Dephosphorylation of neurofilament proteins enhances their susceptibility to degradation by calpain

Harish C. PANT

Laboratory of Neurochemistry, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, U.S.A.

The degradation of phosphorylated and dephosphorylated neurofilament proteins by the Ca^{2+} -activated neutral proteinase calpain was studied. Neurofilaments were isolated from bovine spinal cord, dephosphorylated by alkaline phosphatase (from *Escherichia coli*) and radioiodinated with [^{125}I]-Bolton–Hunter reagent. The radioiodinated neurofilament proteins (untreated and dephosphorylated) were incubated in the presence and absence of calpain from rabbit skeletal muscle, and the degradation rates of large (NF-H), mid-sized (NF-M) and small (NF-L) neurofilament polypeptides were analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. The degradation of dephosphorylated neurofilament proteins occurred at a higher rate, and to a greater extent, than did that of the phosphorylated (untreated) neurofilament proteins. The dephosphorylated high-molecular-mass neurofilament (NF-H_D) was proteolysed 6 times more quickly than the untreated NF-H. The degradation rate of the NF-M and NF-L neurofilament proteins was also enhanced after dephosphorylation, but less than that of NF-H. This indicates that the dephosphorylation of neurofilament proteins can increase their sensitivity to calpain degradation.

INTRODUCTION

Neurofilament proteins (NFP) are highly phosphorylated, major components of the axonal cytoskeleton. They are synthesized and assembled for axonal transport in the cell body (Black *et al.*, 1986), and their phosphorylation occurs principally upon entry into the axon (Oblinger, 1987). Whereas immunocytochemical studies have shown that the highly phosphorylated NFP are largely localized in axons (Goldstein *et al.*, 1983; Sternberger & Sternberger, 1983; Lee *et al.*, 1986; Trojanowski *et al.*, 1986; Cohen *et al.*, 1987), few phosphorylated neurofilaments (or any form of neurofilaments) are found in nerve terminals (Cantino & Mugnaini, 1975; Cohen *et al.*, 1987). In addition, NFP kinases and Ca^{2+} -dependent proteinases are found to be associated with neurofilaments in axons (Brown & Eagles, 1986; Julien *et al.*, 1983; Pant *et al.*, 1986). These observations are consistent with Lasek & Hoffman's (1976) hypothesis that NFP are metabolically stable in the axon during axonal transport, but are rapidly degraded by Ca^{2+} -dependent proteinases in the axon terminals. Studies of cytoskeletal-protein turnover, showing that NFP are degraded relatively rapidly upon entering nerve terminals, lend support to this view (Lasek & Black, 1977; Paggi & Lasek, 1987).

In an extension of this view, Cohen *et al.* (1987) proposed that the degradation of NFP in nerve terminals may also involve dephosphorylation of the NFP. Consistent with this idea is the finding that axoplasm contains little phosphatase activity (Pant *et al.*, 1986), whereas substantially more phosphatase activity is located in nerve terminals (Bass *et al.*, 1987). Furthermore, a recent report by Goldstein *et al.* (1987) indicated that phosphorylation protects neurofilaments against non-specific prote-

olysis. Since the Ca^{2+} -activated neutral proteinase calpain is believed to be the specific enzymic mediator of NFP degradation in nerve terminals (Lasek & Hoffman, 1976; Pant *et al.*, 1982), the present study examined whether the dephosphorylation of NFP would affect their degradation by calpain.

MATERIALS AND METHODS

Materials

Ca^{2+} -activated neutral proteinase (calpain) 80 kDa subunit from rabbit skeletal muscle was obtained from Sigma. Its purity and activity were checked in the laboratory. The proteinase migrated as a single band on SDS/7%-(w/v)-PAGE, and half-maximal activation occurred at 2 mM- Ca^{2+} , with [^{14}C]casein as substrate at 27 °C, for 30 min incubation, in a buffer containing 100 mM-NaCl, 2.0 mM-dithiothreitol (DTT), 0.1 mM-EDTA and 50 mM-Tris/HCl, pH 7.5.

