Chemical cross-linking analyses of ox neurofilaments

Martin J. CARDEN* and Peter A. M. EAGLES†

Department of Biophysics, King's College, University of London, 26–29 Drury Lane, London WC2B 5RL, U.K.

1. Freshly isolated intact ox neurofilaments have been incubated with copper(II)-*o*-phenanthroline complex to induce thiol cross-linking between the two largest (apparent M_r 205000 and 158000) polypeptide components. Subsequent tryptic digestion shows that the thiol bonds formed between these polypeptides are distributed exclusively among 'rod-domain' fragments that remain associated with intact sedimentable filaments. These observations suggest that the polypeptide chains of the two largest neurofilament components are closely arranged within the backbone but are separate from one another in more peripheral regions. 2. Soluble protofilaments derived from neurofilament disassembly at low ionic strength and high pH have also been cross-linked via thiol bonds in order to determine the polypeptide arrangement within these structures. All three neurofilament polypeptides cross-link more readily when in the form of protofilaments than when in the form of fully assembled filaments, and the pattern of cross-linked complexes formed is different. Analysis of one of these complexes shows that at least some of the protofilaments are composed of oligomers containing both the 72000- and the 158000- M_r neurofilament polypeptides arranged in close proximity.

INTRODUCTION

Neurofilaments are found exclusively in neurons, and in common with the polypeptide components of 10 nm-diameter filaments from all other cell types, mammalian neurofilament polypeptides contain an α -helical central domain of $M_r \sim 40000$ (Geisler *et al.*, 1985). Size differences between the three neurofilament polypeptides that in ox have apparent M_r values on SDS/polyacrylamide gels of 205000, 158000 and 72000 (e.g. Carden & Eagles, 1983), arise by differences in the length of domains that are located on the C-terminal side of the central helical, or rod, domain (Geisler *et al.*, 1985). The C-terminal domains of the 205000- and 158000- M_r polypeptides are released upon tryptic digestion of neurofilaments, whereas the filamentous backbone is preserved intact (Chin *et al.*, 1983). Rod domains of the two largest neurofilament components remain associated with the filament backbone and can be separated from the C-terminal tryptic fragments by high-speed centrifugation (Chin *et al.*, 1983). Chemical cross-linking via thiol bonds shows that the 205000- and 158000- M_r polypeptide chains are arranged close together when assembled into ox neurofilaments (Carden & Eagles, 1983). In the present paper we describe experiments involving the tryptic digestion of thiol-linked neurofilaments, the aim of which was to locate more precisely the region of interaction between the two polypeptide components. We also present an analysis of the cross-linking interactions produced between the polypeptides that make up protofilaments [particles that are produced by disassembly of ox neurofilaments at low ionic strength and high pH and are capable of assembling into 10 nm filaments with similar morphology to freshly isolated neurofilaments (Zackroff *et al.*, 1982; Carden & Eagles, 1983)].

METHODS

Materials and methods for neurofilament isolation from ox spinal-nerve roots, cross-linking with copper(II)-*o*-phenanthroline, and electrophoresis were as described previously (Carden & Eagles, 1983; Chin *et al.*, 1983). Protofilaments, assembly-competent particles derived from the disassembly of freshly isolated neurofilaments, were prepared by a method modified from that used previously (Carden & Eagles, 1983). Notably, EDTA and EGTA were omitted from disassembly buffers, since they prevented the cross-linking action of copper(II)-*o*-phenanthroline. Freshly isolated neurofilaments (5 mg/ml) were dialysed for 18 h at 4 °C against 0.01% NaN_3 /1 mM-imidazole/HCl at a final pH of 6.0. These filaments were then disassembled by raising the pH to ~ 8.8 by addition of unbuffered Tris to a final concentration of 5 mM. After the mixture had been left for 1 h on ice, protein was fractionated by sedimentation at 4 °C for 2 h at 200000 g (r_{av} 6.4 cm). Protofilaments remained in the supernatant, which was carefully separated from incompletely disassembled neurofilaments, which pelleted loosely. Samples of protofilaments and of the dialysed intact neurofilaments in low salt, pH 6.0, were taken for cross-linking. Freshly isolated neurofilaments (1 mg/ml) were cross-linked for 30 min at 20 °C in buffer (1.5 M-sucrose/100 mM-NaCl/50 mM-sodium phosphate, final pH 7.0) by using 0.25 mM-copper(II)-*o*-phenanthroline. Thiol groups that had not reacted were blocked with 50 mM-iodoacetamide and the protein was dialysed overnight against 100 mM-NaCl/10 mM-Tris/HCl, final pH 7.0. Samples were digested for 5 and 10 min at 20 °C with trypsin (1.5 $\mu\text{g}/\text{ml}$; diphenylcarbamyl chloride-treated from Sigma) and proteolysis was terminated by adding lima-bean trypsin

