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Proteolytic Fragmentation of *Helix pomatia* α-Hemocyanin: Structural Domains in the Polypeptide Chain†

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ABSTRACT: α-Hemocyanin from the Roman snail *Helix pomatia* is composed of polypeptide chains with a molecular weight of 360 000 ± 30 000. The cylindrically shaped hemocyanin molecule contains 20 of these large chains. The polypeptide chain has been split into components with molecular weights of: 210 000, 154 000, 147 000, 112 000, 120 000, 98 000, 55 000, and 50 000, by gentle proteolysis with enzymes of different specificities. Most of the fragments have molecular weights which are about 50 000 or a multiple of 50 000. Departure from these values, as found in the 112 000 and 120 000 fragments, is probably caused by the high carbohydrate content of these components. A mixture of these fragments has the same oxygen binding properties as the nondigested protein. Subtilisin converts the hemocyanin polypeptide chain, under appropriate conditions, almost completely into fragments of 50 000 and 55 000 daltons with conservation of the oxygen binding properties of the nondigested protein. We conclude from these studies that the polypeptide chain of *Helix pomatia* α-hemocyanin is folded into about seven compact tertiary structures, which are covalently interconnected. This chain of structural domains has been visualized (Siezen and Van Bruggen (1974), *J. Mol. Biol. 90,* 77–89) by electron microscopy, which shows 1/20 hemocyanin molecules to be flexible structures consisting of 7–8 apparently spherical units of 55–60 Å diameter.

The α-hemocyanin of the Roman snail, *Helix pomatia,* is a copper containing, oxygen binding protein, with a molecular weight of approximately 8–9 × 10^6 at neutral pH. A stepwise dissociation of the native molecule into successively 1/2-, 1/10-, and 1/20-size molecules occurs on mild changes in pH, ionic strength, etc. (Konings et al., 1969a; Elliot et al., 1972; Siezen and Van Driel, 1974). Brouwer and Kuiper (1973), who studied the molecular weight of the hemocyanin polypeptide chain, have suggested that this chain consists of separated, compact tertiary structures, so-called structural domains, covalently interconnected by more exposed stretches of the polypeptide chain, as has been found for immunoglobulins (Edelman et al., 1970; Poljak et al., 1972; Schiffer et al., 1973) and bovine serum albumin (Adkins and Foster, 1965; Pederson and Foster, 1969; King and Spencer, 1970). This hypothesis was supported by Siezen and Van Bruggen (1974) who showed by electron microscopy 1/20 hemocyanin molecules to occur as a linear chain of seven to eight globules. Each structural domain is thought to contain one oxygen binding site. If this hypothesis is correct, it may be possible to prepare biologically

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active parts of the polypeptide chain by subjecting the protein to limited enzymatic proteolysis. The loose connecting parts are accessible to the proteases and will be digested at a fast rate, whereas the compact domains will be digested more slowly or not at all. In this paper we show that this formation of large fragments constitutes a proteolytic surgery that produces little debris and preserves the separated parts in their native conformation.

Methods

(*a*) *Preparation of α-Hemocyanin.* α-Hemocyanin of *Helix pomatia* was isolated and stored according to Konings et al. (1969b) as modified by Siezen and Van Driel (1973).

(*b*) *Determination of Protein Concentration.* Protein concentration was determined as described previously (Konings et al., 1969b).

(*c*) *Digestion of α-Hemocyanin with Proteolytic Enzymes.* Hemocyanin molecules (1/10; 5 mg/ml) in 50 mM Tris-HCl-NaCl buffer, pH 8.2, ionic strength 0.1, were incubated at 30 °C with one of the following enzymes: trypsin (pig, three times crystallized, Worthington), chymotrypsin (bovine pancreas, Worthington), thermolysin (three times crystallized, A grade Calbiochem), and subtilisin de Novo (Novo industry). Unless stated otherwise, the enzyme to hemocyanin ratio was 1:1000 (w/w). Samples were taken after 60 min and inhibited as described below. When using trypsin or chymotrypsin as proteolytic enzymes, the digestion was stopped by diluting the samples at room temperature and at 100 °C directly (1:1) with 50 mM Tris-HCl-NaCl buffer, pH 8.0, ionic strength 0.1, containing 2% sodium dodecyl sulfate, 2% mercaptoethanol, and 50 mM EDTA.[1]

Subtilisin was inhibited by addition of a tenfold molar excess of diisopropyl fluorophosphate over subtilisin. Thirty minutes after addition of the inhibitor, the samples were diluted as described above. After dilution the samples were incubated for 2 h at 37 °C and then dialyzed against 10 mM Tris-acetate, pH 8.0, ionic strength 0.01, containing 0.1% sodium dodecyl sulfate.

