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Hemocyanin of *Octopus vulgaris*. The Molecular Weight of the Minimal Functional Subunit in 3 M Urea†

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ABSTRACT: In the presence of 3 M urea *Octopus vulgaris* (Mollusca) hemocyanin dissociates completely, giving a single functional component which reassociates in the original aggregate after removal of urea. The molecular weight of this subunit has been determined by gel filtration, by viscosity measurements, and by ultracentrifugation. The values obtained with the different methods range from 247 000 to 262 000. Electron microscopy shows that the reassociation of the functional subunit after removal of urea is complete and gives the typical cylindrical structure of the native protein. This component is the minimal functional subunit which can be obtained from *Octopus* hemocyanin without splitting covalent bonds. It is suggested that this component is made by five protomers (50 000 daltons) containing one oxygen binding site each bound covalently through, perhaps, the carbohydrate moieties of the protein.

Hemocyanins belong to a large family of chemically and functionally related proteins which reversibly bind oxygen and occur in several classes of Mollusca and Arthropoda.

Molluscan hemocyanins are present in the hemolymph as large aggregates with sedimentation coefficients from 50 S to 100 S or more. Under different experimental conditions (pH, ionic strength, etc.), they dissociate into subunits of different size and shape. The minimal functional subunit containing one oxygen binding site, as calculated from the copper to protein and the copper to oxygen ratios, would have a molecular weight of about 50 000. A component of this size, however, has never been observed among the dissociation products of molluscan hemocyanins.

The molecular weight of the minimal aggregate which can be obtained without splitting covalent bonds is a controversial

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point in the biochemistry of molluscan hemocyanins. The 11S component obtained by pH dissociation, by treatment with 6 M urea, 6 M guanidine hydrochloride, or 1% sodium dodecyl sulfate, has been studied by several authors. Brouwer & Kuiper (1973) found for the hemocyanin of *Helix pomatia* one major subunit of 250 000 daltons together with a minor component of 500 000 daltons. Later, however, a value of 365 000 daltons was assigned to the minimal subunit of the same hemocyanin by Siezen & Van Bruggen (1974). As for another molluscan hemocyanin, that of *Loligo pealei*, Van Holde & Cohen (1964) found by sedimentation equilibrium a molecular weight of 385 000, whereas for the hemocyanin of the marine gastropod *Murex trunculus* Wood & Peacocke (1973) have reported a value of 220 000. Recently, Quitter et al. (1978) have found for *Busycon* hemocyanin that the smallest subunit which can be produced in denaturing solvents has a molecular weight of 300 000.

The hemocyanin of *Octopus vulgaris* has a sedimentation coefficient of 50 S (Eriksson-Quensel & Svedberg, 1936). At the electron microscope this aggregate appears as a right circular cylinder 350 Å in diameter and 170 Å high (Van Bruggen, 1968). According to Eriksson-Quensel and Svedberg, complete dissociation (11 S) of this hemocyanin is achieved only at pH 10.8. We have observed that this dissociation, however, is not completely reversible and, when the pH is lowered, the subunits show a strong tendency to aggregate, giving a polydisperse system with components of higher molecular weight.

Previous experiments carried out in this laboratory have demonstrated that urea at relatively low concentrations is a better dissociating agent for *Octopus* hemocyanin and that the dissociation is completely reversible. In this study the molecular weight of the minimal functional subunit of *O. vulgaris* hemocyanin in the presence of 3 M urea has been determined by gel filtration, by viscosity measurements, and by ultracentrifugation techniques. The reversibility of the dissociation after the removal of urea in the presence of Ca^{2+} has been investigated, and the structure of the aggregate after reassociation has been controlled by electron microscopy. Finally, by viscosity measurements in guanidine hydrochloride the molecular weight of the reduced and cyanoethylated apoprotein has been calculated.

