Influence of the phosphorylation state of neurofilament proteins on the interactions between purified filaments in vitro

Joel EYER and Jean-François LETERRIER*
Centre de Neurochimie du C.N.R.S., 5 Rue Blaise Pascal, 67084 Strasbourg Cedex, France

The extensive enzymic dephosphorylation of neurofilaments determined the progressive loss of their capacity to interconnect in vitro into a reticulated network, measured by the formation of highly viscous gels in purified preparations of neurofilaments [Leterrier & Eyer (1987) Biochem. J. 245, 93–101]. Conversely, a cyclic AMP-dependent activation of the gelation process was obtained by phosphorylation of the neurofilament proteins by the cyclic-nucleotide-dependent protein kinase added to the preparation. These findings argue for a direct relationship between the high phosphorylation level of the neurofilament subunits and the cross-bridging of the polymers in vitro. However, a transient stimulation of the neurofilament viscosity kinetics was also observed during the early steps of dephosphorylation with acid phosphatase, which, moreover, disappeared with longer incubation times before the net inhibition was obtained. In the same way, the calmodulin-dependent brain phosphatase, calcineurin, induced a permanent activation of the phenomenon, correlated with a low dephosphorylation capacity of the neurofilament molecules. Taken together, these results suggest a functional heterogeneity of the numerous phosphate groups of the neurofilament subunits and raise the hypothesis of a highly controlled regulation of the neurofilament cross-bridging by selective phosphorylation–dephosphorylation mechanisms.

INTRODUCTION

Since the discovery by Julien & Mushynski [1] of a high phosphorylation level of neurofilament subunits, several hypotheses were raised concerning the possible role of these phosphate groups in the physiological function of neurofilaments in situ. With the finding of monoclonal antibodies directed against the phosphorylated or dephosphorylated forms of the neurofilament H and M subunits [2], a wide heterogeneity was found in the distribution of these distinct epitopes [2–5]. From immunocytochemical studies, it was assumed that the higher phosphorylation level of neurofilaments within axons is responsible for the higher density and stability of axoskeleton networks [2]. However, this hypothesis has not yet been confirmed by a biochemical approach in vitro. The reassembly of urea-solubilized neurofilament subunits is not modified by their phosphorylation [6], which does affect, by contrast, their interactions with tubulin [7]. These observations support the current working hypothesis that the high phosphate content of the H and M molecules, borne by their arm-like projections standing away from the axis of the filament [8], is essentially involved in regulating the lateral interactions of neurofilaments with other adjacent organelles. The inter-neurofilaments reticulation process in vitro, which we described previously [9], represents a sensitive test for studying this hypothesis, as the formation of highly viscous gels with purified neurofilaments depends upon the conformation of the polymers, and is possibly mediated by the H and M subunits. In the present work, we have studied the relationship in vitro between the phosphorylation state of the neurofilament proteins and the rate of change of the viscosity of the suspension. The experimental results argue for a potent influence of the phosphorylation level of the neurofilament subunits on the interaction between the polymers in vitro, and raise the possibility of a functional heterogeneity of the multiple phosphorylation sites of the neurofilament molecules [10].

MATERIALS AND METHODS

Chemicals

ATP (vanadate-free, magnesium salt, from equine muscle), proteinase inhibitors, potato acid phosphatase type III (53 units/mg), alkaline phosphatase from bovine intestinal mucosa, type VII-T (1000 units/mg of protein at pH 10.4 and 37 °C), calmodulin (1000–3000 units/mg) and cyclic AMP-dependent protein kinase from bovine heart were from Sigma. (γ-32P)ATP (3000 Ci/mmol) was from Amersham International. Affinity-purified calmodulin from frog (Rana) testis was generously given by Professor J. Demaille (University of Montpellier, Montpellier, France). Other chemicals were from Merck.

