Identification of *Limulus polyphemus* haemocyanin messenger RNA

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Total RNA was isolated from cyanoblast-containing tissue taken from behind the compound eye of the horseshoe crab, *Limulus polyphemus*. Poly(A)-containing RNA separated from this by affinity chromatography on oligo(dT)-cellulose was translated in the rabbit reticulocyte haemolysate system in the presence of L-[³⁵S]methionine. By using an antiserum to *Limulus* haemocyanin, polypeptides were isolated from the translation products which had a similar mobility to the authentic *Limulus* haemocyanin polypeptides as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Haemocyanins are the copper-containing oxygen-binding proteins characteristic of certain types of invertebrate, notably Gastropoda and Cephalopoda among the molluscs, and Xiphosura, Arachnida and Crustacea among the arthropods (Wood, 1980). Mollusc and arthropod haemocyanins differ in a number of ways. Although the oxygen-binding site always contains a pair of copper atoms, in mollusc haemocyanins this pair is associated with a protein molecular weight of about 50 000, whereas in arthropod haemocyanins the molecular weight per pair of copper atoms is about 70 000. Mollusc haemocyanins are believed to be constructed from 10, or 20 (depending on species), polypeptide chains each of mol.wt. about 400 000, each being composed of eight linked more-or-less globular oxygen-binding units or 'domains' (Gielens *et al.*, 1980). Arthropod haemocyanins, in contrast, are made up of aggregates of much smaller, non-identical, polypeptide chains, probably one per pair of copper atoms. The aggregates have between 6 and 48 binding sites, depending on species (Wood, 1980). These differences are reflected in markedly distinct and characteristic overall architectures as observed by electron microscopy. However, whether these differences reflect differential post-translational modifications of a common polypeptide, or alternatively different gene products, has yet to be determined.

In addition to structural problems, the site of haemocyanin synthesis remains uncertain. Suggestions have invariably been based either on observations of 'haemocyanin crystals' in cells by electron

microscopy, or, by implication, that tissues with a high copper content were possible sites of biosynthesis. A number of sites have been proposed, including the pore cells of gastropods (Sminia, 1977), the blood-gland of opisthobranchs (Schmekel & Weischer, 1973), the branchial glands of cephalopods (Dilly & Messenger, 1972), and a type of cell called a cyanoblast in the xiphosuran *Limulus polyphemus* (Fahrenheit, 1970) and in the crustacean *Carcinus maenas* (Ghiretti-Magaldi *et al.*, 1973).

In order to resolve these problems we have initiated a series of studies on the biosynthesis of haemocyanins, using the techniques now available for investigating protein synthesis *in vitro*, involving isolation of mRNA and examination of the products of its translation by immunological and electrophoretic methods. In the long term such studies should throw light on such problems as the number of genes, the size of polypeptide chains, and the site and intracellular location of haemocyanin biosynthesis. We report here the isolation of poly-(A)⁺ RNA from *Limulus polyphemus* (horseshoe crab) tissue, its translation in the rabbit reticulocyte haemolysate system, and the detection among the products of translation of polypeptides similar immunologically and in size to authentic *Limulus* haemocyanin.

Materials and methods

Glassware used in the extraction of RNA was heat-sterilized, and all solutions and polyware were autoclaved as described by Craig *et al.* (1979). *Limulus* haemocyanin was purified as described previously (Lamy *et al.*, 1979), and the native haemocyanin dissociated by pH change and separ-

Abbreviations used: poly(A)⁺ RNA, poly(A)-containing RNA; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

ated into five components or zones by chromatography on DEAE-Sephacrose CL-6B (Sullivan *et al.*, 1976). This represents a partial fractionation into the eight subunits thought to constitute the native molecule.

Oligo(dT)-cellulose was obtained from Boehringer Corp. (London) Ltd., and Protein A-Sephacrose was purchased from Pharmacia Fine Chemicals AB. All other chemicals were of analytical grade, and glass-distilled water was used throughout. L-[³⁵S]Methionine (>1000 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Extraction of RNA

