The polypeptide composition of axoplasm and of neurofilaments from the marine worm *Myxicola infundibulum*

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1. Axoplasm from *Myxicola* contains two major polypeptides associated with neurofilaments, together with actin, tubulin and many minor polypeptide components. 2. Some of the minor polypeptides with molecular weights between 140 000 and 50 000 purify with neurofilaments under a variety of conditions and they appear to represent an integral part of the filament structure. 3. Peptide fingerprinting shows that the two major neurofilament polypeptides are almost identical. The fingerprint patterns from these major polypeptides share features with those obtained from the minor components. 4. Peptide fingerprinting has enabled us to propose a scheme for the main sites at which papain cleaves the major neurofilament polypeptides. In addition fingerprinting indicates how the minor components are related to the major polypeptides. 5. It is suggested that many of the minor neurofilament polypeptides could arise by proteolysis *in vivo*.

Axoplasm from the marine worm *Myxicola infundibulum* provides an ideal source for the study of neurofilaments. There are two main reasons for this. The first is that axoplasm from *Myxicola* can be easily and quickly extracted, thereby decreasing the possibility of degradation by proteinases known to exist in axoplasm (Gilbert *et al.*, 1975). The second reason is that neurofilaments constitute more than 70% of the total axoplasmic protein in *Myxicola* and this means that a single worm may be capable of furnishing 2–3 mg of pure neurofilament protein. Because of these advantages we have used *Myxicola* as a model system to study neurofilament structure in the hope that it might not only provide clues to the structure of vertebrate neurofilaments but also help in our understanding of the function of these filamentous proteins.

Previous studies using *Myxicola* have shown that purified neurofilaments are composed of two major polypeptides with molecular weights close to 160 000 and 150 000 (Gilbert *et al.*, 1975; Lasek *et al.*, 1979). These neurofilaments, however, appear to be intricate structures because many minor proteins that have molecular weights ranging from 140 000 to around 50 000 always co-purify with them.

The major question that interested us was whether neurofilaments were really as complex as their composition suggested or whether there was an underlying principle that might relate in some way

the majority of the neurofilament-associated polypeptides. In addition we were interested to find out the nature of some of the other components present in axoplasm.

We report here our studies on the polypeptide composition of axoplasm and of neurofilaments. We show that axoplasm contains the α - and β -components of tubulin and has isoelectric variants of actin. We also show that many of the polypeptides in axoplasm are structurally related to the major neurofilament components and suggest that this could be explained by proteolysis of neurofilaments that had occurred *in vivo*.

Experimental

Materials

Myxicola were dug from the mud in the estuary near Salcombe (Devon, U.K.) and then transported to the laboratory where they were kept for 2–3 months in aerated sea water. Papain (type III), carboxypeptidase, fumarase, creatine kinase, catalase, glutamate dehydrogenase, bovine serum albumin, glyceraldehyde 3-phosphate dehydrogenase, cytochrome *c* and β -galactosidase were purchased from Sigma. Actin was prepared by the method of Spudich & Watt (1971), tubulin by the method of Eipper (1972), phosphorylase by the method of Fischer & Krebs (1962), aldolase by the method of Eagles *et al.* (1969) and triose phosphate isomerase by the method of Putman *et al.* (1972).

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Myosin, paramyosin and troponin were prepared in this department by Dr. G. Offer, Dr. A. Elliott and Dr. E. Morris respectively. Filamin was kindly provided by Miss L. Castellani, spectrin by Mr. V. Ohanian and desmin by Dr. Q. L. Choo; these proteins were also prepared here. All other chemicals were purchased and were of the highest quality available.

Methods

Isolation of neurofilaments. Axoplasm was extracted from worms essentially as described by Gilbert (1972). Neurofilaments were purified from axoplasm by three different methods: (a) repeated cycles of polymerization and depolymerization; (b) precipitation with cytochrome *c*; and (c) chromatography on Sepharose 4B.

