



# Microtubule-independent regulation of neurofilament interactions in vitro by neurofilament-bound ATPase activities

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## ABSTRACT

Neurofilaments (NFs), the major neuronal intermediate filaments, form networks in vitro that mimic the axonal NF bundles. This report presents evidence for previously unknown regulation of the interactions between NFs by NF-associated ATPases. Two opposite effects on NF gelation in vitro occur at low and high ATP concentration. These findings support the hypothesis that NF bundles in situ are dynamic structures, and raise the possibility that ATP-hydrolyzing mechanoenzymes regulate their organization.

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## Introduction

Neurofilaments (NFs), are neuron-specific members of the intermediate filament family and are copolymers of four subunits, NFH, NFM, NFL and internexin [1]. The cellular organization of NFs results from several dynamic events: their preferential transport into axons is based on intermittent motion in the anterograde and retrograde directions interrupted by long pausing periods [2]. These motility events depend upon MTs and are mediated by the molecular motors dynein and members of the kinesin family [3,4]. NFs also form dense multi-polymer bundles in which single NFs are essentially static and crossbridged together through NF projections [5]. Several specific phosphorylation events taking place on NF subunits regulate both the anterograde and the retrograde motion of NFs [6,7], and control the incorporation of moving NFs into bundles [5,7].

The mechanisms of NF bundling have been analyzed in vitro for years [8–13]. Purified NFs form gels which consist of a strong network of interconnected filaments involving the NFH and NFM lat-

eral projections, and do not require any other proteins than those present in the purified NF polymers [8,12,13]. The phosphorylation level of NF subunits modulates the gelation rate of NFs [9,12,13] which is enhanced during aging by the high phosphorylation level of NF projections [11]. These purified NF preparations contain dynein and kinesin activities regularly bound to the polymers, which mediate the bidirectional motion of NFs along MTs in vitro [14]. In the present report, we describe NF-bound ATPases that affect the NF bundling mechanism in the absence of MTs. This unusual situation may reveal previously unexplored mechanisms of the formation and the dynamics of NF bundles.

## Materials and methods

**Chemicals.** MES (morphoethanesulfonic acid), ATP (vanadate free, Mg salt, from equine muscle), GTP (lithium salt), ADP (Na salt), AMP (sodium salt), AMP-PNP (adenosine 5'-( $\beta,\gamma$ -imido) triphosphate tetralithium salt hydrate), oligomycin, ouabain, sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ) and protease inhibitors were from Sigma (L'Isle d'Abeau Chesnes, France). Adenosine 5'-( $\gamma$ - $^{32}\text{P}$ ) triphosphate, trimethylammonium salt (3000 Ci/mmol) was from Amersham (Les Ulis, France). All other reagents were from Merck (Darmstadt, Germany).

**Methods.** NFs were isolated from bovine or rat spinal cord according to the procedures described previously [8,10,12]. Tissues were homogenized at 4 °C in an equal weight of buffer A (Mes 0.1 M,  $\text{MgCl}_2$  1 mM, EGTA 1 mM, pH 6.8 with NaOH) containing protease inhibitors (0.1 mg/ml *N*-p-tosyl-L-arginine methyl ester,

**Abbreviations:** NFs, neurofilaments; NFH, neurofilament high; NFM, neurofilament medium; NFL, neurofilament low; MW, molecular weight subunits; IFs, intermediate filaments; MTs, microtubules; KSP, lysine, serine, proline repeats; IDPN,  $\beta,\beta'$ -imino-3-dipropionitrile.