Experimental Section

Materials

The hemolymph of *O. vulgaris* was collected at the Stazione Zoologica of Naples from the incannulated aorta of live animals. The blood cells were removed by brief low-speed centrifugation, and the clear supernatant was diluted 1:1 with 0.1 M Tris-HCl buffer containing 20 mM $CaCl_2$ at pH 7.4. Hemocyanin was sedimented by centrifugation for 3 h at 120000*g* in a Spinco-Beckman preparative ultracentrifuge, Model 50. The sediment was redissolved in a small volume of the same buffer, dialyzed overnight in the cold (4 °C), and, when not used immediately, quickly frozen after addition of 20% (w/v) sucrose and stored at −40 °C.

Copper-free hemocyanin was prepared as previously described (Salvato et al., 1974). The reduced and cyanoethylated protein was obtained according to Seibles & Weill (1967). The completely denatured hemocyanin was prepared by dissolving copper-free or reduced and cyanoethylated apohemocyanin in 0.2 M Tris-HCl buffer containing 6 M guanidine hydrochloride at pH 7.5.

All measurements in the presence of 3 M urea were carried out in 0.1 M Tris-HCl and 0.1 M methylamine hydrochloride buffer at pH 7.4. Reassociation was obtained by prolonged (48 h) dialysis against the same buffer without urea and containing 20 mM $CaCl_2$.

All reagents were of analytical grade and were used without further purification.

Methods

Protein concentration was determined spectrophotometrically by using an $E_{288}^{0.1\%} = 1.67$ in 0.2 M KOH or an $E_{278}^{0.1\%} = 1.41$ in pH 8 buffer. The absorbancy was corrected for light scattering by using the relation $\Delta A = K(1/\lambda^4)$.

Gel filtration in Bio-Gel A 1.5 M (Bio-Rad; 200–400 mesh) equilibrated with buffered 3 M urea was performed in 1.8 × 110 cm columns at room temperature. The elution rate was 5.5 mL/h; fractions (50 drops) were collected by using a LKB Ultrorac fraction collector, Model 7000. Blue Dextran 2000 (Pharmacia), catalase, horse γ-globulin, bovine serum albumin, ovoalbumin, chymotrypsinogen A, and cytochrome *c* were used for the calibration of the column. Standard proteins and hemocyanin (ca. 10 mg) were dissolved in about 1 mL of the elution buffer.

Viscosity measurements were carried out on native hemocyanin in 3 M urea and on copper-free, reduced and cyanoethylated protein in 6 M guanidine hydrochloride. The protein concentration ranged from 1 to 12 mg/mL. A suspended-level Ubbelohde capillary viscosimeter with an outflow time for water of 114 ± 0.02 s was used at 20 ± 0.01 °C.

The protein was equilibrated with the appropriate buffered solutions by dialysis for 48 h and then centrifuged for 1 h at 30000*g*. The upper part of the supernatant was then carefully collected for the measurements which were performed following the precautions recommended by McKie & Brandts (1972).

The density of the solutions was measured at 20 ± 0.03 °C by using a Mettler H-31/H311 balance adapted as a hydrostatic balance.

Sedimentation and diffusion experiments were performed in a Beckman Spinco analytical ultracentrifuge, Model E, equipped with the electronic speed control and with Schlieren, interference, and absorption optics.

The sedimentation velocity has been determined at 20 °C on native and reassociated hemocyanin at concentrations of 4–5 mg/mL and on dissociated protein at concentrations ranging from 0.2 to 8 mg/mL. The An-D rotor was used at 36 000 or 48 000 rpm for 40–120 min.

Diffusion rates have been determined on 3 M urea dissociated protein by using a single-sector valve-type synthetic boundary cell at 6000 rpm. The diffusion coefficients were calculated with the height–area method from photographs taken at 18-min intervals.

Sedimentation equilibrium was carried out on dissociated hemocyanin by using the interference optics and according to the method of Chervenka (1970). Initial protein concentrations of 2.5 and 5.5 mg/mL have been used at 13 000 and 8000 rpm, respectively. Photographs were taken immediately after reaching maximum speed and after 8, 16, and 24 h.