(a) Axoplasm was homogenized at a concentration of 20 mg/ml in 0.1 M-Tris/HCl, pH 7.4, containing 0.1 M-NaCl, 1 mM-EGTA, 0.01% (w/v) NaN_3 . This process renders filaments from axoplasm and they were spun from solution in 1 h at 4°C and 100 000 *g* (r_{av} 6.3 cm.). The resulting pellet was suspended by gentle homogenization in 1 ml of 0.1 M-Tris/HCl, pH 7.4, containing 0.5 M-NaCl, 1 mM-EGTA, 0.01% NaN_3 to disrupt the filaments into their subunits. The homogenate was then spun for 1 h at 4°C and 100 000 *g* to remove debris and the supernatant from this spin was dialysed against 50 mM-Tris/HCl, pH 7.4, containing 1 mM-EGTA, 0.01% NaN_3 to polymerize the subunits into filaments. These filaments were spun out of solution in 1 h at 4°C and 100 000 *g*. The assembly/disassembly procedure could be repeated two or three times without significant differences being seen in the protein composition of the purified filaments when assayed on gels. The addition of proteinase inhibitors, for example 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'), and phenylmethanesulphonyl fluoride to the buffers did not significantly affect the gel pattern and were therefore omitted.

(b) Precipitation with cytochrome *c* was performed as described previously (Gilbert *et al.*, 1975). Briefly, axoplasm was homogenized in 20 mM-histidine, pH 7.0, and cytochrome *c* (1 mg/ml) added to induce formation of filament rings. The rings were then spun from solution.

(c) Axoplasm was homogenized at a concentration of 50–100 mg/ml in 0.1 M-Tris/HCl, pH 7.4, containing 0.1 M-NaCl, 1 mM-EGTA and 0.01% (w/v) NaN_3 . The homogenate was spun for 5 min at approx. 10 000 *g* (r_{av} 5 cm) to remove easily sedimentable debris and the supernatant applied to a column (1.5 cm diam. $\times$ 25 cm) of Sepharose 4B packed in the same buffer. The column was washed under gravity. The presence of filaments was assayed by electron microscopy and their com-

position assessed by gel electrophoresis. This procedure for preparing filaments is based on one originally developed here by Bell (1978).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and peptide fingerprinting

Gel electrophoresis was performed in the presence of sodium dodecyl sulphate on 5–15% or 5–20% acrylamide gels (acrylamide/bisacrylamide was 50:1.3, w/w) with a discontinuous buffer system (Laemmli, 1970). Peptide mapping was carried out essentially as described by Cleveland *et al.* (1977). Source gels were generally run on gradients containing 5–15% acrylamide and bands cut out after brief staining. Sometimes these bands were re-run to concentrate the proteins before mapping. Mapping was performed on 5–20% acrylamide gels with papain or trypsin. The ratio of proteinase to the polypeptide to be digested was generally between 10^{-2} and 10^{-5} (w/w). Digestion proceeded in the stacking gel and the current was not switched off during the digestion process. Two-dimensional separation of polypeptides after fingerprinting was performed in a similar manner but here a strip (approx. 10 cm in length) was cut from the source gel and overlayed on the 5–20% gel. A solution of papain in 20% glycerol and 10% β -mercaptoethanol was applied evenly to the strip before passing a current through the gel.

To help build a picture of the sites cleaved by the proteinases, peptides released after an initial digestion were often cut out and re-digested.

Isoelectric focusing

This was carried out by the method of O'Farrell (1975), with a pH gradient from 4 to 6.5. After running, the gel was equilibrated for 15 min with sample buffer containing 10% β -mercaptoethanol, before re-running in the second dimension, on a 5–20% acrylamide gradient.

Electron microscopy

Neurofilaments were routinely examined on carbon grids by negative staining in 1% uranyl acetate. The grids were examined in a Philips 300 electron microscope working at an accelerating voltage of 75 kV.