* Present address: Department of Pathology and Laboratory Medicine, Division of Neuropathology, 435 Johnson Pavilion/G2, University of Pennsylvania Medical School, Philadelphia, PA 19104, U.S.A.

† To whom correspondence and reprint requests should be addressed.

inhibitor (Worthington) at a 10-fold molar excess. Digested cross-linked samples were centrifuged in a Beckman Airfuge for 2 h at 148 000 g (r_{av} , 1.32 cm) and at ambient temperature. Supernatants and pellets were carefully separated and dissolved in 1% (w/v) SDS with, or without, addition of 1% (v/v) mercaptoethanol.

RESULTS

Digestion of cross-linked neurofilaments with trypsin

Lane 1 of Fig. 1 shows an oxidized sample of neurofilaments that was cross-linked with copper(II)-*o*-phenanthroline. Comparison with lane 6, which contained the sample in a reduced form, shows that the 205 000- and 158 000- M_r polypeptides in lane 1 were cross-linked, as judged by diminished intensities for the monomer bands. Samples of cross-linked filaments were then digested with trypsin for 5 and 10 min, centrifuged at high speed and the resulting supernatant and pelleted proteins dissolved in SDS, with (lanes 7–10) or without (lanes 2–5) reducing agent. The pattern of bands in the supernatants are almost identical, except that intensities in fractions digested for longer times are higher. The bands in the supernatants are similar to those obtained previously (Chin *et al.*, 1983) and are labelled S_{11} – S_6 . They represent C-terminal fragments of the 205 000- and 158 000- M_r polypeptides (Chin *et al.*, 1983; Geisler *et al.*, 1985). Most importantly, however, the bands S_{11} – S_6 have equivalent patterns and intensities between reduced and oxidized

Fig. 1. SDS/polyacrylamide gel analysis showing limited tryptic digestion of cross-linked neurofilaments

Lane 1 of this SDS/polyacrylamide gel (5–15%, w/v, linear gradient) shows a 25 μ g load of the starting protein; other lanes show pellets (P) and supernatants (S) from similar amounts of filaments digested for 5 or 10 min as indicated. Samples in lanes 1–5 were not reduced; those in lanes 6–10 contained 1% β -mercaptoethanol. The C-terminal fragments of the 205 000- and 158 000- M_r polypeptides released in the supernatants are labelled S_6 – S_{11} according to the system used previously (Chin *et al.*, 1983) and the helical rod regions that pellet are indicated. Apparent M_r values ($\times 10^{-3}$) are indicated by the numbers at the left of the photograph.

samples (compare lane 3 with 8 and lane 5 with 10). Hence they are not cross-linked. Therefore the thiol bonds formed between the 205 000- and the 158 000- M_r neurofilament polypeptides during cross-linking do not occur within these C-terminal domains, implying that the bonds are located in the rod domains of these polypeptides.

After digestion, rod-containing fragments of the two largest ox neurofilament polypeptides sediment with the pelleted material as indicated in lane 9, Fig. 1, and have M_r values ranging from 55 000 to 35 000. The intensities of these rod-containing fragments vary considerably between reduced (lanes 7 and 9) and oxidized (lanes 2 and 4) samples. Diminished intensities of these bands in oxidized samples is accompanied by the appearance of extra, higher- M_r , bands, indicating that the rod-containing fragments are somehow associated in these high- M_r complexes, which must therefore be cross-linked. Thus the 205 000- and 158 000- M_r neurofilament polypeptides are located close together in the regions of their rod domains.

Cross-linking studies on protofilaments

Fig. 2 shows an SDS/polyacrylamide gel used to analyse protein composition in samples from different stages of the disassembly process. Lane 1 contains a sample of the neurofilaments that were dialysed against low-salt buffer at pH 6.0. Under these conditions, neurofilaments remain assembled (Carden & Eagles, 1983). Lane 2 shows a sample of the protofilaments. Band patterns are identical for these two samples, showing that disassembly does not change the relative stoichiometries of the triplet polypeptides.

