Characterization of Domains Obtained from a Mollusc Haemocyanin by Limited Proteolytic Digestion

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The haemocyanin from the freshwater gastropod *Lymnaea stagnalis* was digested with proteolytic enzymes under conditions where it existed as whole (native) molecules (mol.wt. approx. 9×10^6), or as one-tenth molecules. Digestion of whole molecules yielded a fragment of mol.wt. approx. 110000 believed to correspond to the 'collar' of the molecule, and an aggregate some 20–30 times the size of the original native molecule formed by end-to-end polymerization of the molecule after removal of the collar. Digestion of one-tenth molecules yielded a mixture of products that could be separated into three fractions by gel filtration. Analysis of these by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed that they typically contained two or three components. The collar fragment was present as a component of the intermediate-molecular-weight fraction, and it dissociated on sodium dodecyl sulphate/polyacrylamide gels to give two bands corresponding to apparent mol.wts. 65000 and 60000. The c.d. spectra of the separated fractions were recorded and fitted with Gaussian curves by a computer procedure. The fractions each possessed distinct c.d. spectra, by which they could be identified: the collar-fragment c.d. and absorption spectra showed the most striking differences compared with those of the other fragments. The results were interpreted in terms of the postulated existence, within the haemocyanin molecule, of multi-domain structures, each comprising a single polypeptide chain of mol.wt. 200000–300000.

The haemocyanins of molluscs are large copper-containing proteins with molecular weights in the millions, e.g. 9×10^6 in gastropods, which have multiple binding sites for oxygen. Like the giant invertebrate haemoglobins, they are extracellular and show co-operative binding of oxygen, and may in addition have a zero, normal, or even reverse Bohr effect (Redmond, 1968; Hall *et al.*, 1975; Wood *et al.*, 1977). Although it is well established that the functional oxygen-binding site contains two copper atoms in a protein mol.wt. of about 50000, a functional subunit of this size has so far only been obtained by treatment with proteolytic enzymes (Lontie *et al.*, 1973; Wood & Mosby, 1976). In contrast, treatment of the native protein with reagents normally used to produce polypeptide chains from multisubunit proteins (1% SDS, 6M-guanidinium chloride etc.) fails to produce a subunit of mol.wt. less than 200000–300000 (Wood & Peacocke, 1973; Brouwer & Kuiper, 1973). Such a polypeptide chain would contain perhaps five or more oxygen-binding domains. On the basis of these and other studies, it has been proposed (Van Breemen *et al.*, 1977) that the gastropod haemocyanin molecule is built up of 20 multi-domain polypeptide chains arranged helically to form the cylindrical structure observed in electron micrographs of the negatively

stained material. Such a structure could conceivably have arisen by gene duplication, but at present detailed information, including sequences, on the structure of the 50000-mol.wt. subunit or domain is lacking.

The aim of the present work has been to identify the different types of domain that comprise the haemocyanin of the freshwater gastropod, *Lymnaea stagnalis*, produced by the use of proteolytic digestion. The fragments so obtained have been partially separated and characterized by physicochemical methods.

Materials and Methods

Isolation and partial dissociation of haemocyanin

Specimens of *L. stagnalis* were obtained and bled as described previously, and the haemocyanin was purified by at least two cycles of preparative ultracentrifugation (Hall *et al.*, 1975). In the haemolymph of the animal the haemocyanin molecules exist in the so-called 'whole' state, having a sedimentation coefficient approx. 100S corresponding to a mol.wt. of about 9000000. At pH values greater than about pH 7.5 in the absence of Ca^{2+} or Mg^{2+} , partial or complete dissociation occurs into one-tenth, and at much higher pH values one-twentieth, molecules

Abbreviation used: SDS, sodium dodecyl sulphate.

(Hall *et al.*, 1975). Thus by controlling the concentration of Ca^{2+} or Mg^{2+} at a pH of 8.2 it is possible to arrange for proteolytic enzymes to act on either whole or one-tenth molecules.