Results

The polypeptide composition of Axoplasm from Myxicola

Fig. 1(a) shows a typical gel of whole axoplasm. It is seen that two major polypeptides with mol.wts. 172 000 and 155 000 dominate this pattern. These polypeptides are components of the neurofilaments (Gilbert *et al.*, 1975; Lasek *et al.*, 1979). Other polypeptides are present that from their molecular

Fig. 1. Polyacrylamide-gel patterns of (a) axoplasm and (b) neurofilaments

This Figure shows typical polyacrylamide gels (5–15% acrylamide) of whole axoplasm (a) and of purified neurofilaments (b) that were run in sodium dodecyl sulphate. The axoplasm shown here was extracted in less than 10s and plunged into boiling 5% sodium dodecyl sulphate. The neurofilaments were purified by cytochrome *c* precipitation. The molecular weights of the components are given ($\times 10^{-3}$) together with the regions where actin and tubulin are found. The proteins listed under 'Materials' were used as molecular-weight markers.

weight suggested they might be actin (mol.wt. 42000) and tubulin (mol.wt. 50000–55000). This was confirmed by isoelectric focusing and by peptide fingerprinting.

A typical isoelectric focusing gel is represented in Fig. 2 and shows the position of actin and tubulin. In

this system actin showed two variants with pI values between 5.7 and 5.85; the more basic variant was the major one. These variants were cut from a gel and mapped separately with papain using rabbit muscle actin as a control. Both variants gave identical maps that were indistinguishable from the control. Tubulin was resolved into three components: α_1 , α_2 and β with pI values between 5.4 and 5.6. Bands from the tubulin region were fingerprinted and showed a strong resemblance to tubulin prepared from pig brain.

Apart from actin, tubulin and the major neurofilament components, however, many other polypeptides are present in axoplasm. A number of them focus in an arc (see Fig. 2) and tend to streak across the gel as do the bands at mol.wts. 172000 and 155000. There are some polypeptides, however, that focus into discrete spots, for example those with mol.wts. 90000, 74000 and 50000, and others that streak but lie outside the arc, such as the one with mol.wt. 118000. As we shall see below, peptide mapping suggests that the polypeptides that focus on the arc are all neurofilament in origin.

It is interesting that the components at mol.wt. 50000 have similar pI values to desmin. In fact the more acidic component has the same isoelectric point and position as the more basic component of chicken desmin. Unfortunately we have not as yet been able to obtain enough material to determine the fingerprint patterns of these polypeptides from *Myxicola*.

The polypeptide composition of neurofilaments

In an attempt to identify more fully some of the minor axoplasmic components, especially those associated with neurofilaments we have isolated filaments by a variety of procedures, all of which gave similar results.

Fig. 1(b) shows a gel of purified neurofilaments prepared by cytochrome *c* precipitation. The two major polypeptides are present together with many minor ones. Filaments prepared by the other methods described gave essentially the same pattern. Thus the complex array of polypeptides with mol.wts. between 172000 and about 47000 that appear to constitute purified filaments are indeed neurofilament in origin and are not contaminants arising out of the purification procedure.

It may be argued that the array of polypeptides is the result of proteolysis during extraction but this is not the case for the following reasons. (a) Axoplasm can be extracted directly into boiling sodium dodecyl sulphate in less than 10s and this procedure always results in the same protein bands being present with the same relative intensities. If proteolysis were occurring during this procedure to produce those bands one would not expect the patterns to be so reproducible. (b) Cleanly extracted axoplasm can be

Fig. 2. Isoelectric focusing of axoplasmic components

A typical isoelectric-focusing gel of whole axoplasm from *Myxicola* is shown with approximate pH values and molecular weights ($\times 10^{-3}$). The smaller photograph shows a better resolved region around tubulin and actin. The numbers in the photographs indicate molecular weight ($\times 10^{-3}$).

left at room temperature for up to 1 h without any noticeable changes in the gel pattern. If the array were the result of proteolysis during extraction one would expect proteolysis to continue after extraction. No evidence of such a process is seen in the gel patterns. (c) The gel pattern arising from purified

filaments was distinctly different from Fig. 1(b) when axoplasm was accidentally contaminated with blood during extraction or when EGTA was not employed in the buffer during isolation. These changes were, however, almost exclusively the result of activation of the Ca^{2+} proteinase, which took at least 2 h to cause

significant alterations in the gel pattern. This indicates that although proteinases are present in axoplasm they are not active enough during extraction to account for the gel pattern in Fig. 1(b).

On the basis of these results it is concluded that purified neurofilaments from *Myxicola* are composed of two major polypeptides with mol.wts. $172\,000 \pm 2\,000$ ($n = 5$) and $155\,000 \pm 3\,000$ ($n = 5$), together with about 15 minor polypeptide components.