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0.05 U/ml aprotinin, 1  $\mu$ M pepstatin, 1  $\mu$ M leupeptin, 1 mM phenylmethane sulfonylfluoride, 0.1 mM chloroquin, 0.1 mM *N*-tosyl-L-lysine chloromethyl ketone and 10 nM soybean trypsin inhibitor, prepared as a 100-fold concentrated mixtures, and added to the experimental buffer immediately before use). Homogenates were centrifuged at 78,000g for 1 h at 4 °C (50Ti rotor, Beckman). Supernatants were carefully collected and adjusted to 4 M glycerol and kept in ice for 3 h before centrifugation at 78,000g for 1 h at 4 °C. The pellet contains the crude NF preparation (70% of total NFs in the soluble extract, Fig. 1), which is resuspended in buffer A + protease inhibitors and loaded on 1.5 M sucrose and 0.8 M sucrose in buffer A before centrifugation for 3 h 30 min at 200,000g at 4 °C. Purified NFs (pellets) resuspended in buffer B (buffer A containing 0.8 M sucrose and protease inhibitors) were dialyzed against the same buffer containing 1 mM PMSF at 4 °C for 24 or 48 h.

Viscosity measurements of 100  $\mu$ l NF samples (following the specific precautions described in [12]) were performed according to [15] as previously reported [10].

The KCl extraction of purified NFs was made by incubation with 0.8 M KCl for 30 min at 4 °C, before sedimentation at 78,000g for 2 h at 4 °C on buffer B. Control NFs were submitted to the same procedure without KCl. Pellets were resuspended in buffer B and dialyzed overnight at 4 °C against buffer B + protease inhibitors.

The ATPase activity of NF preparations was measured using the micromethod described in [16]. Incubations were performed at 30 or 35 °C in a 100  $\mu$ l assay containing NFs in buffer B + 6 mM  $MgCl_2$ , protease inhibitors, and 0.2–0.3 mM  $\gamma^{32}P$ -ATP (0.5  $\mu$ Ci/assay). Samples (20  $\mu$ l) were loaded on 3MM filters (Whatman) at the indicated times, and processed as described in [16]. The radioactivity was measured in water (Cerenkov counting, 40% efficiency). Controls were run systematically in the absence of molybdate to determine any incorporation of  $^{32}P$  into proteins (almost undetectable at the low ATP specific activity used).

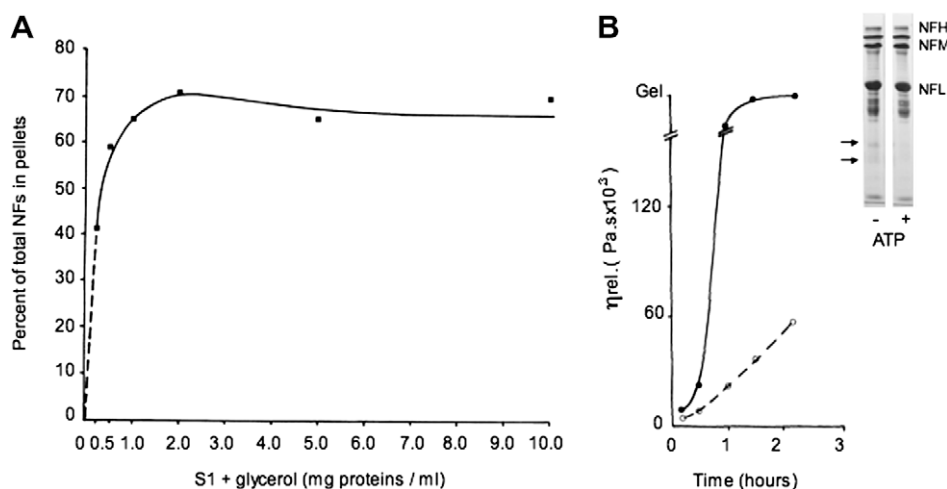
The activity of the NF-bound protein kinase was measured under the same conditions, using 50  $\mu$ M ATP (0.5  $\mu$ Ci/assay), by autophosphorylation of NFs by their NF-bound kinase. Samples (20  $\mu$ l) were loaded onto 2  $\times$  2 cm 3MM Whatman papers previously soaked with 50  $\mu$ l TCA 20% in water, and further washed 1  $\times$  in 10% TCA, 3  $\times$  in 5% TCA, 1  $\times$  in 90% ethanol and 1  $\times$  in ethylester before drying and counting in water.