Digestion with proteinases and separation of products

Trypsin and pig plasmin were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Digestion of whole molecules was carried out in 0.1M-Tris/HCl buffer, pH 8.2, containing 25mM- CaCl_2 , for up to 2h at 32°C. Digestion of one-tenth molecules was carried out in 50mM-Tris/HCl, pH 8.2, containing 50mM-NaCl under similar conditions. Enzyme:haemocyanin ratios of 1:20 to 1:1000 (w/w) were employed. The products of digestion were separated from each other and from proteolytic enzyme by gel filtration on a column of Sephadex G-150 equilibrated with digestion buffer. When whole molecules were digested, the products were of such widely different sizes that an initial ultracentrifugation step before gel filtration was used. It appears that part of the molecule, the so-called 'collar' observed by electron microscopy (Mellema & Klug, 1972), is removed, and the remaining cylindrical structures undergo an indefinite end-to-end polymerization to give structures some 20–30 times the size of the original molecule (Wood, 1977). These aggregates are easily sedimented, leaving collar and digestive enzyme in the supernatant, which may be separated by gel filtration. The kinetics of the process were also studied by measuring the turbidity increase at 400nm as digestion of whole molecules proceeded (see Fig. 1 legend). Dissociation of the aggregate into small fragments was achieved by removing Ca^{2+} by resuspension in 0.1M-Tris/HCl buffer, pH 8.2, containing no Ca^{2+} . The fragments were separated by gel filtration as described above.

After gel filtration, tubes containing protein were pooled and the protein was concentrated by adsorption on and elution from small columns of DEAE-cellulose. The concentrated fractions were re-chromatographed on Sephadex G-150 once or twice as necessary.

Characterization of digestion products

Calibration of the Sephadex G-150 column with marker proteins of known molecular weight gave an indication of the molecular weights of the digestion products. Absorption spectra were recorded by using a Unicam SP.1800 spectrophotometer, and c.d. spectra were obtained with a Jouan Dichrographe III instrument. Several path lengths and protein concentrations were used in order to take maximal advantage of the range and sensitivity of the instruments. Protein concentrations were determined from the A_{280} by using a specific absorption coefficient,

$A_{1\text{cm}}^{1\%}$, of 15.6 obtained by dry-weight measurements. It was assumed that all the fractions had the same absorption coefficient at 280nm, although this is probably only approximately true. C.d. was expressed as $\Delta\epsilon$, the difference in molar absorption for left- and right-circularly polarized light, and ellipticities were calculated as described previously (Wood & Peacocke, 1973) by using as the molar basis 25000, the molecular weight containing one copper atom. In the visible and near-u.v. region c.d. data were expressed as a function of wavenumber, σ , and the data were fitted by using the equation:

$$\Delta\epsilon_j = \sum_{i=1}^{i=n} c_{3i} \cdot \exp \left[-\frac{1}{2} \left(\frac{\sigma_j - c_{3i-2}}{c_{3i-1}} \right)^2 \right] \quad (1)$$

where n is the number of Gaussian curve components each characterized by three parameters, c_{3i} , c_{3i-1} , c_{3i-2} . Initial values of the parameters were estimated by examining plots of the data and were used to calculate values of $\Delta\epsilon$, according to eqn. (1). The sum of squares of the differences between calculated values, $\Delta\epsilon_j$, and experimental values were minimized by using the procedure of Marquardt (1963), embodied in a computer program. The rotational strength, R , of c.d. bands was evaluated by the expression (Woldbye, 1965):

Fig. 1. Digestion of whole *L. stagnalis* haemocyanin molecules with trypsin

The inset shows a typical progress curve for the rate of development of turbidity measured at 400nm in a 10mm cell. The conditions for the digestion were 100mM-Tris/HCl buffer, pH 8.2, containing 25mM- CaCl_2 , at 32°C. Initial rates were taken from a series of progress curves at different haemocyanin concentrations, by using the slope at the point of inflection, and plotted against haemocyanin concentration. Extrapolation to zero turbidity gave a critical haemocyanin concentration for tubular aggregate formation, c_r , of approx. 0.8mg/ml.

$$R = 2.45 \times 10^{-39} \Delta \epsilon_0 (2\delta) / \sigma_0 \quad (2)$$

Sedimentation-velocity experiments were performed as described previously (Wood & Mosby, 1975), and sedimentation-equilibrium experiments were performed on purified digestion products by the high-speed method (Yphantis, 1964). A value of 0.726 ml/g was assumed for the partial specific volume, $\bar{v}$, which is a typical value for gastropod haemocyanins (Wood *et al.*, 1971).

Detergent/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970) on 1 mm-thick slab gels, and the gels were stained in Coomassie Brilliant Blue R250.

Electron microscopy was performed with a Philips 300 instrument on samples negatively stained with uranyl acetate.

Results

Digestion of whole L. stagnalis haemocyanin molecules

Digestion of whole haemocyanin molecules resulted in the appearance, within a few minutes, of a silky turbidity. There was an initial lag phase (see Fig. 1, inset), and, after a period during which the turbidity increased, a plateau was reached. It was shown by electron microscopy that the aggregated material exists as tubules some 20–30 times the length of the native haemocyanin molecule (Plate 1, and Wood, 1977). As shown in Plate 1, in the undigested molecule some structure can be seen at the ends of the cylindrical (native) haemocyanin molecules. In contrast, after digestion, when molecules are seen end-on, this structure, presumably the collar of the molecule, has disappeared.