Thermolysin was inhibited by diluting the samples (1:1) with 0.2 M citrate, pH 3.5, containing 2% sodium dodecyl sulfate, 2% mercaptoethanol, and 50 mM EDTA. Under these conditions the samples precipitated. After incubating for 1 h at room temperature, the samples were heated at 110 °C for 3 min and then dialyzed against Tris-acetate, pH 8.0, ionic strength 0.01, containing 0.1% sodium dodecyl sulfate and 1% mercaptoethanol until the samples were completely dissolved. The denatured samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, to determine the molecular weight of the components present in the various proteolytic digests.

(*d*) *Sodium dodecyl sulfate–polyacrylamide gel electrophoresis* was performed essentially as described previously (Brouwer and Kuiper, 1973), using gel concentrations ranging from 3.5 to 6% with 2.5% cross-linking. Gels were calibrated with carboxymethylated samples of chymotrypsinogen A, ovalbumin, bovine serum albumin, phosphorylase A, β-galactosidase and with carboxymethylated samples of dimethylsuberimidate cross-linked ovalbumin and serum albumin (Carpenter and Harrington, 1972). Gels were stained for proteins with a solution of 0.5% amido black in 7% acetic acid. For glycoproteins the periodic acid–Schiff reagent was used as described by Zacharius (1969), with the following modifications. After fixation in trichloroacetic acid, the gels were extensively washed with 7% acetic acid for at least 24 h. After treatment with periodic acid, the gels again were washed for 24 h with distilled water. The gels were scanned at 675 nm for amido black and at 560 nm for the periodic acid–Schiff stain. The mobility of marker proteins relative to chymotrypsinogen A was plotted vs. the natural logarithm of their molecular weight.

The free electrophoretic mobility of the proteolytic fragments and of albumin and ovalbumin was calculated from Ferguson plots, obtained by plotting the values of the logarithm of the relative mobility at five different gel concentrations vs. the gel concentration (Ferguson, 1964; Banker and Cotman, 1972).

(*e*) *Analytical Ultracentrifugation.* Sedimentation velocity experiments were carried out as described by Konings et al. (1969a).

(*f*) *Measurement of the Oxygen Binding Properties.* Protein was incubated overnight in the presence of tenfold molar excess of hydroxylammonium chloride over copper to regenerate possibly aged protein (Van Driel, 1973). Hydroxylammonium chloride was removed by dialysis against 50 mM Tris-HCl-NaCl, pH 8.2, ionic strength 0.1. Oxygen binding curves were determined in this buffer as described by Konings et al. (1969b).

(*g*) *Amino Acid Analysis.* Amino acid analyses were performed with a Technicon Sequential Multisample amino acid analyzer on protein samples hydrolyzed in 6 N HCl at 110 °C in vacuo for 24 h.

(*h*) *Isoelectric Focusing.* Isoelectric focusing in thin layers of polyacrylamide gels was carried out as described by Bours (1971). "Ampholine" carrier ampholytes, pH range 3–10, were used. The pH gradient was directly measured with a combined microsurface glass electrode (Ingold, A. G., Zürich, Type 403-30-M8).

Results

(a) *Molecular Weights of the Proteolytic Fragments Obtained by Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis.* When plotting the relative mobilities of the standard proteins on 4% polyacrylamide gels vs. the natural logarithm of their molecular weight, a straight line, with a correlation coefficient of −0.998, was obtained. Ferguson plots of the marker proteins ovalbumin and serum albumin, together with the hemocyanin polypeptide chain and its proteolytic fragments present in a tryptic digest, showed straight lines. The free electrophoretic mobilities of the various protein–sodium dodecyl sulfate complexes, obtained by extrapolation to zero gel concentration, were approximately the same: 1.07 ± 0.06.