Fig. 2 also shows the gel patterns of these same samples in non-reduced form (lanes 7 and 5). Cross-linking is present in each, as judged by reduced intensities for the triplet bands and the presence of new bands ranging in apparent M_r from 160 000 to 400 000 or more, at the top of the stacking gel. It is likely that this cross-linking arose from oxidation during analysis. Disassembly did not change the pattern of air-induced cross-linking in protofilaments from that present in intact neurofilaments (compare lanes 5 and 7).

Copper(II)-*o*-phenanthroline was used to induce further cross-linking in the samples. The resulting cross-linking pattern of intact neurofilaments in low salt, pH 6.0 (lane 6, Fig. 2), is essentially identical with that reported previously for freshly isolated filaments (Carden & Eagles, 1983), and second-dimension cross-linking analysis of interactions confirmed this. The low pH and low ionic strength of buffers used for cross-linking did not, therefore, affect the reaction of intact neurofilaments with copper(II)-*o*-phenanthroline.

Lanes 3 and 4 of Fig. 2 show the cross-linking patterns in disassembled neurofilament protein samples after their incubation with copper(II)-*o*-phenanthroline for 1 and 10 min respectively. There are two major differences between these patterns and that produced from the intact filaments (compare lanes 3 and 4 with lane 6). First, a band doublet, labelled H and H', appears at a position that indicates cross-linked complexes with apparent M_r values of ~ 240 000. Secondly, the extent of cross-linking for all three neurofilament polypeptides is greater in protofilaments than in intact filaments. Thus these polypeptides are more readily thiol-linked after neurofilament disassembly. Cross-linking of protofilaments was

Fig. 2. One-dimensional SDS/polyacrylamide-gel analysis of neurofilament disassembly and cross-linking

Protofilaments were prepared as described in the Methods section. Lanes 1 and 7 of this 3–12%-(w/v)-polyacrylamide gradient gel contain samples of the neurofilaments that were dialysed against low-salt buffer at pH 6.0 in reduced and non-reduced forms respectively. Protofilament samples, prepared from these neurofilaments, were also separated under reducing (lane 2) and non-reducing conditions (lane 5). Protofilaments were cross-linked with 0.25 mM-copper(II)-*o*-phenanthroline for 1 min (lane 3) and 10 min (lane 4) at 20 °C, whereas the intact filaments, at low salt and pH 6.0, were cross-linked with 0.25 mM-copper(II)-*o*-phenanthroline for 30 min (lane 6). Bands that are intense in the cross-linking pattern from disassembled neurofilament protein but not in that of intact filaments are indicated (H and H') and those produced in the normal filament sample (lane 6) are labelled as previously (Carden & Eagles, 1983).

rapid, occurring in less than 1 min (lane 3) compared with the 30 min required for samples of intact filaments (lane 6). Moreover, although increasing the period of incubation with copper(II)-*o*-phenanthroline to 10 min led to further decrease in triplet band intensities, the pattern of cross-linked bands did not change (lane 4). Cross-linked complexes are therefore formed rapidly, suggesting close alignment of thiol groups in suitable conformations between polypeptides making up the same protofilament.

Two-dimensional gels were used to analyse the cross-linking pattern obtained from protofilaments. Fig.

Fig. 3. Two-dimensional SDS/polyacrylamide-gel analysis of cross-linked complexes produced from disassembled neurofilament protein

Cross-linked protein was separated on a 5% -polyacrylamide gel without any stacking gel. A vertical strip was cut from this first-dimension separating gel and thiols were cleaved and re-run as described previously (Carden & Eagles, 1983). The 6% -polyacrylamide second-dimension gel is shown here, and a sample of reduced, disassembled neurofilament protein was run at the left-hand side as a reference standard. The arrow shows the top of the first-dimension gel. Note that the cross-linked complex J produces only one spot, which migrates with the same M_r as the 72000- M_r polypeptide and that the complex E contains only the 158000- M_r component; H and H' contain both neurofilament components. A complex, K, contains only the 56000- M_r polypeptide that is always present in neurofilament preparations and persists as a component of protofilaments. This complex (K) is also formed during thiol linking of assembled neurofilaments.