Aggregate formation occurred only above a minimum haemocyanin concentration of about 0.8 mg/ml (Fig. 1). This 'critical concentration', c_c , may be determined by extrapolating either the plateau turbidities or the initial rate of development of turbidity to zero turbidity. The former is formally more precise, but the latter was in practice the more reliable way to make the extrapolation in the present case. The existence of a critical concentration suggested that polymerization took place by a process similar to that described by Oosawa & Higashi (1967), and which occurs with tubulin (Lee & Timasheff, 1977). It can be shown that for such a system the observed critical concentration approximates to the reciprocal of the association constant, K_p , for the addition of a monomeric unit to a growing tubular or helical polymer. In the present work the molecular weight of the polymerizing unit is uncertain, but is likely to be that of whole molecules from which a number of oxygen-binding domains (i.e. collar) have been removed by the proteolysis, say 8×10^6 instead of 9×10^6 . Making this assumption we may use the association constant, K_p , to calculate the apparent free energy of polymer propagation, ΔG_p^0 . Under the conditions used for estimating the critical polymerizing concentration (Fig. 1), a value of very approximately -40 kJ/mol could be calculated for ΔG_p^0 .

Removal of the tubular aggregates from the digestion mixture by centrifugation left in the supernatant solution a species of comparatively low molecular weight along with proteolytic enzyme and small peptides, which were separated by gel filtration. The molecular weight of the haemocyanin fragment in the supernatant (collar) was 100 000–120 000, by

Fig. 2. SDS/polyacrylamide-gel electrophoresis with *L. stagnalis* haemocyanin and fragments produced by proteinase digestion. Slab gel electrophoresis was by the method of Laemmli (1970), by using 5% acrylamide with an acrylamide/methylene-bisacrylamide ratio of 37.5:1 (w/w), and stained with Coomassie Brilliant Blue. Samples (30–50 μ l) were applied containing approx. 10 μ g of protein. Track 1, native haemocyanin; track 2, fraction P1; track 3, fraction P2; track 4, fraction P3; track 5, collar fragment; track 6, dissociated aggregate. The scale at the side is derived from a set of marker proteins of known molecular weight.

sedimentation equilibrium, suggesting that it is composed of two oxygen-binding domains. The observed sedimentation coefficient of 6.5S (uncorrected) is in reasonable agreement with this molecular weight. However, the molecular weight obtained by gel filtration on a calibrated Sephadex column was 80000. This is possibly explained by the fact that haemocyanin is a glycoprotein (Hall *et al.*, 1977), or that partial dissociation to two domains of mol.wt. 50000–60000 took place on the column, or by shape factors (see the Discussion section), or all of these. Analysis by detergent/gel electrophoresis revealed one major band along with a minor band of slightly higher mobility (Fig. 2). The molecular weights assigned to these bands, based on calibration of the gel by using proteins of known molecular weight, were 65000 (major) and 60000 (minor).

Concentrated preparations of collar fragment had a strong purple colour, in contrast with the blue of native haemocyanin, and this was associated with an absorption maximum at 540nm, compared with 570nm for undigested haemocyanin. In addition, the absorption maximum in the near u.v. was shifted from 346 to 342nm. The absorption spectrum of dissociated aggregate was practically identical with that of the native protein.

Fig. 3 shows the c.d. spectra of native *L. stagnalis* oxyhaemocyanin, collar and aggregate in the visible and near-u.v. regions. To obtain a spectrum of the aggregate, the material was dissociated by removing Ca^{2+} ions. The c.d. of the native oxyhaemocyanin was closely similar to that of other gastropod haemocyanins (Nickerson & Van Holde, 1971; Bannister & Wood, 1972) and in this region could be well fitted by four Gaussian components with maxima at 692 (+), 563 (–), 484 (+) and 349nm (–). The c.d. data, including intensities and rotational strengths, are summarized in Table 1. In contrast, the c.d. of the collar fragment did not show negative extrema in the 560nm region, and over the region 400–700nm could be fitted by two (positive) Gaussian components centred at 604 and 493nm. Although the second of these resembled band III of native oxyhaemocyanin in intensity and λ_{max} , it was necessary to use a further small, negative Gaussian component centred at 398nm to fit the spectrum. The major negative band (IV) had a similar intensity