Protein measurements were conducted according to [17], using bovine serum albumin as a standard. Proteins were resolved by 7.5% or 10% acrylamide SDS-PAGE according to [18].

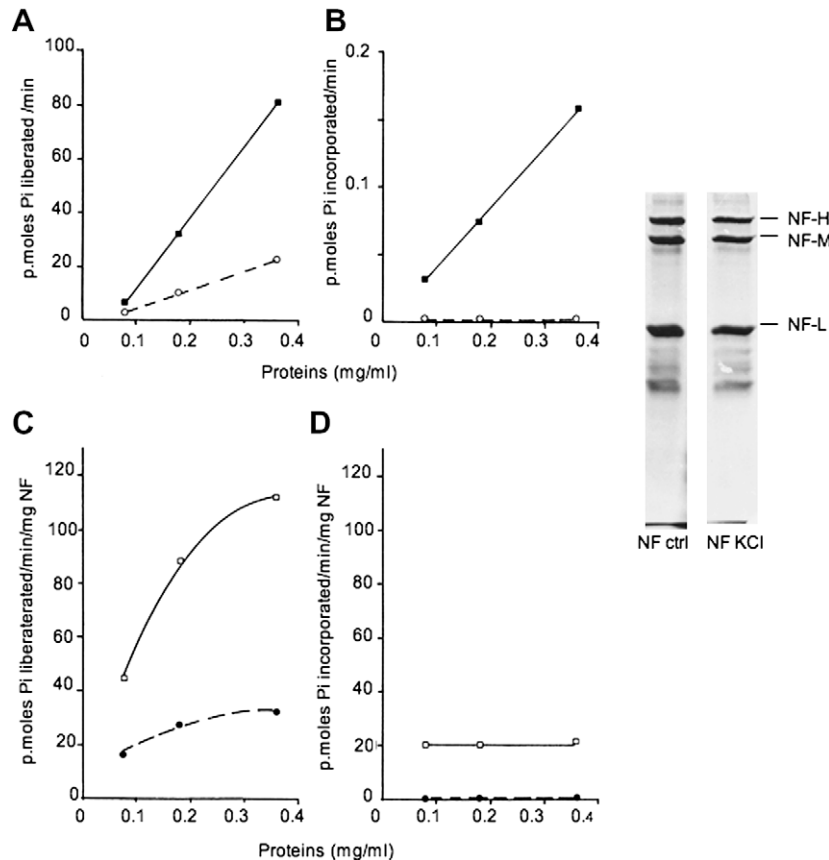
## Results

The purification of NFs from soluble extracts of spinal cord homogenates is based on the self-association of single filaments in the presence of glycerol at 4 °C [8,19]. A significant fraction ( $\approx$ 30%) of the NFs solubilized during homogenization remain in the supernatant after centrifugation at 78,000g of the cold incubate in the presence of glycerol, independent of the protein concentration of the sample (Fig. 1A). Investigating the conditions for an optimum recovery of NFs, we found that the addition of ATP to the buffer used for homogenization of spinal cord fragments at 4 °C resulted in a significant decrease in the yield of NFs sedimenting in the crude NF pellet after incubation at 4 °C for 3 h. An average of  $74.8 \pm 11.2\%$  (three preparations) of the amount of NFs solubilized in homogenates made in the absence of ATP was obtained if 2 mM ATP was present during homogenization. In contrast, addition of AMP-PNP in the homogenization step resulted in a 25% increase in the recovery of NFs as compared to the control without added nucleotide (not shown), suggesting that almost all solubilized NFs could be sedimented under these conditions. The purified NFs from homogenates  $\pm$  ATP differ both by their protein composition and their gelation activity (Fig. 1B). Minor polypeptides (MW  $\approx$  40 and 30 kDa, respectively) which otherwise contaminate purified NFs obtained in the absence of ATP were systematically released when ATP was included in the homogenization step (Fig. 1B, Inset). These later preparations exhibit much slower gelation kinetics when compared to that of control NFs at the same concentration (Fig. 1B).