Neurofilament purification and dephosphorylation

Neurofilaments were isolated from bovine spinal cord as previously reported [9], the procedure of Delacourte et al. [11] being used. The final neurofilament pellet was resuspended in 1.2 M-sucrose in buffer A [0.1 M-Mes (adjusted to pH 6.8 with 5 M-NaOH)/1 mM-MgCl₂/1 mM-EGTA), stored at 4 °C and used within 24 h. The enzymic dephosphorylation of neurofilaments was performed by incubation of the freshly purified

Abbreviations used: PMSF: phenylmethanesulphonyl fluoride; neurofilaments subunits: H, 200 kDa; M, 150 kDa; L, 68 kDa, apparent molecular masses on SDS/polyacrylamide-gel electrophoresis.

* To whom correspondence and reprint requests should be sent.
preparation (4.5 mg/ml) with acid or alkaline phosphatase (5 units/ml) under dialysis at room temperature against buffer A containing 0.8 M-sucrose and 1 mM-PMSF. Alternatively the enzymes were dialysed alone against the same buffer at 4°C, and they were added in the same ratio to neurofilaments immediately before the measurement of the viscosity kinetics, in the presence of a "cocktail" of proteinase inhibitors [N-p-tosyl-L-arginine methyl ester (Sigma; 0.1 mg/ml), aprotinin (0.05 Unit/ml), pepstatin (10⁻⁴ M), leupeptin (10⁻⁴ M), PMSF (1 mM), chloroquin (0.1 mM) and soybean trypsin inhibitor (10 nm)]. Calcinurin (60 μg/ml) and calmodulin (1 μM) were used in the presence of 2.5 mM-MnCl₂ and 2.5 mM-CaCl₂ to obtain the maximum phosphatase activity of the brain enzyme [12].

**Viscosity measurements**

The formation of gels in neurofilament preparations was monitored as previously described [9] by measuring the viscosity of the samples with the falling-ball apparatus described by MacLean-Fletcher & Pollard [13]. A mixture of proteinase inhibitors (see above) was systematically added to all samples before the incubation at 22°C.

**Phosphorylation of neurofilaments with in vitro [γ⁻³²P]ATP**

Phosphorylation kinetics of neurofilaments (0.4-4 mg/ml) were carried out at 30°C in buffer A, containing 0.8 M-sucrose, in the presence of 5 mM-MgCl₂, 5 x 10⁻⁵ M-ATP (0.1 μCi/nmol) and the mixture of proteinase inhibitors described above. The neurofilaments were incubated alone (phosphorylation by the associated protein kinase) or in the presence of cyclic AMP-dependent protein kinase (60 μg/ml) plus or minus 5 x 10⁻⁴ M-cyclic AMP. The time course of incorporation of ³²P into proteins was monitored by precipitation of 20 μl aliquots of the incubation mixture on to 2 cm x 2 cm square papers (Whatman 3MM) previously loaded with 50 μl of 10% (w/v) trichloroacetic acid. The papers were further washed in 10% and 5% trichloroacetic acid, ethanol and diethyl ether as described elsewhere [14], and their radioactivities were determined by Čerenkov counting in 10 ml of water.

**Other procedures**

The protein concentration was determined as described by Lowry et al. [15], with bovine serum albumin as standard. The protein composition of neurofilaments was analysed by electrophoresis on 7.5%-acylamide 10 cm x 10 cm slab gels (1.5 mm width) in the presence of 0.1% SDS, by the method of Laemmlli [16], and protein bands were revealed by Coomassie Blue R250 (0.8%, w/v) staining in acetic acid/methanol/water (1:5:4, by vol.). The gels were destained in acetic acid/methanol/water (1:1:8, by vol.). Polyacrylamide gels of ³²P-labelled neurofilaments were further dried and exposed for radiography with XR O-Matic films (Kodak).