Fig. 3. Visible- and near-u.v.-region c.d. spectra of *L. stagnalis* haemocyanin and the products from the digestion of whole molecules

Composite spectra for a number of path lengths and protein concentrations are shown. The vertical scale is marked in divisions of ellipticity units of 5×10^{-3} degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ with the zero line displaced by ± 10 units for clarity; horizontal scale is linear in wavenumber. The Gaussian components fitted by a computer procedure are shown as dashed lines. (b) Native haemocyanins (undigested); (c) collar fragment; (a) tubular aggregate (dissociated to avoid problems with turbidity). All were in the oxy form and ellipticities were calculated on the basis of one Cu atom (mol.wt. 25000).

to that in native oxyhaemocyanin, but was centred about 6nm further towards the u.v. (cf. the absorption

EXPLANATION OF PLATE I

Electron micrograph of L. stagnalis haemocyanin and tubular aggregates produced by tryptic digestion

(a) Native haemocyanin. The cylindrical molecule, dimensions approx. 30nm diam. by 32nm height, shows evidence of a 'collar' (see the text) in most instances (arrowed). Occasionally molecules can be seen which appear to lack collar. These are thought to be one-half molecules which happen to have landed 'upside down', revealing the inner cavity of the molecule. (b) and (c) show the tubular aggregates at different magnifications, in which it is virtually impossible to see where one molecule ends and the next one begins. (d) In such a digest, molecules which happen to land so that they can be seen end-on now lack collar (arrowed).

Table 1. Gaussian analysis of the c.d. spectra of *Lymnaea haemocyanin* and digestion products

All the samples were in the oxy form, and P1, P2 and P3 represent fragments from digestion of one-tenth haemocyanin molecules with plasmin (see the text). The molecular-weight basis was 25 000 (=1 Cu atom) throughout, and by making the assumption that the A_{280}/g of protein had not been changed by digestion. Values given are means $\pm$ S.E.M.

	Band	λ_0 (nm)	σ_0 (cm ⁻¹)	δ (cm ⁻¹)	$\Delta\epsilon_0$ (litre.mol ⁻¹ .cm ⁻¹)	$10^{39} \times R$ (c.g.s. units)
Native oxyhaemocyanin	I	692	14440 $\pm$ 178	818 $\pm$ 163	0.92 $\pm$ 0.05	2.56 $\pm$ 0.53
	II	563	17750 $\pm$ 118	876 $\pm$ 138	-0.79 $\pm$ 0.15	-1.92 $\pm$ 0.47
	III	484	20650 $\pm$ 98	1726 $\pm$ 91	2.57 $\pm$ 0.05	1.06 $\pm$ 0.09
	IV	349	28665 $\pm$ 14	1970 $\pm$ 17	-9.26 $\pm$ 0.06	-3.12 $\pm$ 0.03
Collar	Ia	604	16560 $\pm$ 42	693 $\pm$ 44	0.47 $\pm$ 0.02	0.10 $\pm$ 0.01
	III	493	20280 $\pm$ 17	1410 $\pm$ 20	2.96 $\pm$ 0.02	1.01 $\pm$ 0.02
	IIIa	398	25100 $\pm$ 159	1540 $\pm$ 169	-0.82 $\pm$ 0.06	-0.24 $\pm$ 0.03
	IV	343	29140 $\pm$ 19	1820 $\pm$ 15	-10.60 $\pm$ 0.04	-3.23 $\pm$ 0.03
Aggregate	I	695	14380 $\pm$ 383	845 $\pm$ 385	0.41 $\pm$ 0.05	1.19 $\pm$ 0.56
	II	569	17560 $\pm$ 387	920 $\pm$ 387	-0.31 $\pm$ 0.11	-0.78 $\pm$ 0.43
	III	477	20960 $\pm$ 523	1500 $\pm$ 843	0.66 $\pm$ 0.05	2.30 $\pm$ 1.31
	IIIb	429	23300 $\pm$ 389	770 $\pm$ 490	0.29 $\pm$ 0.35	0.47 $\pm$ 0.64
Fraction P1	IV	347	28840 $\pm$ 14	2060 $\pm$ 15	-6.19 $\pm$ 0.03	-2.17 $\pm$ 0.02
	I	680	14700 $\pm$ 96	600 $\pm$ 103	0.61 $\pm$ 0.03	0.12 $\pm$ 0.02
	II	572	17480 $\pm$ 50	1031 $\pm$ 66	-0.85 $\pm$ 0.03	-0.25 $\pm$ 0.02
	III	461	21690 $\pm$ 51	1600 $\pm$ 65	1.17 $\pm$ 0.03	0.42 $\pm$ 0.02
Fraction P2	IV	350	28600 $\pm$ 8	1890 $\pm$ 9	-8.08 $\pm$ 0.03	-2.61 $\pm$ 0.02
	I	681	14680 $\pm$ 165	820 $\pm$ 181	0.42 $\pm$ 0.03	0.12 $\pm$ 0.03
	II	564	17710 $\pm$ 74	730 $\pm$ 86	-0.62 $\pm$ 0.07	-0.13 $\pm$ 0.02
	III	486	20554 $\pm$ 48	1570 $\pm$ 50	2.20 $\pm$ 0.03	0.82 $\pm$ 0.02
Fraction P3	IV	347	28790 $\pm$ 10	1990 $\pm$ 10	-8.57 $\pm$ 0.04	-2.90 $\pm$ 0.02
	I	669	14960 $\pm$ 583	568 $\pm$ 676	0.22 $\pm$ 0.44	0.04 $\pm$ 0.09
	II	644	15540 $\pm$ 6770	1656 $\pm$ 3566	-0.12 $\pm$ 0.30	-0.06 $\pm$ 0.20
	III	508	19680 $\pm$ 189	975 $\pm$ 206	1.06 $\pm$ 0.10	0.26 $\pm$ 0.06
	IIIb	466	21590 $\pm$ 320	758 $\pm$ 180	0.53 $\pm$ 0.19	0.09 $\pm$ 0.04
	IV	351	28460 $\pm$ 11	2055 $\pm$ 12	-6.93 $\pm$ 0.03	-2.45 $\pm$ 0.02

