Reassembly of wall domains of Roman-snail (*Helix pomatia*) β -haemocyanin

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(Received 19 August 1980/Accepted 12 December 1980)

β -Haemocyanin molecules consist of 20 very large polypeptide chains. These chains are composed of eight structural domains. So-called 'collar' domains can be removed by trypsinolysis of the native cylindrical molecule, resulting in an association of the remaining hollow cylinders into large tubular polymers. Dissociation of the tubular polymers gives one single- and four multi-domain fragments. The role of these fragments in the reassembly process of these tubular polymers was investigated. The two-domain fragment could form tubular polymers. The other domain fragments were not able to form tubular polymers unless in the presence of the two-domain fragment. Tubular polymers with enlarged diameter and ribbon-like structures were observed in the reassembly products when the one-domain fragment was omitted.

The haemocyanin of the Roman snail (*Helix pomatia*) consists of 75% of an α -component and 25% of a β -component; the α -component dissociates into two halves in 1 M-NaCl, but the β -component does not (Heirwegh *et al.*, 1961). Great functional differences exist between the α -component and the β -component (Kuiper *et al.*, 1976; Zolla *et al.*, 1978). Both components are huge molecules (mol.wt. 9000000) consisting of 20 polypeptide chains. These polypeptide chains can be dissociated from each other by raising the pH to about 10 and removing bivalent cations. Lowering the pH back to about 7 leads to reassociation of the native cylindrical molecule (Siezen, 1974); the process is promoted by adding bivalent cations. The polypeptide chain consists of eight domains, each of which carries one oxygen-binding site.

Trypsin treatment of the undissociated molecule leads to the formation of long tubes (van Breemen *et al.*, 1975); closer inspection reveals that the so-called 'collar' is removed, leaving hollow cylinders, which then polymerize. Three-dimensional image reconstruction with electron micrographs of the tubes shows six domains per polypeptide chain located in the wall (van Breemen *et al.*, 1977).

The tubes dissociate on raising the pH to 8.5 in the presence of EDTA. This results in a heterogeneous mixture of multidomain fragments (van der Laan *et al.*, 1981; Gullick *et al.*, 1979).

SDS/polyacrylamide-gel electrophoresis reveals Abbreviations used: Bistris, 2-[bis-(2-hydroxyethyl)-amino-2-(hydroxymethyl)propane-1,3-diol; SDS, sodium dodecyl sulphate.

five fragments, which comprise six, four, three, two and one domain respectively (van der Laan *et al.*, 1981). The six-domain fragment is the polypeptide chain without the two 'collar' domains. The four-domain fragment arises by a cut in the six-domain fragment between the fourth and fifth domains from the *N*-terminal end, yielding also a two-domain fragment. The four-domain fragment in turn may yield a three-domain fragment and a one-domain fragment (Torensma *et al.*, 1980).

Here we report on the role of these fragments in the reassembly of the tubular polymers.

Materials and methods

Helix pomatia β -haemocyanin was isolated as described by Gielen *et al.* (1975). Trypsinolysis of the undissociated molecules was performed by using the procedure of van Breemen *et al.* (1975), as modified by van der Laan *et al.* (1981). Trypsinolysis of dissociated tubular polymers was performed in 0.02 M-borate/HCl, pH 8.5, containing 1 mM-EDTA for 30 min. The enzyme/haemocyanin ratio was 1:200 (w/w). The fragments constituting the tubular polymers were separated as described by van der Laan *et al.* (1981). Reassembly of the fragments was performed by dialysis at 4°C against two changes of the appropriate buffers for 48 h. Protein concentrations ranged from 10 to 20 mg/ml. Reassembly products were fractionated by size on Sepharose CL-4B. The column (50 cm $\times$ 1 cm) was eluted with a flow rate of 15 ml/h with the same buffer as that used for reassembly. The con-

stituents of the different peaks were analysed by detergent/polyacrylamide-slab-gel electrophoresis, which was performed by the method of Laemmli (1970). Reassembly was also detected by electron microscopy as described by Siezen & van Bruggen (1974).

Results

Reassembly of dissociated tubes

Isolated tubular polymers dissociate in borate buffer, pH 8.5, yielding five domain fractions (Fig. 1) (van der Laan *et al.*, 1981; Torensma *et al.*, 1980). The fragment mixture could be 50% reassembled by lowering the pH from 8.5 to 7.0 by dialysis against 0.02 M-Bis-Tris/0.02 M-HCl, pH 7.0. Further reassembly was achieved by adding bivalent cations to the

reassembly buffer; beyond 50 mM-CaCl₂, no further reassembly could be detected. The reassociated material was separated from the remaining dissociated fragments by gel filtration on Sepharose CL-4B. The peak area indicated over 90% reassembly at this stage. Electron micrographs of the reassociated material revealed tubular structures, most of which possessed rectangular ends (Plate 1), similar to the undissociated tubular polymers. Detergent/polyacrylamide-gel electrophoresis showed that the reassociated material consisted of the same five domain fragments as the undissociated polymers. The unreassociated material, however, comprised only three-, two- and one-domain fragments.

