Neurofilaments from ox spinal nerves

Isolation, disassembly, reassembly and cross-linking properties

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1. An isolation procedure for neurofilaments from ox spinal nerves is described where the triplet polypeptides (which have molecular weights of 205 000, 158 000 and 72 000) constitute more than 80% of the preparation. Soon after purification, the neurofilaments form a gel that is stable for many weeks. 2. The purified neurofilaments disassemble in low-salt buffers at pH > 7.0 into soluble particles that contain all of the triplet polypeptides. Greater than 90% of the protein can reassemble to form filaments. 3. The thiol-containing residues in the filaments can be cross-linked. Analyses of the complexes formed show that in the filament the 205 000-mol.wt. components are arranged so that they can be cross-linked to themselves and to the 158 000-mol.wt. polypeptides, and that the 72 000-mol.wt. components are arranged so that their thiol groups can be cross-linked together.

Mammalian neurofilaments are composed of three distinct polypeptides, generally called the neurofilament triplet, having molecular weights around 200 000, 155 000 and 70 000 (e.g. Lasek & Hoffman, 1976; Schlaepfer & Freeman, 1978; Willard & Simon, 1981). Their structural arrangement within the neurofilament is not clear, although immunoelectron microscopy (Willard & Simon, 1981; Sharp *et al.*, 1982) has shown a characteristic location for each of the triplet polypeptides along the filament. The patterns indicated that the 70 000-mol.wt. polypeptide is associated with the core of the filament, whereas the 200 000-mol.wt. component is located peripherally, with a periodic and probably helical repeat. The disposition of the 155 000-mol.wt. polypeptide was found to be intermediate between these two extremes. Studies with the purified 70 000-mol.wt. polypeptide (Geisler & Weber, 1981; Liem & Hutchison, 1982) support these conclusions, and show that this polypeptide can assemble into filaments in the absence of the other two triplet components.

The present study was undertaken to define more

precisely the location of the polypeptides with respect to each other, within the neurofilament. To achieve this, we have made use of the fact that chemical oxidation of neurofilaments causes disulphide links to be formed between adjacent polypeptides and this allows analysis of contacts to be relatively easily performed (for a review of the use of cross-linking, see Freedman, 1979).

First, we describe a method for isolating neurofilaments from ox intradural nerves. This method yields neurofilaments that have not been subjected to denaturation during preparation, are substantially free of contaminating cytoskeletal proteins and are in sufficient quantities for detailed structural analyses.

We have also characterized some of the properties of these neurofilaments, and examined aspects of their disassembly at high pH and low ionic strength.

Lastly, we describe our cross-linking studies on the arrangement of the triplet polypeptides, within the filament, which show that the 72 000-mol.wt. polypeptide can be cross-linked to form a homodimer, and that the 158 000- and 205 000-mol.wt. components can be cross-linked to each other.

Materials and methods

Abbreviations used: CuP₂, (*o*-phenanthroline)₂-Cu(II) complex; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide-gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('tosyl-lysine chloromethyl ketone'); Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one ('tosylphenylalanyl chloromethyl ketone').

β -Mercaptoethanol, DTT and 1,10-phenanthroline were obtained from BDH. Bovine serum albumin, bovine trypsin, iodoacetamide, PMSF, Tos-Lys-CH₂Cl, Tos-Phe-CH₂Cl and SDS were obtained

from Sigma. Lima-bean trypsin inhibitor was from Worthington. All other chemicals were of the highest purity available.

Preparation of neurofilaments

Ox spinal cords were transported to the laboratory on ice and the intradural nerve roots were dissected out and placed in extraction buffer containing 5 mM-Tris/HCl, 1 mM-EGTA, 0.1 mM-DTT, 100 mM-NaCl, 0.01% (w/v) NaN_3 and 0.1 mM-PMSF at a final pH of 7.8 at 4°C. Nerves (40 g) were quickly, but carefully, cleaned in ice-cold buffer. They were then finely chopped in a mincer and homogenized in a glass homogenizer with a loosely-fitting pestle; 17.5 ml of the extraction buffer was used per g (wet wt.) of tissue. The homogenate was stirred at 4°C for 1 h and was centrifuged at 2500 g ($r_{\text{av.}} = 7.5$ cm) for 5 min at 4°C. Then 65 ml of this supernatant was carefully layered on to 35 ml of buffer containing 50 mM-sodium phosphate, 1 mM-EGTA, 0.1 mM-DTT, 100 mM-NaCl, 1.5 M-sucrose, 0.01% (w/v) NaN_3 and 0.1 mM-PMSF at a final pH of 7.0. This was centrifuged at 75 000 g ($r_{\text{av.}} = 7.6$ cm) for 16 h at 4°C. Careful removal of the supernatant yielded pellets that were pooled, homogenized and stored at 4°C. For the studies described in the present paper, neurofilaments were generally used within 1 week of preparation. Neurofilaments from cat were prepared by essentially the same method.