The partial specific volume was calculated from the amino acid composition according to McMeekin & Marshall (1952). For technical reasons the eventual effect of urea and of the carbohydrate content of the protein has not been taken into account.

Electron Microscopy. The negative staining technique has been used. Protein solutions were diluted immediately before use to concentrations of 20–50 μg/mL and mixed with the staining solution on carbon-coated Butwar B-98 films. After 30 s the excess solution was removed. Uranyl oxalate was

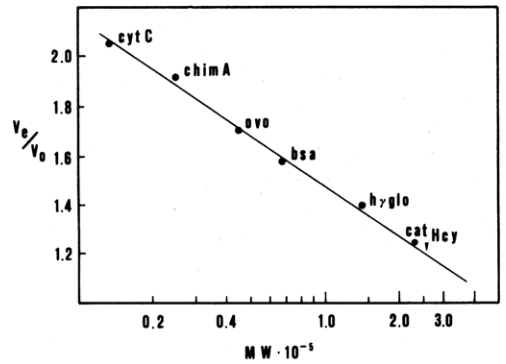

FIGURE 1: Calibration plot of the Bio-Gel A 1.5 M column in the presence of 3 M urea.

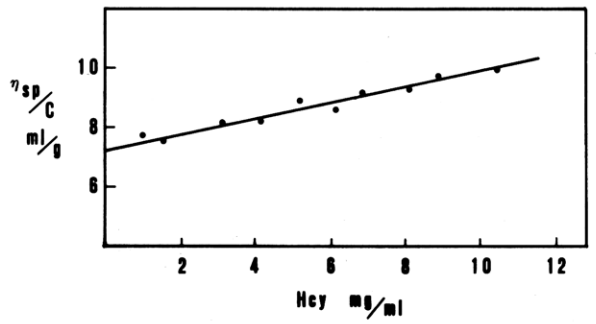

FIGURE 2: Viscosity measurements of native hemocyanin in 3 M urea.

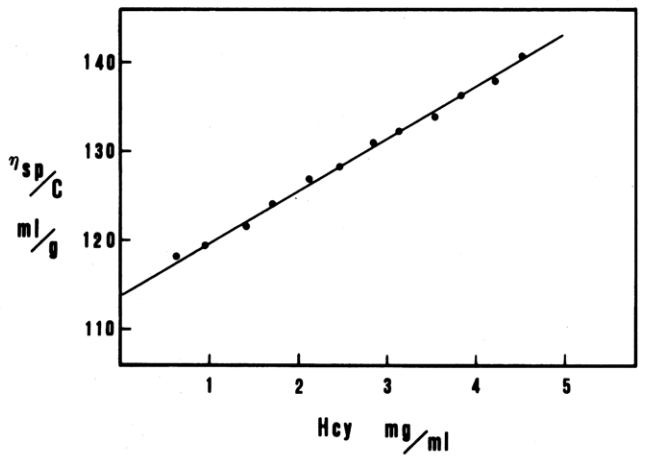

FIGURE 3: Viscosity measurements of reduced and cyanoethylated hemocyanin in 6 M guanidine hydrochloride.

prepared from uranyl acetate by dissolving 0.5% acetate in 12 mM oxalic acid; the solution was brought to pH 5.0 by addition of diluted ammonia. Observations were made on a Philips EM 300 at 80 kV and at a magnification of 42 000×.

Results

When *Octopus* hemocyanin is filtered through a column of Bio-Gel in the presence of 3 M urea, it appears as a single symmetrical peak in a greater elution volume than Blue Dextran 2000. The incubation of hemocyanin for 10 days in 3 M urea at room temperature does not alter the elution pattern. When the incubation is prolonged for 20–30 days, together with a reduction of the copper–oxygen absorption band, a new component appears which has an elution volume near the void volume and which amounts to about 10–20% of the total protein.

The molecular weight of the hemocyanin dissociation product, as evaluated from a plot of V_e/V_0 (elution volume/void volume) vs. log of molecular weight obtained for the standard proteins, is equal to about 250 000 (Figure 1).