To determine the molecular weight of the intact hemocyanin polypeptide chain, hemocyanin was incubated for different times at different temperatures (up to 100 °C) in sodium dodecyl sulfate + 2-mercaptoethanol, with and without inhibitors of proteolytic enzymes, such as diisopropyl fluorophosphate and phenylmethanesulfonyl fluoride. All these methods gave the same molecular weight of the hemocyanin polypeptide chain, indicating that we are dealing with an intact polypeptide chain and not with a proteolytic fragment. The molecular weight of the polypeptide chain amounts to 360 000 ± 30 000.

Figure 1 shows a spectrophotometric tracing at 670 nm of sodium dodecyl sulfate–polyacrylamide gels obtained from a 60-min tryptic digest of 1/10 hemocyanin molecules. Further incubation had no effect on the digestion pattern, except for a very slow decrease of the 210 000 fragment, concomitant with a very slow increase of the 98 000 fragment. The molec-

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

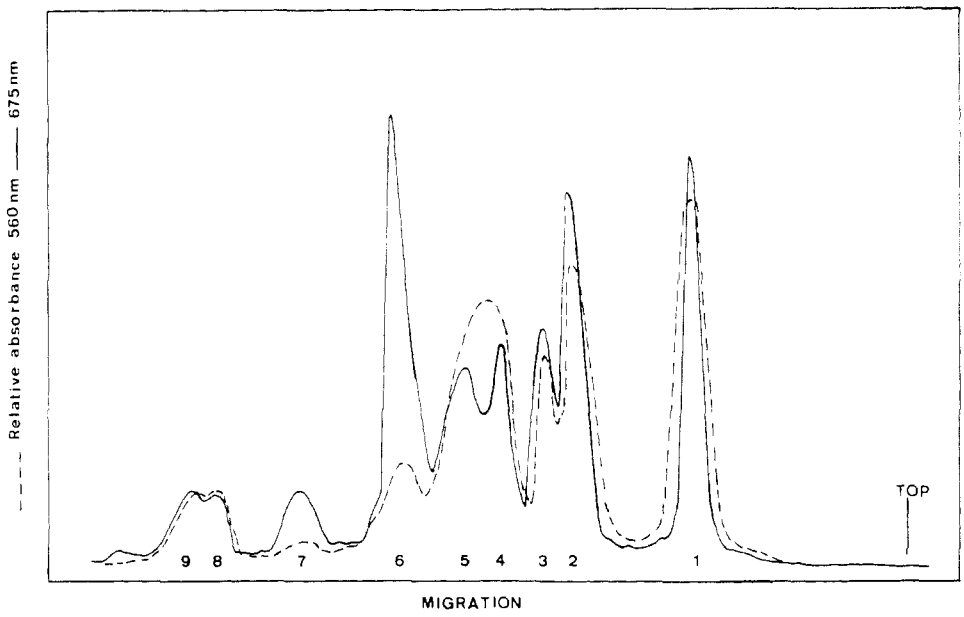

FIGURE 1: Spectrophotometric scan of the electrophoretic pattern on sodium dodecyl sulfate gels of the products formed during trypsin digestion. Hemocyanin molecules ($^1/_{10}$) at pH 8.2, ionic strength 0.1, were treated for 60 min at 30 °C with 0.1% trypsin (w/w). The molecular weights of the fragments are: (1) 210 000; (2) 154 000; (3) 147 000; (4) 120 000; (5) 112 000; (6) 98 000; (7) 73 000; (8) 55 000; (9) 50 000. (Full line) Protein; (broken line) glycoprotein.

