

Assembly and Exchange of Intermediate Filament Proteins of Neurons: Neurofilaments Are Dynamic Structures

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Abstract. We have explored the dynamics of intermediate filament assembly and subunit exchange using fluorescently labeled neurofilament proteins and a fluorescence resonance energy transfer assay. Neurofilaments (NFs) are assembled from three highly phosphorylated proteins with molecular masses of 180 (NF-H), 130 (NF-M), and 66 kD (NF-L) of which NF-L forms the structural core. The core component, NF-L, was stoichiometrically labeled at cysteine 321 with fluorescein, coumarin, or biotin-maleimide to produce assembly-competent fluorescent or biotinylated derivatives, respectively. Using coumarin-labeled NF-L as fluorescence donor and fluorescein-labeled NF-L as the fluorescence acceptor, assembly of NF filaments was induced by rapidly raising the NaCl concentration to 170 mM, and the kinetics was followed by the decrease in the donor fluorescence. Assembly of NF-L subunits into filaments does not re-

quire nucleotide binding or hydrolysis but is strongly dependent on ionic strength, pH, and temperature. The critical concentration of NF-L, that concentration that remains unassembled at equilibrium with fully formed filaments, is 38 $\mu\text{g/ml}$ or 0.6 μM . Under physiological salt conditions NF-L filaments also undergo extensive subunit exchange. Kinetic analysis and evaluation of several possible mechanisms indicate that subunit exchange is preceded by dissociation of subunits from the filament and generation of a kinetically active pool of soluble subunits. Given the concentration of NF-L found in nerve cells and the possibility of regulating this pool, these results provide the first information that intermediate filaments are dynamic structures and that NF-L within the NF complex is in dynamic equilibrium with a small but kinetically active pool of unassembled NF-L units.

NEURONS are highly asymmetric cells. The axon that emanates from the cell body can extend as a very long process often hundreds of times longer than the length of the cell body. Electron microscopic examination of the neuronal processes has shown that neurons, like other cells, have a network of interwoven strands appearing like filamentous material. These strands are composed of microtubules (MTs),¹ microfilaments (MFs), and neurofilaments (NFs). Unlike MTs and MFs, much less is known about the biochemistry and cell biology of NFs and the interactions with other cytoskeletal components in determining cell shape and function. NFs belong to the heterogeneous class of intermediate filaments (IFs), a group of proteins that are tissue specific and developmentally regulated (18). Because of their poor solubility properties in physiological buffers, IFs have often been considered as totally passive and static structures. However, there are several indications that IFs are dynamic structures. For example, some IF antibodies do not detect IFs in interphase. However, antibodies to vimentin that react

only with migrating fibroblasts (4), or antibodies to a desmin epitope that is expressed with the alignment of myofibrils (5), hint that IFs may be dynamic structures whose assembly may be somehow regulated by cell cycle events. For a highly polarized cell such as a neuron, it seems reasonable that during cell cycle events such as axonal extension or regeneration, IFs must be very plastic and respond by assembly and disassembly. In neurons, assembly of the IF network takes on additional importance since the intrinsic asymmetry of these cells requires either that these proteins are assembled by addition at the proximal end and disassembled at the distal end, or are turned over by replacement at internal sites along the filament (2). Whether the incorporation and replacement occurs at the site of synthesis in the perikaryon (2, 17), at other points along the axon, or at filament ends remains an important question for neuronal cell biology. Recent pulse labeling measurements have shown that NF proteins are nonuniformly integrated into the NF lattice and suggest that exchange of subunits with filaments is one potential mechanism for NF turnover rather than the addition of subunits to the proximal end and transport of the entire NF matrix (23, 24).