The Bio-Gel method in our conditions is orientative. It has been used under the assumption that the influence of 3 M urea on the hydrodynamic volume is about the same in all protein samples.

By extrapolation to 1 of V_e/V_0, an excluded molecular weight of 500 000 is obtained in our conditions which is much lower than the theoretical value of 1.5×10^6 for Bio-Gel 1.5 M in water.

The reduced viscosities of native hemocyanin in 3 M urea, as a function of protein concentration, are reported in Figure 2. The intrinsic viscosity value, as calculated with the method of least squares from the experimental data, is equal to 7.26 mL/g. The reduced viscosities of copper-free, reduced and cyanoethylated protein in 6 M guanidine hydrochloride are reported in Figure 3; the calculated intrinsic viscosity gives a value of 114 mL/g. An intrinsic viscosity of 90 mL/g was obtained for copper-free, nonreduced protein in guanidine hydrochloride.

From the intrinsic viscosity of the reduced and cyanoethylated hemocyanin a number of 2169 ± 100 amino acid residues have been calculated by using the general equation of Mark-Houwink (Yang, 1961) as modified by Tanford et al. (1967) for linear randomly coiled polypeptide chains in 6 M guanidine hydrochloride

$$[\eta] = 0.716n^{0.66}$$

where n is the number of amino acid residues.

Native *Octopus* hemocyanin in the presence of Ca^{2+} is sedimented as a single peak with a sedimentation coefficient of 49.5 S (Figure 4). The same hemocyanin, when treated with 3 M urea, sediments as a slower single symmetrical peak (Figure 5). The sedimentation coefficients vs. protein concentration are plotted in Figure 6. The linearity of the plot with values of s which increase at lower protein concentrations indicates that only one component is present having a homogeneous molecular weight. By extrapolation to zero protein concentration an $s^0_{20} = 7.92$ S is obtained for the dissociation product in 3 M urea.

When urea is removed by dialysis against buffer containing Ca^{2+}, the protein reassociates, giving a sedimentation pattern identical with that obtained for the native hemocyanin. If the dialysis is carried out with a buffer or lower ionic strength (containing Ca^{2+} or Mg^{2+}) the protein associates, giving a 24S

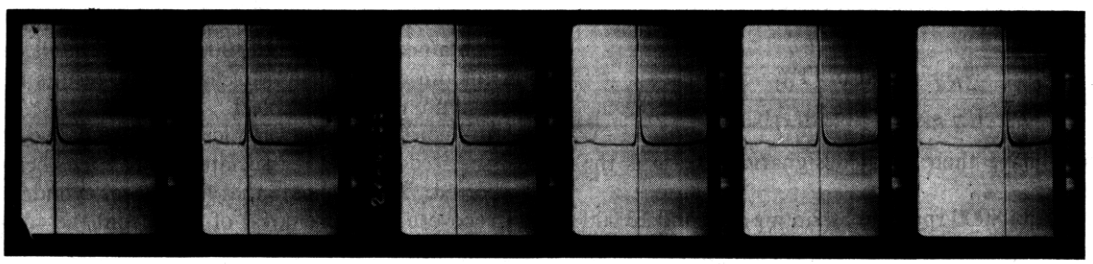

FIGURE 4: Sedimentation pattern of native hemocyanin in the presence of $CaCl_2$. Protein concentration was 4 mg/mL.

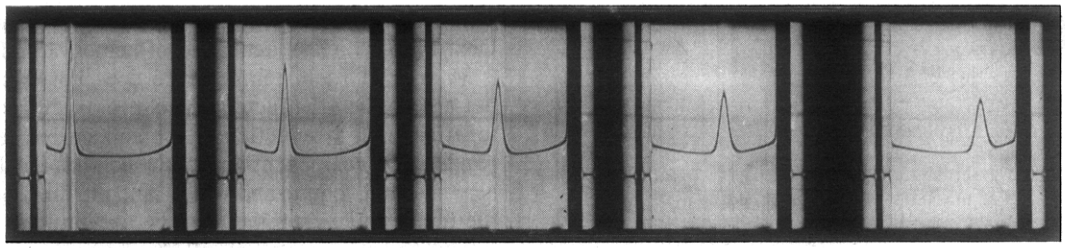

FIGURE 5: Sedimentation pattern of native hemocyanin in the presence of 3 M urea.