Peptide fingerprinting

In an attempt to find out whether common features exist between the neurofilament polypeptides the technique of fingerprinting in sodium dodecyl sulphate with papain and trypsin (Cleveland *et al.*, 1977) was employed.

Initially the two major polypeptide components were studied. Source gels of purified neurofilaments were run and the components at mol.wts. 172 000 and 155 000 were removed and fingerprinted with increasing amounts of papain. Fig. 3 shows the results of this experiment.

Two features were immediately obvious from this gel pattern. The first was that the fingerprints of the components at mol.wts. 172 000 and 155 000 that were present in the high-molecular-weight region (mol.wt. >90 000) were apparently quite different. The second was that below mol.wt. 90 000 the fingerprints of these two components were almost identical. These patterns suggested to us the possibility that the two major neurofilament polypeptides were overall very similar and that the apparent differences in the fingerprints were due to preferential cleavage at particular sites. To test this possibility these fingerprint patterns were analysed further.

The first major peptide (A) (see Fig. 3) to be released from the component with mol.wt. 172 000 had mol.wt. approx. 135 000, which suggested that this component was cleaved to give a polypeptide of mol.wt. 135 000 plus one of about 37 000. By similar reasoning the component with mol.wt. 155 000 appeared to be cleaved into a component of mol.wt. 119 000 (A^1) plus one of about 36 000. The most likely candidates for the smaller polypeptides were E and E^1 (Fig. 3), both of which had mol.wt. 35 000. Further support for this idea comes from the fact that during the initial stage of digestion the polypeptides with mol.wt. 35 000 appear to increase in intensity as A and A^1 increase in intensity (see for example Fig. 6). To confirm this proposed scheme for digestion components A, A^1 , E and E^1 were removed from gels and fingerprinted again.

Fig. 4 shows the results of fingerprinting components A and A^1 compared with a control of the polypeptide with mol.wt. 172 000. If the above scheme for digestion is correct then neither A nor A^1

should contain the fragment with mol.wt. 35 000. This is observed and is shown in Fig. 4. Moreover no fragment with mol.wt. 35 000 has been found in maps of A or A^1 , even after prolonged digestion. Although A and A^1 in Fig. 4 are digested to slightly different extents, it is clear from these patterns that these components have very similar maps with a number of common polypeptides (for example those at mol.wts. 31 000, 22 000 and 19 000). In addition more extensive digestion of A and A^1 shows them to have almost indistinguishable fingerprints.

To see if the fingerprints of E and E^1 were similar they were removed from digests like Fig. 3 and re-digested. Fig. 5 shows that, to the resolution of this technique, components E and E^1 are identical, producing similar peptides with mol.wts. of 31 000, 30 000, 22 000 and 17 000.

These studies therefore show that (a) initially papain cleaves the component with mol.wt. 172 000 into two polypeptides with mol.wts. approx. 135 000 (A) and 35 000 (E) and likewise the component with mol.wt. 155 000 is cleaved into polypeptides with mol.wts. 119 000 (A^1) and 35 000 (E^1), (b) components A and A^1 are very similar and (c) components E and E^1 are identical.

These studies also suggest that E and E^1 come from similar regions of the major neurofilament polypeptides. This may be inferred from the difference in molecular weight between A and A^1 (16 000), which is similar to the difference between 172 000 and 155 000 (17 000), indicating that the initial cleavage that results in the appearance of the fragment with mol.wt. 35 000 is at the opposite end of the molecule to that where the peptide with mol.wt. 17 000 is found. For convenience we have called the end of the molecule that gives rise to components E or E^1 the tail and the opposite end the head (see diagram, Fig. 8).