Analysis of ATP-consuming enzymes in purified NF preparations reveals that in addition to the NF-bound kinase phosphorylating the NF subunits previously described [20,21], an additional ATPase activity is regularly present (Fig. 2). While all kinase activity is released from the NF polymers by 0.8 M KCl [21] (Fig. 2B and D), a variable fraction (40–70%) of the total ATPase activity remains associated with the KCl-treated NFs (Fig. 2A and C). In addition, measurements of the specific activity of the NF-bound ATPase at



**Fig. 1.** ATP affects the yield and properties of purified NFs. (A) Increasing concentrations of the soluble extract of spinal cord in buffer A incubated with 4 M glycerol at 4 °C for 3 h (see Materials and methods) were centrifuged at 4 °C for 30 min at 176,000g (Airfuge Beckman). The relative fraction of sedimented NFs versus total NFs of the soluble extract (estimated by quantitative measurements of the triplet NFH + NFM + NFL by SDS-PAGE) is expressed as a function of the protein concentration of the extract in assays. (B) Viscosity kinetics of 3 mg/ml purified NFs obtained simultaneously from homogenates  $\pm$  2 mM ATP. A strong gelation rate of NFs purified without ATP (filled circles and plain line) contrasts with that of NFs purified + 2 mM ATP (open circles and dotted line). Inset: 10% acrylamide SDS-PAGE of NFs purified without (-) or with (+) 2 mM ATP (40  $\mu$ g loaded). Two minor proteins (arrows: apparent MW  $\approx$  40 kDa and 30 kDa) regularly present in the purified NFs in the absence of ATP are released by ATP.



**Fig. 2.** The NF-associated ATPase activity is distinct from a couple kinase-phosphatase. Purified NFs (control and KCl-extracted) were analyzed at 35 °C for the hydrolysis of ATP and the phosphorylation of NF proteins in buffer B + 6 mM  $\text{MgCl}_2$ . (A) ATPase activity (initial velocities of Pi release. NFs in buffer B + 6 mM  $\text{MgCl}_2$  + 0.2 mM  $\gamma\text{-}^{32}\text{P}$ -ATP) measured for increasing concentrations of control (plain line and filled squares) and KCl-extracted (dotted line and open circles) NFs. (B) Incorporation of  $^{32}\text{P}$  into proteins (in buffer B + 6 mM  $\text{MgCl}_2$  + 0.05 mM  $\gamma\text{-}^{32}\text{P}$ -ATP) of increasing concentrations of control (plain line, filled squares) and KCl-extracted (dotted line and open circles) NFs. (C) Specific ATPase activities of control (plain line and open squares) and KCl-extracted (dotted line and filled circles) NFs from experiments of (A). (D) Specific autophosphorylation activity of control (plain line and open squares) and KCl-extracted NFs (dotted line and filled circles) from experiments of (B). Inset: Coomassie blue staining of control and KCl-extracted NFs analyzed on 10% SDS-PAGE (20  $\mu\text{g}$  proteins loaded).