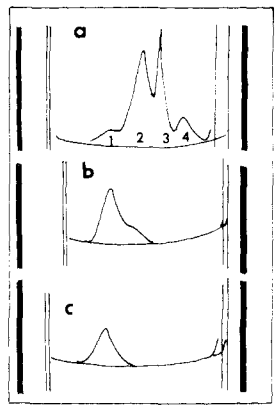

FIGURE 2: Sedimentation velocity runs of proteolytic hydrolysates of hemocyanin. (a) Hemocyanin molecules ($^1/_{10}$) at pH 8.2, ionic strength 0.1, were treated for 60 min with 0.1% trypsin (w/w) at 30 °C. The reaction was stopped by adding soybean trypsin inhibitor in a twofold excess (w/w) over trypsin: (1) 3.6 S; (2) 5.7 S; (3) 6.9 S; (4) 8.7 S. (b) Hemocyanin at pH 9.6, ionic strength 0.01, was treated with 1% (w/w) subtilisin, at 30 °C for 60 min. The reaction was stopped by adding a tenfold molar excess diisopropyl fluorophosphate over subtilisin. The digest was put directly in the ultracentrifuge cell ($s_{20,w}$ = 3.7 S). (c) Subtilisin digest, as prepared under b, after removal of the large material on Sephadex G-100 ($s_{20,w}$ value 3.6 S).

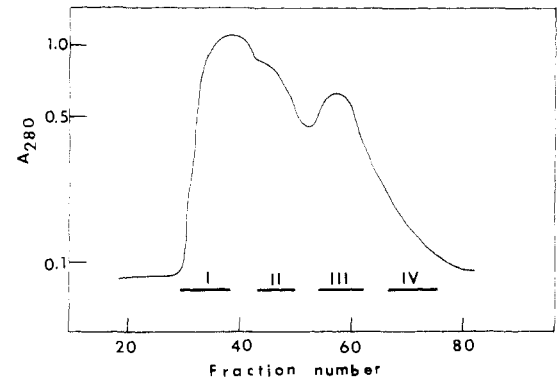

FIGURE 3: Gel filtration of a 60-min tryptic digest of $^1/_{10}$ hemocyanin molecules (Tris-HCl–NaCl, pH 8.2, ionic strength 0.1, protein concentration 30 mg/ml, 0.1% (w/w) trypsin) on Sephadex G-100 and G-200 (3.2 × 100 cm, connected in tandem). Fractions collected and concentrated are indicated by the bars I, II, III, and IV.

ular weights, averages of at least 15 determinations, of the fragments are: 210 000, 154 000, 147 000, 112 000, 120 000, 98 000, 73 000, 55 000, and 50 000.

Staining for glycoproteins reveals that the carbohydrate moiety of hemocyanin is not evenly distributed among the proteolytic fragments. Treatment of $^1/_{10}$ hemocyanin molecules with thermolysin, chymotrypsin, or subtilisin yields similar results.

(b) *Analytical Ultracentrifugation and Gel Filtration of a Tryptic Digest.* Figure 2a shows a Schlieren diagram obtained after ultracentrifugation of a 60-min tryptic digest. The digestion was stopped in this case by adding soybean trypsin inhibitor in twofold excess (w/w) over trypsin. The $s_{20,w}$ values (protein concentration 5 mg/ml) of the sedimenting boundaries are: 3.6, 5.7, 6.9, and 8.7 S, respectively. Figure 3 shows the corresponding elution pattern, obtained after gel filtration of the 60-min tryptic digest on two columns of Sephadex G-100 and G-200 (3.2 × 100 cm, connected in tandem). The 3.6, 5.7, 6.9, and 8.7 S boundaries could be purified from fractions IV, III, II, and I, respectively (Figure 3), by another gel filtration step, followed by ion-exchange chromatography on DEAE-Sephadex A-50 (1 × 60 cm), using a linear salt gradient generated from 250 ml of Tris-HCl–NaCl, pH 8.2, ionic strength 0.1, and 250 ml of this buffer containing 0.8 M NaCl. The boundaries with sedimentation coëfficients of 5.7 and 3.6 S correspond to components with molecular weights of 98 000 and 50 000–55 000, respectively, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figures 4a and 4B). The relative amounts, in molar units, of the fragments present in the 6.9 and 8.7 S boundaries (Figures 4c and 4d) were calculated by dividing the measured peak area of the fragments, expressed as percentage of the total peak area per gel, by their estimated molecular weights. The purified 6.9 S boundary contained the 154 000, 147 000, and 112 000–

120 000 fragments in equimolar amounts (Figure 4c). The 8.7 S boundary contained the 210 000, 154 000, and 112 000–120 000 fragments in equimolar amounts. Therefore we conclude that the main fragments present in a tryptic digest after sodium dodecyl sulfate denaturation are also present in the digest prior to sodium dodecyl sulfate denaturation. The contents of tubes 80–120 (Figure 3) were collected and lyophilized. The amounts of amino acids and peptides present in this lyophilisate were neglegible, indicating that no smaller fragments are formed during proteolysis.