One of the major impediments to the examination of IF dynamics has been the lack of suitable optical probes like 5-(4,6-dichlorotriazinyl)amino fluorescein-tubulin and py-

1. *Abbreviations used in this paper:* CPM, 3-(4-maleimidylphenyl) 7-diethylamino-4-methylcoumarin; FM, fluorescein-5-maleimide; IF, intermediate filament; MF, microfilament; MT, microtubules; NF, neurofilament; NF-H, neurofilament of high molecular mass (180 kD); NF-M, neurofilament of medium molecular mass (130 kD); NF-L, neurofilament of low molecular mass (66 kD).

rene-actin. Fluorescent cytoskeletal protein analogues have contributed greatly to our understanding of the dynamics of these proteins *in vitro* and in living cells (6, 15, 30, 31, 36, 38). To apply these methods to elucidate the structural requirements and regulation of assembly, and to monitor the dynamics of the neuronal cell cytoskeleton during axonal growth, we have prepared fluorescent derivatives of the low molecular mass (66 kD) (NF-L) protein that forms the core of the NF complex. Under physiological salt conditions, NFs assemble in the absence of nucleotide binding or hydrolysis, and rapid and extensive subunit exchange between filaments occurs. The features of assembly and subunit exchange could permit rapid changes in NF structure during cellular events and challenge previous concepts that NFs/IFs are not dynamic structures. Furthermore, the methods and findings provide a basis upon which NF assembly in growing and regenerating neurons can be examined and have general implications for IF assembly.

Materials and Methods

Materials

Staphylococcus aureus V8 protease, (MES) α -chymotrypsin, urea, sucrose, and 2-(*N*-morpholino)ethanesulfonic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Fluorescein-5-maleimide (FM) and 3-(4-maleimidylphenyl) 7-diethylamino-4-methylcoumarin (CPM) were purchased from Molecular Probes Inc. (Junction City, OR). Biotin-maleimide was from Calbiochem-Behring Corp. (San Diego, CA). DE-52 was purchased from Fisher Scientific Co. (Pittsburgh, PA) and hydroxylapatite was from Bio-Rad Laboratories (Cambridge, MA). All other reagents were the highest commercial quality available.

Protein Purification

NF-L protein from bovine spinal cord was purified by sequential chromatography on DE-52 and hydroxylapatite as previously described (32) with minor modifications including a prior batch purification using hydroxylapatite (39). Purified NF-L protein was analyzed by SDS-PAGE and selected fractions were pooled and concentrated to 1.5–4.0 mg/ml by ultrafiltration in preparation for labeling with fluorescent or biotinylated probes.

Labeling of NF-L with Fluorescent or Biotinylated Probes

Since NF-L contains a single cysteine at residue 321 (19) the sulfhydryl-specific fluorescent maleimides, FM, CPM, and biotin-maleimide were used. Chemical modification of freshly prepared NF-L was performed in either the dissociated state or the assembled state. Protein was labeled at a ratio of 1:10 (protein/fluorophore) for 22°C for 60 min in either 150 mM sodium phosphate and 8 M urea, pH 7.4, or in a buffer composed of 0.1 M MES, 0.17 M NaCl, pH 8.1. The reaction was terminated by 1 mM DTT, and was dialyzed at 37°C for 12 h against either 20 mM Hepes-Tris, pH 7.2, 10 mM NaCl, and 1 mM MgCl₂ (assembly buffer) to remove excess fluorophore and urea. Centrifugation and pelleting of the filaments was avoided since pelleted filaments resulted in extensive aggregation. NF-L protein so labeled was used directly for experiments and stored at 4°C for no longer than 3 d.

Electron Microscopy

Filaments were negatively stained with 1% uranyl acetate and examined by EM in an electron microscope (model 301; Philips Electronic Instruments, Inc., Mahwah, NJ) operated at 80 kV. Grids were examined at a magnification of 14,000. A large number of filaments with identifiable ends were used for quantitative measurements of filament length distribution.

Spectroscopic Measurements

The dye-to-protein ratio of fluorescently labeled NF was determined spec-

trophotometrically with a scanning spectrophotometer (model 210 UV-VIS; Cary Varian Instruments, Palo Alto, CA). In Hepes buffer, the extinction coefficients were $\epsilon_{385\text{ nm}} = 30,200 \text{ M}^{-1}\text{cm}^{-1}$, and $\epsilon_{495\text{ nm}} = 75,000 \text{ M}^{-1}\text{cm}^{-1}$ for the cysteine conjugates of CPM and FM, respectively.