Disassembly experiments

Protein was deemed to be soluble if it remained in the supernatant under the following centrifugal conditions; either 2 h at 4°C at 200 000 g ($r_{\text{av.}} = 6.4$ cm), or for 1½ h at ambient temperature at 150 000 g ($r_{\text{av.}} = 1.3$ cm) in a Beckman Airfuge.

Studies on the pH-dependence of disassembly entailed the dialysis at 4°C of neurofilaments (50 µg/ml or 1 mg/ml) after treatment with proteinase inhibitors. After dialysis, filaments were sedimented in an Airfuge, and the pellets and supernatants were analysed for protein content by the paper-assay, and for composition by SDS/PAGE. The buffer used throughout these studies was the universal buffer of Britton & Robinson (1931), in which each component was at 1.43 mM, and to which was added 0.1% (w/v) NaN_3 , 0.1 mM-EGTA and 0.1% (v/v) β -mercaptoethanol. This buffer was used neat, with 100 mM-NaCl or with 500 mM-NaCl.

Preparation of disassembled neurofilament protein at low salt and high pH

Disassembled neurofilament protein was prepared by the following method; 5 mg of neurofilament preparation was homogenized with proteolytic inhibitors at the following concentrations: 0.1 mM-PMSF, 0.1 mM-Tos-Lys- CH_2Cl , 0.1 mM-

Tos-Phe- CH_2Cl , 0.1 mM-iodoacetamide and 0.1 mg of trypsin inhibitor/ml, to which was added 1.0 mM-EGTA, 0.1 mM-EDTA and 0.1% (w/v) NaN_3 . After 1 h at 4°C, the treated filaments were exhaustively dialysed at 4°C against 1 mM-imidazole/HCl, 0.01% (w/v) NaN_3 , 0.1% (v/v) β -mercaptoethanol, 0.1 mM-EGTA, 0.1 mM-EDTA and 0.01 mM-PMSF at a final pH of 6.0. After dialysis, disassembly was induced by the addition of unbuffered Tris to 5 mM, which raised the pH to approximately 8.8. The preparation was then centrifuged at 4°C for 2 h at 200 000 g ($r_{\text{av.}} = 6.4$ cm). The supernatant was carefully removed, stored at 4°C, and used for analysis within 3 days of preparation.

Reassembly experiments

The reassembly of filaments was generally achieved by dialysis and protein concentrations were greater than 200 µg/ml. Either imidazole/HCl or $\text{NaH}_2\text{PO}_4/\text{NaOH}$ was used to buffer over the pH range 6.0 to 7.2. Buffers always included 0.1% (w/v) NaN_3 and salt concentration was varied using NaCl or KCl in the range 0–500 mM. In some experiments 1 M-sucrose, 0.1 mM-PMSF, 2 mM-ATP, 5 mM- MgCl_2 or 0.1% (v/v) β -mercaptoethanol was present in the reassembly buffers. Reassembly was performed for 18 h at temperatures varying between 4 and 37°C. Control experiments involved the dialysis or incubation of protein with disassembly buffer under otherwise identical conditions. Reassembly was monitored by electron microscopy and by SDS/PAGE of the pellets and supernatants after sedimentation in an Airfuge (1½ h at ambient temperature, 150 000 g, $r_{\text{av.}} = 1.3$ cm).

Cross-linking of neurofilaments

Thiol-group cross-linking in neurofilaments was induced using CuP_2 . A stock solution containing 2.5 mM- CuSO_4 and 5 mM-1,10-phenanthroline was freshly prepared by the method of Kobashi (1968). Neurofilaments were routinely cross-linked with CuP_2 at final concentrations between 0.7 mM and 7 µM for 30 min at 4°C or room temperature. The reaction was terminated by the addition of iodoacetamide and EDTA each to a final concentration of 50 mM; after 10 min, samples were prepared for SDS/PAGE.

For the determination of the effect of protein concentration upon the cross-linking pattern, filaments were first homogenized, and diluted to between 4 mg/ml and 50 µg/ml with 50 mM-sodium phosphate/100 mM-NaCl/0.1 mM-EGTA/0.1% (w/v) NaN_3 /10 mM-DTT/1.0% (v/v) β -mercaptoethanol at a final pH of 7.0 in order to reduce existing disulphide bonds. The filaments were then dialysed at 4°C for 18 h against this buffer, which had been degassed, and without reducing agents. Dialysed protein was cross-linked as described

above. Samples were then adjusted to the same protein concentration with buffer and filaments harvested by centrifugation. The pellets were used for SDS/PAGE.