(c) *Oxygen Binding Properties of the Tryptic Digest.* The Hill plot of the oxygen binding curve of a tryptic digest of $^1/_{10}$ molecules is a straight line. The slope of the plot (n = 0.83), the oxygen affinity, expressed as the oxygen pressure at half saturation (p_{50} = 5.2 mmHg), and the oxygen binding capacity, expressed as the difference between the optical density at 346 nm of oxy- and deoxyhemocyanin per mg of protein (ΔE_{346}/mg = 0.340–0.360), are identical with those of untreated $^1/_{10}$ molecules (Van Driel, 1974).

(d) *Complete Conversion of Hemocyanin to Fragments with a Molecular Weight of 50 000.* After incubation of $^1/_{20}$ hemocyanin molecules in ethanolamine buffer at pH 9.6, ionic strength 0.01, for 60 min with 1% (w/w) subtilisin at 30 °C, the analytical centrifuge shows one peak with a sedimentation coefficient of 3.7 S. The peak is asymmetrical toward the bottom of the ultracentrifuge cell (Figure 2b). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis shows a double band with estimated molecular weights of 55 000 and 50 000. Above the 55 000 band, a faintly colored smear is present, showing the presence of some heterogeneous material with molecular weights larger than 55 000. This large material can easily be removed by gel filtration on Sephadex G-100. The 50 000 molecular weight component obtained in this way sediments as a single boundary (Figure 2c), which yields upon isoelectric focusing at least 18 components with isoelectric points varying from 4.75 to 6.20. The oxygen binding curves of the subtilisin digest and the untreated hemocyanin at pH 9.6, ionic strength 0.01, were measured immediately after stopping the reaction with diisopropyl fluorophosphate and are straight lines. The Hill coefficient for both curves is identical, 0.86. The oxygen pressure at half saturation is 5 mm for the untreated protein and about 6.5 mm for the subtilisin digest. The oxygen binding capacity of the untreated protein (ΔE_{346}/mg) is 0.270, while that of the subtilisin digest is 0.230. Note that in this case the protein has not been regenerated with hydroxylammonium chloride to avoid any possible proteolysis by subtilisin during the prolonged regeneration procedure, possibly explaining the low oxygen binding capacities.