Steady-state fluorescence measurements were performed in a spectrofluorimeter (model 8000; SLM Instruments, Inc., Urbana, IL) interfaced to a computer (model XT; IBM Instruments, Inc., Danbury, CT) using 0.3 × 0.3 cm microcuvettes. Fluorescence spectra were corrected for wavelength-dependent variation in light source output, phototube response, and monochromator efficiency (1). Quantum yields, emission anisotropies, excited state lifetimes, overlap integrals, and Forster critical distances (R_0) were measured as described (1). The quantum yield, ϕ , of fluorescently labeled NF-L was determined by comparison with the quantum yield of fluorescein in 0.1 N NaOH.

Filament Assembly

Fluorescence resonance energy transfer was used to monitor assembly. Before assembly studies, we established an energy transfer quench curve for donor-labeled protein as a fraction of the mole fraction of acceptor labeled NF-L (fluorescein) co-assembled in filaments. To determine the efficiency with which labeled NF-L competes with unlabeled NF-L in filament assembly, increasing amounts of fluorescent NF-L in 8 M urea were added to decreasing amounts of unlabeled NF-L in 8 M urea. The relative concentrations of each sample were adjusted so that the total protein concentration remained constant. The urea was removed, the resulting hybrid NF-L filaments were collected by rapid gel filtration on Sephadex G-10 and then assayed for fluorescence. Alternatively, CPM-labeled NF-L in assembly buffer (20 mM Hepes-Tris, pH 7.2, 10 mM NaCl, 1 mM MgCl₂) was added to a mixture containing various proportions of FM-labeled NF-L and unlabeled NF-L. Filaments were then assembled by the addition of NaCl to a final concentration of 170 mM. Final concentrations were 0.25 mg/ml CPM-labeled NF-L and 1.0 mg/ml of combined FM-labeled and unlabeled NF-L. The fluorescence quenching of CPM at 495 nm was monitored and the percent fluorescence quench calculated by

$$\% \text{ quench} = \frac{F_i - F_{\text{eq}}}{F^*} \times 100. \quad (1)$$

where F_{eq} = the final fluorescence intensity at equilibrium; F_i is the fluorescence intensity immediately after mixing; and F^* is the fluorescence intensity of the fully dissociated or randomized sample. F^* was determined after assembly was complete, as evidenced by no further decreases in donor fluorescence and by disruption of the filaments with 1% SDS.

Sedimentation was also used to confirm that the critical concentration of fluorescent NF-L was the same as labeled NF-L. Polymerized fluorescent NF-L or unlabeled NF-L (100 μ l) at varying total protein concentrations were sedimented for 60 min at 150,000 *g* in an airfuge (Beckman Instruments, Inc., Palo Alto, CA). Aliquots (30 μ l) were removed from the supernatant, and the concentration of protein was compared to a standard curve of known concentration of unlabeled NF-L (27).

NF-L filament formation was monitored by fluorescence energy transfer using CPM-labeled NF-L as the donor and FM-labeled NF-L as the acceptor. Donor- and acceptor-labeled NF-L were mixed in assembly buffer at 37°C. Baseline fluorescence of the mixture was measured for 5–10 min, and the assembly was initiated by the addition of a concentrated aliquot of NaCl to rapidly raise the ionic strength to 170 mM NaCl. Dilution was <10%. Filament assembly was continuously monitored, with the excitation shutters closed between measurements, by the decrease in donor fluorescence at 495 nm. When no further changes in fluorescence quench were observed, assembly was determined to be complete. The extent of assembly was determined by using eq. 1, while the kinetics were analyzed using the Guggenheim method (11) for a first-order reaction approaching equilibrium. In addition, at the conclusion of the experiment, formation of 10-nm filament morphology was assessed by negative stain EM.