Methods

Neurofilament isolation, dephosphorylation and radioiodination. Bovine neurofilament-enriched extract was produced from fresh bovine spinal cords enriched from white matter as described by Carden *et al.* (1985). The final neurofilament-enriched pellet was washed in the presence of neurofilament wash buffer (150 mM-NaCl/1 mM-EGTA/1 mM-EDTA/2 mM-DTT/10 mM-sodium phosphate, pH 7.0). The NFP were dephosphorylated by incubation with alkaline phosphatase (*Escherichia coli* type III-S from Sigma) as described by Carden *et al.* (1985) with slight modifications. In brief, the neurofilament pellet containing 4 mg of protein was homogenized in 4 ml of 100 mM-NaCl/1 mM- ZnSO_4 /leupeptin

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; NF-H, NF-M and NF-L, large, mid-sized and small neurofilament polypeptides; NFP, neurofilament proteins; DTT, dithiothreitol; NF-H_D etc., dephosphorylated NF-H etc.

Fig. 1. Dephosphorylation of bovine NFP with alkaline phosphatase from *E. coli*

Coomassie Blue staining patterns of untreated and dephosphorylated NFP are shown. A portion (0.5 ml) of neurofilaments at 4 mg/ml in buffer containing 1 mM- ZnSO_4 , 100 mM- NaCl , 50 mM- Tris/HCl at pH 8.0 and leupeptin (0.1 mg/ml) were incubated with 80 units of alkaline phosphatase type III (from *E. coli*) from Sigma at 36 °C in a final volume of 0.7 ml. The untreated samples contained 50 mM-sodium phosphate, pH 7.3. After 18 h of incubation, 0.35 ml of ice-cold 1 M-sodium phosphate buffer, pH 7.3, was added and centrifuged in a Beckman Microfuge for 15 min. The pellet was washed twice in neurofilament wash buffer (150 mM- NaCl /1 mM-EGTA/1 mM-EDTA/2 mM-DTT/10 mM-sodium phosphate, pH 7.0) and finally with a buffer containing 100 mM- KCl , 10 mM- MgCl_2 , 1 mM-EGTA and 20 mM-Hepes, pH 7.0. The final pellet was resuspended in 10 mM- NaCl /1 mM-EGTA/20 mM- Tris/HCl , pH 7.6. Aliquots of both untreated and dephosphorylated samples were used for SDS/6.5%-PAGE. The gels were stained with Coomassie Blue. Molecular masses of identified proteins are shown on the left. H, M and L designate 200 kDa large (NF-H), 150 kDa mid-sized (NF-M) and 68 kDa small (NF-L) polypeptide components respectively of NFP.

(0.1 mg/ml)/50 mM- Tris/HCl , pH 7.5. Aliquots were incubated in the presence (untreated) or absence (dephosphorylated) of 80 mM-sodium phosphate containing alkaline phosphatase (20 units/mg of NFP) at 36 °C for 18 h. The reaction was terminated with ice-cold buffer [100 mM- NaCl /50 mM-sodium phosphate (pH 7.0)/10 mM-EDTA] and centrifuged on a Microfuge at 10000 rev./min (r_{av} , 2.0 cm) for 15 min. The pellet was resuspended in the neurofilament wash buffer and washed twice more. This procedure completely removed the alkaline phosphatase (checked by SDS/PAGE). Radioiodination of the NFP was performed with [^{125}I]Bolton-Hunter reagent as described elsewhere (Pant *et al.*, 1978; Bennett, 1983; Paggi & Lasek, 1984).

Proteolytic degradation of neurofilament proteins. Aliquots (100 μg of protein) of radioiodinated NFP (untreated and dephosphorylated) were incubated with 0.2 unit of calpain in a buffer containing 100 mM- NaCl , 2 mM-DTT, 0.1 mM-EDTA and 50 mM- Tris/HCl ,

Fig. 2. Degradation of dephosphorylated and untreated neurofilament protein by Ca^{2+} -activated neutral proteinase (calpain II)

Dephosphorylated and untreated NFP were radioiodinated by using [^{125}I]Bolton-Hunter reagent as described in the Materials and methods section. Aliquots were incubated in the presence (2 mM- CaCl_2) or absence (no added CaCl_2 plus 1 mM EGTA) of Ca^{2+} in a buffer containing 100 mM- NaCl , 50 mM- Tris/HCl , pH 7.5, 2 mM-DTT, 1 mM-EDTA and 0.1 unit of calpain from Sigma in a final volume of 0.1 ml. The mixture was incubated at 27 °C for 5 min. The reaction was terminated by adding SDS (2% final concn.). The samples were boiled for 1 min and then 1% (v/v) β -mercaptoethanol was added and run on a 9%-acrylamide slab gel. The gels were stained with Coomassie Blue, dried and autoradiographed. This Figure shows the autoradiograph of untreated and dephosphorylated samples in the (2 mM) or absence of Ca^{2+} . I_1 and I_2 represent the major proteolytic breakdown products of NFP.

pH 7.5, at 27 °C for 10 min. The activation of proteinase was initiated by adding CaCl_2 solution to a final concentration of 2–4 mM in a total reaction volume of 100 μl . The reaction was terminated at different times by adding SDS [2% (w/v) final concn.] to the mixture and transferring it to an ice/water bath at 0 °C. The degradation of NFP was analysed by SDS/PAGE and autoradiography (Neville, 1971; Paggi & Lasek, 1984). The quantification of NF-H, NF-M and NF-L degradation was done by cutting out each individual band from the gel and then measuring its radioactivity with a γ -radiation counter.