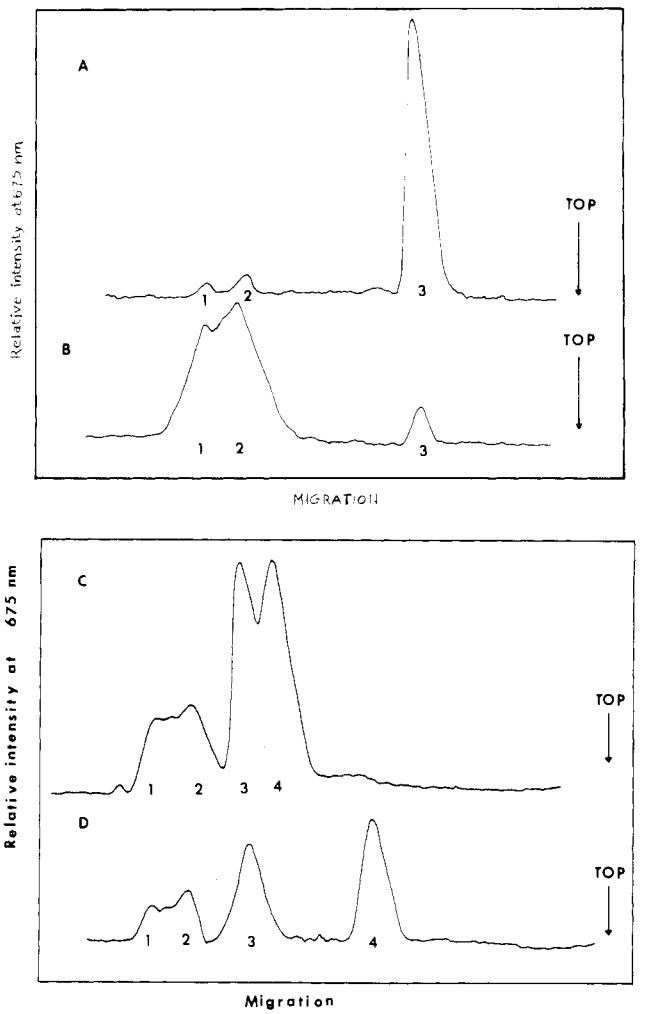

FIGURE 4: Spectophometric scans of the electrophoretic patterns on sodium dodecyl sulfate–polyacrylamide gels of fractions I–IV (Figure 3), after chromatography on DEAE-Sephadex. (a) Fraction III on a 7.5% polyacrylamide gel: (1) 50 000; (2) 55 000; and (3) 98 000. (b) Fraction IV on a 7.5% gel: (1) 50 000; (2) 55 000; (3) 98 000. (c) Fraction II on a 4% gel: (1) 112 000; (2) 120 000; (3) 147 000; (4) 154 000. (d) Fraction I on a 4% gel: (1) 112 000; (2) 120 000; (3) 147 000; (4) 210 000.

Discussion

Validity of Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis for Molecular Weight Determination of Hemocyanin and Its Proteolytic Fragments. The usual type of calibration curve used in sodium dodecyl sulfate–polyacrylamide gel electrophoresis is only approximately linear. The actual curves of log molecular weight vs. relative mobility are sigmoidal in nature (Neville, 1971). Therefore, extrapolations into ranges not covered by markers should be avoided. Since very few large molecular weight markers are readily available and their molecular weights are not known with great accuracy, we used covalently cross-linked ovalbumin and serum albumin as marker proteins. As we have found that these cross-linked markers fall on the same calibration curve as do chymotrypsinogen, ovalbumin, serum albumin, phosphorylase A, and β-galactosidase, it seems justified to use these cross-linked proteins as markers in gel electrophoresis. In this way we have been able to show that for 4% gels the calibration line was straight up to 400 000, completely covering the range of the molecular weights of hemocyanin and its proteolytic fragments. Although most proteins migrate according to their molecular weight on sodium dodecyl sulfate gels, some glycoproteins do not (Bretscher, 1971; Segrest et al., 1971). As hemocyanin is a glycoprotein (Dijk et al., 1970), the sodium dodecyl sulfate method for estimating polypeptide molecular weights may be questionable in this case. Since Segrest et al. (1971) have demonstrated that the anomalous behavior of glycoproteins during gel electrophoresis results from a decreased binding of sodium dodecyl sulfate, which leads to a very low free electrophoretic mobility (Banker and Cotman, 1972), we determined the free electrophoretic mobility of hemocyanin and its proteolytic fragments.

The values of the free electrophoretic mobilities of the intact hemocyanin polypeptide chain, its proteolytic fragments, ovalbumin and serum albumin, are very close to each other (1.07 ± 0.06), as has been found for at least 13 other protein–sodium dodecyl sulfate complexes (Neville, 1971; Banker and Cotman, 1972). Thus the basic assumption of a constant value

of the free electrophoretic mobility for marker and unknown protein–sodium dodecyl sulfate complexes, which is involved in calculating molecular weights from a value of the relative mobility in sodium dodecyl sulfate electrophoresis (Neville, 1971), appears to be valid for hemocyanin and its proteolytic fragments.

The most conclusive evidence that the molecular weights determined by sodium dodecyl sulfate gel electrophoresis are correct resides in the fact that sedimentation equilibrium yields the same value for the molecular weight of native $1/20$ molecules (365 000 ± 20 000; Siezen and Van Bruggen, 1974) as does gel electrophoresis (360 000 ± 30 000).