As the fragment with mol.wt. 35 000 is located at one end of the molecule and as it is preferentially cleaved off by papain during digestion one can investigate whether peptides derived from neurofilament fingerprints originate from head or tail regions. For example, a characteristic feature of fingerprints from both the components with mol.wts. 172 000 and 155 000 is a polypeptide of mol.wt. 53 000 that always appears as a sharp, though not strong, band (D, Fig. 3) and disappears at high papain 'loads' (lane 5, Fig. 3). Excision of this band followed by fingerprinting shows it to break down to give the fragment with mol.wt. 35 000 (plus characteristic products) together with a peptide of mol.wt. approx. 19 000. The cleavage that gives rise to the component with mol.wt. 53 000 is therefore toward the tail-region of the polypeptide (see diagram Fig. 8). Another example is seen with maps from components B, B^1 , C and C^1 (Fig. 3), which always appear blurred on gels. The absence of the fragment with

Fig. 3. *Peptide fingerprinting of major neurofilament polypeptides*

The digestion of the major neurofilament polypeptides with papain and trypsin (lanes 1, 3, 5, 7 and 9 for the 172000-mol.wt. component and lanes 2, 4, 6, 8 and 10 for the component 155000-mol.wt.). Source gels of purified neurofilaments were run and the major polypeptides removed. These were then digested (approx. 20 μ g of protein/sample) with the proteinase the amount of which is shown in the Figure. Although the 172000-mol.wt. component shows the presence of a minor polypeptide it is not a common feature of these fingerprints. Numbers to the left of the photograph and in the body of the Figure indicate molecular weights ($\times 10^{-3}$).

Fig. 5. *Peptide fingerprinting of 35 000-mol.wt. fragment*
Digestion with papain of isolated components E and E¹ compared with parts of the fingerprint profile from the major neurofilament polypeptides. Lanes 1, 2 and 3 correspond to E, E¹ and E, lanes 4, 5 and 6 to E¹, E and E¹ but with higher papain loads and lanes 7 and 8 to relevant regions from the complete digestion of the mol.wt.-172000 and -155000 polypeptides respectively. Numbers to the left of the photograph indicate molecular weight ($\times 10^{-3}$).

mol.wt. 35000 from these maps suggests that they originate, like components A and A¹, from the head-region. This is also inferred from the differences in molecular weight between B (or B¹) and C (or C¹), which vary between 16000 and 20000, close to the value of 17000 expected if the head-regions were preserved throughout these transitions.

The exact origin of the blurred components B, C, B¹ and C¹ (Fig. 3) is uncertain. Fragment C appears to originate from B and B from A and a

Fig. 4. *Peptide fingerprinting of A and A¹*

The digestion with papain of components A and A¹ derived from polypeptides with mol.wts. 172000 and 155000 respectively compared with the complete digestion profile of the mol.wt.-172000 polypeptide. Note the absence in A and A¹ of the mol.wt.-35000 fragment and the presence of a mol.wt.-17000 peptide in A but not A¹ even though A¹ is more digested. The hole in the profile is where the mol.wt.-135000 component was removed for re-digestion. Numbers to the left of the photograph indicate molecular weight ($\times 10^{-3}$).

similar transition ($A^1 \rightarrow B^1 \rightarrow C^1$) can be detected with peptides from the component with mol.wt. 155 000. Blurring of the bands makes precise measurements difficult although the molecular-weight differences ($A - B = \text{approx. } 22\,000$, $B - C = \text{approx. } 18\,000$ and $A^1 - B^1 = \text{approx. } 26\,000$, $B^1 - C^1 = \text{approx. } 17\,000$) might suggest that similar sites on the parent polypeptides were being cleaved. Further evidence for this idea comes from the fingerprints of A and A^1 (Fig. 4). Major digestion products that are common to A and A^1 are seen with mol.wt. approx. 22 000 and approx. 19 000 and these could arise from the $A \rightarrow B$ ($A^1 \rightarrow B^1$) and $B \rightarrow C$ ($B^1 \rightarrow C^1$) transitions. From these data we suggest that the proteolytic digestion of both the major neurofilament polypeptides follows a similar pattern. After the initial cleavage of the chains, which removes fragments with mol.wt. 35 000, the resulting head-regions are subsequently cleaved, releasing peptides with mol.wt. 22 000–26 000, which are situated adjacent to the '35 000-mol.wt.' site and peptides with mol.wt. 18 000 situated next to the '22 000–26 000-mol.wt.' site (see diagram, Fig. 8).