Reassembly of isolated domain fragments

The four- and six-domain fragments can be regarded as partial splitting products (Torensma *et al.*, 1980). We were unable to purify the six-domain fragment because of its low concentration in the mixture and because it was slowly degraded during the isolation procedure into its component domain fragments. The four-domain fragment also appeared to be unstable during the isolation procedure; therefore the dissociated tubular polymers were digested further with trypsin to convert the four- and six-domain fragments into their constituent domain fragments, which are relatively stable against the action of trypsin (Gielens *et al.*, 1975). By means of ion-exchange chromatography the resulting fragment mixture was separated into virtually pure one-, two- and three-domain fragments, which have unique positions in the polypeptide chain (van der Laan *et al.*, 1981; Torensma *et al.*, 1980). The one-domain fragments alone did not form tubular structures at pH 7.0 or 5.5 in the presence of 50 mM-CaCl₂, as was deduced from gel filtration, analytical ultracentrifugation and electron microscopy. The three-domain fragments, too, failed to form tubular polymers by themselves on lowering the pH, although by using the same buffer composition and the same protein concentration a slight increase in the value of the $s_{20,w}$ from 7.5 to 8.7S was detected. The two-domain fragments, on the contrary, formed large tubular polymers, which could be easily separated from residual dissociated material by gel filtration (Fig. 2). Electron micrographs of the reassembled material showed tubular polymers almost indistinguishable from undissociated polymers (Plate 2). Lowering the pH to 5.5 in the presence of 50 mM-CaCl₂ gave the same results. Reassembly of a mixture of two-, three- and four-domain fragments at pH 7 in the presence of 50 mM-CaCl₂ yielded tubular polymers indistinguishable from undissociated polymers (Plate 3a). Reassembly was not complete, as was deduced from gel filtration on Sepharose CL-4B. Detergent/poly-

Fig. 1. Location of the domains in the wall Schematic front view of a half molecule of *Helix pomatia* haemocyanin of which the 'collar', consisting of two domains, is omitted (van Breemen *et al.*, 1977). The domains are indicated with different symbols. The position of the polypeptide chain in the wall of the cylindrical molecule is indicated by the hatched symbols. The connections between the domains are linkages between the different domains. The closed connections are possible splitting places for trypsin.

- △-□-▽-○-△ Six-domain fragment
- △-□-▽ Four-domain fragment
- △-□ Three-domain fragment
- △ Two-domain fragment
- ▽ One-domain fragment

The 'collar' domains are linked to the two-domain fragment.

EXPLANATION OF PLATE 1

Electron micrograph of reassembled polymers

Tubular polymers were dissociated into a multidomain mixture. This mixture was reassembled by dialysis against 0.02 M-Bistris/0.02 M-HCl, pH 7.0, containing 50 mM-CaCl₂. Magnification $\times 156\,000$.

EXPLANATION OF PLATE 2

Electron micrograph of reassembled two-domain fragments

The reassembly procedure is the same as described in the legend to Plate 1. Magnification $\times 156\,000$.

EXPLANATION OF PLATE 3

Electron micrographs of reassembled mixtures: (a) two-, three- and four-domain fragments, and (b) two- and three-domain fragments
(a) The reassembly procedure was as described in the legend to Fig. 2. Magnification $\times 156\,000$. (b) The reassembly was performed by dialysis against 0.02 M-sodium acetate/0.05 M-CaCl₂, adjusted to pH 5.5 with acetic acid. Magnification $\times 156\,000$. The arrow indicates a normal tubular polymer.

EXPLANATION OF PLATE 4

Electron micrograph showing ribbon-like structures

Reassembly of a mixture of two-domain and three-domain fragments resulted in structures shown in Plate 3(b) and in structures shown in this Plate. The reassembly procedure was the same as that mentioned in Plate 3(b). Magnification $\times 156\,000$.

Fig. 2. Separation of reassembled and non-reassembled two-domain fragments

A sample containing the reassembled and non-reassembled two-domain fragments was applied to a Sepharose CL-4B column (50 cm $\times$ 1.0 cm) and eluted as described in the Materials and methods section. Peak A, reassembled material; peak B, non-reassembled material.