3 shows a typical result. The composition of the cross-linked complexes, H and H', are clearly indicated. Both appear to be composed of a combination of 158000- and 72000- M_r polypeptides; therefore bands H and H' are heteromers. The 158000 M_r polypeptide linked into complex H' migrates just below that in complex H. The altered mobility may be the result of slight degradation by proteolysis, owing to the omission of EDTA and EGTA from disassembly buffers. (These were effective in reducing band splitting, but both interfered with copper(II)-*o*-phenanthroline-induced cross-linking.) Despite resolution of the 158000- M_r band as a doublet, the protofilaments were able to reassemble into 10 nm filaments and therefore represent structural intermediates in the assembly of neurofilaments.

The gel in Fig. 3 also shows a cross-linked complex, band E, of apparent M_r 350000 that appears to be composed solely of the 158000- M_r neurofilament component. This complex co-migrates with one formed during cross-linking of intact filaments (lane 6, Fig. 2). Our earlier analyses showed that band E contained both the 205000- and the 158000- M_r polypeptides and that,

under conditions that produced extensive thiol-bond formation, the intensity of band E diminished, but not to zero (Carden, 1983; Carden & Eagles, 1983). This decrease in intensity was accompanied by loss of only the 205000- M_r component from the complex, presumably because it was further cross-linked into higher- M_r complexes. Since the 205000- M_r neurofilament polypeptide is very extensively cross-linked in the sample used for the gel in Fig. 3 (note its almost complete absence from the non-cross-linked 'diagonal'), we believe that a similar process explains the result obtained here for protofilament cross-linking.

DISCUSSION

Tryptic digestion of intact cross-linked neurofilaments shows that thiol bonds formed between the two largest neurofilament components by the action of copper(II)-*o*-phenanthroline are the results of interaction within the filament backbone. Thus the 205000- and 158000- M_r polypeptides are arranged close together along domains that mediate their anchorage in this part of the neurofilament. In more peripheral domains, however, these polypeptides do not appear to interact, as judged by the failure of copper(II)-*o*-phenanthroline to produce thiol cross-linking within or between C-terminal domains. This could merely reflect the absence of cysteine residues in these domains, or that all thiol groups are distant from one another or that thiols are unsuitably aligned for bond formation. However, repeated attempts to cross-link isolated C-terminal fragments with the rather non-specific reagent glutaraldehyde have also been unsuccessful so far (Chin *et al.*, 1983), suggesting the absence of an interaction. As to the function of these regions, the evidence is little, but it has been proposed that the C-terminal portion of the 205000- M_r polypeptide projects from the neurofilament backbone to form side arms that could aid interaction of filaments with other neuronal components (e.g. Chin *et al.*, 1983; Julien & Mushynski, 1983; Hirokawa *et al.*, 1984; Geisler *et al.*, 1985).

Cross-linking of the particles derived from ox neurofilament disassembly produces cross-linked oligomers composed of both the 158000- and 72000- M_r neurofilament components. Since protofilaments were obtained by disassembly of freshly isolated (i.e. native) neurofilaments, both polypeptides must be arranged close together in single filaments, as suggested previously from reassembly (e.g. Minami *et al.*, 1984) and antibody-decoration experiments (e.g. Hirokawa *et al.*, 1984, and references therein). However, the 158000- and 72000- M_r components do not cross-link in significant quantities when fully assembled neurofilaments are thiol-linked (Carden & Eagles, 1983). There are a number of possible explanations for this observation. First, thiol groups used to form band H (and H') in protofilament units might also be capable of other interactions when protofilaments are assembled into whole filaments. In assembled filaments, therefore, the formation of band H might be masked by, and in competition with, formation of inter-protofilament interactions. Since the latter interactions would be almost absent after disassembly into individual units, formation of band H might be expected to increase. The second possible reason for increased formation of band H in protofilaments is that disassembly might relax packing restraints on polypeptide chains.