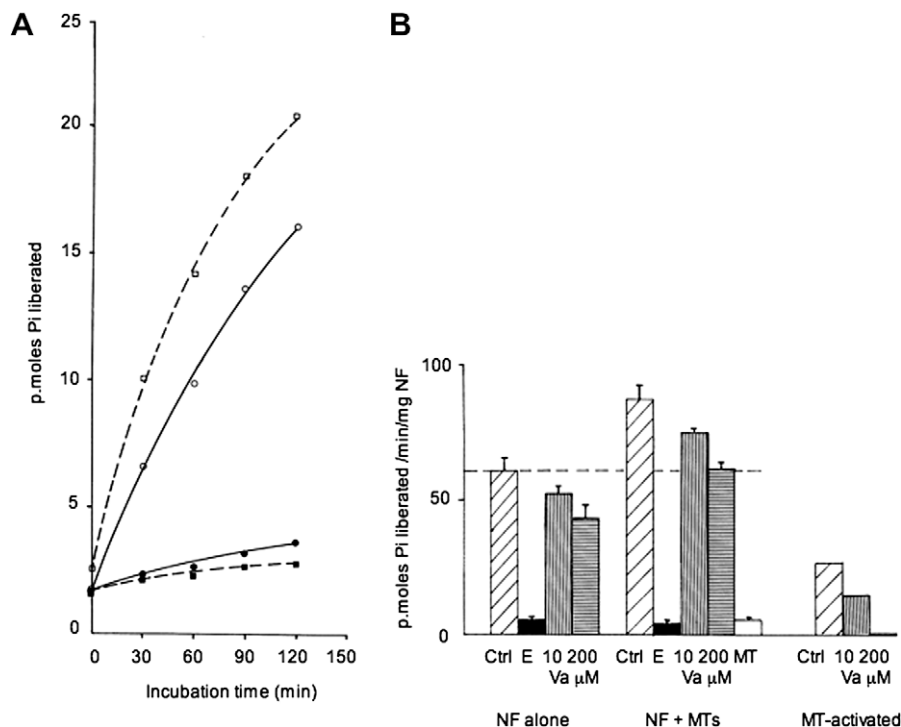
increasing protein concentrations revealed that it is not constant, like the kinase activity (Fig. 2D), but increases with the NF concentration toward a plateau reached around 0.5 mg NF/ml (Fig. 2C). This later observation suggests that the ATPase activity is affected by concentration-dependent interactions between filaments in the suspension, similar to the gelation process *in vitro* which requires 0.8–1.0 mg/NF/ml to occur [8].

The identity of the enzymes responsible for the ATPase activity measured in purified NF suspensions could be partly assigned to the molecular motors kinesin and dynein bound to NFs and responsible for their bidirectional translocation along MTs *in vitro* [14] and *in vivo* [4]. The ATPase activity of both kinesin and dynein is stimulated by MTs and is inhibited by sodium orthovanadate [22–24]. Fig. 3 shows ATPase activities of NFs measured in the presence or the absence of pure tubulin MTs stabilized by taxol. These data demonstrate that a fraction of the total ATPase measured in NFs is MT-dependent and inhibited by saturating amounts of sodium orthovanadate. However, the major part of the NF-associated ATPase activity remains unaffected by MTs or by 200  $\mu\text{M}$  Vanadate (Fig. 3B), suggesting that other unknown enzymes, distinct from the molecular motors, are bound to purified NFs. Measurement of the dependence of NF-bound ATPase activities upon divalent cations and ATP concentration showed that the NF-associated ATPase(s), with an apparent  $K_m$  for ATP  $\approx 30 \mu\text{M}$ , require  $\text{MgCl}_2$  above 2 mM for their maximal activity, and are inhibited by Ca ions (not shown).

The purification of NFs could involve the contamination of the polymers during homogenization by membrane-bound ATPases (Na-dependent plasma membrane ATPase, mitochondrial F1 ATPase). We investigated in parallel experiments the effect of ATP and specific inhibitors of these enzymes on the ATP hydrolysis of NFs and their gelation in the presence or the absence of ATP: the ouabain- or oligomycin-inhibited ATPases are essentially absent from the purified NF preparations (Table 1). Furthermore, the gelation of NFs is activated by 0.2 mM ATP, this effect being unaffected by the inhibitors, but abolished by high vanadate (Table 1).

The specificity for ATP of both the ATPase and the activation of NF gelation was estimated by using a series of nucleotides at identical concentrations (Table 2). The results show that neither the addition of GTP, AMP, ADP nor AMP-PNP at 0.2 mM could mimic the activation of NF gelation by 0.2 mM ATP (Table 2). In parallel, the addition at the same concentration (0.2 mM) of these nucleotides to the ATPase assays (0.4 mg/ml NF,  $\gamma\text{-}^{32}\text{P}$ -ATP 0.2 mM) resulted in the partial inhibition of the ATPase activity (Table 2). These data are indicative of the specificity of the NF-associated ATPase(s) for ATP, which are unrelated to the GTPase previously found in NF preparations [25].