Electrophoresis

SDS/PAGE and isoelectric focusing were routinely performed by the method of Laemmli (1970) and O'Farrell (1975) respectively. Sometimes a high-resolution mini-gel system was used (see Chin *et al.*, 1983). Cross-linked proteins were dissolved in 2% (w/v) SDS/10% (v/v) glycerol/0.1% (w/v) NaN_3 /60 mM-Tris/HCl/0.001% (w/v) Bromophenol Blue at a final pH of 6.8. They were then boiled for 10 min and centrifuged for 10 min in an Eppendorf benchtop centrifuge. Gels were stained with Coomassie Blue R250 and densitometry performed at 560 nm.

For the analysis of cross-linked products, proteins were first separated by non-reducing SDS/PAGE. Whole strips representing approx. 5 μg of protein, or single protein bands with less than 5 μg were excised from the gel. Other lanes containing the same sample were stained in order to detect visually the separated protein bands. To cleave the cross-links, the slices were reduced by incubation for 20 min at room temperature, in a buffer containing 1% (v/v) β -mercaptoethanol, 10% (v/v) glycerol, 10 mM-DTT, 2% (w/v) SDS, 0.1% (w/v) NaN_3 , 0.001% (w/v) Bromophenol Blue and 60 mM-Tris/HCl at a final pH of 6.8. The slices were rinsed briefly in fresh buffer immediately before they were loaded on to a second gel. Second-dimension cross-linking gels were stained with silver, by the method of Morrissey (1981). Molecular weights were determined with marker proteins as described previously (Eagles *et al.*, 1981). In addition to these, higher molecular weights were estimated by using phosphorylase *a* that had been cross-linked with glutaraldehyde to give dimers (mol.wt. 196 000), trimers (mol.wt. 294 000) and tetramers (mol.wt. 392 000).

Measurement of protein concentration

Protein was measured by a modification of the paper-assay of Bramhall *et al.* (1969). The method entails the staining of protein that had been applied to filter paper, with Coomassie Brilliant Blue R250. After destaining, the bound dye was eluted with methanol, and quantified by spectrophotometry at 585 nm. Bovine serum albumin was used as standard. With care, this procedure can be made sensitive to less than 1 μg of protein, and is linear up to 10 μg with an accuracy of $\pm 5\%$.

A more direct method for determining protein by measuring the absorbance at 210 nm (Tombs *et al.*, 1959) was also used.

Electron microscopy

Preparations were routinely examined unfixed on carbon-coated grids after negative-staining with uranyl acetate, in a Philips 200 electron microscope. Disassembled neurofilament protein was also observed after shadowing with platinum. Protein was sprayed at a concentration of 50 $\mu\text{g}/\text{ml}$ on to freshly-cleaved mica, and shadowed by the method of Elliott & Offer (1978).

Ultracentrifugation

Protein samples at a concentration between 0.8 and 3.0 mg/ml were analysed in a single-sector cell of an An-D type rotor accelerated to 59 780 rev./min in a Spinco model E ultracentrifuge, and run for about 2 h at 20°C. Sedimentation peaks were recorded with Schlieren optics at regular intervals during the run.

Results

Purification of neurofilaments from ox intradural nerve roots

A batch of 40 g of nerve roots routinely yielded about 25 mg of protein at a concentration of about 2 mg/ml. Fig. 1(a) is the electrophoretic profile of the isolated ox neurofilaments. This shows that they contain three major polypeptides with mol.wts. 205 000, 158 000 and 72 000 that form more than 80% of the total protein. The preparation always contained several minor components with mol.wts. around 40 000–60 000 and the amounts of these polypeptides appeared constant between preparations.

Examination of the neurofilaments in the electron microscope after negative-staining with uranyl acetate (Fig. 1b) showed smooth-sided filaments of diameter around 10 nm and length greater than 3 μm . If careful cleaning of the nerve roots was not performed, collagen fibrils were occasionally observed.

The neurofilament preparation upon storage at 4°C in the sucrose preparation buffer became extremely gelatinous, an effect that is demonstrated in Fig. 1(c). This gel was highly hydrated, since upon high-speed centrifugation a clear pellet of less than one-thirtieth the original volume was produced. The gel represents a weak association of filaments, being disrupted if the temperature is raised to around 20°C. Disruption also occurs if the gel is taken to pH 5.0, where the neurofilaments upon centrifugation form a whitish precipitate distinct from the usually clear pellet obtained at pH 7.0.