spectrum). In spite of these shifts in spectrum, the collar fragment was still capable of binding oxygen (Wood, 1977).

The c.d. of dissociated aggregate in the region 400–700nm is also shown in Fig. 3. It is somewhat reminiscent of that of native oxyhaemocyanin, except that an extra positive Gaussian band (IIIb) centred at 429nm was required for the fitting, albeit a poor one. It is possible that in the native protein this positive band wholly or partly neutralizes the negative band (IIIa) required to fit the collar c.d. spectrum in this region of the spectrum. The relative intensities of bands I–IV of the c.d. spectrum of the aggregate were different from those in the spectrum of native oxyhaemocyanin, and overall the intensities were smaller.

In the 250–300nm region the c.d. spectrum of native oxyhaemocyanin was again very similar to that observed with other gastropod haemocyanins (Fig. 4). The main features are an intense positive band centred at about 252nm, and a less-intense positive doublet in the 280–290nm region. Although collar fragment showed the same general features as this, the intensities of the bands were much lower; the band at 280nm was considerably weaker than that at approx. 270nm. Collar could therefore be

distinguished quite clearly by its c.d. spectrum in this region, from native haemocyanin, as well as from the products of digestion of one-tenth molecules (see below).

Dissociated aggregate sedimented in the analytical ultracentrifuge as a mixture of components with major sedimentation coefficients approx. 6.5 and 8.5S (Fig. 5), but, when subjected to gel filtration on Sephadex G-150, a pattern of three peaks was observed. The peak of intermediate molecular weight (see Fig. 6) was decreased in area in comparison with the pattern of three peaks obtained by gel filtration of the products from the digestion of one-tenth molecules. This indicated that the collar fragment was a component of the intermediate peak, having been separated in this case from the tubular aggregate by the preliminary ultracentrifugation step. SDS/polyacrylamide-gel electrophoresis seemed to confirm that this was the case (Fig. 2).

Digestion of L. stagnalis haemocyanin one-tenth molecules

At pH values in excess of pH 8.0 in the absence of Ca²⁺ or Mg²⁺ ions, *L. stagnalis* haemocyanin forms one-tenth molecules, which in SDS/polyacrylamide-

gel electrophoresis dissociated further and moved as a single band with apparent mol.wt. about 250000. This would suggest a five-domain structure for the polypeptide chain of this haemocyanin.

Fig. 4. U.v.-region c.d. spectra for *L. stagnalis* haemocyanin and the proteinase-digestion products

Conditions were as in Fig. 3. Spectra: —, native (undigested) haemocyanin; ····, collar fragment; ----, fraction P1; - · - · - ·, fraction P2; - - - -, fraction P3. Scale is linear in wavelength. All were in the oxy form.

