The location of phosphorylation sites and Ca^{2+} -dependent proteolytic cleavage sites on the major neurofilament polypeptides from *Myxicola infundibulum*

Peter A. M. EAGLES, David S. GILBERT* and Alison MAGGS

Department of Biophysics and M.R.C. Cell Biophysics Unit, King's College, 26/29 Drury Lane, London WC2B 5RL, U.K.

(Received 28 April 1981/Accepted 16 June 1981)

1. When axoplasm is incubated with [^{32}P]P_i the main phosphorylated components are the neurofilament polypeptides. 2. Activation with Ca^{2+} of the proteinase present in axoplasm causes degradation of these neurofilaments and the peptides produced by this reaction have been analysed by fingerprinting. 3. Fingerprinting shows that initially the Ca^{2+} -activated proteinase cleaves the neurofilament polypeptides at three major sites producing polypeptides with mol.wts. 70 000, 50 000 and 47 000. 4. These polypeptides sediment with filaments, originate from the tail-region of the molecule and contain a little radioactive label. 5. As these polypeptides are produced, other polypeptides that come from the head-region of the molecule are liberated as soluble products that contain the bulk of the radioactivity. 6. Fingerprinting therefore shows that at least two regions on the molecule are phosphorylated and that the major one is located towards the head-end of the polypeptides.

Neurofilaments from *Myxicola infundibulum* comprise two major polypeptide chains (Gilbert *et al.*, 1975; Lasek *et al.*, 1979), which have mol.wts. of 172 000 and 155 000. About 15 minor components always co-purify with these major polypeptides. We have shown recently (Eagles *et al.*, 1981) that the major polypeptides are almost identical and that all the polypeptides of purified neurofilaments have a common structural feature; i.e. the presence of a polypeptide fragment with mol.wt. 35 000 situated at one end of the molecule. We have called this end the tail and the opposite end the head. The fragment is readily cleaved from the intact molecule by papain and its presence in peptide fingerprints enables polypeptides that come from the tail-region of the molecule to be identified. The classification of polypeptides by this method has enabled us to locate regions on the neurofilament molecule that are modified *in vitro* by two enzymic processes, namely Ca^{2+} -dependent proteolysis and phosphorylation.

The presence of a Ca^{2+} -dependent proteinase in axoplasm was first observed by Gilbert *et al.* (1975) using giant axons from the fan worm *Myxicola* and

the squid *Loligo forbesii*. An enzyme with similar properties has now been found in vertebrate nervous tissue (Schlaepfer & Micko, 1979). Although the function of these enzymes is unknown, it has been suggested that they might play a role in the degradation of neurofilaments (Lasek & Hoffman, 1976).

Myxicola neurofilaments can also be modified by another enzymic process, phosphorylation. A specific kinase is present in axoplasm to accomplish this (Eagles *et al.*, 1978). Phosphorylation appears to be a common feature of these filamentous proteins, as it has also been observed in squid and vertebrate neurofilament preparations (Pant *et al.*, 1978; Eagles *et al.*, 1980; Runge *et al.*, 1981). Like the Ca^{2+} -dependent proteinase activity, the function of this phosphorylation has yet to be determined.

The studies that we report in the present paper were embarked upon in an attempt to elucidate the function of these enzymic processes by initially locating the major sites that they modify on the neurofilament polypeptides. We show that when the Ca^{2+} -dependent proteinase is activated it cleaves these polypeptides predominantly at three sites. In addition we show that at least two regions are phosphorylated in the molecule, the major one being located towards the head-end.

Abbreviation used: SDS, sodium dodecyl sulphate.

*Deceased December 1979.

Materials and methods

Materials

Myxicola were collected and axoplasm was extracted from them as described previously (Eagles *et al.*, 1981). Papain (type III) was obtained from Sigma. [γ - ^{32}P]ATP (sp. radioactivity $>5000\text{ Ci/mmol}$) and [^{32}P]P_i (carrier-free, 1 mCi/ml) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of the highest purity available. Kodirex EP X-ray film was obtained from Kodak. Phosphocellulose paper (P81) was purchased from Whatman.

Methods

Polyacrylamide gels (5–15% and 5–20% acrylamide) were run in the presence of SDS and peptide fingerprinting was performed as described previously (Eagles *et al.*, 1981). After electrophoresis, gels containing radioactively labelled polypeptides were first stained and destained, then blotted dry, and finally placed in a plastic bag that was then sealed. The gel was held flat against a glass plate together with a sheet of X-ray film. The film was developed after 1–20 days exposure. The labelled areas on it were then matched with the corresponding Coomassie Blue-stained polypeptides on the gel.