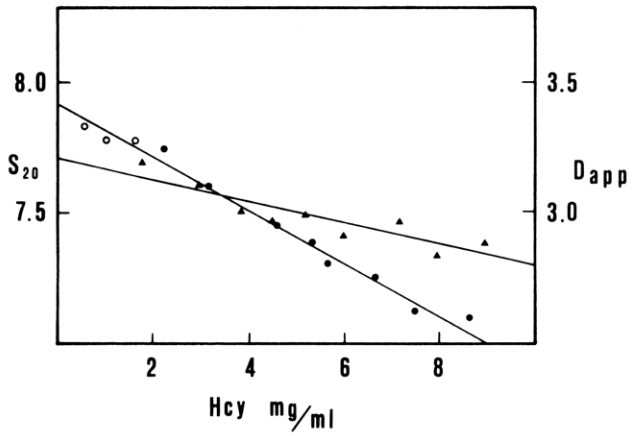

FIGURE 6: Left scale: protein concentration dependence of the sedimentation coefficient. (●) Schlieren optics. (○) Absorption optics. Right scale: protein concentration dependence of the apparent diffusion coefficient (▲).

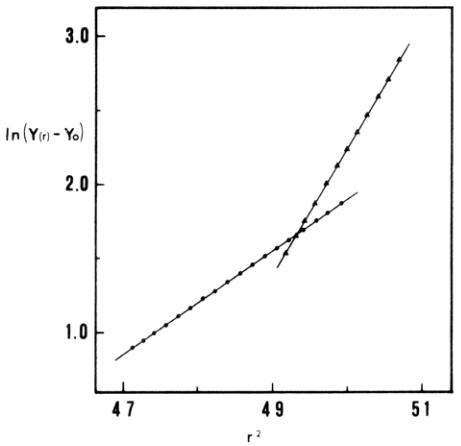

FIGURE 7: Sedimentation equilibrium of native hemocyanin in 3 M urea. Triangles: 2.5 mg/mL hemocyanin at 12 000 rpm. Circles: 5 mg/mL hemocyanin at 8000 rpm.

component which has never been observed among the dissociation products of *Octopus* hemocyanin. Also, in Figure 6 the D_{app} is reported as a function of protein concentration. By extrapolation to zero protein concentration, a $D^0_{20} = 3.2 \times 10^{-7}$ cm^2 s^{-1} is obtained. No monotonic drift has been evidenced in the calculation of D_{app} for each protein concentration as a function of diffusion time.

Assuming that hemocyanin does not preferentially bind urea and that, therefore, the system can be treated as having only two components, we can calculate $s^0_{w,20}$ and $D^0_{w,20}$ by the equations

$$s^0_{w,20} = s^0_{20}\frac{\eta_{solv}}{\eta_w}\frac{1 - \rho_{w,20}\bar{\nu}}{1 - \rho_{solv}\bar{\nu}}$$

$$D^0_{w,20} = D^0_{20}\frac{\eta_{solv}}{\eta_w}$$

where η_{solv} and η_w are the viscosities of the solvent and water at 20 °C, $\rho_{w,20}$ and ρ_{sol} are the densities of water and solvent at 20 °C, and $\bar{\nu}$ is the partial specific volume of the protein. In this way values of $s^0_{w,20} = 11.25$ S and $D^0_{w,20} = 3.9 \times 10^{-7}$ cm^2 s^{-1} are obtained for the dissociation product of hemocyanin in 3 M urea.

