DIMORPHISM OF CANCER MAGISTER HEMOCYANIN SUBUNITS

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SUMMARY: Hemocyanin from Cancer magister is composed of two different subunits with molecular weights of 76,000 and 84,000 as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. These two subunits may play a functional role in the binding of oxygen by hemocyanin.

INTRODUCTION: Native hemocyanin has been shown to be heterogeneous in its susceptibility to dissociation by salt (1) and pH (2,3). Differences have also been observed in the electrophoretic behavior of undissociated (4,5) as well as dissociated hemocyanin molecules (6,7). Whether this heterogeneity is due to differences in primary structure of hemocyanin subunits or to variability in conformations of subunits or aggregates has not been conclusively established at the level of the smallest oxygen-binding subunit. In the present study we have observed two such small subunits of Cancer magister hemocyanin which differ in molecular weight by 10% according to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

MATERIALS AND METHODS: Cancer magister hemolymph freed of clotted protein and particulate matter was centrifuged 7 hours at 150,000 x g to obtain a blue hemocyanin containing pellet (8). The pellet material was judged to be greater than 95% hemocyanin by polyacrylamide gel electrophoresis. Apohemocyanin was prepared by dialysis of hemocyanin against 0.01 M KCN, 0.01 M Tris-Cl (pH 8.5). Carbohydrate content was estimated by a modified orcinol procedure for the determination of neutral sugars (9).

High resolution polyacrylamide gel electrophoresis (Ortec, Inc., Oak Ridge, Tenn.) was performed in Tris buffer containing 0.001 M EDTA and 0.2% sodium dodecyl sulfate (SDS) (pH 8.0). Gels were polymerized with 6 - 10% acrylamide,

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0.15 - 0.25% bis-acrylamide, 0.06% TEMED, and 0.05% ammonium persulfate. The gel buffer contained 0.35 M Tris-sulfate while the tank buffer contained 0.03 M Tris-acetate. Protein samples were dissolved in 0.03 M Tris-sulfate containing 40% sucrose, 1% SDS, 1% mercaptoethanol, and heated 10 minutes at 65°. Electrophoresis was run for two hours at 300 volts, 150 ma at 10°, then stained with Coomassie blue in iso-propanol - acetic acid solvent (10).

RESULTS AND DISCUSSION: When Cancer magister hemocyanin is subjected to SDS gel electrophoresis, a method which separates proteins on the basis of their molecular weights (11), more than 95% of the protein is found in two bands of similar mobility (Fig.1). Calibration of the gels with proteins of known molecular weight (Fig.2) yields values of 76,000 and 84,000 for the molecular weights of the two hemocyanin species. The range of error for these average values (6 experiments) is within ±10%, the experimental error of the method (12).

The two hemocyanin subunits are present in equal amounts and neither the relative distribution nor molecular weights are affected by sample treatment or electrophoresis conditions. Similar results are obtained whether or not the sample is heated in the presence or absence of mercaptoethanol, although heating in mercaptoethanol does decrease the amount of residual high molecular weight (MW > 200,000) material. Results are also unaffected by varying gel concentration from 6 to 10%, varying SDS concentration from 0.1 to 1%, varying gel buffer/tank buffer composition from Tris sulfate/acetate (pH 8) to Tris sulfate/borate (pH 8 or 9) or Tris acetate/acetate (pH 9), or prior electrophoresis of gels to remove catalysts and unpolymerized acrylamide. Furthermore, the distinctness of the two species is preserved upon excision of the two bands from one gel and electrophoresis into a second gel. These experiments indicate that the polymorphism observed in the ca. 80,000 MW subunit is not an artifact of electrophoresis.

Heterogeneity of hemocyanin subunits could be due to variable carbohydrate content or the occurrence of different hemocyanins in the organisms pooled for a particular preparation (13). However, our preparation of Cancer magister hemocyanin contains less than 1% neutral sugar, compared to over 5% observed