Isoelectric focusing was performed by the method of O'Farrell (1975) with minor modifications. Electron microscopy was performed as described previously (Eagles *et al.*, 1981).

Phosphorylation. When [γ - ^{32}P]ATP was used to phosphorylate neurofilaments, axoplasm was first homogenized at a concentration of 40 mg/ml in a medium containing 100 mM-Tris/HCl , 10 mM-MgCl_2 , 1 mM-EGTA , $1\text{ mM-}\beta$ -mercaptoethanol at a final pH of 8.5. [γ - ^{32}P]ATP was then added to give a concentration of $1\text{--}10\text{ }\mu\text{Ci/mg}$ of axoplasm and the mixture was incubated for 15–30 min at 25°C . Under these conditions, maximum labelling of the polypeptides was obtained with minimum interference from proteolytic side-reactions. The reaction was terminated by adding SDS to a concentration of 1% then boiling for 5 min.

Neurofilament phosphorylation could also be achieved by using [^{32}P]P_i together with buffers designed to maintain functional mitochondria. Two buffer systems were used: buffer A, which kept the neurofilament network intact, consisted of $500\text{ mM-potassium glutamate}$, 10 mM-MgCl_2 , 10 mM-Tris/HCl , $10\text{ mM-sodium succinate}$ at a final pH of 7.2 and buffer B, which partially disrupted the neurofilament network, consisted of 300 mM-KCl , 400 mM-sucrose , $10\text{ mM-sodium succinate}$, 10 mM-MgCl_2 , 100 mM-Tris/HCl (or 20 mM-histidine) at the same pH value. To preserve the delicate mitochondria, axoplasm ($5\text{--}10\text{ mg}$) after extraction was not homogenized but gently dispersed by pipette in

either buffer A or B. [^{32}P]P_i was then added to give a concentration of $1\text{--}10\text{ }\mu\text{Ci/mg}$ of axoplasm and the samples were incubated at 25°C or 37°C for 1–2 h. Despite the absence of EGTA in the buffers, little proteolysis could be detected at the end of the incubation when samples were assayed on polyacrylamide gels. Neurofilament phosphorylation was stopped by inhibiting mitochondrial phosphorylation. This was accomplished by (1) adding $50\text{ }\mu\text{l}$ of $10\text{ mM-dinitrophenol}$ and (2) rapidly freezing and thawing the sample three times.

By using [^{32}P]P_i, a quantitative assay for measuring neurofilament phosphorylation was developed. Axoplasm dispersed in buffer B (containing 1 mM-EGTA) was generally used for this assay but buffer A could also be used after minor modifications to the procedure. [^{32}P]P_i was added after dispersion and the sample was incubated. Portions ($10\text{ }\mu\text{l}$) were taken from this sample at timed intervals, diluted with $10\text{ }\mu\text{l}$ of $0.5\text{ M-sodium phosphate}$, pH 7.0, and spotted on to phosphocellulose paper that had been treated with 10% (w/v) trichloroacetic acid. The paper was dried briefly, then thoroughly washed with four to five changes of 4% trichloroacetic acid containing $10\text{ mM-phosphoric acid}$, and finally dried in an oven. The samples were cut from the paper and counted for radioactivity (Čerenkov method) with an efficiency of around 30%.

Ca²⁺-dependent proteolysis. The activity of this enzyme is discussed below only as it affects these particular experiments. Generally, the proteinase was activated in an EGTA-free medium by adding $1\text{--}20\text{ mM-Ca}^{2+}$ and incubating at 25°C for 30–40 min. Phosphorylation of the neurofilaments did not affect their ability to be cleaved by the proteinase, so neurofilaments were generally phosphorylated before activating this enzyme. Proteolysis was stopped by adding EGTA and the samples were then centrifuged at $120\,000\text{ g}$ (r_{av} , 6.5 cm) for 2 h at 10°C . This procedure separated cleanly the pellet from the supernatant. Each fraction was removed, boiled with 1% SDS containing 1% β -mercaptoethanol and run on polyacrylamide gels. After electrophoresis, gels were stained briefly and subjected to autoradiography. Labelled polypeptides were subsequently excised for fingerprinting.