The results obtained from the sedimentation equilibrium experiments are reported in Figure 7. The plot is linear at both protein concentrations and velocities. The values of weight-average apparent molecular weight have been obtained by the equation

$$M_{w,app} = \frac{2RT}{1 - \rho\bar{\nu}}\frac{1}{\omega^2}\frac{\mathrm{d}\ln[Y_{(r)} - Y_0]}{\mathrm{d}(r)^2}$$

where R and T have their usual designation and ω is the angular velocity.

At 5 mg/mL and 8000 rpm and at 2.5 mg/mL and 13 000 rpm, values of 259 000 and 256 000, respectively, have been obtained for the molecular weight of the hemocyanin subunit.

The partial specific volume, as calculated from the amino acid composition, is equal to 0.726 mL g^{-1}. Using this value, together with s^0_{20} and D^0_{20} in Svedberg's equation

$$M = \frac{RTs}{D(1 - \rho\bar{\nu})}$$

where R and T have their usual designation, ρ is the solvent density, and $\bar{\nu}$ is the partial specific volume, we have calculated a molecular weight of 265 000 for the hemocyanin subunit.

From s^0_{20} and the intrinsic viscosity $[\eta]$ in 3 M urea in the Flory–Mandelkern equation (Mandelkern & Flory, 1942)

$$M^{2/3}\beta(1 - \rho\bar{\nu}) = Ns^0[\eta]^{1/3}\eta_0$$

where N is Avogadro's number, η_0 is the solvent viscosity, and β is a constant with an assumed value of 2.5×10^6, the calculated molecular weight is equal to 262 000.

From the values of D^0_{20} and $[\eta]$ in 3 M urea in the equation

$$\frac{kT}{D_0} = 6\pi\eta\frac{f}{f_0}\left(\frac{3M[\eta]}{4\pi N\bar{\nu}}\right)^{1/3}$$

and by using for $(f_0/f)\,\bar{\nu}^{1/3}$ the proportional term β with the same value as in the Flory–Mandelkern equation, we find that the molecular weight is equal to 247 000.

From the number of amino acid residues calculated by Tanford's equation (2169 ± 100), using the average molecular weight of 119.86 as calculated from the amino acid composition of *Octopus* hemocyanin (Ghiretti-Magaldi et al., 1966), we obtained a value of 260 000 for the subunit considered as a random-coil polypeptide chain. The results here reported are summarized in Table I.

In Figure 8 the electron micrographs of native (a), 3 M urea dissociated (b), and reassociated (c) *Octopus* hemocyanin are reported. They indicate that the typical cylindrical structure is recovered after removal of urea from the completely dissociated form, as shown also from the sedimentation experiments.

Table I: Some Hydrodynamic Parameters and Calculated Molecular Weights of *Octopus vulgaris* Hemocyanin in 3 M Urea, in Guanidine Hydrochloride, and in Buffer

	native	dissociated	reassociated
s_{20}	49.6		49.8
s^0_{20} (3 M urea)		7.92	
$s^0_{w,20}$		11.25	
D^0_{20} (3 M urea)		3.2×10^{-7} cm^2 s^{-1}	
$D^0_{w,20}$		3.9×10^{-7} cm^2 s^{-1}	
η_{int} (3 M urea)		7.26 mL g^{-1}	
η_{int} (Gdn·HCl)		90 mL g^{-1}	
η_{int} (Gdn·HCl + R–SH)[a]		114 mL g^{-1}	
[illegible] of residues per chain		2169 ± 100	
molecular weight			
gel filtration		~250 000	
s_0 and D_0		256 000	
s_0 and η_{int}		262 000	
D_0 and η_{int}		247 000	
η_{int} in Gdn·HCl		260 000	
SE,[b] 5 mg/mL		259 000	
SE, 2.5 mg/mL		256 000	

[a] Reduced and cyanoethylated apohemocyanin. [b] SE, sedimentation equilibrium.