Specimens of *Limulus polyphemus* which had been maintained in tanks of circulating sea water were anaesthetized by immersing them in crushed ice. Tissue was dissected out from behind the compound eye and immediately frozen in liquid nitrogen. Tissue collection was done in July, when it is reported (Fahrenbach, 1970) that the concentration of cyanoblasts is highest. The frozen tissue was crushed to a powder with a pestle and mortar and then homogenized. Total RNA was extracted by the phenol/chloroform method as described previously (Wood *et al.*, 1979). After precipitation with 3.0 M-sodium acetate, pH 5.2, poly-(A)⁺ RNA was separated by two cycles of affinity chromatography on oligo(dT)-cellulose. The RNA was applied to the column equilibrated in 0.5% (w/v) SDS/0.5 M-NaCl/1 mM-EDTA/10 mM-Tris/HCl buffer, pH 7.4. After a wash in the same buffer lacking SDS, the column temperature was raised to 60°C and the poly(A)⁺ RNA eluted with 10 mM-Tris/HCl buffer, pH 7.4, and precipitated with ethanol and recovered by centrifugation (see Craig *et al.*, 1979). Routinely 1 g of tissue yielded 15–20 µg of poly(A)⁺ RNA.

The poly(A)⁺ RNA so obtained was translated for 90 min in the micrococcal-nuclease-treated rabbit reticulocyte-haemolysate system in the presence of [³⁵S]methionine (Pelham & Jackson, 1976). The incorporation of [³⁵S]methionine into protein was determined as described by Evans & Rosenfeld (1976).

Analysis of translation products

Antibodies to *Limulus* haemocyanin were raised in rabbits and the IgG fraction was purified by (NH₄)₂SO₄ fractionation followed by DEAE-cellulose chromatography (Harboe & Inghild, 1973). Proteins immunologically identical with *Limulus* haemocyanin were isolated from the mRNA-directed cell-free system by adding purified antibody essentially as described by Amara *et al.* (1980), except that Protein A-Sephacrose (Payvar & Schimke, 1979) was used rather than *Staphylo-*

coccus aureus cells. Typically, to 60 µl of total translation products was added 30 µl of antibody buffer (50 mM-Tris/HCl, pH 7.6, containing 0.5% Nonidet P40, 150 mM-NaCl, 5 mM-EDTA, 2 mM-L-methionine, 1 mM-Na₂N₃) followed by 6 µl (30 µg) of pre-immune IgG. This mixture was incubated for 30 min at room temperature and 30 min at 4°C, and then 2 mg of Protein A-Sephacrose was added suspended in 10 µl of antibody buffer. After incubation for 1 h at room temperature with agitation, the mixture was layered over a 1 ml cushion of 1 M-sucrose in antibody buffer in a plastic tube. After centrifugation (10000 g for 20 min) the tube was frozen in liquid N₂ and the tip containing the pellet cut off. The pellet was washed a further three times in this way. The supernatant was treated with 30 µl of anti-(*Limulus* haemocyanin) IgG and Protein A-Sephacrose in the same way. Immune and pre-immune pellets were suspended in SDS-gel buffer (see below) and heated to 100°C for 5 min. After addition of 2-mercaptoethanol and urea to 5 M, the samples were centrifuged (3000 g for 10 min) and the supernatants applied to gradient slab SDS/polyacrylamide gels for electrophoresis in the buffer system of Laemmli (1970); the stained gels were photographed and then fluorographed (Bonner & Laskey, 1974).

Results and discussion

The addition of *Limulus* poly(A)⁺ RNA (0.6 µg) to a reticulocyte-lysate cell-free system resulted in a 60–70-fold stimulation over the control of incorporation of [³⁵S]methionine into protein after a 90 min incubation. Examination of the translation products by SDS/polyacrylamide-gel electrophoresis followed by fluorography revealed that a wide spectrum of proteins was being synthesized (Fig. 1a), ranging from 13 000 to 140 000 in molecular weight. As a means of identifying haemocyanin, the pooled translation products from a number of incubations were treated with pre-immune serum and then with anti-(*Limulus* haemocyanin) IgG. The final immunoprecipitates were solubilized and then applied to SDS/polyacrylamide gels, and the [³⁵S]methionine-containing immunoprecipitable material was identified by fluorography. This represented 0.25% of the total [³⁵S]methionine incorporated into protein, and electrophoresed as one predominant and two or three more weakly radio-labelled polypeptides (Fig. 1a). All were within the expected molecular-weight range for the *Limulus* haemocyanins (see below). The predominant band moved with a mobility corresponding to a molecular weight of about 71 000, and the weaker bands corresponded to molecular weights of about 69 000, 70 000 and 71 500.