RESULTS AND DISCUSSION

The Coomassie Blue staining patterns of untreated (control) and dephosphorylated neurofilament proteins on SDS/6.5%-PAGE are shown in Fig. 1. The gel mobilities of the two largest neurofilament polypeptides, NF-H (200 kDa) and NF-M (170 kDa), increases markedly with the degree of dephosphorylation, whereas that for the low-molecular-mass component, NF-L (68 kDa), was unaffected. In order to improve the resolution between NF- H_D and NF- M_D , a higher concentration (9%) of acrylamide was used for subsequent

Fig. 3. Rates of proteolytic degradation of dephosphorylated and untreated NFP by calpain

NFP (dephosphorylated and untreated) were radioiodinated using [125 I]Bolton–Hunter reagent as described in the Materials and methods section. Aliquots ($\sim 20 \mu\text{g}$ of protein) were incubated in a buffer containing 100 mM-NaCl, 50 mM-Tris/HCl, pH 7.5, 2 mM-DTT, 1 mM-EDTA and 0.1 unit of calpain in a final volume of 0.1 ml. Proteolytic reaction was started by adding CaCl_2 (4 mM final concn.) to the reaction mixture and terminated by adding SDS (2% final concn.) at different times. The radioiodinated proteins of these samples were analysed as described in Fig. 2. The protein bands from the gel corresponding to ^{125}I -labelled proteins in the autoradiograph were excised and counted for radioactivity in a γ -radiation counter (LKB-1282 Compugamma). The ordinate represents percentage degradation of the neurofilament polypeptides, and the abscissa represents the incubation time. (a), (b) and (c) represent the degradation of large (NF-H), mid-sized (NF-M) and small neurofilament polypeptides [untreated ($\circ$) and dephosphorylated ($\bullet$) respectively].

SDS/PAGE analyses. Fig. 2 shows the autoradiograph of untreated and dephosphorylated NFP that had been labelled by [^{125}I]Bolton–Hunter reagent. Although the mobilities of the dephosphorylated polypeptides are decreased in the 9% gel system, the separation between NF-H_D and NF-M_D is improved. Since it was necessary to excise the protein bands (NF-H_D and NF-M_D) from the gel for analysis, an SDS/9% PAGE system was used in the following experiments. The data shown in Fig. 2 also compare the degradation patterns of untreated and dephosphorylated NFP by calpain in the presence of 2 mM- CaCl_2 . The proteolysis of the NFP was allowed to proceed at 27 °C for 5 min in the presence (2 mM- CaCl_2) or absence (no added CaCl_2 , +1 mM-EGTA) of CaCl_2 . The degradation of the NF_D polypeptides appeared to be more effective compared with the untreated (non-dephosphorylated) neurofilament polypeptides under similar conditions. I₁ and I₂ represent the major degradation products of NFP.

The time course of degradation of the neurofilament polypeptides by calpain is shown in Fig. 3. The dephosphorylated and untreated neurofilament preparations were radioiodinated as described in the Materials and methods section. Samples were incubated at 27 °C in the presence of calpain, and the reaction was started by adding CaCl_2 (4 mM final concn.) and stopped at different times by the addition of SDS (2% final concn.). SDS/9% PAGE was performed as described in the Materials and methods section. The individual polypeptide bands were excised after autoradiography and counted for radioactivity. The autoradiographs as well as the Coomassie Blue staining patterns were similar in both the absence of added Ca^{2+} (+1 mM EGTA) or in the presence of 4 mM- Ca^{2+} plus leupeptin (0.1 mg/ml) (results not shown). The rates of degradation of NF-H and NF-H_D by calpain are shown in Fig. 3(a). NF-H_D were degraded 6 times faster than the (untreated) NF-H. The time courses of degradation of NF-M and NF-M_D, and of NF-L and NF-L_D, are shown in Figs. 3(b) and 3(c) respectively. Both M_D and L_D were degraded twice as fast as untreated polypeptides. These data indicate that the dephosphorylation of NFP affects the rate of their proteolytic degradation by calpain. It is also clear from the present study that the extent of the degradation of NF-H was significantly enhanced by dephosphorylation.