A number of preparations were stored at 4°C in sucrose buffer for 3–6 months to examine the effects of residual proteolytic activity. In a few of these samples a little degradation had taken place. The

Fig. 1. Isolated neurofilaments from ox spinal nerves

(a) Electrophoretic profile of ox neurofilaments analysed on a 5–15% linear acrylamide gradient gel at a loading of approx. $10\mu\text{g}$ of protein. Molecular weights ($\times 10^{-3}$) are indicated. (b) Electron micrograph of freshly-prepared neurofilaments that were negatively-stained with 1% uranyl acetate. The bar represents $0.05\mu\text{m}$. (c) Neurofilaments at a concentration of about 2mg/ml after storage at 4°C for 1 day in preparation buffer. They are sufficiently gelatinous and cohesive to remain at the bottom of an inverted container.

filaments were otherwise well-preserved, as judged by electron microscopy. If the sucrose was removed by dialysis, the proteolytic activity was enhanced. Therefore filaments were not stored for long periods after dialysis.

Isoelectric focusing was routinely performed on various preparations, and Fig. 2 shows a typical pattern. The triplet polypeptides have acidic pI values, and the associated polypeptides are also acidic.

Disassembly of ox neurofilaments

Initial studies showed that filaments were disassociated in 6M-urea containing 1% (v/v) β -mercapto-ethanol, to form individual polypeptides that could be separated on a column of Sepharose 4B-CL, although they were not disrupted in 1% (w/v) Tween-80, Nonidet NP-40, Lubrol or Triton X-100.

Lowering the ionic strength also caused filament disassembly. Fig. 3 is the disassembly profile of neurofilaments at different pH values and salt concentrations. This profile shows that there is a region of stability between pH 4.0 and pH 6.0 in low salt. In 100mM-NaCl, the filaments are not disassembled to any great extent until the pH rises above 10.0. The nature of the species produced in the supernatant at $\text{pH} < 4.0$ and low salt was not easy to investigate, because it was extremely cohesive. For example, it became bound to dialysis tubing, and glassware.

At any pH value above 6.0, the yield of protein in the supernatant from low-salt buffers varied between

Fig. 2. Two-dimensional gel electrophoresis of neurofilaments

A 5–15% linear polyacrylamide gradient gel was used. Approx. $25\mu\text{g}$ of protein was loaded. Note that each triplet polypeptide displays a series of isoelectric variants. Approximate pH values generated in the first dimension are indicated below the gel, and molecular weights ($\times 10^{-3}$) are shown at the side of the photograph.

preparations; at pH 8.0 in Universal buffer, values for the disassembly have ranged from 40 to 60%. The extent of disassembly was independent of the

Fig. 3. *The disassembly profile of neurofilaments* Neurofilaments were dialysed at a concentration of 50 µg/ml or 1 mg/ml against Universal buffer at the pH values shown, as described in the Materials and methods section. Samples were then centrifuged and the protein in both the pellet and the supernatant was assayed. The protein remaining in the supernatant is expressed as a percentage of the total. The open symbols represent experiments performed with a single preparation of neurofilaments, which was dialysed at 50 µg/ml and measured with the paper-assay method. The closed symbols were from a different preparation, dialysed at 1 mg/ml, and values were obtained by densitometry of gel-lanes. O and ●, disassembly in buffer with no salt added; Δ and ▲, disassembly in the presence of 100 mM-NaCl; □, in the presence of 500 mM-NaCl. In this last experiment with 500 mM-NaCl, the disassembly was not investigated above pH 4.0. From a number of other studies at pH values above 4.0, however, the disassembly in 500 mM-NaCl followed the relationship shown here for 100 mM-NaCl.

protein concentration within the range 10 µg/ml to 5 mg/ml.

Attempts were made to increase the amount of disassembled protein by, for example, including reducing agents like β-mercaptoethanol and DTT, though these did not significantly affect the yield.

Disassembly of neurofilaments was often accompanied by splitting of the triplet bands owing to proteolysis. If PMSF, Tos-Lys-CH₂Cl, Tos-Phe-CH₂Cl, iodoacetamide and trypsin inhibitor were used together they eliminated band-splitting, although the disassembly characteristics of the neurofilaments remained the same, indicating that band-splitting had little effect on neurofilament disassembly. Also when band-splitting was eliminated, there was no preferential release of any of the triplet components, since both the supernatant and the residue remaining contained the triplet polypeptides in about the same ratio as in neurofilaments, over the pH range 8.0–11.0.