The ATP-stimulated NF gelation was investigated further using KCl-extracted NFs devoid of bound kinase [21] and control NFs, in the presence of increasing concentrations of ATP or AMP-PNP (Fig. 4). ATP stimulates the gelation of both KCl-treated and control NFs within a narrow concentration window, between 0.05 and



**Fig. 3.** Effect of microtubules and vanadate on the NF-bound ATPase activity. The ATPase activity of 0.25 mg/ml NFs was analyzed in buffer B + 6 mM  $\text{MgCl}_2$  at 35 °C in the presence of 0.3 mM  $\gamma\text{-}^{32}\text{P}$ -ATP alone or in the presence of 1 mg/ml taxol-stabilized tubulin polymers. (A) Time course of ATPase of NFs (plain line, open circles) and NFs + MTs (dotted line, open squares). ATPase was measured for NFs + 3 mM EDTA and 1 mM  $\text{MgCl}_2$  (plain line, filled circles) and for MTs + 6 mM  $\text{MgCl}_2$  (dotted line, filled squares). (B) Histogram of the initial velocities of ATPase activities of NFs (0.28 mg/ml) alone or + 1 mg/ml MTs. Measurements in triplicate  $\pm$  SD for NFs  $\pm$  MTs alone (Ctrl), +EDTA 3 mM (E) or 10 and 200  $\mu\text{M}$   $\text{Na}_3\text{VO}_4$  (Va). Differences between values obtained  $\pm$  MTs are shown on the right panel.

0.5 mM (Fig. 4A). Surprisingly, concentrations of ATP higher than 0.5 mM inhibit gelation in control and KCl-treated NFs, although much less in the later (Fig. 4A). The two preparations differ in their response to AMP-PNP, which activates the gelation of control NFs above 0.1–0.2 mM (Fig. 4B-II) and is inactive on KCl-extracted NFs (Fig. 4B-I). These observations raise the possibility that two distinct ATP-dependent mechanisms coexist in NF preparations, of opposite effects on NF gelation, with respective efficient concentration ranges of 0.05–0.5 mM ATP and above 0.1–0.2 mM ATP.

## Discussion

These data indicate that ATP hydrolysis induces complex behaviors of NF suspensions, as the result of the ATPase activity of several enzymes associated with the purified polymers. This activity

**Table 1**

Effect of ATP and ATPase inhibitors on the NF-bound ATPase activity and the ATP-stimulated gelation of purified NFs. Left: ATPase initial velocity (% of control) of 0.4 mg/ml NFs  $\pm$  inhibitors (30 °C in buffer B + 6 mM  $\text{MgCl}_2$  and 0.2 mM  $\gamma\text{-}^{32}\text{P}$ -ATP). Right: initial gelation velocities (% of control without ATP) of 1.6 mg/ml NFs  $\pm$  0.1 mM ATP and ATPase inhibitors (30 °C in buffer B + 6 mM  $\text{MgCl}_2$ ).