The above observations indicate that dephosphorylation of NFP can decrease their resistance to proteolysis by calpain. The most significant effect was found for NF-H, which was degraded significantly more rapidly and extensively after dephosphorylation. The relative efficacy of dephosphorylation on the changes in the NF-H, NF-M and NF-L sensitivity to calpain (Fig. 3) may relate to the fact that such dephosphorylation procedures are incomplete (Georges *et al.*, 1986) and are more effective for NF-H compared with NF-M and NF-L. It is possible that a more complete dephosphorylation of these proteins could render them even more susceptible to calpain degradation. These data suggest that one function of the extensive phosphorylation of NFP in the axon is to increase their resistance to calpain digestion and therefore to prevent premature degradation. Although these data are consistent with the idea that a dephosphorylation mechanism, as well as a calpain-mediated mechanism, is involved in neurofilament degradation in nerve terminals, evidence for both these mechanisms from studies *in vivo* is still needed.

I thank Dr. Harold Gainer for helpful discussion and critical review of the manuscript before its submission.

REFERENCES

- Bass, M., Pant, H. C., Gainer, H. & Soderling, T. R. (1987) *J. Neurochem.* **49**, 1116–1123
- Bennett, V. (1983) *Methods Enzymol.* **96**, 313–324
- Black, M. M., Keyser, P. & Sobel, E. (1986) *J. Neurosci.* **6**, 1004–1012
- Brown, A. & Eagles, P. A. M. (1986) *Biochem. J.* **239**, 191–197
- Cantino, D. & Mugnaini (1975) *J. Neurocytol.* **4**, 505–536
- Carden, M. J., Schlaepfer, W. W. & Lee, V. M.-Y. (1985) *J. Biol. Chem.* **260**, 9805–9817
- Cohen, R. S., Pant, H. C., House, S. & Gainer, H. (1987) *J. Neurol. Sci.* **7**, 2056–2074
- Georges, E., Lafebre, S. & Mushynski, W. E. (1986) *J. Neurochem.* **47**, 477–483
- Goldstein, M. E., Sternberger, L. A. & Sternberger, N. H. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3101–3105
- Goldstein, M. E., Sternberger, N. H. & Sternberger, L. A. (1987) *J. Neuroimmunology* **14**, 149–160
- Julien, J. P., Smoluk, G. D. & Mushynski, W. E. (1983) *Biochim. Biophys. Acta* **755**, 25–31
- Lasek, R. J. & Black, M. (1977) in *Mechanisms, Regulations, and Special Functions of Protein Synthesis in the Brain* (Roberts, E., Lajtha, A. & Gispen, W. H., eds.), pp. 161–169, Elsevier/North-Holland, New York
- Lasek, R. J. & Hoffman, P. M. (1976) in *Cell Motility* (Goldman, T. R., Pollard, T. & Rosenbaum, E., eds.), pp. 1021–1049, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Lee, V. M.-Y., Carden, M. J. & Trojanowski, J. W. (1986) *J. Neurosci.* **6**, 850–858
- Neville, D. (1971) *J. Biol. Chem.* **246**, 6328–6334
- Oblinger, M. M. (1987) *J. Neurol. Sci.* **7**, 2510–2531
- Paggi, P. & Lasek, R. J. (1984) *Neurochem. Int.* **6**, 589–597
- Paggi, P. & Lasek, R. J. (1987) *J. Neurosci.* **7**, 2397–2411
- Pant, H. C., Terakawa, S., Baumgold, J., Tasaki, I., Matsumoto, G. & Gainer, H. (1978) *Biochim. Biophys. Acta* **513**, 132–140
- Pant, H. C., Gallant, P. E., Gould, R. & Gainer, H. (1982) *J. Neurosci.* **2**, 1579–1587
- Pant, H. C., Gallant, P. E. & Gainer, H. (1986) *J. Biol. Chem.* **261**, 2968–2977
- Sternberger, L. A. & Sternberger, N. H. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 6126–6130
- Trojanowski, J. Q., Walkenstein, N. & Lee, V. M.-Y. (1986) *J. Neurosci.* **6**, 650–660

Received 27 June 1988/12 September 1988; accepted 19 September 1988