Fig. 4(a) is an electron micrograph of the

Fig. 4. *Analysis of disassembled ox neurofilament protein and its reassembly*

(a) Electron micrograph of the disassembled ox neurofilament protein prepared as described in the Materials and methods section. This sample was negatively stained with uranyl acetate. The scale bar represents 0.05 µm. (b) Electrophoretic profile of disassembled ox neurofilament protein analysed on a 5–15% polyacrylamide gradient gel. Molecular weights ($\times 10^{-3}$) are indicated to the side of the photograph. (c) Electron micrograph of reassembled filaments obtained by dialysis of disassembled ox neurofilament protein at a concentration of 1 mg/ml against 100 mM-imidazole/HCl/100 mM-NaCl/0.1% NaN₃ at a final pH of 6.4. The sample was negatively stained with uranyl acetate without being centrifuged. The scale bar represents 0.05 µm. (d) Electrophoretic profile of reassembled filament pellet. The filaments shown in (c) were sedimented in an Airfuge and the pellet was prepared for SDS/PAGE analysis on a 5–15% polyacrylamide gradient gel. Molecular weights ($\times 10^{-3}$) are indicated to the side of the photograph.

disassembled neurofilament protein. Irregularly-shaped particles were consistently seen. Studies on similar preparations that were platinum-shadowed

Fig. 5. Dependence of neurofilament cross-linking upon the concentration of CuP_2 reagent

Samples were cross-linked at 20°C for 5 min at a protein concentration of 1 mg/ml in preparation buffer, by the addition of CuP_2 at various concentrations. Samples were then separated on a minigel with a 3–12% polyacrylamide gradient. Lanes are as follows: (a) freshly-prepared neurofilaments; (b) filaments as in (a) were cross-linked with CuP_2 at a final concentration of $4\ \mu\text{M}$, (c) $7\ \mu\text{M}$, (d) $14\ \mu\text{M}$, (e) $36\ \mu\text{M}$, (f) $71\ \mu\text{M}$, (g) $143\ \mu\text{M}$, (h) $357\ \mu\text{M}$ and (i) $715\ \mu\text{M}$. Lane (j) shows neurofilaments as in (a), except that 1% β -mercaptoethanol was added. A–K are the major cross-linked bands. Band J is composed of only the 72000-mol.wt. polypeptide. Over the range of conditions used to produce lanes (c)–(i), the 72000-mol.wt. polypeptide was decreased in intensity by about 25% at maximum: not more than 15% was cross-linked into band J, and the remainder (approx. 10%) was distributed in high-molecular-weight complexes. Filaments at 2 mg/ml, cross-linked with $2.5\ \text{mM}$ - CuP_2 for 12 h in phosphate buffer at 4°C , showed a reduction of around 60% in the intensity of the 72000-mol.wt. polypeptide. Under these conditions, however, there was no comparable increase in the intensity of band J and most of the 72000-mol.wt. polypeptide that was cross-linked (45%) was found in high-molecular-weight complexes that were formed by interfilamentous cross-linking (see Table 1). Molecular weights ($\times 10^{-3}$) are indicated at the side of the photograph.

indicated the presence of globular particles that appeared more regular than those observed under negative-staining conditions, and had diameters around 20 nm. Attempts were made to separate the particles that were seen by microscopy. However, they were not resolved on native acrylamide or agarose gels nor could they be fractionated on Sepharose 4B-CL. Thus disassembly at high pH does not produce individual polypeptides.

Ultracentrifugation was also used to assess the extent of heterogeneity in various preparations after disassembly. The results showed a major peak that

Fig. 6. Second-dimension analysis of neurofilament cross-linking

Ox neurofilaments at 1 mg/ml were cross-linked with $0.25\ \text{mM}$ - CuP_2 for 30 min at room temperature in sucrose-containing preparation buffer. After electrophoresis of the sample, a slice was removed from the gel and reduced for electrophoresis in the second-dimension. The gel is stained with Coomassie Blue. The major spots in the pattern are lettered in accordance with the identification system described in the text. For example the spots c, d and e, which lie in a horizontal line at mol.wt. 205000, come from cross-linked complexes containing the 205000-mol.wt. triplet component, which were resolved as bands C, D and E respectively in the first-dimension. Molecular weights ($\times 10^{-3}$) are indicated at the side of the photograph.

contained between 50 and 70% of the material. In disassembly buffer, this peak sedimented with a value varying from 2.5 to 4.5 S and remained sharp during the run. In addition to the major peak, there was also a minor, faster sedimenting peak and a dispersed leading peak, both of which varied in extent between different preparations of neurofilaments.

Reassembly of neurofilaments

When the disassembled neurofilament protein was dialysed against buffers of raised ionic strength at pH 6.0 to 7.2, filaments were formed that could be easily sedimented from solution. Factors affecting yield, appearance and length of the reassembled filaments were studied.

