemocyanin is initially 2.4 s^{-1} and slows to 1.5 s^{-1}. In contrast to this rather slow process, the dissociation of *Helix* α-hemocyanin occurs too rapidly to be observed by the rapid-mixing method employed in this investigation. It seems rather surprising that the cylindrically shaped molluscan hemocyanin molecule, which is closed at both ends by a "collar" and whose walls are composed of huge polypeptide chains folded in separate compact domains, is so much more labile with respect to EDTA-induced dissociation at high pH. As a matter of comparison, we note that the 3×10^6 M_r hemoglobin from the earthworm, *Lumbricus terrestrus*, containing 192 hemes (Wiechelman & Parkhurst, 1972), dissociates much more slowly than do the

hemocyanins studied here. The pH-induced dissociation of the oxyhemoglobin at 21 °C can be described with two rate constants having values of 0.26 and 0.011 s^{-1}, respectively (Goss et al., 1975).

Stopped-flow and temperature-jump experiments with *H. pomatia* α-hemocyanin (van Driel et al., 1978) suggest that at low levels of ligand binding the rate of the allosteric transition from a low- to a high-affinity state (T → R transition) is slow, on the order of 20–120 s^{-1}. This is rather slow compared with the rate of quaternary structural change in hemoglobin, which is reported to be 2500 s^{-1} for the T → R transition for triply liganded hemoglobin at 22 °C (Ferrone & Hopfield, 1976) and 6400 s^{-1} for the R → T transition for ligand-free hemoglobin at 20 °C (Sawicki & Gibson, 1977). We consider that the differences in rates reported in the literature may be due to the fact that the rate of the transition depends upon the direction of the transition (R → T or T → R) and on the degree of oxygenation at which the conformational transition occurs.

In general, it seems that the rates of ligand-linked conformational changes in hemoglobin are much faster than ligand binding (Antonini & Brunori, 1971). Similarly, when *Limulus* or *Helix* deoxyhemocyanins are mixed with oxygenated dissociating buffer, the combination with oxygen is complete within the dead time of the stopped-flow apparatus, and the observed dissociation processes are those characteristic of the quaternary condition of fully oxygenated hemocyanin (Figures 5 and 7). These experiments lead to the conclusion that the oxygen-linked transitions from T states to R states for both hemocyanins are complete within the dead time and have first-order rate constants with a minimum value of 2000 s^{-1}. The marked contrast with the value reported by van Driel et al. (1978) (20–120 s^{-1}) for *Helix* hemocyanin seems to be a direct indication that the experimental conditions which influence the allosteric equilibrium also influence the rate of the quaternary conformational transition.

References

Antonini, E., & Brunori, M. (1971) *Front. Biol. 21*, 145–150.

Bates, R. G. (1973) *Determination of pH: Theory and Practice*, Wiley Press, New York.

Bonaventura, J., Bonaventura, C., & Sullivan, B. (1975) *J. Exp. Zool. 194*, 155.

Bonaventura, J., Bonaventura, C., & Sullivan, B. (1977a) *Oxygen Physiol. Funct., Proc. Am. Physiol. Soc. Symp., 1976*, 177–220.

Bonaventura, J., Bonaventura, C., & Sullivan, B. (1977b) *Struct. Funct. Haemocyanin, [Proc. Eur. Mol. Biol. Int. Workshop], 5th, 1976*, 206–216.

Brouwer, M., & Kuiper, H. A. (1973) *Eur. J. Biochem. 35*, 428.

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

Brouwer, M., Bonaventura, C., & Bonaventura, J. (1977) *Biochemistry 16*, 3897.

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

Brouwer, M., Wolters, M., & van Bruggen, E. F. J. (1979) *Arch. Biochem. Biophys. 193*, 487.

Brouwer, M., Bonaventura, C., & Bonaventura, J. (1980) in *Invertebrate Oxygen-Binding Proteins: Structure, Active Site and Function* (Lamy, J., & Lamy, J., Eds.) Marcel Dekker, New York (in press).

Colosimo, A., Brunori, M., & Wyman, J. (1974) *Biophys. Chem. 2*, 338.

DePhillips, H. A., Nickerson, K. W., Johnson, M., & Van Holde, K. E. (1969) *Biochemistry 8*, 3665.

DePhillips, H. A., Nickerson, K. W., & Van Holde, K. E. (1970) *J. Mol. Biol. 50*, 471.

Er-El, Z., Shaklai, N., & Daniel, E. (1972) *J. Mol. Biol. 64*, 341.

Ferrone, F. A., & Hopfield, J. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4497.

Gielens, C., Preaux, G., & Lontie, R. (1975) *Eur. J. Biochem. 50*, 271.

Gielens, C., Preaux, G., & Lontie, R. (1977) *Struct. Funct. Haemocyanin [Proc. Eur. Mol. Biol. Int. Workshop], 5th, 1976*, 85–94.

Goss, D. J., Parkhurst, L. J., & Görisch, H. (1975) *Biochemistry 14*, 5461.

Hendrickson, W. A. (1977) *Trends Biochem. Sci. [Pers. Ed.] 2*, 108.

Johnson, M. L., & Yphantis, D. A. (1978) *Biochemistry 17*, 1448.

Ma, J. K. H., Luzzi, L. A., Ma, J. Y. C., & Li, N. C. (1978) *J. Pharm. Sci. 66*, 1684.

Mellema, J. E., & Klug, A. (1972) *Nature (London) 239 (5368)*, 146.

Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88.

Nickerson, K. W., & Van Holde, K. E. (1971) *Comp. Biochem. Physiol. B 39*, 855–872.

Riggs, A. F., & Wolbach, R. A. (1956) *J. Gen. Physiol. 39*, 585.

Sawicki, C. A., & Gibson, Q. H. (1977) *J. Biol. Chem. 252*, 5783.

Shaklai, N., & Daniel, E. (1970) *Biochemistry 9*, 564.

Siezen, R. J., & van Driel, R. (1973) *Biochim. Biophys. Acta 295*, 131.

Siezen, R. J., & van Driel, R. (1974) *J. Mol. Biol. 90*, 91.

Siezen, R. J., & van Bruggen, E. F. J. (1974) *J. Mol. Biol. 90*, 77.

Sullivan, B., Bonaventura, J., & Bonaventura, C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2558.

Van Driel, R., Brunori, M., & Antonini, E. (1974) *J. Mol. Biol. 89*, 103.

Van Driel, R., Kuiper, H. A., Antonini, E., & Brunori, M. (1978) *J. Mol. Biol. 121*, 431.

Van Holde, K. E., & Cohen, L. B. (1964) *Biochemistry 3*, 1803.

Van Holde, K. E., & van Bruggen, E. F. J. (1971) *Biol. Macromol. 5 (Part A)*, 1.

Wiechelman, K. J., & Parkhurst, L. J. (1972) *Biochemistry 11*, 4515.

Zolla, L., Kuiper, H. A., Vecchini, P., Antonini, E., & Brunori, M. (1978) *Eur. J. Biochem. 87*, 467.