Another common product from A and A^1 maps is a peptide with mol.wt. 31 000, the position of which on the main chain has not yet been determined. It is interesting that this peptide can be phosphorylated after reacting neurofilaments with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. This fact should enable it to be located without confusion with the component with mol.wt. 31 000 resulting from the breakdown of the non-phosphorylated 35 000-mol. wt. fragment.

From a molecular weight of about 90 000 and below, the fingerprints of the components with mol.wt. 172 000 and 155 000 are almost identical. This must mean that at or before this point in the digestion the peptide with mol.wt. 17 000 that differentiates these components is lost. It is tempting to suggest that this peptide is in fact band H in Fig. 3 because it is consistently stronger in maps of the 172 000-mol.wt. component, and it may also be the peptide with mol.wt. 17 000 found in maps of A but not in maps of A^1 (Fig. 4). If this peptide is removed in the early stages of digestion it might be possible to show directly that the component at mol.wt. 155 000 can be generated from that at 172 000. An attempt to achieve this is shown in Fig. 6.

This Figure shows the results from repeated fingerprinting of the component with mol.wt. 172 000. After this procedure (see legend to Fig. 6), the component still generates a faint polypeptide with mol.wt. 155 000, which is unlikely to arise by contamination. Unfortunately too little of this polypeptide was formed to enable it to be collected and mapped with the authentic component with mol.wt. 155 000. Therefore we cannot at the present time unambiguously determine whether the site cleaved here comes from the head or tail region.

Fig. 6. Peptide fingerprinting of 172 000-mol.wt. polypeptide

Source gels of axoplasm were run and the mol.wt.-172 000 polypeptide removed. This component was re-run, and showed no contaminating polypeptide with mol.wt. 155 000. It was then cut out and fingerprinted with papain (lane a). The undigested polypeptide at mol.wt. 172 000 from a sample similar to lane (a) was cut out, re-run and showed no polypeptide at mol.wt. 155 000 but a polypeptide with this molecular weight appeared after digestion of this re-run material (lane b), indicating that a polypeptide with mol.wt. 155 000 can be generated from the one at mol.wt. 172 000. The numbers to the left of the photograph indicate molecular weight ($\times 10^{-3}$).

Two-dimensional fingerprinting of axoplasm from Myxicola

Typical two-dimensional separations of axoplasmic components after fingerprinting are shown in Fig. 7.

Fig. 7. Two-dimensional fingerprinting of axoplasmic components

This Figure shows two-dimensional separations after fingerprinting polypeptides from whole axoplasm with (a) low and (b) high papain loads. The first dimension was constructed from a strip taken from a source gel of whole axoplasm. To construct the second dimension papain was added to this strip after placing it lengthwise across the gel top, and electrophoresis was then performed. (a) Digestion with papain (approx. 1 ng/ml) of axoplasmic polypeptides shows the presence of the mol.wt.-35000 fragment in many of them. Cleavage of these polypeptides at the '35000'-mol.wt. site also results in a second diagonal being formed from the head-regions. Some of the axoplasmic components do not have characteristic neurofilament fingerprints, for example those at mol.wts. 118000, 90000, 74000, 50000 and 42000. (b) Same as for (a) but with more papain (approx. 100 ng/ml), which results in the complete loss of intact neurofilament polypeptides. Numbers on the photographs and to the right of the photographs refer to molecular weight ($\times 10^{-3}$).

One feature immediately obvious from these gels is the presence of the fragment at mol.wt. 35 000 and of its breakdown products in many axoplasmic components between mol.wts. 140 000 and 50 000. Cleavage from these components of the fragment

mol.wt. 35 000 results in a second diagonal on the gel from mol.wt. 110 000–20 000. Many of the components between mol.wts. 140 000 and 50 000 therefore seem to be related to the major neurofilament components.

Fig. 8. The cleavage sites by papain on the major neurofilament polypeptides

Diagram showing some of the main cleavage sites for papain in sodium dodecyl sulphate on the two major neurofilament polypeptides. The polypeptides are represented as bars with width indicating their abundance and the length their molecular weight. Only the head of the mol.wt.-172 000 polypeptide is shown, which is the region of non-identity between the chains. The triangles mark the proteolytic cleavage sites and the numbers indicate molecular weight ($\times 10^{-3}$) of the peptides that would be generated.