The best conditions for reconstituting filaments were dialysis at a protein concentration of $\geq 1\ \text{mg/ml}$ at 37°C against three changes of 100 mM-imidazole/HCl/100 mM-NaCl/0.1% (w/v) NaN_3 at a final pH of 6.4 over a period of 18 h. This resulted in more than 90% of the protein being pelleted after centrifugation in the form of filaments approx. $1\ \mu\text{m}$ in length. Fig. 4(c) is an electron micrograph of these reassembled filaments.

Fig. 7. Selection of regions from second-dimension gels of neurofilament cross-linking

(A)–(F) show areas from the 205 000- and 158 000-mol.wt. region; (H)–(J) show the 72 000-mol.wt. region; (G) shows both regions. Linear 5–15% polyacrylamide gradient gels were used. The gel loading for (A), (B), (G), (H), (I) and (J) was approx. $4 \mu\text{g}$ of protein and for (C), (D), (E) and (F) it was approx. $2 \mu\text{g}$ of protein. The position of the top of the first-dimension gel slice is indicated (∇). All gels were stained with silver and the diagonals run from top left to bottom right. Each gel is labelled with the protein concentration at which cross-linking took place, the method of oxidation and the buffer. 'Phosphate' refers to 50 mM-sodium phosphate/100 mM-NaCl/0.1% (w/v) NaN_3 at a final pH of 7.0, and 'Sucrose' refers to the sucrose-containing preparation buffer described in the Materials and methods section. In the experiments where CuP_2 was used, cross-linking was performed at room temperature for 30 min at a final concentration of CuP_2 of $71 \mu\text{M}$. In (A) and (B) filaments were first reduced with mercaptoethanol and then cross-linked with CuP_2 at different protein concentrations. (D) shows the state of cross-linking in filaments immediately after preparation. These filaments were dialysed in phosphate buffer when further air-induced cross-linking took place (F). The filaments used in (D) and (F) were then cross-linked further by using CuP_2 to produce samples shown in (C) and (E) respectively. In (G), (H), (I) and (J) filaments were first reduced with mercaptoethanol and then cross-linked with CuP_2 at different protein concentrations. In a number of the gels minor diagonals were seen connecting non-triplet components as shown in (I) and (J). The molecular weights ($\times 10^{-3}$) from the second-dimension are marked on the individual photographs to aid identification.

Table 1. *Summary of the disulphide interactions between ox neurofilament polypeptides*

The Table gives the band identification (A–K), its apparent cross-linked molecular weight, its composition and spot characterization, the interaction forming the band and notes on the formation of the band. Symbols used represent the following: *, interaction was found on all gels; †, interaction was found only after extensive oxidation; ‡, interaction decreased after extensive oxidation; §, interaction was independent of protein concentration; ||, interaction was dependent upon protein concentration; ¶, the assigned interaction was confirmed by analysis of the cross-linked band after excision.

Band	$10^{-3} \times$ Apparent cross-linked mol.wt.	Polypeptide composition	Interaction	Notes
A	>400	205a, 158a, 72a	Entrapment	†, §
B	>400	205b, 158b, 72b	Unclear	†, §
C	~400	205c	Homologous	†, §
D	~400	205d, 158d	Heterologous	*, ‡, §, ¶
E	~350	205e, 158e	Unclear	*, ‡, §, ¶
F	~290	205f, 72f	Heterologous	†,
G	~280	205g, 72g	Heterologous	†,
H	~270	158h, 72h	Heterologous	†, , ¶
I	210–240	205i, 158i	Most probably intra-polypeptide cross-linking	*, ‡, §
J	200 (± 20)	72j	Homodimer	*, §, ¶
K	145 (± 10)	56k	Homologous	*, , ¶

Disulphide bonds in freshly-isolated neurofilaments

Fig. 5 shows that isolated neurofilaments contain disulphide-linked bands (compare lanes *a* and *j*) and the bands were labelled A–K. Densitometry showed that about 20% of the 205 000-mol.wt. component and less than 10% of the 158 000- and 72 000-mol.wt. components had been linked. Two possibilities could account for the formation of these bands: either the neurofilaments were already cross-linked *in vivo* or they had become cross-linked during their isolation. In an attempt to distinguish between these possibilities, filaments were prepared from nerve roots under conditions that would limit disulphide bond formation. Roots from cats were used as these could be dissected out within 30 min of death; they were immediately homogenized in the presence of 5 mM-iodoacetamide in degassed buffers. Buffers used for isolation were similar to those described for the standard preparation except that they contained no reducing agent and iodoacetamide (5 mM) was added to them.

Our analyses showed that neurofilaments isolated under these conditions were not cross-linked, although control filaments isolated at the same time using standard buffers were. This argues that the cross-linking observed in the ox neurofilaments arose from cross-link formation during isolation, by air-oxidation and suggests that neurofilaments *in vivo* are not significantly stabilized by disulphide bridges.