**RESULTS**

**Effect of enzymic dephosphorylation of neurofilaments on their gelation kinetics**

Several dephosphorylation conditions were studied. First, the freshly purified filaments were incubated under dialysis with the phosphatases for several hours before testing their activity. A gel was slowly formed during the

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**Fig. 1. Decrease of the neurofilament gelation capacity with increasing times of dialysis in the presence of acid phosphatase**

Freshly purified neurofilaments (4.5 mg/ml) were incubated at 20°C under dialysis against buffer A containing 0.8 M-sucrose and 1 mM-PMSF alone (△) or in the presence of acid phosphatase (5 units/ml; ●). Samples were removed after 10 h (a) or 24 h (b). The gelation experiments were performed at 22°C with dialysed neurofilaments (3.9 mg/ml) in buffer A containing 0.8 M-sucrose, 5 mM-MgCl₂ and a mixture of proteinase inhibitors. (c) 30 μg of neurofilaments dialysed 24 h with (II) or without (I) phosphatase were analysed by SDS/7.5%-polyacrylamide-gel electrophoresis. \( \eta_{rel} \) is the relative viscosity of the gel.
dialysis period against buffer A. The dialysed preparations were dissociated by mild homogenization before testing the viscosity kinetics in the presence of 5 mM-MgCl₂. A considerable decrease in the velocity of the viscosity kinetics was obtained in the samples incubated with acid phosphatase (Fig. 1). The extent of inhibition was proportional to the time of dialysis of the preparation with the enzyme. A 50% decrease in the viscosity change was obtained after 10 h at 20 °C (Fig. 1a), and the neurofilaments became almost totally inactive after 24 h incubation under the same conditions (Fig. 1b). Under the conditions adopted, a significant inhibition was obtained with alkaline phosphatase only after 24 h (results not shown). The analysis by SDS/polyacrylamide-gel electrophoresis of the dialysed samples showed a characteristic shift of the migration of the H and M subunits induced by dephosphorylation (Fig. 1c).

In an alternative protocol, the phosphatases were added at the same concentration as above, immediately before recording the viscosity kinetic of the mixture. In contrast with the preceding results, a strong activation of the reaction was obtained in the presence of acid phosphatase, and to a lower extent with alkaline phosphatase (Fig. 2a). If vigorously sheared, the neurofilament gels were disrupted and their viscosity returned to the basal level. Several successive gelation kinetics can be recorded [9]. However, the efficiency of the reaction decreased progressively, as illustrated by the three kinetics shown in Fig. 2(b). The longer lag time observed after twice shearing the gelled sample depends upon the number of gelation cycles, suggesting that the reversibility of the reticulation was not complete, probably because neurofilaments were not fully dissociated from each other by shearing. Under these conditions, the stimulation induced initially by the presence of acid phosphatase turned into a slight inhibition during the second kinetic, which was enhanced in the course of the third one (Fig. 2b). Furthermore, a stimulation of the gelation process was systematically found with calcineurin, a calmodulin-dependent phosphatase from brain, when assayed with neurofilaments in the presence of Ca²⁺ and Mn²⁺, which permitted its full activation by calmodulin [12] (Fig. 3). At the low neurofilament concentration adopted in the experiment of Fig. 3, no gel was obtained in the control sample. The presence of calcineurin determined the reticulation of the neurofilament suspension under the critical gelation concentration previously found above, namely 1 mg/ml [9]. Calmodulin at 1 μM stimulated the reaction efficiently. At higher neurofilament concentrations, the stimulation of the viscosity kinetics induced by calcineurin and calmodulin was about 300% of the control value. In contrast with the effect of acid phosphatase, the activation by calcineurin remained constant over several cycles of gelation (Table 1). No change in the electrophoretic mobility of the H and M subunits was associated with the effect of calcineurin on neurofilament gelation, even after several hours of incubation with the brain phosphatase (Fig. 3, inset). This observation suggests that the enzyme did not dephosphorylate the filament proteins to the same extent as that obtained with acid phosphatase.