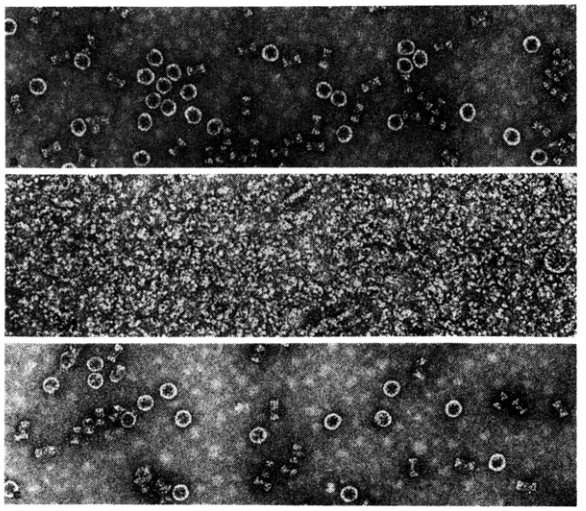

FIGURE 8: Electron micrographs of native hemocyanin. (a) Associated protein in the presence of $CaCl_2$ (58 500×); (b) dissociated in 3 M urea (112 500×); (c) reassociated after removal of urea and addition of $CaCl_2$ (58 500×).

The dissociated molecules are not sufficiently resolved, but occasionally they show a "necklace" structure similar but smaller to those described by Siezen & Van Bruggen (1974) for the smallest subunit of *Helix pomatia* hemocyanin.

Discussion

As known, urea has a double effect upon proteins: it induces dissociation of the oligomeric aggregates and modifications of the secondary and tertiary structure. At high urea concentrations these modifications may lead to complete denaturation (Tanford, 1968).

These effects occur at very different concentrations of urea on the hemocyanin of *O. vulgaris*. Whereas complete dissociation into minimal subunits is observed at 3 M urea, denaturation is induced at concentrations higher than 6 M as shown by viscosity measurements. When studying the action of increasing concentrations of urea on *Octopus* hemocyanin, Costantino et al. (1971) found a sudden increase of the intrinsic viscosity of about 20% around 2–3 M urea which remains unchanged up to 6 M where a second transition is observed which induces a 10-fold increase of viscosity. This indicates that, at least from the hydrodynamic point of view, the dissociation induced by 2–3 M urea does not imply gross conformational changes of the protein structure since the observed low increase of viscosity probably is the result of the swelling of the protein molecule.

The relative constancy of the secondary and tertiary structure is further demonstrated by the results of spectrophotometric, fluorometric, and circular dichroism studies (Tamburro et al., 1976; Salvato and Jori, unpublished experiments). In the range 240–700 nm the absorption spectra of *Octopus* hemocyanin in absence and in presence of urea are essentially identical; the CD spectra show only little modifications in the near-UV probably due to minor modifications limited to the region of the active site. The calculated percent of the ordered structure is the same both in the presence and in the absence of urea. Finally, the ability of the protein to combine reversibly with oxygen does not change in 3 M urea (Salvato & Tallandini, 1977); the properties of the oxygen dissociation curves can be easily interpreted on the basis of the protein swelling which leaves the internal structural relationships essentially unaltered. Also, the electron microscope observations reported here indicate that the swelling effect induced by urea is completely reversible at least in the sense that it does not compromise the ability of the protein to resume the native quaternary structure.

The dissociation effect of urea on *O. vulgaris* hemocyanin produces functional subunits which reassociate completely after the removal of the denaturing agent. The consistency of the results obtained with the different methods used allows us to assert that these subunits have a molecular weight of about 250 000; i.e., they are a pentamer of the protomer containing one oxygen binding site.