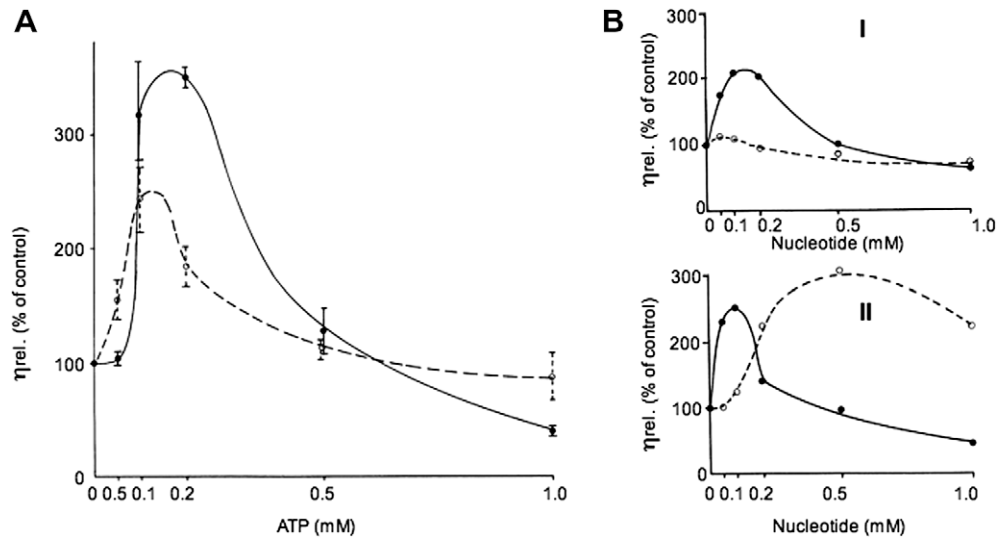
| Incubation conditions                        | ATPase (initial velocity) (% of control) | Rel. viscosity (initial velocity) (% of control) |
|--|--|--|
| Control                                      | –  | 100  |
| +ATP 0.2 mM                                  | 100                                      | 310  |
| +ATP 0.2 mM                                  | 97.5                                     | 306  |
| +Oligomycin 25 $\mu\text{g}/\text{ml}$       |  |  |
| +ATP 0.2 mM                                  | 83.5                                     | 310  |
| +Ouabain 100 $\mu\text{M}$                   |  |  |
| +ATP 0.2 mM                                  | 63.5                                     | 176  |
| + $\text{Na}_3\text{VO}_4$ 100 $\mu\text{M}$ |  |  |
| +ATP 0.2 mM                                  | 41.5                                     | 105  |
| + $\text{Na}_3\text{VO}_4$ 200 $\mu\text{M}$ |  |  |

is independent of a putative cycle of a NF-associated kinase + phosphatase since the kinase is totally solubilized from KCl-treated NFs (Fig. 2) and the activity of a NF-associated phosphatase in purified NFs, measured by using in vitro  $^{32}\text{P}$ -labeled NFs as a substrate, was very weak (not shown). In addition, the specific activity (initial velocity of  $^{32}\text{P}$  release/mgNFs) of the NF-associated ATPase(s) increases with the NF concentration, an observation that suggests a direct stimulation of the enzyme(s) by inter-NF interactions. Previous studies of the protein kinase and ATPases activities associated with purified NFs from rats in young versus aged animals [11], and control versus IDPN intoxicated animals [10], demonstrated that they are differentially affected in two physiological situations associated with alterations of the subcellular organization of NFs. While the NF-associated kinase increases by 73% in NFs from IDPN-treated versus control rats with no significant change in the NF-associated ATPase measured at 0.2 mg/ml [10], an opposite situation was found during aging: the ATPase of 0.2 mg/ml NFs

**Table 2**

Effect of various nucleotides on the NF-bound ATPase and the gelation of NFs. Left: ATPase initial velocity (% of control) of 0.36 mg/ml NFs  $\pm$  unlabeled nucleotides (30 °C in buffer B + 6 mM  $\text{MgCl}_2$  and 0.2 mM  $\gamma\text{-}^{32}\text{P}$ -ATP). Right: initial gelation velocities (% of control without ATP) of 1.5 mg/ml NFs  $\pm$  0.2 mM ATP or other nucleotides at 0.2 mM (30 °C in buffer B + 6 mM  $\text{MgCl}_2$ ).

| Incubation conditions                     | ATPase (initial velocity) (% of control) | Rel. viscosity (initial velocity) (% of control) |
|---|--|--|
| Control                                   | –  | 100  |
| $\gamma\text{-}^{32}\text{P}$ -ATP 0.2 mM | 100                                      | –  |
| +ATP 0.2 mM                               | 45                                       | 206.7  |
| +ADP 0.2 mM                               | 47                                       | 106.7  |
| +AMP 0.2 mM                               | 75                                       | 77.8   |
| +AMP-PNP 0.2 mM                           | 59                                       | 114.4  |
| +GTP 0.2 mM                               | 80                                       | 111.1  |