**Relationship between the phosphorylation in vitro of neurofilaments and their gelation activity**

Neurofilaments are phosphorylated in vitro by the protein kinase associated with the preparation [17]. However, attempts to establish a relationship between the two events meet a major difficulty: the addition of 0.1 mM-ATP to the preparation resulted in an activation of the viscosity kinetics, which might be the result of the
activity of the neurofilament-associated protein kinase (results not shown). However, we found that an ATPase activity was constantly detected in the filament preparation that might also participate to this effect of ATP (J. Eyer & J.-F. Leterrier, unpublished work). For this reason, the choice was made of studying the gelation phenomenon in the presence of known amounts of exogenous cyclic AMP-dependent protein kinase, in the presence or the absence of the cyclic nucleotide.

As previously reported [14,18], a cyclic AMP-dependent phosphorylation of the neurofilament proteins was recorded with $\gamma^{32}\text{P}ATP$ (Fig. 4a). The basal incorporation of radioactivity by the endogenous kinase was only slightly increased by the addition of the enzyme without cyclic AMP, the cyclic nucleotide inducing a 320% maximum stimulation of the reaction within 10 min. However, a fraction of these phosphorylated sites was slowly hydrolysed with time, and the acid-stable radioactivity level attained after 60 min (ratio + cyclic AMP/– cyclic AMP = 2) remained unchanged over several hours (results not shown). Under these conditions, the

Table 1. Ratio of the initial velocities of three successive neurofilament gelation kinetics performed in the presence of acid phosphatase or calcineurin with calmodulin versus the control values

<table>
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<tr>
<th>Gelation cycle</th>
<th>Initial velocity of the gelation kinetics (% of control)</th>
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<tr>
<td></td>
<td>Acid phosphatase</td>
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<tr>
<td>I</td>
<td>305</td>
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<tr>
<td>II</td>
<td>73</td>
</tr>
<tr>
<td>III</td>
<td>33</td>
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M neurofilament subunit was the preferential substrate of the cyclic AMP-dependent phosphorylation (Fig. 4b). Nevertheless, a significant incorporation of the label also occurred in the L and H components in the order M > L > H). In addition to the neurofilament subunits, several minor polypeptides were also phosphorylated in a cyclic AMP-dependent manner, including the contaminating microtubule-associated proteins (Fig. 4b). The neurofilament gelation kinetics were further measured under the standardized cyclic AMP-dependent phosphorylation conditions determined as above, ATP (at 5 × 10$^{-6}$ M) being included in all samples. The results showed a significant stimulation by cyclic AMP (200%) of the initial velocity of the viscosity kinetics (Fig. 5).

DISCUSSION

The present work demonstrates clearly that the velocity of the gelation kinetics reflecting the association of neurofilaments in vitro is strongly affected by the phosphorylation state of their subunits and suggests the existence of a direct relationship between the two events. The cyclic AMP-dependent phosphorylation of neurofilaments (Fig. 4) leads to a cyclic AMP-dependent activation of the viscosity kinetics (Fig. 5). The M subunit is the major substrate of the cyclic AMP-dependent phosphorylation in vitro [14,18; Fig. 4] and might be the target of the stimulation of the gelation by the protein kinase in the presence of cyclic AMP. This possibility is supported by the finding that the purified molecule inhibits the interaction, suggesting its participation in the inter-filament cross-bridges [9]. However, other proteins of the filament preparation (including the H and L neurofilament subunits) were also labelled in a cyclic AMP-dependent fashion (Fig. 4b). Their involvement in the effect of the enzyme on the neurofilament reticulation cannot be excluded at the present time.