Authentic *Limulus* haemocyanin runs on SDS/

Fig. 1. (a) SDS/polyacrylamide-gel electrophoresis and fluorography of the products of translation of *Limulus* mRNA and (b) electrophoretic behaviour of *Limulus* haemocyanin and its separated subunits in the presence of SDS

(a) Pooled translation mixture was treated with pre-immune IgG, followed by anti-(*Limulus* haemocyanin) IgG. The immune complexes were isolated by using Protein A-Sepharose and solubilized before being applied to the gel. Track 1 shows electrophoresis of authentic *Limulus* haemocyanin running as a set of closely spaced bands. All the

polyacrylamide gels as a set of closely spaced bands (Fig. 1b), which may be partially separated by ion-exchange chromatography into six to eight different bands of molecular weights in the range 67000–72000 (see Fig. 1b). In contrast with the material synthesized *in vitro*, the native haemocyanin peptides contain a preponderance of lower-molecular-weight material, corresponding to bands I and II and especially IV, compared with the higher-molecular-weight bands III and V. Since all the subunits have approximately the same methionine content (Sullivan *et al.*, 1976), differences in labelling intensity would not be expected as a result of differential incorporation of [35 S]methionine. Consequently it is possible that the discrepancy observed in molecular-weight distribution may be accounted for by translational modifications *in vivo* which do not occur *in vitro*.

The results described above provide interesting insights into the site and manner of synthesis of *Limulus* haemocyanin. Cyanoblasts, the proposed site of haemocyanin biosynthesis, appear to become entrapped in the sinusoidal spaces pervading the neural plexus of the compound eye (Fahrenbach, 1970). The present work shows that tissue from behind the compound eye contains mRNA which directs the biosynthesis *in vitro* of polypeptides similar to haemocyanin in both size and immunological determinants. These observations substantiate previous conclusions based on electron microscopy (Fahrenbach, 1970), histoimmunofluorescence (Ghiretti-Magaldi *et al.*, 1973) and optical diffraction (Ghiretti-Magaldi *et al.*, 1977) that the cyanoblasts of arthropods may be the site of haemocyanin biosynthesis. The antiserum used in the present work reacted equally well with all of the subunits of native *Limulus* haemocyanin. Presumably, therefore, the pattern of bands in the material

other tracks are fluorographs. Track 3 represents total translation products and track 5 the supernatant remaining after immune precipitation. Track 4 shows the product obtained by treatment with pre-immune IgG, and track 2 shows the material obtained by treatment with immune IgG. Authentic *Limulus* haemocyanin was run in every alternate track, e.g. between tracks labelled 1 and 2 in the fluorograph shown, etc., and its position can be identified by the 'necking' it caused in tracks 3 and 5. (b) W, whole, and unfractionated haemocyanin (I–V, subunits I–V separated by ion-exchange chromatography). M, marker proteins (the arrow shows the position of the bovine serum albumin marker, of mol.wt. 67000); the other calibration markers were β -galactosidase (130000), phosphorylase *a* (94000), pyruvate kinase (57000), glutamate dehydrogenase (53000), alcohol dehydrogenase (41000), aldolase (40000) and carbonic anhydrase (29000).

isolated from the total translation products represents a mixture of the six to eight polypeptide chains found in native haemocyanin, or precursors of these. The fact that a preponderance of heavier bands (by some 1000–2000 daltons) could be identified among the translation products by using this antiserum therefore implies either that these represent polypeptide precursors that need to be processed by peptidases, a feature common to secretory proteins (Blobel & Dobberstein, 1975*a,b*), or alternatively that the peptides synthesized *in vitro* lack specific post-translational modifications, thereby affecting their mobility on SDS/polyacrylamide gels.

The question as to whether the different polypeptides represent different gene products remains to be resolved. It has been established that, although the subunits are closely similar to one another in amino acid composition, each showed a different and distinct fragmentation pattern after cleavage with trypsin or with CNBr (Sullivan *et al.*, 1976). Furthermore, immunological distinctions also exist between the different subunits (Lamy *et al.*, 1979). Thus structural studies, and our own identification of multiple polypeptides after mRNA-directed protein synthesis, would tend to weight the evidence in favour of some at least of the subunits being individual gene products.

Although our studies are preliminary, they demonstrate that the application of established techniques of molecular biology should ultimately resolve many of the questions relating to the structure of haemocyanins. Thus it would be interesting to discover the relationships, if any, between arthropod and mollusc haemocyanins, since a fundamental difference lies in the number of oxygen-binding sites per polypeptide chain (one in arthropods and eight in molluscs). Preliminary experiments suggest that a very large mRNA species (>30S) is responsible for directing the biosynthesis of mollusc haemocyanin(s) (Wood & Siggins, 1981), in contrast with arthropod haemocyanins (see above), which presumably are encoded by a somewhat smaller mRNA species.

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