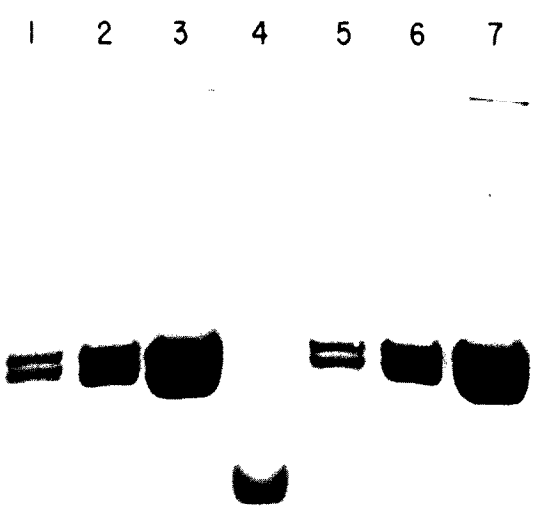

FIGURE 1: Electrophoresis of hemocyanin and bovine plasma albumin on a 6% gel. Columns 1 - 3 and 5 - 7 contained hemocyanin samples separately purified from two crabs and applied in 1, 5, and 20 μg quantities, respectively. Column 4 contained 5 μg bovine plasma albumin with 66,000 MW main band and aggregate bands of 132,000 MW and higher.

for Helix pomatia hemocyanin (13). This small amount of carbohydrate could not account for the 10% difference in the molecular weights of the Cancer hemocyanin subunits. Furthermore, the polymorphism is observed in hemocyanin isolated from a single animal (Fig. 1). That the heterogeneity is not the result of protein degradation is evidenced by the presence of only two distinct bands on the gel with little material below 75,000 MW.

The existence of two protein subunits close to 80,000 MW in crustacean hemocyanin agrees with the cellulose acetate experiments on Carcinus maenas hemocyanin (7). However, the functional significance of these two forms is uncertain. Since our hemocyanin preparations exhibit maximum oxygenation ($A_{340}/A_{280\ nm} = 0.195$ at pH 8.5), both subunits must be active in binding oxygen. This polymorphism of hemocyanin subunits could be functional in the cooperative interaction with oxygen or the Bohr effect as with the α and β protein chains of hemoglobin, or it might be related to developmental processes.

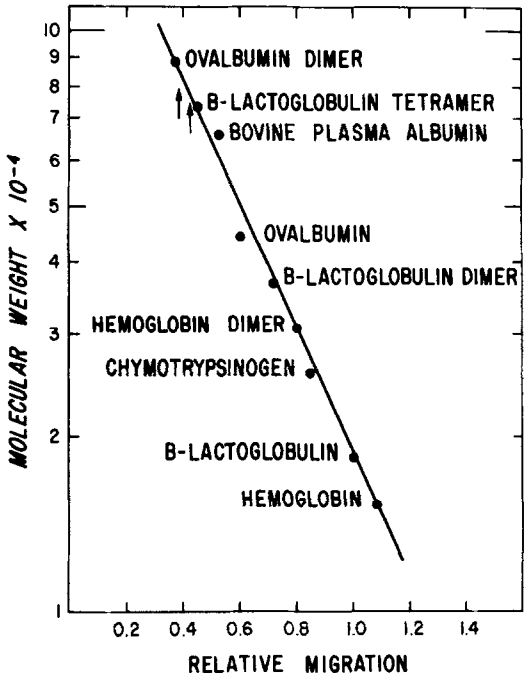

FIGURE 2: Relationship of molecular weight to migration distance in SDS gel electrophoresis of purified proteins. The arrows indicate the position of the hemocyanin subunits. Data averaged from two 8% gels.

It would be of interest to know whether the ca. 80,000 MW subunits observed by SDS gel electrophoresis represent single polypeptide chains or extremely stable aggregates of smaller subunits. End group analyses have indicated that the 80,000 MW species are intact protein molecules in crustaceans (14,15). Lower molecular weight protein components in Cancer magister hemocyanin only appear in aged samples. Protein degradation is probably also responsible for the lower molecular weight species observed after treatment of hemocyanin with formic acid (13). If smaller subunits do exist as has been indicated for mollusc and crustacean hemocyanin after acetylation and SDS gel filtration (16), they must form very strong aggregates. For example, removal of copper from Cancer hemocyanin makes the protein much more susceptible to denaturation, yet it has no effect on the SDS gel electrophoresis pattern. In any case, since the functional unit of hemocyanin contains two contiguous copper atoms (17,18), the functional 80,000 MW subunits must have these copper atoms suitably arrang-

ed. It is possible, then, that a single polypeptide binds these atoms.

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