Conversely, long-term incubation of neurofilaments with acid phosphatase resulted in a time-dependent inhibition of their interaction (Fig. 1). The conditions adopted in our experiments are not optimal for the full
activity of either the acid or the alkaline phosphatases, because of the nearly neutral pH that is required for the gelation of neurofilaments in vitro [9]. For example, a 5-fold lower velocity of the viscosity kinetics was obtained by increasing to 7.8 the pH of the dialysing buffer. This low gelation activity was further inhibited by the presence of alkaline phosphatase during an overnight incubation at 20 °C, an effect similar to that of acid phosphatase at pH 6.8 (J. Eyer & J.-F. Leterrier, unpublished work). As a consequence of this choice of a neutral pH, an incomplete dephosphorylation of the neurofilament subunits occurred even after 24 h of incubation with acid phosphatase (Fig. 1). Furthermore, this enzyme was shown to dephosphorylate the M subunit preferentially to the H molecule [19], which could explain the small shift of the electrophoretic mobility of the latter protein under our conditions compared with the situation where the dephosphorylation was performed with other enzymes [3]. Nevertheless, the partial removal of phosphate groups from the neurofilament subunits significantly affected the neurofilament gelation kinetics, suggesting that an extensive dephosphorylation of the same molecules might abolish the interaction in vitro between the polymers. However, an activation of the neurofilament gelation process also occurred when the enzymes were added to the neurofilaments immediately before recording the gelation kinetics (Figs. 2 and 3; Table 1). This suggests that the stimulation of the velocity of the process was the consequence of early dephosphorylation events. Several experimental results argue for this eventuality. First, the activation initially induced by acid phosphatase was labile and was reversed after several hours of incubation and two viscosity kinetics (Fig. 2b). Secondly, a strong activation occurred with calcineurin that was enhanced by calmodulin (Fig. 3). This result was obtained with neurofilaments at a sub-critical concentration at which no gelation usually occurred [9]. In contrast with the effect of acid phosphatase, the activation induced by calcineurin persisted over several gelation cycles separated by shearing (Table 1). Calcineurin dephosphorylates microtubule proteins in vitro [20] and is known to affect

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**Fig. 4. Phosphorylation in vitro of neurofilaments by an exogenous cyclic AMP-dependent protein kinase**

(a) The incorporation of $^{32}$P into purified neurofilaments (0.6 mg/ml) was performed at 30 °C in a 125 μl assay mixture (buffer A containing 5 mM-MgCl$_2$, 0.8 m-sucrose and the mixture of proteinase inhibitors) in the presence of [γ-$^{32}$P]ATP ($5 \times 10^{-5}$ M; 0.1 μCi/nmol). Neurofilaments were assayed alone (■) or in the presence of cyclic AMP-dependent protein kinase (60 μg/ml) plus (▲) or minus (△) $5 \times 10^{-5}$ M-cyclic AMP. The kinetic of phosphorylation of the neurofilament proteins was monitored by the precipitation, at the times indicated, on trichloroacetic acid-impregnated papers, of 20 μl of the incubation mixture. (b) A portion (25 μl) of each incubation mixture in (a) was removed after 40 min incubation, mixed with denaturation buffer and analysed by electrophoresis on SDS/7.5% polyacrylamide gels. The gel was then dried and autoradiographed for 48 h. Lane I, autophosphorylated neurofilaments; lane II, same sample with the added protein kinase and without cyclic AMP; lane III, neurofilaments phosphorylated by the cyclic AMP-dependent kinase fully activated by the nucleotide. Arrows indicate the position of the three neurofilament subunits.
Fig. 5. Stimulation of the viscosity kinetics of neurofilaments by cyclic AMP-dependent phosphorylation

Control neurofilaments (2.5 mg/ml) were incubated at 22 °C in buffer A containing 1.2 mM-sucrose, 5 mM-MgCl₂, the proteinase inhibitors and 5 x 10⁻⁶ M-ATP (▲). To identical samples was added the cyclic AMP-dependent protein kinase (60 μg/ml), alone (●) or with 5 x 10⁻⁴ M-cyclic AMP (■), immediately before incubation. The assays were performed in triplicates, and mean values are represented. η_rel. is the relative viscosity of the gel.

suggests a functional heterogeneity of the numerous phosphorylation sites of the same molecules, shown to be differently accessible to phosphatases [10]. In the light of these observations, the recent finding of a differential turnover of the phosphate groups between the three neurofilament subunits during their transport into the axon [24] might reflect a ‘fine tuning’ of the cross-bridges interconnecting the filaments in situ and consequently of the viscosity of the axoplasm.

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