Results

Isoelectric focusing

Isoelectric focusing of whole axoplasm that has been incubated with [γ - ^{32}P]ATP shows that nearly all of the radioactive label is associated with the two major neurofilament polypeptides (Fig. 1). Some of the minor neurofilament components that form an arc on this gel are also weakly labelled. Thus apart from the polypeptides that originate from neurofilaments, there is no labelling of other axoplasmic

Fig. 1. Isoelectric focusing gel (a) and corresponding autoradiograph (b) of phosphorylated neurofilaments. Neurofilaments were phosphorylated as described in the Materials and methods section with [γ -³²P]ATP. The positions of actin and tubulin are marked in (a) together with the positions of three other axoplasmic components (mol.wts. 118 000, 90 000 and 74 000).

components under the phosphorylation conditions employed.

As the two major polypeptides are almost identical the labelling detected here presumably

means that similar sites are being modified on the common regions of these chains. Moreover, as label can be detected on the minor components down to a molecular weight of approx. 70 000 at least one site

must be present on that half of the molecule that incorporates the fragment with mol.wt. 35 000 towards the tail-region. To establish the location of these phosphorylation sites more precisely, peptide fingerprinting with papain was employed.

Fingerprinting of ^{32}P -labelled neurofilament polypeptides

An initial attempt to locate some of the phosphorylation sites on the major polypeptides is shown in Fig. 2. Neurofilaments were first labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described under 'Methods' and source gels were run from which the major polypeptides were removed. They were then digested with papain. Figs. 2(a) and 2(b) show that peptide fingerprints of these phosphorylated components have some similarity. For example, both maps contain high-molecular-weight peptides that are heavily labelled. They also show that the tail-region fragment (mol.wt. 35 000) of each polypeptide is not phosphorylated and that a common component with mol.wt. 31 000 is strongly labelled. The labelling

pattern in the maps indicates that similar sites are being phosphorylated on both the major neurofilament polypeptides and that these sites exist on the components A and A' (mol.wts. 135 000 and 119 000) that are formed after cleaving the major polypeptides at the '35 000-mol.wt.' site (Eagles *et al.*, 1981).

The phosphorylated polypeptides A and A' were isolated and fingerprinted separately in an attempt to locate on them the labelled sites; these patterns are shown in Figs. 2(c)–2(f). Most of the peptides produced from both components A and A' in the high-molecular-weight region down to about mol.wt. 70 000 are heavily labelled. Radioactive label is also found at mol.wt. 31 000 and occasionally faint label is seen at mol.wts. 27 000 and 22 000 after prolonged digestion. In the preceding paper (Eagles *et al.*, 1981) on the papain digestion of neurofilaments we suggested that components A and A' were digested preferentially at sites adjacent to the '35 000-mol.wt.' site, thus preserving the head-regions on high-molecular-weight polypeptides.

Fig. 2. *Peptide fingerprints and autoradiographs of phosphorylated neurofilament polypeptide*

Source gels were run of phosphorylated neurofilaments and the major polypeptides (mol.wts. 172 000 and 155 000) were removed. These were digested with papain to remove the fragment with mol.wt. 35 000 at the tail-region, so producing component A (mol.wt. 135 000) from the 172 000-mol.wt. polypeptide and A' (mol.wt. 119 000) from the 155 000-mol.wt. polypeptide. The Figure shows typical fingerprints (c–f) and autoradiographs (c'–f') of these components together with controls (a, b, and corresponding autoradiographs a' and b') that were all run on the same gel. The polypeptide samples are: (a) 172 000-mol.wt. control; (b) 155 000-mol.wt. control; (c) A, mol.wt. 135 000; (d) the same as (c) with more digestion; (e) A', mol.wt. 119 000; (f) the same as (e) with more digestion. Notice that the tail-region fragments (mol.wt. 35 000) in samples (a) and (b) are not phosphorylated and that a phosphorylated component (mol.wt. 31 000) is common to the major neurofilament polypeptides and the components A and A'.

Therefore, the pattern of labelling found here indicates that major phosphorylation sites are located towards the head-region of the molecule.

The origin of the common, phosphorylated, peptide with mol.wt. 31000 is not clear from these experiments. The spot at this molecular weight on the autoradiograph is related in position and in intensity to a peptide in the gel pattern. As shown below, cleavage with the Ca^{2+} -dependent proteinase suggests that this peptide probably resides near to the '35000-mol.wt.' site.