Table 1. Polypeptide composition of axoplasm from *Myxicola*

Axoplasmic polypeptides that originate wholly or in part from neurofilaments are italicized. P signifies that the polypeptide has been found phosphorylated after incubating whole axoplasm with [γ - 32 P]ATP. Where brackets have been inserted for example around 135 000 and 131 000 this means that it was difficult to distinguish which component was phosphorylated. The presence of a polypeptide in purified neurofilament preparations is marked by 'a' if it was present after precipitation with cytochrome c, 'b' if it was found after repeated polymerization and depolymerization cycles and 'c' if it was found eluting with filaments (assayed by electron microscopy) after chromatography on Sepharose 4B. * signifies that there was uncertainty regarding the presence of a component because it was too faint. Abbreviations used: ND, not detected. Some polypeptides, for example those with mol.wt. ≤ 42 000, were not found in purified preparation of neurofilaments. The presence of the fragment with mol.wt. 35 000 and of its characteristic breakdown products as revealed by peptide fingerprinting of the polypeptide is marked by + if they were indicated by two-dimensional fingerprinting, and $\checkmark$ if they were found after the polypeptide had been isolated from gels and mapped separately.

$10^{-3} \times$ Molecular weight of polypeptide in axoplasm	Presence of polypeptide in purified preparation of neurofilaments	Presence of fragment with mol.wt. 35 000 and breakdown products	Comments
172 P	a,b,c	+	Major neurofilament polypeptides
155 P	a,b,c	+	
140	a	+	
135 } P	a,b,c*	+	
131 } P	a,b*	+	
129 } P	a,b,c*	+	
120 } P	a,b,c*	+	Neurofilament polypeptide not major axoplasmic component
118	a,b,c	+	
108 P	a,b*,c	+	
100 P	a,b*,c	+	
96 P	a,b*	+	Neurofilament polypeptide not the major axoplasmic component
90 P	a,b,c	+	
82			Bands too weak to be accurately analysed
76			
74 P	a,b,c	+	Neurofilament polypeptide not the major axoplasmic component
70 P	a,b,c	+	Neurofilament polypeptide plus another
64 P	a,b,c,	+	
60 P	a	+	
55	a,b,c	+	
51 P	a,b*,c*	+	Tubulin regions α_1 , α_2 and β (desmin may also be present here)
47	a,b,c*	+	
42	(abc) ND	ND	
<35 P	(abc) ND	ND

Fig. 9. The polypeptide composition of purified neurofilaments

Diagrammatic representation of the polypeptide composition of purified neurofilaments from the data presented in Table 1. Each bar represents a polypeptide the length of which corresponds to its molecular weight (measured from the tail-region) and its thickness gives an indication of its abundance. The numbers indicate the molecular weight ($\times 10^{-3}$) of the polypeptides. The two arrows at the tail represent the sites at which papain cleaves off the fragment of mol.wt. 35 000.

The simplest interpretation for this result is that these polypeptides are derived from the component with mol.wt. 172 000 by the loss of residues from the head-region, the tail of the molecule being intact as indicated by the presence of the fragment with mol.wt. 35 000. If a number of the minor polypeptides in axoplasm are indeed structurally related to major neurofilament components then they could well represent part of the intact filament and this might explain the host of polypeptides associated with purified neurofilament preparations (see Fig. 1). In view of these findings the minor components between mol.wt. 140 000 and 50 000 were subject to more rigorous scrutiny.

Table 1 is compiled from studies on polymerization/depolymerization, precipitation, column chromatography and peptide mapping and shows that there are good reasons for believing that as many as 15 polypeptides between mol.wt. 140 000 and 47 000 are related to the neurofilament component with mol.wt. 172 000. A diagrammatic representation of the way in which the minor components seem to be related to one another is shown in Fig. 9.

Table 1 also indicates that some of the polypeptide bands present in axoplasm are composed of more than one component, examples being at mol.wts. 118 000, 90 000 and 74 000, where the major axoplasmic component is not neurofilament. It is interesting that these bands do not focus on the arc that appears to characterize many of the neurofilament components (see Fig. 2).