Cross-linking of ox neurofilaments

Addition of CuP_2 to freshly-isolated filaments increased the intensities of nearly all the bands (A–K) shown in Fig. 5. During short incubations

(≤ 20 min) with high CuP_2 concentrations ($> 100 \mu\text{M}$) densitometry showed that more than 95% of the 205 000-, about 50% of the 158 000- and about 25% of the 72 000-mol.wt. components were cross-linked. All the cross-links formed by the addition of CuP_2 could be cleaved with mercaptoethanol.

Two-dimensional analyses were used to assess the composition of the cross-linked bands and Fig. 6 shows a typical gel. The polypeptides composing each band form spots in the second dimension, which were labelled according to their origin; for example band D produced spots of 205d and 158d. Areas from similar gels are shown in Fig. 7.

Our main objective was to investigate which of the cross-linked bands were produced by interaction between polypeptides lying within the filament. Intrafilamentous interactions are independent of protein concentrations, whereas interfilamentous cross-linking is not. We therefore investigated the effect of protein concentration on polypeptide cross-linking. To achieve this we first reduced the disulphide links in freshly-isolated filaments with mercaptoethanol and DTT; then the filaments were diluted to the required concentration, briefly dialysed to remove reducing agents and then cross-linked with CuP_2 . Prolonged dialysis induces cross-linking by air oxidation and the interactions produced in this way were also analysed. The results of all the analyses are summarized in Table 1. Generally, oxidation of neurofilaments by either air (Fig. 7F) or CuP_2 (Figs. 7A and 7B) produced similar cross-linking patterns. Furthermore the presence of most spots is independent of protein concentration (compare Figs. 7A and 7B). Fig. 7(D) shows the cross-linking pattern of freshly-isolated neurofila-

ments and the extent of cross-linking here can be increased either by incubation with CuP_2 (Fig. 7C) or with air by dialysis (Fig. 7F). Extensive cross-linking causes disappearance of some spots (compare 205 d and 158 d in Fig. 7E with those in Fig. 7F) showing that bands can be further cross-linked.

The pattern of cross-linking is clearly complex, although a preliminary interpretation of the origins of the bands can be attempted (Table 1). For example, band C contains only the highest-molecular-weight triplet component, has an apparent molecular weight of around 400 000 and is present in patterns over a wide range of protein concentrations (compare Figs. 7A and 7B). This interaction is therefore homologous, most probably dimeric, and arises within the filament. By similar reasoning band D represents a heterologous interaction between the 205 000- and 158 000-mol.wt. polypeptides, which is also within the filament and probably indicates a 1:1 complex. The origin of band E is more complex and probably represents a mixture of homologous and heterologous interactions. This is shown in Figs. 7E and 7F, where extensive cross-linking disproportionately reduces spot 205 e compared with 158 e. Bands F, G and H represent interactions between the 72 000-mol.wt. component and the two higher-molecular-weight polypeptides, but as they were only clearly present at high protein concentrations these interactions are most likely to be interfilamentous. Band I was always diffuse and its molecular-weight range (210 000–240 000) probably indicates a variety of intrapolypeptide complexes. Band J is the major homologous interaction of the 72 000-mol.wt. component that arises within the filament and for reasons discussed later is dimeric. Lastly, band K is the major complex of the non-triplet polypeptides and represents a homologous interaction between species with mol.wt. 56 000.

In summary, these studies show that within the filament the 205 000-mol.wt. components are arranged so that they can be cross-linked to themselves and to the 158 000-mol.wt. polypeptides and that the 72 000-mol.wt. components are arranged so that their thiol groups can also be cross-linked together.

Discussion

The procedure described here for the isolation of neurofilaments from intradural nerve roots has distinct advantages. For example, it routinely yields large amounts of material compared with those obtained from peripheral nervous tissue of smaller animals (e.g. Schlaepfer & Freeman, 1978; Liem *et al.*, 1978; Mori & Kurokawa, 1980; Autilio-Gambetti *et al.*, 1981), and has little tubulin and glial fibrillary acidic protein, which are usually purified

with neurofilaments isolated from brain or spinal cord (Thorpe *et al.*, 1979; Delacourte *et al.*, 1980; Runge *et al.*, 1981). More importantly for the structural studies here, the neurofilaments are preserved in a near-native state during their preparation, which involves neither disassembly nor denaturation.