Proteolytic digestion of phosphorylated neurofilaments with the Ca^{2+} -dependent proteinase

To identify more fully the location of the phosphorylation sites it was clear that another proteinase with different specificity must be used. For a number of reasons, both technical and otherwise, we chose the Ca^{2+} -dependent proteinase. This proteinase, for instance, is easily activated with Ca^{2+} and is readily inhibited with EGTA. In addition, by using this enzyme to uncover how it cleaves neurofilaments something might be learnt about its role *in vivo*. We therefore investigated the following scheme to probe the phosphorylation sites and Ca^{2+} -dependent proteinase cleavage sites.

First, axoplasm was incubated with [γ - ^{32}P]ATP to phosphorylate the neurofilament polypeptides. Secondly, Ca^{2+} was added to activate the proteinase and digest the filaments. Thirdly, this digest was separated into polypeptides by gel electrophoresis and their composition was analysed by fingerprinting with papain followed by autoradiography.

In this scheme it was clearly important to ensure that (1) neurofilaments were highly phosphorylated and (2) no phosphorylation occurred after cleavage by the proteinase. For these reasons, during the phosphorylation reaction we chose [^{32}P]P_i as the labelled donor that was converted by the axoplasmic mitochondria into [γ - ^{32}P]ATP. By using this method neurofilament phosphorylation was efficient and could be easily stopped by disrupting the mitochondrial phosphorylating system.

To find the best way of inhibiting mitochondrial phosphorylation in axoplasm, various inhibitors were tested and a set of typical results is given in Table 1. Treatment of axoplasmic samples with cyanide or dinitrophenol or by freezing and thawing effectively inhibited phosphorylation whilst allowing the Ca^{2+} -dependent proteinase to be activated. In the studies reported in the present paper phosphorylation was stopped by using a combination of freezing and thawing and treatment with dinitrophenol, which was found to inhibit by more than 96%.

After establishing that the proteinase could still work after phosphorylation was stopped, we were then in a position to investigate the structure of the peptides produced by the proteinase. Initially the results from gels were difficult to interpret because of the large number of peptides formed. A preliminary fractionation by centrifugation was therefore employed to separate some of the polypeptides and undegraded filaments before subjecting them to further analysis.

Throughout our procedures the ionic strength of the buffers was such that the filaments did not disassemble significantly into subunits and they could be readily sedimented by centrifugation at 100000g for 2h. Therefore we anticipated that centrifuging filaments that had been subjected to Ca^{2+} -dependent proteolysis would still pellet filamentous structures. These would be composed of partially degraded polypeptides, leaving polypeptides that had been cleaved from the filaments free in the supernatant fraction. Fig. 3(a) shows the gel analysis of such an experiment, where neurofilaments were first phosphorylated, then cleaved by the proteinase and finally fractionated by centrifugation.

If we consider first the changes that occur in the pelleted fractions we see that without Ca^{2+} (sample A) most of the neurofilaments are sedimented by centrifugation. Then as the Ca^{2+} concentration is raised to increase activation of the proteinase (samples B, C and D) so there is a gradual reduction in the major neurofilament polypeptides in the pellet.

Table 1. *Effect of inhibitors on neurofilament phosphorylation*

Freshly extracted axoplasm (20mg) was gently dispersed in 0.5ml of buffer B (see the Materials and methods section). This was then divided into five equal portions and 5 μCi of [^{32}P]P_i was added to each. The five samples were treated respectively as follows: (a) was stopped immediately; (b) was incubated with no additions; (c) and (d) were treated with KCN and 2,4-dinitrophenol respectively and then incubated; and (e) was frozen and thawed three times with acetone at -30°C , and then incubated. Incubations lasted for 40min at 25°C after which samples were treated as described in the Materials and methods section.

Sample	Conditions	Radioactivity in sample (d.p.m.)
(a)	Axoplasm (zero-time control)	366
(b)	Axoplasm (after incubation for 40min)	33847
(c)	Axoplasm + 10 mM-KCN	383
(d)	Axoplasm + 3 mM-2,4-dinitrophenol	5028
(e)	Axoplasm (sample frozen and thawed three times)	1666

Fig. 3. The polypeptide composition of ^{32}P -labelled neurofilaments after digestion with the Ca^{2+} -dependent proteinase (a) and the corresponding autoradiograph (b)