Molecular Weight of the Intact Polypeptide Chain. There is a clear discrepancy between the molecular weight of 360 000 ± 30 000 found for the intact polypeptide chain in these studies, and that of 265 000 determined in our previous studies (Brouwer and Kuiper, 1973). This difference may have been caused by proteolytic damage to the preparations used previously for molecular weight determinations in 6 M guanidine hydrochloride. These preparations were heterogeneous in sedimentation equilibrium. To obtain homogeneous preparations, the denatured protein was chromatographed in 6 M guanidine hydrochloride on Sepharose 4B. By this chromatography we may inadvertently have separated the intact polypeptide chain from a proteolytic fragment and, hence, have determined the molecular weight of a large fragment instead of the intact polypeptide chain. In this connection it is interesting to mention that we have observed a very rapid formation of a fragment with a molecular weight of 255 000 during the time course of trypsinolysis. The previous value of 300 000 daltons determined by sodium dodecyl sulfate gel electrophoresis is also too low since it was determined on 5% gels for which our present studies show the calibration curve to be linear only up to 250 000. Therefore, we conclude that the molecular weight of the intact polypeptide chain of hemocyanin amounts to 360 000 ± 30 000.

The molecular weight reported here is nearly identical with the molecular weight obtained by sedimentation equilibrium, for $1/20$ hemocyanin molecules of *Helix pomatia* and for $1/10$ hemocyanin molecules of *Loligo pealei* (Van Holde and Cohen, 1964). It is, however, 10–20% lower than $1/20$ the molecular weight of the whole hemocyanin molecule, obtained by other methods (Wood et al., 1971; Cox et al., 1972).

Composition of Proteolytic Digests of Hemocyanin. Brouwer and Kuiper (1973) have suggested that the polypeptide chain of *Helix pomatia* hemocyanin consists of compact tertiary structures, so-called structural domains, covalently interconnected by more exposed stretches of the polypeptide chain. Each domain is thought to contain one oxygen binding site. As hemocyanin contains 2.41 μg of Cu/mg of protein and as one oxygen is bound per two copper ions (Konings et al., 1969b), an average molecular weight of about 50 000 is calculated for a structural domain. It is to be expected that mild proteolysis will convert such a polypeptide chain preferentially into fragments having molecular weights of approximately 50 000 and multiples of 50 000, with conservation of the oxygen binding properties of the nondigested protein. Hemocyanin molecules ($1/20$) which represent completely dissociated hemocyanin, seem to be of most interest in studying the covalent substructure of the hemocyanin polypeptide chain. However, as preliminary experiments showed that limited proteolysis of $1/20$ molecules with trypsin, chymotrypsin, subtilisin, and thermolysin, at an enzyme to protein ratio of 1:1000 (w/w), gave rise to the same proteolytic fragments as $1/10$ molecules, we preferred to investigate the proteolysis of α-hemocyanin under the mild conditions at which $1/10$ molecules are present (pH 8.2, ionic strength 0.1), rather than at the high pH (pH 9.6) at which $1/20$ molecules occur.

A small amount of a specific enzyme was used (trypsin, 0.1% w/w) to effect the cleavage of the polypeptide chain, in order to avoid heterogeneity of the proteolytic fragments caused by aspecific cleavages, which is likely to occur when an aspecific enzyme at higher levels is used. Such artificial heterogeneity is expected to complicate the purification and chemical characterization of the various proteolytic fragments.

Sodium dodecyl sulfate gel electrophoresis of proteolytic digests of hemocyanin shows the presence of fragments with molecular weights of: 210 000, 154 000, 147 000, 120 000, 112 000, 98 000, 73 000, 55 000, and 50 000 (Figure 1). Proteases are often stable for some times under conditions which denature their substrates. Termination of proteolytic action by addition of sodium dodecyl sulfate and 2-mercaptoethanol might therefore expose further cleavage positions for still reactive proteases. In our experiments this possibility can be excluded for the following reasons. (1) Boiling the samples, immediately after the addition of sodium dodecyl sulfate and 2-mercaptoethanol, does not change the digestion pattern. (2) Inhibition of trypsin with diisopropyl fluorophosphate, phenylmethanesulfonyl fluoride, or soybean trypsin inhibitor, prior to the addition of sodium dodecyl sulfate and 2-mercaptoethanol, does not change the digestion pattern. (3) Four enzymes, with different specificities, all give rise to the same proteolytic fragments.