Indeed, maximal thiol-linking of α -tropomyosin, which is organized as a double-stranded coiled coil, as has been proposed for neurofilament polypeptides (Geisler *et al.*, 1985), requires local separation of polypeptide chains around the region containing the single cysteine residue (Lehrer *et al.*, 1981). The third, and last, explanation for increased formation of band H in protofilaments might be that the thiol-containing rod regions of the 72000- and 158000- M_r polypeptides are mostly buried when protofilaments are assembled together into neurofilaments. They would therefore be hidden from the oxidizing environment created by copper(II)-*o*-phenanthroline, but would become exposed after disassembly. The idea that the rod-containing regions of these two neurofilament components might be mostly buried in assembled neurofilaments is consistent with current models (e.g. Zackroff *et al.*, 1982; Chin *et al.*, 1983; Geisler *et al.*, 1983; Julien & Mushynski, 1983; Hirokawa *et al.*, 1984; Minami *et al.*, 1984; Geisler *et al.*, 1985).

Partial amino-acid-sequence data is now available for all three porcine neurofilament polypeptides (Geisler *et al.*, 1983, 1985) and for the 72000- M_r polypeptide from mouse (Lewis & Cowan, 1985). Comparing the two 72000- M_r polypeptides from mouse and pig, each contains a single cysteine residue, and extensive homology exists around this position. It seems likely, therefore, that the equivalent ox polypeptide has its cysteine residue at the same position. It also seems most probable that the cysteine residues in the other neurofilament components of ox are in similar positions to those found for the porcine polypeptides. By using this information, some of our cross-linking data derived here and previously (Carden & Eagles, 1983) can be interpreted further.

First, the single cysteine residue in the 72000- M_r polypeptide would, from structural prediction methods, face the 'seam' of the coiled coil (Geisler *et al.*, 1985). Thus, rod pieces of this polypeptide would have to be arranged in complete register for homodimer formation as observed in the cross-linking patterns of both intact neurofilaments and protofilaments. Geisler *et al.* (1983) were able to isolate a fragment of the porcine 72000- M_r component that is C-terminal to the rod (domain b) and that cross-linked as a dimer. Hence the 72000- M_r polypeptide is most likely packed into neurofilaments as a double-stranded coiled coil in which chains are in parallel, rather than anti-parallel, orientations and in which chains are in complete alignment, i.e. without stagger. There are two cysteine residues in the rod piece of the porcine equivalent of the ox 158000- M_r component (Geisler *et al.*, 1985). However, these are located more than ten amino acid residues away from the position of cysteine in the 72000- M_r component and, furthermore, would face outward from the coiled coil (Geisler *et al.*, 1985). Therefore the second consequence of sequence data and our cross-linking studies on the interaction between the 158000- and 72000- M_r ox polypeptides in protofilaments is that, if these polypeptides are bound together in the same coiled coil, then the polypeptides must be staggered relative to one another. Alternatively, if the polypeptides form only single-species coiled coils, then the cross-linking observed must occur between two different coiled coils.

We thank the Medical Research Council and the Wellcome Trust for support (to P.A.M.E.) and the Science and

Engineering Research Council for a postgraduate training award (to M.J.C.). We are also most grateful to Professor W. Schlaepfer for helpful comments on our results.

REFERENCES

- Carden, M. J. (1983) Ph.D. Thesis, University of London
Carden, M. J. & Eagles, P. A. M. (1983) *Biochem. J.* **215**, 227–237
Chin, T. K., Eagles, P. A. M. & Maggs, A. M. (1983) *Biochem. J.* **215**, 239–252
Geisler, N., Kaufmann, E., Fischer, S., Plessmann, U. & Weber, K. (1983) *EMBO J.* **2**, 1295–1302
Geisler, N., Fischer, S., Vandekerckhove, J., Van Damme, J., Plessmann, U. & Weber, K. (1985) *EMBO J.* **4**, 57–63
Hirokawa, N., Glicksman, M. A. & Willard, M. B. (1984) *J. Cell Biol.* **98**, 1523–1536
Julien, J. P. & Mushynski, W. W. (1983) *J. Biol. Chem.* **258**, 4019–4025
Lehrer, S. S., Graceffa, P. P. & Betteridge, D. (1981) *Ann. N.Y. Acad. Sci.* **366**, 285–299
Lewis, S. & Cowan, N. J. (1985) *J. Cell. Biol.* **100**, 843–850
Minami, Y., Endo, S. & Sakai, H. (1984) *J. Biochem. (Tokyo)* **96**, 1481–1490
Zackroff, R. V., Idler, W. W., Steinert, P. M. & Goldman, R. D. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 754–757

Received 8 November 1985/13 December 1985; accepted 18 December 1985