In this experiment 160 mg of freshly extracted axoplasm was gently dispersed in 1 ml of buffer B (see the Materials and methods section), which contained $300\ \mu\text{Ci}$ of $[^{32}\text{P}]\text{P}_i$. The sample was then incubated at 37°C for $1\frac{1}{2}$ h. The phosphorylation reaction was stopped by adding dinitrophenol and freezing and thawing the sample three times. The sample was then split into four equal aliquots and Ca^{2+} was added to initiate proteolysis. The final Ca^{2+} concentration was 0, 4.5 mM, 12.5 mM and 20 mM for samples (A), (B), (C) and (D) respectively. After incubating these samples for $1\frac{1}{2}$ h they were centrifuged at $120\,000\ g$ (r_{av} , 6.5 cm) for 2 h. The supernatant was then carefully separated from the pelleted fraction and both were solubilized in 1% sodium

Concomitant with this is the appearance of intense, new, polypeptide bands (1, 2 and 3) at mol.wts. 70 000, 55 000 and 47 000, together with many minor ones between mol.wts. 50 000 and 42 000. After extensive digestion of the major neurofilament components other polypeptides are formed in the pellet, particularly one at mol.wt. 66 000 (not prominent in Fig. 3a).

Changes also occur in the polypeptide composition of the supernatant fraction that parallel these events. Initially, without Ca^{2+} the supernatant contains mainly actin and tubulin, together with some low-molecular-weight peptides ($<20\,000$). During proteolysis, however, four densely staining polypeptide bands (4, 5, 6 and 7) appear in the supernatant at mol.wts. 125 000, 108 000, 94 000 and 78 000. Blurring of these bands makes exact measurement of their molecular weights difficult. Band 4 is the weakest and appears only after short digestion times. It soon disappears as digestion continues. Bands 6 and 7, however, increase in intensity and tend to dominate this pattern.

The autoradiograph of this gel is represented in Fig. 3(b). Initially most of the radioactive label is on the major neurofilament polypeptides and this is decreased as digestion proceeds. Some of the new polypeptides that are found in the pelleted fraction are labelled (bands 1 and 2); but it is clear from this Figure that after digestion the majority of the label that was originally on the major neurofilament polypeptides is now found on polypeptides in the supernatant (bands 4, 5, 6 and 7). The changes that occur in the pattern of labelling during digestion mimic the changes seen in Coomassie staining.

We interpret the main features of these patterns in the following way. As the two major neurofilament polypeptides are almost identical in structure it is likely that they will be cleaved by the Ca^{2+} -dependent proteinase in a similar manner. Band 1 (mol.wt. 70 000) could therefore arise by cleavage of the component with mol.wt. 172 000 and would generate an additional polypeptide with mol.wt.

dodecyl sulphate/1% β -mercaptoethanol and run on the gel. For each sample the pellet (P) is in the left lane and the supernatant (S) in the right. The new polypeptides that arise after proteolysis are numbered 1, 2 and 3 in the pellet and 4, 5, 6 and 7 in the supernatant. After electrophoresis this gel was stained, destained and autoradiographed as described in the Materials and methods section. The results are shown in (b). Polypeptides 1 and 2 are weakly labelled and most of the ^{32}P resides on polypeptides 4, 5, 6 and 7. In both (a) and (b), the numbers to the left and right of the photographs represent $10^{-3} \times$ molecular weight.

approx. 100 000 (band 5 or 6). Band 1 could also arise by cleavage of the 155 000-mol.wt. component and so produce another polypeptide with mol.wt. approx. 85 000 (band 6 or 7). Likewise the molecular weights of the polypeptides generated from the major neurofilament polypeptides together with band 2 (mol.wt. 55 000) would be 117 000 and 100 000 and those with band 3 (mol.wt. 47 000) 125 000 and 108 000. The composition of the supernatant bands could therefore be explained, at least in part, by combinations of these high-molecular-weight polypeptides. The intensities of the bands would be proportional to the intensity of the component from which they arise. Thus polypeptides generated from the component with mol.wt. 155 000 would prevail in these patterns and be about three times as intense as those from the 172 000-mol.wt. component.

If digestion does follow this path then because the polypeptides in band 1 are radioactively labelled, albeit weakly, together with the high-molecular-weight polypeptides in bands 5, 6 and 7 the implication is that there exist at least two phosphorylation sites on the molecule. Furthermore, most of the label is localized on one half of it.