Fig. 5. Sedimentation of tubular aggregate after dissociation

Conditions: protein concentration approx. 4 mg/ml in 50 mM-borate/NaOH buffer, pH 9.2, containing 50 mM-NaCl, at 22°C. Speed, 52640 rev./min, 12 mm cell, direction of migration left to right, schlieren phase-plate angle 70°; picture taken after 88 min of sedimentation. Uncorrected sedimentation coefficients were 6.5 and 8.5 S.

Digestion of *L. stagnalis* haemocyanin one-tenth molecules followed by gel filtration yielded three fractions (Fig. 6) corresponding to apparent mol.wts. 127000–130000, 80000–90000 and 50000. These are referred to as T1, T2, T3 (in order of decreasing molecular weight) if derived from a tryptic digest, and P1, P2, P3, if derived from a plasmin digest, respectively. All three fractions were capable of binding oxygen reversibly.

The c.d. spectra of the three fractions differed from one another, especially in the visible region (Fig. 7), but there were also differences in the u.v. region (Fig. 4). The highest-molecular-weight fraction (P1 or T1) had a c.d. spectrum closely similar to that of undigested haemocyanin, with alternating positive and negative extrema at 680 (+), 572 (–), 486 (+) and 350 nm (–). The spectrum was well fitted by four Gaussian components whose intensities and relative intensities were similar to but not identical with those in the spectrum of native oxyhaemocyanin. Fraction P2 (which is believed to contain collar) showed the first two of these bands, but with decreased intensities, whereas the third (+) band had almost double the intensity and was shifted approx. 15 nm into the red. The spectrum of fraction P3 showed the first two bands in the visible region at lower intensities, whereas the third band required two positive components (III and IIIb) in order to obtain a fit, albeit a poor one. Although this general shape of band III plus IIIb is reminiscent of that observed in the c.d. of the dissociated aggregate (Fig. 3), the maxima were at 508 and 466 nm, instead

Fig. 6. Fractionation of digestion products on Sephadex G-150

Dimensions of columns were 1.8 cm × 125 cm; flow rate was 15 ml/h. Buffer was 50 mM-Tris/HCl, pH 8.2, containing 50 mM-NaCl; fraction volume was approx. 2 ml. ▲, Fractionation of products derived from the digestion of one-tenth haemocyanin molecules with plasmin (plasmin/haemocyanin ratio 1:33, w/w); △, fractionation of dissociated tubular aggregate; ■, purified collar fragment.

Fig. 7. Visible- and u.v.-region c.d. spectra of fractions resulting from plasmin digestion of one-tenth haemocyanin molecules

Details were as in Fig. 3. Fractions are numbered in order of decreasing size after separation on Sephadex G-150: thus P3 is the smallest fraction, of mol.wt. approx. 50000. Gaussian components are shown as dashed lines.

of at 477 and 429 nm. In addition, the c.d. spectra of fractions P1 and P3 showed inflections near to 300 nm not observed in the spectrum of fraction P2. At this stage it is not possible to make anything more than a crude comparison of these spectra. Apart from making the (probably unjustified) assumption that all the fractions have the same A_{280} , it must also be recognized that the fractions are not pure species, as was revealed by detergent/gel electrophoresis (see below). The fitting of Gaussian bands is also to some extent an artificial procedure, and the errors (Table 1) show that some of the fits are comparatively poor. It does, however, provide a way of classifying the spectra and of identifying or distinguishing the different components resulting from digestion procedures.

Fractions P1 and T1 had a molecular weight by sedimentation equilibrium of approx. 150000 (cf. 127000–130000 by gel filtration) and by this criterion appeared to be pure in that a straight line was obtained in a plot of log (fringe displacement) versus

(radial distance)². They would therefore appear to be composed of three linked oxygen-binding domains of mol.wt. 50000, the links between which are not susceptible to digestion by the proteolytic enzymes used. In detergent/polyacrylamide-gel electrophoresis two major bands were found with apparent mol.wts. 140000 and 90000, as well as a minor band at about 65000. It is thus possible that a three-domain and a two-domain unit are present, but are in close association in the undenatured material.

Fractions P2 and T2 had apparent mol.wts. of 90000–100000 by sedimentation equilibrium and somewhat lower ones by gel filtration, but it is known that this fraction contains the collar fragment as well as other component(s). In detergent/gel electrophoresis, three major bands were observed with apparent mol.wts. 98000, 65000 and 62000. The two lower molecular weights are those observed for collar fragment obtained by digestion of whole haemocyanin molecules, although it may be noted that the relative intensities of the two bands were not the same. Thus, in the collar fragment the 65000-mol.wt. band was much the stronger, whereas in fraction P2 the 60000-mol.wt. band was slightly the more intense (see Fig. 2). It seems fairly certain therefore that there is a two-domain unit present in this fraction (mol.wt. 98000), but that collar is also present, perhaps digested in a slightly different way.

