MOLECULAR WEIGHT DETERMINATION OF POLYPEPTIDE CHAINS OF MOLLUSCAN AND ARTHROPOD HEMOCYANINS*

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1. Introduction

As is known, hemocyanins (Hc) are large molecular aggregates at physiological pH values and their aggregation state is dependent on H^+ concentration, ionic strength and the presence of Ca^{2+} and Mg^{2+} ions. The Hc from molluscs and arthropods shows its highest aggregation state from pH 4 to a maximum of about 10 in the absence of divalent cations [1]. The dissociation which occurs at higher values, however, is not complete. The smallest subunits which can be observed by ultracentrifugation have dissociation constants of about 11 S and 14 S in molluscs, i.e. 1/10 or 1/20 of the original molecular weight; the Hc from arthropods goes down to about 5 S which corresponds to approx. 75,000 daltons [1, 10]. It is also known that the molecular weight calculated from the copper to protein and the copper to oxygen ratio is equal to 50,000 and 75,000 daltons for molluscan and arthropod Hc, respectively. Yet, all attempts for obtaining homogeneous solutions of these subunits by physical or chemical methods have failed, also in the case of the arthropod Hc.

The aim of this work was to find the appropriate conditions in which the elementary polypeptide chains could be obtained for the purpose of determining the molecular weight of the chains and their number in the functional subunit containing two copper atoms. The Hc of *Octopus vulgaris* and of *Carcinus moenas* which were extensively studied in this laboratory, have been used as representative proteins of the phyla of Mollusca and Arthropoda.

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2. Experimental

Preliminary experiments were carried out by the gel electrophoresis technique [2–4] but it was found that Hc does not dissociate completely after treatment with sodium dodecyl sulfate (SDS) and mercaptoethanol. The same occurred when the proteins has been previously succinylated [5, 6]. A higher degree of dissociation was observed after acetylation, but also in this case the formation of a number of bands indicated that complete interaction with SDS had not been obtained. The interesting result, however, was that the lowest subunits formed have the same molecular weight, both in Octopus and Carcinus Hc.

It was decided therefore to apply to this study the gel filtration method using a Sephadex G-200 column 110 cm long and 1.2 cm wide. As shown in fig. 1, from the acetylated Hc from Carcinus in 0.1% SDS and Tris-HCl buffer (μ 0.1, pH 8.6) three subunits of 75,000, 50,000 and 25,000 daltons can be separated. In the same conditions the acetylated Hc from Octopus gives two components of 200,000 and 50,000 daltons.

Since Octopus Hc contains at least 5 disulfide bridges per functional subunit [7], the acetylated protein was reduced with dithioerythritol (DTE) or mercaptoethanol, then cyanoethylated with acrylonitrile [8]. When passed on the column as above, two new components were obtained of 100,000 and 25,000 daltons, respectively.

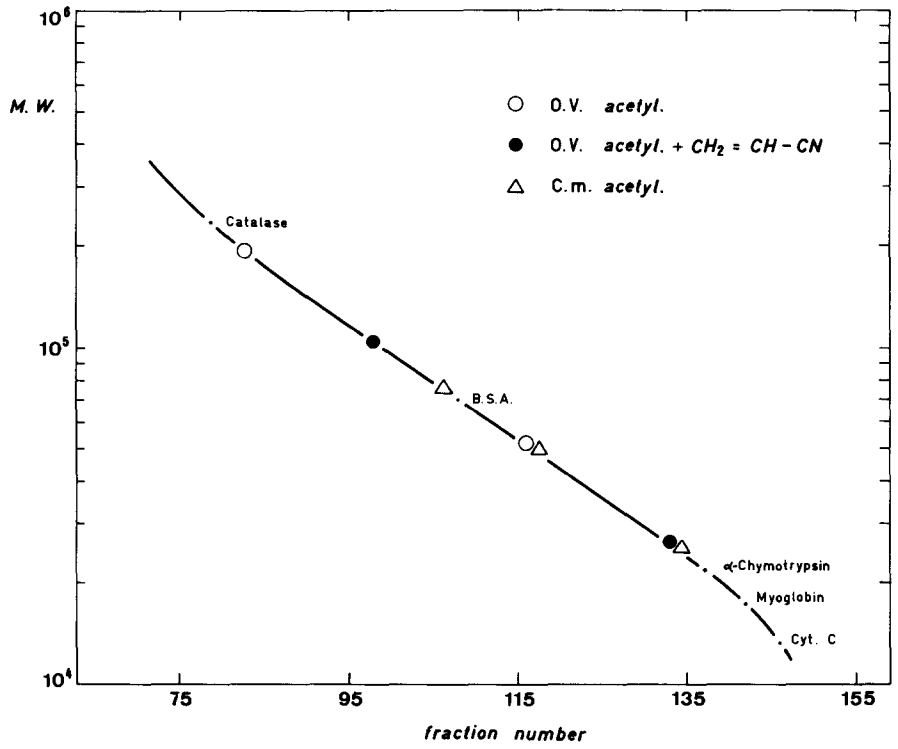

Fig. 1. Molecular weight determination of the subunits obtained from hemocyanin by gel filtration. A column of Sephadex G-200 (110 × 1.2 cm) with an elution rate of 4–5 ml/hr/cm^2 was eluted with 0.1% SDS in Tris-HCl buffer (μ 0.1 pH 8.6). Since blue Dextran cannot be employed for measuring the excluded volume, the calibration curve was worked out with the same column using as standard proteins: Cyt. *c* (12,000), myoglobin (17,000), α-chymotrypsin (22,500), ovalbumin (43,400), bovine serum albumin (67,000) and catalase (230,000). The Hc from Carcinus had been acetylated before treatment with SDS and mercaptoethanol. The acetylated Hc from Octopus was also reduced with DTE, then cyanoethylated with acrylonitrile.

3. Results and discussion

This is the first experimental demonstration that the Hc from molluscs and arthropods are composed of polypeptides having the same molecular weight. The hypothesis that arthropod Hc could be made up by three subunits, two of which carrying copper and one "structural", has been advanced recently by van Holde et al. [10] who proposed a model for the Hc of *Cancer magister* in which the main component of 16 S could be made up by 18 subunits of 26,000 daltons each.

Our findings strongly support this hypothesis. Arthropod and molluscan hemocyanins seem not to be unrelated proteins which have evolved independently from separate precursors. The similarity in the amino acid composition [7, 11] also indicates that they may have formed by a common set of genes. Our recent results [12] on the amino terminal residues (aspartic acid in Octopus Hc and 2:1 aspartic acid and glycine in Carcinus Hc) are a further support for the assumption that in molluscan Hc the functional subunit is made up by two polypeptide chains of 25,000 daltons containing one copper atom each, whereas in arthropod Hc the single site unit (75,000 daltons) is formed by three chains having the same (25,000) molecular weight, two of which are "functional" and are linked by a disulfide bridge [9], and one is "structural", i.e. without copper.

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