To progress further with the identification of these phosphorylation sites clearly requires an understanding of where in the intact molecule the polypeptides that constitute these bands are located. One way of approaching this problem is to remove the bands cleaved by the Ca^{2+} -dependent proteinase and subject them to further digestion by papain since the papain fingerprints of neurofilament polypeptides have been well analysed. Consequently bands 1–7 were removed from gels and fingerprinted in this way.

Papain fingerprints of the polypeptides in bands 1, 2 and 3

Fig. 4. shows a gel where the polypeptides from bands 1, 2 and 3 have been fingerprinted. It appears that each band contains a dominant species of polypeptide and it is clear that each of these polypeptides contains the fragment at mol.wt. 35 000. Fig. 4 shows that as polypeptide 3 (mol.wt. 47 000) is progressively digested, the 35 000-mol.wt. fragment first increases in intensity and is then further digested to its breakdown products at mol.wts. 31 000, 30 000 and 20 000. Together with these changes, a peptide at mol.wt. 12 000 increases in intensity. Therefore the pattern is consistent with the idea that polypeptide 3 is cleaved to produce this 12 000-mol.wt. peptide together with the tail-region fragment. By similar reasoning, the molecular weights of the peptides cleaved initially from polypeptide 2 (mol.wt. 55 000) appear to be 35 000 and approx. 22 000 and those from polypeptide 1 (mol.wt. 70 000) 35 000 and approx. 31 000. The polypeptides

Fig. 4. Fingerprinting with papain of the pelleted and phosphorylated polypeptides in bands 1, 2 and 3

In this gel the polypeptides in bands 1, 2 and 3 with mol.wts. 70 000, 55 000 and 47 000 respectively have been mapped, together with the two major neurofilament polypeptides (mol.wts. 172 000 and 155 000). From left to right the samples, which are in lots of three, correspond to bands 3, 2 and 1. Each sample has been digested with three different concentrations of papain. At the extreme right of the gel are the fingerprints of the major neurofilament polypeptides. The phosphorylated peptides in these fingerprints are marked above with an asterisk and this assignment represents the accumulated results from four quite separate experiments. Polypeptides from band 3 were not found to be strongly labelled. Polypeptides from bands 2 and 1 were digested to give as their major phosphorylated breakdown products peptides at mol.wts. 22 000 and 31 000 respectively. Further controls for all these samples were obtained from gels on which had been run neurofilaments that had not been exposed to the Ca^{2+} -dependent proteinase. Regions at the same molecular weight as the samples were cut from these gels and used for the controls.

forming bands 1, 2 and 3 therefore appear to come from the tail-region of the molecule (see Fig. 8).

Of the polypeptides 1, 2 and 3 only 1 and 2 are phosphorylated significantly. As all three originate from the same region of the molecule a phosphorylation site must exist between the end of polypeptide 2 and the end of polypeptide 3. This region has been called P_2 (see Fig. 8) and would form part of the phosphorylated peptide with mol.wt. 22 000 from fingerprints of polypeptide 2. It would also form part of the phosphorylated peptide with mol.wt. 31 000 from polypeptide 1 (Fig. 4). The label

on the 22000- and 31000-mol.wt. peptides appears to account for the majority of that which was originally present in bands 1 and 2.

Peptide fingerprints of the polypeptides in bands 4, 5, 6 and 7

Typical fingerprints and autoradiographs of these polypeptides are shown in Figs. 5, 6 and 7. Unfortunately band 4 (mol.wt. 125000) was never present in sufficient quantities for its polypeptides to be mapped successfully. However, fingerprints from the other bands all showed similar features. These were (1) the complete absence of the fragment (mol.wt. 35000) at the tail of the molecule, suggesting that the supernatant polypeptides belonged to the head-region, (2) the presence of intensely labelled high-molecular-weight polypeptides that account for the bulk of the radioactivity in the original bands and (3) the absence of the phosphorylated peptide (mol.wt. 31000) found in digests of whole polypeptides and digests of A and A' (see Fig. 2), implying that this peptide has either been lost or modified during Ca^{2+} -dependent proteolysis.

Other features were common amongst certain bands. Polypeptides from bands 6 and 7, for example, showed similarities during fingerprinting at certain papain loads (Figs. 5c and 5d) and polypeptides from bands 5 and 6 generally produced non-phosphorylated components around mol.wt. 17000. As it is likely that bands 5, 6 and 7 are complex mixtures of polypeptides resulting from the breakdown of both the 155000-mol.wt. component and that at mol.wt. 172000, detailed interpretations of the patterns have not, as yet, been done.