Fractions P3 and T3 had mol.wts. of about 50000 by both sedimentation equilibrium and gel filtration and would seem to be single-domain structures. However, the c.d. spectra (Fig. 7) were peculiar and a doublet of bands was observed in detergent/gel electrophoresis. The apparent molecular weights corresponding to these bands were 51000 and 53000.

Discussion

The structure of the gastropod haemocyanin molecule presents an intriguing problem. Although chemical analysis shows that the functional unit is that weight of protein containing 2g-atoms of copper, namely approx. 50000g, discrete fragments of mol.wt. 50000 may only be obtained by limited proteolytic action (Lontie *et al.*, 1973; Pearson & Wood, 1974). Otherwise all the available evidence points to a minimum polypeptide-chain mol.wt. of between 200000 and 300000 (Wood & Peacocke, 1973; Brouwer & Kuiper, 1973), which would correspond to four to six functional domains. The minimum polypeptide-chain molecular weight of *L. stagnalis* haemocyanin appears to be of the order of 250000, in agreement with values for other haemocyanins. Quittner *et al.* (1978) found that the smallest subunit from *Busyon* haemocyanin had mol.wt. 300000 by sedimentation equilibrium, but 250000 by SDS/polyacrylamide-gel electrophoresis. It seems highly probable that there are different

types of domain within single polypeptide chains, such as the 'collar domains', with distinct properties. The present work has been aimed at dissecting the molecule with proteolytic enzymes and trying to characterize the products. Notable success has been achieved by using this technique with immunoglobulins.

Of the gastropod haemocyanins so far studied, only two or three, including those from *Lymnaea* species, and the β -type of haemocyanin from *Helix pomatia* (Van Breemen *et al.*, 1975), form tubular aggregates after proteinase digestion. Under similar conditions other gastropod haemocyanins are simply digested to smaller fragments. We have partially characterized the process of indefinite polymerization which occurs when the collar fragment is removed. Our preliminary studies allow the calculation of a value for ΔG_p^0 of about -40 kJ/mol. By comparison, Lee & Timasheff (1977) reported a value for ΔG_p^0 for tubulin polymerization of about -31 kJ/mol. Any interpretation of the process observed with *L. stagnalis* haemocyanin must await further studies of the polymerization reaction.

One of the major problems in separating and characterizing the products of limited proteinase digestion of *L. stagnalis* haemocyanin has been the uncertainty in molecular-weight values obtained by SDS/polyacrylamide-gel electrophoresis and gel filtration. This may result from the fact that *L. stagnalis* haemocyanin is a glycoprotein and may bind SDS anomalously in comparison with 'standard' proteins (Quittner *et al.*, 1978; Salvato & Zatta, 1978). Added to this is the general problem, examined by Tanford's group (Nozaki *et al.*, 1976), of using such methods to estimate the size of large asymmetric particles. They found, for example, that native fibrinogen, which has a true Stokes radius of 10.8 nm, appeared to have a radius of only 7.1 nm as determined by gel filtration based on calibration with globular proteins. Possibly asymmetric particles are retarded on the column because of end-on insertion into the gel pores. It is significant that the haemocyanin fragments, with the exception of the smallest (mol.wt. 50000), all appeared to have lower molecular weights by gel filtration than by sedimentation equilibrium. One could envisage a protein composed of a number of roughly globular domains held loosely together by stretches of more flexible polypeptide chains like a string of beads, behaving as Nozaki *et al.* (1976) suggest, leading to underestimates of their Stokes radius and hence molecular weight. The electron micrographs by Siezen & Van Bruggen (1974) give much support to the string-of-beads structure for dissociated haemocyanin. The smallest unit (mol.wt. 50000) appeared to behave normally, and even this separated into two closely running bands on detergent/gel electrophoresis. It might nevertheless be expected that this unit would

be a single, globular structure, and this seems essentially to be confirmed. Other fractions that were uniform in size by repeated gel filtration on Sephadex G-150 were observed to be made up of a number of components by SDS/polyacrylamide-gel electrophoresis. Although one ought to be able to classify fractions as one-domain (mol.wt. 50000), two-domain (100000), and so on, on the basis of the copper-to-protein ratio, the uncertainty in the molecular-weight values has made this somewhat difficult.