Fig. 3. Detergent/polyacrylamide-gel electrophoresis of *Helix pomatia* and *Octopus vulgaris* haemocyanin. Slab-gel electrophoresis was performed as described by Laemmli (1970); 4%-polyacrylamide gels were used. Tracks 1 and 2, *Helix pomatia* haemocyanin; tracks 3 and 4, *Octopus vulgaris* haemocyanin. The arrow indicates the direction of the electrophoresis.

ments. The latter mixture could be reassembled, with a yield of about 30%, by lowering the pH to 5.5 in the presence of 50 mM-CaCl₂. However, in this case, electron micrographs revealed structures different from those observed in Plate 3(a). Besides normal-looking tubular polymers, tubular polymers with enlarged diameter (Plate 3b) and ribbon-like structures (Plate 4) were seen. Both reassembled and residual dissociated material consisted of two- and three-domain fragments.

Discussion

Gastropod haemocyanin polypeptide chains are believed to be folded in separated compact domains connected by more open regions of the polypeptide chain. Although a variety of molecular weights have been found (Wood & Peacocke, 1973; Salvato *et al.*, 1979; Quittner *et al.*, 1978; van der Laan *et al.*, 1981), there is evidence for eight domains, each with a mol.wt. around 55 000, which are liberated by proteolytic digestion of one-tenth molecules (Gielens *et al.*, 1981) and of whole molecules (van Breemen *et al.*, 1975, 1977). There were marked functional differences between the domains in the isolated domain fragments (van der Laan *et al.*, 1981; Torensma *et al.*, 1980). The two-domain fragments, however, contained two functionally equivalent oxygen-binding sites. These two domains play an important part in the reassembly process, as shown in the present paper.

Undissociated β -haemocyanin is a cylindrically shaped molecule. The wall of this cylinder is composed of 12 stacked rings of protein subunits (van Breemen *et al.*, 1977). As Fig. 1 shows, in the native molecule, all equivalent domains are located in a single ring. It is therefore to be expected that reassembly of the two-domain fragments results in a ring consisting of two layers of protein subunits (domains). In contrast, tubular polymers were observed. Apparently the two-domain fragments are able to polymerize by forming a large stack of rings. Each ring has one face equal to the reassembly interface between the 'collarless' cylinders, which are associated into tubular polymers. The other end of the ring must have the same reassembly properties to make the assembly into tubular polymers possible. Both domains in the two-domain fragment thus have similar reassembly properties and also similar functional properties.

Reassembly of two- and three-domain fragments at pH 5.5 resulted in three types of aggregated material. The type with a normal appearance probably consisted of two-domain fragments, as shown by reassembly of two-domain fragments by themselves. Therefore the other two types, tubes with enlarged diameter and ribbon-like structures, were probably aggregates comprising both two- and three-domain

acrylamide-gel electrophoresis revealed the presence of four-, three- and two-domain fragments in the reassembled material (Fig. 3). The non-reassembled material contained only three- and two-domain frag-

fragments. 'Ribbons' were not observed when four-domain fragments, which consist of a three-domain fragment covalently linked to a one-domain fragment, were present; in this case the normal appearance was observed.

Haemocyanin of the common octopus (*Octopus vulgaris*) resembles half molecules of gastropod haemocyanin. However, half molecules of gastropod haemocyanin have a sedimentation value of 60S (Eriksson-Quensel & Svedberg, 1936), unlike that of the common octopus, which has a sedimentation value of 50S (Eriksson-Quensel & Svedberg). Apparently octopus haemocyanin has a lower molecular weight. This proposed lower molecular weight for 'whole' octopus haemocyanin is a reflection of the lower molecular weight of the polypeptide chain as demonstrated by SDS/polyacrylamide-gel electrophoresis (Fig. 3). Octopus haemocyanin, although having a smaller polypeptide chain, is still able to form a cylindrical molecule.

Our study demonstrated that the two-domain fragment alone could form cylindrical structures. Therefore it is likely that a smaller haemocyanin, like that of the octopus, still possesses the domains of the two-domain fragment, in order to form a cylindrical molecule. Any domains missing in this haemocyanin should be one or more of those in the three-domain fragment. This would also explain why these haemocyanins are not capable of forming whole molecules with a sedimentation value of 100S, namely because the contacts involved in binding two halves together are located in this part of the molecule.

This work was supported by the Netherlands Foundation for Chemical Research (S. O. N.), with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z. W. O.). We thank Dr. R. N. Campagne and Dr. M. Brouwer for critical reading of the manuscript, Dr. G. Nardi (Naples) for generously providing *Octopus vulgaris* haemocyanin, Mr. N. Panman for drawing the Figures, Mr. J. F. L. van Breemen for taking the electron micrographs, Mr. K. Gilissen for

printing the photographs and Miss T. Uneken for typing the manuscript.

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