In summary, therefore, analyses of the supernatant fractions show that most of the polypeptides that are liberated into solution during Ca^{2+} -dependent proteolysis are highly phosphorylated and are derived from the head-region of the neurofilament molecule.

Discussion

The results reported in the present paper are an initial attempt to identify the regions that are enzymically modified by phosphorylation and Ca^{2+} -dependent proteolysis on the major neurofilament polypeptides from *Myxicola*. These neurofilaments can be selectively phosphorylated in axoplasm and this has enabled two of the labelled sites to be characterized by proteolysis with papain and with the axoplasmic Ca^{2+} -dependent proteinase.

Digestion with the Ca^{2+} -dependent proteinase has also enabled a preliminary identification to be made of the sites at which it cleaves the neurofilament molecule. Initially this proteinase cleaves at three major sites, producing polypeptides with mol.wts. 70000, 55000 and 47000, which are sedimentable

Fig. 5. Peptide fingerprints of supernatant polypeptides from bands 5, 6 and 7

Bands 5, 6 and 7 were removed from a gel similar to that shown in Fig. 3 and their polypeptides were fingerprinted with papain. The neurofilaments used in this particular experiment were not phosphorylated. The samples, which were all run on the same gel, are as follows: (a) control of major neurofilament polypeptide with mol.wt. 155000; (b) band 5 (mol.wt. 108000); (c) band 6 (mol.wt. 94000); and (d) band 7 (mol.wt. 78000). Note the absence of the tail-region fragment with mol.wt. 35000 in the fingerprints of bands 5, 6 and 7.

and which form part of a filamentous precipitate. The three polypeptides come from the tail-region of the molecule, because they all contain the 35000-mol.wt. fragment and characteristic breakdown

Fig. 6. Peptide fingerprints of ^{32}P -labelled supernatant polypeptides from bands 5, 6 and 7

Bands 5, 6 and 7 prepared from ^{32}P -labelled neurofilaments were removed from a gel similar to that shown in Fig. 3 and the polypeptides were fingerprinted with papain. All the samples were run on the same gel and are as follows: (a) and (b), controls of major neurofilament polypeptides with mol.wts. 155 000 and 172 000 respectively; (c) band 7 (mol.wt. 78 000); (d) control of ^{32}P -labelled polypeptide A (mol.wt. 135 000) produced after removal of the tail-region fragment from the polypeptide with mol.wt. 172 000; (e) band 6 (mol.wt. 94 000); and (f) band 5 (mol.wt. 108 000). Samples (a')–(f') are the corresponding autoradiographs. Note the absence in fingerprints of polypeptides from all the bands, of tail-region fragments, polypeptides at mol.wt. 30 000–31 000 and the ^{32}P -labelled components at mol.wt. 31 000. Further controls for all these samples were obtained from gels on which had been run neurofilaments that had not been exposed to the Ca^{2+} -dependent proteinase. Regions at the same molecular weight as the samples were cut from these gels and used for the controls.

products. When these polypeptides appear in the pelleted fraction new peptides, representing the rest of the molecule and therefore containing the head-regions, appear in the supernatant. Lasek *et al.* (1979) have concluded that the Ca^{2+} -dependent proteinase cleaves the neurofilament molecule into two fractions of one-third and two-thirds of the molecules' length, results clearly in agreement with those reported in the present paper.

In the preceding paper (Eagles *et al.*, 1981) we examined the structure of some of the minor polypeptides associated with purified neurofilaments. Three of these polypeptides have mol.wts. 70 000, 50 000 and 47 000 and originate from the tail-region of the molecule. It is interesting that the Ca^{2+} -dependent proteinase cleaves the neurofilament *in vitro* to release apparently structurally similar peptides. This raises the interesting possibility that the proteinase may have been responsible for the production *in vivo* of some of the minor

polypeptides; the reason for this is not clear at present.

It is also intriguing that these three polypeptides, which come from the tail-region of the molecule, sediment with undigested neurofilaments. This could mean that these polypeptides still have a structural feature that allows them to form a filament and would imply that the 35 000-mol.wt. tail-region fragment, which is common to them, is involved in this.