We may compare the findings with *L. stagnalis* with those of Gielens *et al.* (1975) with *H. pomatia* β -type haemocyanin. It has been shown (Hall *et al.*, 1975) that *L. stagnalis* haemocyanin behaves in some ways as a β -type haemocyanin. However, the digestion procedure and the fractionation systems used by Gielens *et al.* (1975) were somewhat different from those used in the present work. Indeed, they may be criticised inasmuch as they used Sephadex G-100 for the initial fractionation of digestion products, and also that molecular weights were calculated from sedimentation coefficients by the use of an empirical formula, rather than by sedimentation-equilibrium methods. In view of what has been said above about the probable asymmetric and flexible structure of most of the fragments, it seems unlikely that calculations from sedimentation coefficients could yield reliable molecular-weight values. The initial fractionation on Sephadex G-100 of digestion products of *H. pomatia* β -type haemocyanin resulted in separation into one large peak corresponding to an apparent mol.wt. of about 100000, and two very much smaller peaks corresponding to apparent mol.wts. approx. 75000 and 50000 respectively. In our work with *L. stagnalis* haemocyanin, the fractionation on Sephadex G-150 allowed separation of the heavier components resulting from the digestion with apparent mol.wts. approx. 130000 and 80000–90000. In comparison with the *H. pomatia* work, however, it is striking that the low-molecular-weight fraction resulting from the digestion of *L. stagnalis* haemocyanin one-tenth molecules, having a fairly clear mol.wt. of 50000, made up over one-quarter of the total protein. With *H. pomatia* β -haemocyanin the corresponding fraction represented only about 10% of the total protein applied to the Sephadex G-100 column. It is not clear whether digestion had proceeded to completion. Gielens *et al.* (1975) subjected their high-molecular-weight fraction to a further separation by ion-exchange chromatography on DEAE-cellulose and so obtained three components which they called T1A, T1B and T1C. By comparing the c.d. spectra of these with the c.d. spectra recorded for the *L. stagnalis* fractions, it is possible to identify their intermediate fraction (T1B) with the collar fragment obtained in the present work by digestion of whole haemocyanin molecules.

Fraction T1B had mol.wt. approx. 110000, and dissociated into smaller units with apparent mol.wt. 66000 in detergent/polyacrylamide-gel electrophoresis. We have not isolated the collar fraction after digestion of one-tenth *L. stagnalis* molecules, but we identify it as a component of the intermediate peak in gel filtration. It is also possible to conclude that fraction T1C of Gielens *et al.* (1975) is similar to our fraction P3 as judged by the c.d. spectra. Here again, however, the molecular weights were discrepant. Whereas fraction P3 from *L. stagnalis* had mol.wt. approx. 50000 or a little greater by all the methods used, Gielens *et al.* (1975) obtained a value of 105000, even in detergent/polyacrylamide-gel electrophoresis. It will be recalled that our fraction P3 in fact ran as two components on detergent/polyacrylamide-gel electrophoresis with apparent mol.wts. 53000 and 51000. The possibility of interactions between domains leading to association even in the presence of SDS cannot be ruled out. Such interactions have been observed with certain membrane proteins.

The present findings are also relevant to c.d. measurements undertaken to try to investigate the structure of the copper-oxygen moiety in haemocyanins. Gastropod as well as cephalopod haemocyanins have been studied from this point of view by several groups. Nickerson & Van Holde (1971) published c.d. data for the haemocyanins of *Busycon canaliculatum* and *Loligo pealei*, Takesada & Hamaguchi (1968), Tamburro *et al.* (1976) and Van Holde (1967) for that from *Octopus*, Bannister & Wood (1972) for that from *Murex trunculus*, and Gielens *et al.* (1975) for that from *H. pomatia*. In terms of general shape of spectra and rotational strengths etc., the present observations on native *L. stagnalis* haemocyanin agree very well, and the general significance of the observed bands in relation to electronic transitions in the copper-oxygen complexes cannot be disputed. It is, however, clear that the observed spectra for native haemocyanin represent the sums of spectra for individual components or domains whose c.d. spectra differ from one another. Indeed Gielens *et al.* (1975) summed the c.d. spectra for their fragments, appropriately weighted, and were able to arrive at the spectrum of the native material to a very good approximation. This strongly suggests that the differences referred to already exist in the subunits in the native haemocyanin and do not result from changes occurring during the digestion process. It would seem appropriate therefore that, when c.d. studies are undertaken with the aim of obtaining information about the state of the copper atoms in the copper-oxygen complex, such as the magnetic-c.d. studies of Mori *et al.* (1975), account should be taken of the fact that the observed spectra are almost certain to be mixtures of spectra of the different types of oxygen-binding domains present.

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