The identities of the bands in the 40 000–60 000-mol.wt. region are unknown. That they are closely associated with filaments is demonstrated by their co-purification, their reassembly together with the triplet components into filaments and by the fact that a few of them cross-link to the 72 000-mol.wt. polypeptide. Some of these components could be derived from the two higher-molecular-weight triplet polypeptides. This is suggested by the appearance of bands of about this molecular weight after digestion of neurofilaments with proteinases (Chin *et al.*, 1983).

If the bands in this molecular weight region were derived from the triplet components, then antibodies raised to the three major mammalian neurofilament polypeptides might be expected to bind to them. Autilio-Gambetti *et al.* (1981) have indeed described a polypeptide of mol.wt. 57 000 that was associated with rat peripheral nerve neurofilaments and that reacted with antisera raised against all three triplet polypeptides.

Bands with molecular weights identical with these non-triplet polypeptides are also found in samples of nerve roots, when precautions are taken to minimize proteolysis, suggesting that these components may exist *in vivo*. It is interesting in this context that, in neurofilaments from *Myxicola*, proteolytic fragments of the major polypeptides are found as integral components of the filament (Eagles *et al.*, 1981).

After purification of the neurofilaments, the formation of a gel readily occurred upon storing them at temperatures around 4°C and at protein concentrations greater than 1 mg/ml. This is not a unique feature of vertebrate neurofilaments, as filaments from *Myxicola* and squid (*Loligo forbesi*) also form gels. Studies on these gels may provide clues to possible cross-connections occurring between neurofilaments.

Isoelectric focusing of ox neurofilament polypeptides produced pI values similar to those found for rabbit triplet by Czosnek *et al.* (1980). On some gels, a series of distinct spots was resolved for each of the ox triplet. As the triplet components can be phosphorylated (Eagles *et al.*, 1980; Shecket & Lasek, 1982), and as isolated ox neurofilaments have been shown to contain phosphate (Jones & Williams, 1982), the isoelectric variants observed here might be due to differences in the state of phosphorylation of these polypeptides. Indeed, Julien & Mushynski (1982) have shown that dephosphoryla-

tion of neurofilaments altered the pI values of the triplet components.

Disassembly of ox neurofilaments occurs at low ionic strength under conditions similar to those used by Schlaepfer (1971, 1978) for rat neurofilaments. However, unlike rat neurofilaments, ox filaments remained intact for many weeks in 100 mM-NaCl at neutral pH.

Ox neurofilament disassembly produces particles that resemble those seen by Schlaepfer (1977); these particles reassemble into filaments of around 10 nm diameter, which retain the original ratios of the triplet polypeptides. Similar results have been found by Zackroff *et al.* (1982) and Steinert *et al.* (1982). In addition, our studies provide more details on the conditions for disassembly and the nature of the particles formed during this process.

In the cross-linking technique used here, residues must be aligned within a few Ångström units of each other, in order that an intrafilamentous thiol linkage be formed. Therefore the absence of an observed interaction between two polypeptides does not preclude the possibility of their proximity within the filament. It is clear from our studies, however, that in ox neurofilaments some of the thiol groups of the 205 000-mol.wt. polypeptides are close together, and some are within a few Ångström units of thiol groups in the 158 000-mol.wt. polypeptides. Digestion of cross-linked neurofilaments with proteinases shows that the regions of the 205 000- and 158 000-mol.wt. polypeptides responsible for these interactions are located towards the filament backbone and are thus within the tail-region of these molecules, as defined in Chin *et al.* (1983). Our studies also show that each of these polypeptides contains several cysteine residues, in agreement with the studies of Chiu & Norton (1982) and Hogue-Angeletti *et al.* (1982). Because the 72 000-mol.wt. polypeptide can interact with these two higher-molecular-weight triplet components in an apparently interfilamentous manner, not all the cysteine residues of these polypeptides are buried.

Since in the filament only a proportion of the 72 000-mol.wt. polypeptides can interact by disulphide formation with others of this type, these polypeptides are not all equivalently related. The evidence of Geisler *et al.* (1982) shows that this polypeptide contains a single cysteine residue. Thus the interaction we observe must be dimeric. The apparent high molecular weight of the dimer (calibrated at 200 000) probably reflects an altered mobility, which is sometimes seen for cross-linked bands when using the Laemmli (1970) system (see also Quinlan & Franke, 1982).

Because of such difficulties in accurately determining molecular weights of cross-linked complexes, it is important to stress that at this stage the stoichiometries that we have suggested for the other polymers must be regarded as tentative.

In conclusion, the data support a model for the structure of the mammalian neurofilament in which a population of the thiol groups of the 205 000- and 158 000-mol.wt. polypeptides are within a few Ångström units of each other in the filament, and in which the packing arrangement of the 72 000-mol.wt. polypeptides allows some of them to interact at their cysteine residues.

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