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Amino Acid Sequence of California Quail Lysozyme. Effect of Evolutionary Substitutions on the Antigenic Structure of Lysozyme†

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ABSTRACT: To examine the effect of amino acid substitutions in lysozyme on the binding of antibodies to lysozyme, we purified lysozyme from the egg whites of California quail and Gambel quail. Tryptic peptides were isolated from digests of the reduced and carboxymethylated lysozymes and subjected to quantitative analysis of their amino acid compositions. The two proteins were identical by this criterion. Each peptide from the California quail lysozyme was then sequenced by quantitative Edman degradation, and the peptides were ordered by homology with other bird lysozymes. California quail lysozyme is most similar in amino acid sequence to bobwhite quail lysozyme, from which it differs by two substitutions: arginine for lysine at position 68 and histidine for glutamine at position 121. California and bobwhite quail lysozymes were antigenically distinct from each other in quantitative microcomplement fixation tests, indicating that substitutions at one or both of these positions can alter the antigenic structure of lysozyme. Yet neither of these positions is among those claimed to account for the precise and entire antigenic structure of lysozyme [Atassi, M. Z., & Lee, C.-L. (1978) *Biochem. J. 171*, 429–434]. Two possible explanations for this discrepancy are discussed.

Although much progress has been made in our understanding of the antigenic structure of globular proteins, this understanding is incomplete in important respects. Lysozyme *c* is an example of a globular protein which has been studied particularly extensively by immunochemists. Especially notable are recent studies by Atassi and co-workers, who claim to have delineated precisely the complete antigenic structure of lysozyme[1] (Atassi & Habeeb, 1977; Atassi & Lee, 1978). According to these authors the number of sites at which antibodies bind to lysozyme is only three, regardless of whether the antisera are produced in rabbits or in goats. The sites comprise residues at the following positions: site I, residues 5, 7, 13, 14, and 125; site II, residues 33, 34, 113, 114, and 116; site III, residues 62, 87, 89, 93, 96, and 97 (Atassi & Lee, 1978). These sites were delineated by studies with chemically modified lysozyme and with peptides isolated from lysozyme or synthesized to simulate certain surface parts of the molecule (Atassi & Lee, 1977, 1978; Lee & Atassi, 1977).

Investigators from other laboratories have also contributed to the knowledge of the antigenic structure of lysozyme. Evidence from these other laboratories is consistent with the possibility that lysozyme has additional antigenic sites (Maron et al., 1972; Arnon et al., 1974; Fainaru et al., 1974; Matthyssens et al., 1974; Fujio et al., 1974; Wilson & Prager, 1974).

We now present further evidence that evolutionary substitutions at positions outside the sites proposed by Atassi and co-workers alter the antigenic structure of lysozyme. This evidence was obtained by sequencing lysozyme from the California quail and comparing it immunologically with other lysozymes of known amino acid sequence, especially the lysozyme of the bobwhite quail.

Materials and Methods

Eggs and Lysozymes. Freshly laid California quail (*Lophortyx californicus*) and Gambel quail (*Lophortyx gambeli*) eggs were obtained from A-1 Game Birds, Ashland, Oregon. The egg whites were separated from the yolks and stored frozen at −10 °C.

Prior to lysozyme purification, individual California quail egg whites were tested for the presence of electrophoretic variants by cellulose acetate electrophoresis at pH 8.6 (Ibrahimi, 1977). No variants were found. Lysozyme was then purified from 1800 mL of pooled egg white, derived from 477 eggs, according to procedures previously described (Arnheim et al., 1969; Prager & Wilson, 1971a). The purity of the lysozyme obtained was tested by electrophoresis in different media at several pH values (Prager & Wilson, 1971a; Ibrahimi, 1977; Prager et al., 1978), by immunodiffusion, and by amino-terminal analysis.

Gambel quail lysozyme was purified from 570 mL of pooled egg white, derived from 133 eggs, in the same manner as was California quail lysozyme, and its purity was evaluated by the same criteria. Gambel quail lysozyme was included in this study because taxonomists (Leopold, 1978) consider the Gambel quail to be the closest living relative of the California quail, and we therefore hoped to obtain lysozymes differing at only one or two positions.

All other lysozymes used in this investigation were those previously purified and characterized (Prager & Wilson, 1971a; Jollès et al., 1976).

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[1] Abbreviations used: lysozyme, lysozyme *c*; CM (Cm in figure), carboxymethyl.

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