Discussion

The results presented here show that neurofilaments purified from *Myxicola* are composed of two major polypeptides with mol.wts. 172 000 and 155 000 together with 15 minor components. The minor polypeptides are not contaminants arising out of the purification procedure nor are they the result

of proteolysis occurring during the extraction of the axoplasm. They therefore represent part of the normal structure of these neurofilaments.

Peptide fingerprinting of the major neurofilament polypeptides suggests that they are almost identical, the major structural difference between them being an additional peptide of mol.wt. 17 000 at the head-region of the larger polypeptide. In addition fingerprinting shows that the larger polypeptide (mol.wt. 172 000) is cleaved by papain initially at a site near one end of the molecule, releasing polypeptides with mol.wts. 35 000 and approx. 135 000. The end of the molecule that gives rise to the smaller fragment has been called the tail-region. The other major neurofilament polypeptide (mol.wt. 155 000) is cleaved in a similar manner, releasing polypeptides with mol.wts. 35 000 and approx. 119 000. The reason for the susceptibility of the site towards the tail-region to digestion is not clear but may reflect a particular conformation of the molecule that exists in the presence of sodium dodecyl sulphate. After the fragment with mol.wt. 35 000 has been cleaved, the peptide fingerprints of the resulting head-regions suggest that they are susceptible to digestion at sites adjacent to this cleavage site. Further work, however, is necessary to establish the exact sequence of the peptides released.

The fact that the major neurofilament components can be easily identified by their fingerprint patterns has enabled the identification of other axoplasmic components that share part of this pattern: the presence of the fragment of mol.wt. 35 000 and of its degradation products. This fragment appears to be conserved in many of the axoplasmic polypeptides between mol.wt. approx. 140 000 and 50 000, which also co-purify with the major neurofilament polypeptides. It appears therefore that these minor components are generated from the major neurofilament polypeptides by removal of peptides from the head-regions of these molecules.

It might be argued that the minor components are not fragments of some precursor molecule but in fact represent separate gene products and that their peptide resemblance indicates similarity through, for example, gene duplication. Although this argument cannot at the moment be completely ruled out, recent studies show that it is unlikely for the following reason. Many of the minor components terminate at positions that appear to represent sites cleavable by proteinases. For example, in the absence of sodium dodecyl sulphate the major neurofilament polypeptides of intact neurofilaments are cleaved *in vitro* by the Ca^{2+} -dependent proteinase yielding polypeptides with mol.wts. 47000, 55000 and 70000.

Moreover each of these cleaved polypeptides has a peptide fingerprint that is similar to the fingerprint of the minor component having the same molecular weight.

If these minor neurofilament components are generated by proteolysis then this must have occurred *in vivo* before extraction of the axoplasm. The proteinases responsible for this have not yet been identified, but in view of the studies referred to above it seems likely that the Ca^{2+} -dependent proteinase is involved.

It is interesting to speculate on the question of why, in isolated neurofilaments, there are no neurofilament polypeptides with molecular weight less than approx. 47000. One reason for this might lie in the way the neurofilament polypeptides are arranged within the filament. If the head-regions of the polypeptides were exposed and the tail-regions protected by being wrapped in the core of the filament then one might expect proteinases to act by cleaving preferentially at exposed head-region sites. This would suggest that the minor component with mol.wt. 47000 is the smallest structure compatible with an intact filament.

Another possibility is that the sites cleaved by these proteinases may indicate regions between stretches of α -helices. It is well known that highly α -helical regions in proteins such as myosin (Lowey *et al.*, 1969) and keratin (Steinert, 1978) resist proteolytic breakdown. If this were also true of neurofilaments that have approx. 50% α -helix, then

the minor component of mol.wt. 47000 may be left intact simply because it is rich in α -helix. As the region covered by this component is towards the tail of the molecule one might expect an increase in the α -helical content of peptides removed from this region.

Some support for this idea comes from recent studies where we have measured the α -helical content of polypeptides removed from intact molecules by enzymic digestion. These studies indicate that polypeptides that are approximately two-thirds of the total molecule and that arise from the head region have less than 20% of the total α -helix, implying that the remaining 80% is located towards the tail of the molecule.

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