**Fig. 4.** ATP and AMP-PNP influence NF gelation in opposite concentration-dependent fashions. (A) Viscosity measurements of 4 mg/ml purified control (plain line, filled circles) and 0.8 M KCl-extracted NFs (dotted line, open circles) in the presence of increasing ATP (buffer B + 6 mM  $MgCl_2$ ). Values after 2 h at 30 °C from three preparations are expressed as a %  $\pm$  SD of the viscosity of samples without ATP. (B) Comparison of the effect of increasing ATP (plain line, filled circles) and AMP-PNP (dotted line, open circles) on the viscosity of 3 mg/ml KCl-extracted (I) and control (II) NFs, after 2 h at 37 °C (buffer B + 6 mM  $MgCl_2$ ). Values are expressed as a % of the viscosity of samples without nucleotide.

from 36 month versus 3 months old rats increases by  $175.4 \pm 39.6\%$ , with a non-significant change in the kinase activity ( $109 \pm 20\%$  of 3 month old rat NFs) (three preparations) (unpublished results). These observations suggest that the two types of enzymes differ in their association to NFs in aging (hyperphosphorylation of subunits of NF aggregates in axons) and in the NF tight bundles excluding MTs in axonal swellings induced by IDPN [26,27].

The putative contribution of the NF-associated protein kinase to NF gelation by phosphorylation of NF subunits can be excluded in KCl-treated NFs, which exhibit a behavior close to that of control NFs in the presence of ATP (Fig. 4). Thus, the ATPase activity remaining in KCl-extracted NFs is assumed to be involved in the bell shape activation curve of NF gelation by low ATP, between 0.05 and 0.5 mM, while the stronger activation in control NFs might result from the combined activities of the NF-bound protein kinase and this same ATPase. Furthermore, another component of NF-associated ATPases activities, lost in KCl-extracted NFs, inhibits the gelation of control NFs at ATP concentrations above 0.5 mM, with an opposite activation by AMP-PNP (Fig. 4). This is inconsistent with the protein kinase activity, which stimulates inter-NF interactions in direct correlation with the phosphorylation level of NF subunits [9,11–13]. These data support the hypothesis that a complex of ATPases associated to NFs allows the modulation of NF interactions in opposite directions and in an ATP concentration-dependent manner.

These results motivate several questions with regard to the nature of ATP-hydrolyzing enzymes and of the molecular mechanisms involved in the observed phenomena. The only ATPases previously identified in purified NFs are the molecular motors dynein and kinesin [14], bound in vivo to NFs [3,4]. Our present data confirm that a fraction of the total ATPase activity present in NF preparations is stimulated by MTs and is inhibited by sodium orthovanadate (Fig. 3), both properties characteristic of these molecular motors [22–24]. However, it has not been shown, yet, that the organization of intermediate filaments (IFs) into bundles could be regulated through the activity of molecular motors in the absence of MTs. Instead, IFs of all types have been shown to behave as (inert) cargoes of such motors carried along MTs through the ATP-dependent activity of these molecular motors [4,28,29].

Beyond this largely accepted concept, our experimental results open new avenues for the possible regulation in vivo of IF organization by means of MT-independent mechanisms involving ATP-hydrolyzing enzymes. The evidence that the activation of NF gelation by ATP is abolished by sodium orthovanadate (Table 1) favors the contribution of NF-bound molecular motors in this effect. Recent experimental findings demonstrated that NF bundles formed in situ, specifically consisting of highly phosphorylated NFs [3,5], contain also NF-bound dynein [30]. These indications, together with our data, point at the unexpected hypothesis that the NF-associated dynein and other NF-associated ATPases could contribute to the organization and the dynamics of NF bundles. These new findings should contribute to a better understanding of the complex mechanisms controlling the dynamic organization of NF networks and bundles in axons.

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