Hemocyanin is known to contain 8.15% carbohydrate (Dijk et al., 1970). Figure 1 shows that the carbohydrate content of the various proteolytic fragments is different. It is especially striking that the fragment with a molecular weight of 98 000 does not or hardly does not contain any carbohydrate. This observation is supported by direct chemical analysis for the amino sugar glucosamine. Hemocyanin contains an average of 4 mol of glucosamine per 50 000 mol wt (Dijk et al., 1970). Amino acid analysis of the purified 98 000 molecular weight fragment shows this fragment essentially to be devoid of glucosamine. This means that the molecular weight of the protein part of a structural domain amounts to about 49 000. This also implies that fragments must be present with relatively high carbohydrate contents and hence relatively high molecular weights. It is clear from Figure 1 that these are the fragments with molecular weights of 112 000–120 000. So the departure from a molecular weight, which has the value of a multiple of 50 000, possibly is caused by the relatively high carbohydrate contents of these fragments.

These results indicate that the protein parts of the majority of the components present in proteolytic digests of $1/10$ and $1/20$ hemocyanin molecules have molecular weights of 50 000 or multiples of 50 000. Therefore we can conclude that only certain regions of the hemocyanin polypeptide chain, which are very reactive toward different proteolytic enzymes and are most likely located on flexible or loosely structured regions of the molecule, are attacked. These cleavages do not influence the oxygen binding properties.

Up to now, only one condition has been found, which leads to an almost complete conversion of the hemocyanin polypeptide chain to 50 000 fragments, without disturbing the oxygen binding properties of the digest. This condition is met, when $1/20$ molecules at pH 9.6, ionic strength 0.01, are digested with 1% subtilisin (w/w) for 60 min at 30 °C.

The results presented here are in very good agreement with the hypothesis that the polypeptide chain of *Helix pomatia* α-hemocyanin is composed of structural domains with a mo-

lecular weight of about 50 000. From our studies we can conclude that each chain consists of seven domains. However, in view of the uncertainties in the determination of the molecular weight of the intact polypeptide chain (360 000 ± 30 000) and the subunits (50 000 and 55 000) the presence of six or eight domains per chain cannot be excluded.

Strong support for this model has been delivered by Siezen and Van Bruggen (1974), who showed by electron microscopy that $1/20$ hemocyanin molecules appear as flexible structures of 7–8 apparently spherical units of 55–60 Å diameter, sometimes clearly seen as a unbranched chain of globules, a "necklace" structure.

Preliminary experiments with Busycon hemocyanin show that the largest polypeptide chain has a molecular weight of about 360 000 and that this chain can be converted into fragments with a molecular weight of 100 000 and 150 000 by mild trypsinolysis. This suggests that the model we propose for *Helix pomatia* may apply to other gastropod hemocyanins as well.

Nevertheless, the presence of a fragment with a molecular weight of about 73 000 in a proteolytic digest of $1/10$ hemocyanin molecules, and to a slight extent in the subtilisin digest of $1/20$ molecules, suggests that the model we propose here may be an oversimplification and that the structure of the hemocyanin polypeptide chain may be more complex than presented here. Our model is also in agreement with the results of Lontie et al. (1973) who were able to prepare functionally active components with molecular weights of 110 000 and 50 700, respectively, by proteolysis of *Helix pomatia* hemocyanin with subtilisin.

The findings of Makino et al. (1972) that hemocyanin of *Dolabella auriculata* can be dissociated into functionally active components with molecular weights of 60 000–70 000 and 150 000–160 000, by prolonged treatment with DEAE-cellulose, may be explained by the occurrence of proteolysis during the incubation.

Similarity of the Various Structural Domains. At this moment we cannot decide whether the domains are chemically and functionally alike or not, except that there is a clear-cut difference in carbohydrate content and isoelectric point between the subunits. It must be borne in mind, however, that the use of the relatively aspecific enzyme subtilisin, partly may have contributed to the observed heterogeneity in isoelectric points.

To shed more light upon the question whether all domains are similar or not, studies are now in progress, to characterize the isolated proteolytic fragments as to their physico-chemical and functional properties.

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