After neurofilaments have been phosphorylated, digestion with papain of the major polypeptide components shows that the fragment at the tail-region is not labelled. It is not known whether this is because the fragment contains no susceptible sites or because potential sites are rendered inaccessible to phosphorylation by, for example, the assembly of these regions into filaments. A fragment with mol.wt. 31 000 is, however, phosphorylated on both the major neurofilament polypeptides in intact fila-

Fig. 7. *Peptide fingerprints of ^{32}P -labelled supernatant polypeptides from bands 6 and 7*

The polypeptides from bands 6 and 7 were fingerprinted as in Fig. 6, but with various concentrations of papain. All samples were run on the same gel and are as follows: (a)–(c) band 6 (mol.wt. 94 000) with increasing papain; (d) and (e) band 7 (mol.wt. 78 000) with increasing papain. Samples (a')–(e') are the corresponding autoradiographs.

Fig. 8. *Diagrammatic representation of the location of phosphorylation regions and sites cleaved by the Ca^{2+} -dependent proteinase on the major neurofilament polypeptides in *Myxicola**

The polypeptides are represented as bars, the length of which indicates their molecular weight and the height of which indicates the abundance. The polypeptide with mol.wt. 172 000 is partly shown at the head-region; apart from this area it is believed to be identical with the component with mol.wt. 155 000. The position of the 35 000-mol.wt. tail-region fragment is hatched. The 15 small arrowheads beneath the 155 000-mol.wt. polypeptide represent the regions where the minor neurofilament polypeptides terminate (Eagles *et al.*, 1981). The large triangles indicate the sites where the Ca^{2+} -dependent proteinase cleaves the molecule. The numbers 70 000, 55 000 and 47 000 represent the molecular weights of the peptides (measured from the tail) that would be generated by cleavage at these sites. The ^{32}P -labelled regions are marked P_1 and P_2 . Most of the label is found in the region P_1 but the number of residues phosphorylated here have not yet been determined. The probable position of the phosphorylated peptide with mol.wt. 31 000 is shown as a line of dashes adjacent to the 35 000-mol.wt. fragment.

ments, but digestion with papain alone has not enabled its position to be unambiguously determined. Further information on the location of this phosphorylation site has, however, been obtained by analysing the peptides produced after digestion with the Ca^{2+} -dependent proteinase.

When neurofilaments have been phosphorylated and cleaved with the Ca^{2+} -dependent proteinase the majority of the radioactivity is found on the soluble polypeptides, which originate from the head-region of the molecule. For convenience we have designated this region P_1 (Fig. 8) and we have an indication of its maximum extent. As cleavage at the 70 000-mol.wt. site results in most of the label being found on these soluble polypeptides, site P_1 must be within a region of mol.wt. approx. 90 000. As yet we do not know how many residues are phosphorylated here.

Peptide fingerprinting of the soluble polypeptides shows that they contain no phosphorylated component with mol.wt. 31 000. This component is, however, present in papain digests of intact molecules and digests of A and A'. These facts would suggest that this peptide is situated at a region that is cleaved by the Ca^{2+} -dependent proteinase, i.e. adjacent to the 35 000-mol.wt. site. It is likely therefore that this peptide somehow incorporates the phosphorylated site P_2 . This would explain the fact that the phosphorylated peptide with mol.wt. 70 000 that appears in the precipitate after Ca^{2+} -dependent proteinase cleavage can be digested with papain into a labelled component (mol.wt. approx. 31 000) together with the non-phosphorylated tail-region fragment. Again we do not know how many residues are phosphorylated at this site.

Neurofilament phosphorylation is an intriguing phenomenon and may well play an important physiological role. Some support for this idea comes from the fact that the maximum phosphorylation rate *in vitro* (approx. 0.1 nmol of P_i transferred/h per mg of axoplasm) occurs under conditions that approximate those in axoplasm. Furthermore, phosphate can be removed from neurofilaments *in vitro* by phosphatases, implying that similar enzymes *in vivo* may be responsible for phosphate turnover.

This phenomenon has recently been observed in squid, where experiments show that ATP can 'chase out' ^{32}P from labelled neurofilaments. Additional work will, however, be needed to clarify the nature of the reactions involved.

The studies that are reported in the present paper, although preliminary, are beginning to show how neurofilaments are modified by two major enzymic reactions found in axoplasm. In addition these studies are enabling us to build up a molecular picture of the neurofilament and it is hoped that in the long term this will lead to a better understanding of the filaments' cytoskeletal role.

We thank Professor M. H. F. Wilkins for support and Mr. A. N. Mills, Mr. M. J. Carden and Mr. C. Wais-Steider for advice. We also thank the British Foundation for Age Research, The Mental Health Foundation and the Medical Research Council for financial assistance.

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