Filament Subunit Exchange

Two types of energy transfer experiments were used to examine subunit exchange. In the first, CPM-labeled subunits (donor) were copolymerized into filaments with FM-labeled subunits (acceptor) at a 1:2 ratio (donor/acceptor). The copolymer was mixed at 37°C with an excess of unlabeled filaments, and the relief of CPM quenching at 495 nm with 410 nm excitation was monitored over 30-s to 1-min intervals. As CPM- and FM-labeled subunits exchange with unlabeled protein, the average distance between CPM- and FM-labeled subunits within the filament increases and the fluorescence in-

creases as a result of the relief of CPM quenching through energy transferred to fluorescein. The copolymer assay, in effect, measures the dissociation of acceptor-labeled subunits since the signal change is based on acceptor subunits leaving the copolymer filament.

The extent of subunit exchange was determined by

$$\% \text{ exchange} = \frac{F_{\text{eq}} - F_i}{F^* - F_i} \times 100. \quad (2)$$

where F_i is the fluorescence of the CPM-FM-labeled copolymer before mixing (at 1:2 donor/acceptor or the maximum percent quench at the mole fraction of acceptor); F_{eq} is the fluorescence of the filament mixture after the exchange period; and F^* is the fluorescence of dissociated subunits.

We also used a second energy transfer assay, or mixed polymer, to examine subunit exchange where CPM-labeled filaments were mixed with FM-labeled filaments. FM-labeled NF-L and CPM-labeled NF-L filaments were assembled separately and then mixed at 37°C. The fluorescence intensity was monitored simultaneously at 520 nm for the relief of fluorescein self-quenching (excitation 495 nm, channel A) and at 495 nm for the quenching of coumarin donor fluorescence (excitation, 410 nm; emission, 495 nm; channel B). Using this assay, as subunits dissociate, the fluorescein signal increases as a result of relief of fluorescein quenching (that arises from energy transfer between adjacent fluorescein subunits), while no change in the CPM fluorescence signal is observed as CPM-labeled subunits dissociate from these filaments. However, as FM-labeled NF-L subunits incorporate into CPM-labeled filaments to form CPM-FM NF-L copolymers, the CPM fluorescence is quenched by energy transfer to the now adjacent FM-labeled subunits. The rate of change of CPM (donor) quenching reflects the rate of subunit incorporation. Thus, this assay is used to resolve the rates of subunit dissociation and incorporation steps.

The extent of subunit exchange based on the reduction of CPM donor fluorescence by acceptor incorporation was calculated from the fluorescence observed immediately after mixing donor- and acceptor-labeled filaments, F_i , at the end of the exchange period, F_{eq} , and in the quenched state where CPM donor subunits would have an adjacent acceptor, F^* .

$$\% \text{ exchange} = \frac{F_i - F_{\text{eq}}}{F_i - F^*} \times 100. \quad (3)$$

Although the quenched state could be determined by the mole fraction of acceptor subunits at the mixing ratios used, at the conclusion of each experiment, we verified and obtained F^* by randomizing the filament at the end of the exchange period with 8 M urea and reforming the CPM-FM copolymer by dialysis.

Results

Chemical Modification of NF-L

Sequence analysis of NF-L (10, 19) shows only one cysteine at position 321. Using sulfhydryl-specific maleimides (35), fluorescent and biotinylated derivatives of NF-L were prepared. The reaction between protein sulfhydryl and the fluorophore is rapid, specific, and stoichiometric. Pretreatment of NF-L with 1 mM *N*-ethylmaleimide for 120 min completely blocked the incorporation of either fluorescent or biotinylated label. For each of the labels used, the final labeling stoichiometry approaches 1:1 when NF-L is labeled as a dissociated molecule in 8 M urea. We found, however, that labeling of NF-L in the filamentous form yields fluorophore-to-protein ratios that never exceeded 0.25. Longer times did not increase the extent of labeling. This suggests that the assembly of NF-L polypeptides leads to protection of the otherwise accessible sulfhydryl group located in the protein's rod regions. To maximize the magnitude of the signal change, in most experiments, only NF-L molecules where labeling led to 1 mol fluorophore/1 mol protein were used. However, both the kinetics and extent of the exchange was independent of the level of labeling.

Unstained SDS-polyacrylamide gels of the labeled pro-

teins shows that only the NF-L protein is fluorescent—no other contaminating or degradation product bands were present—and that removal of unconjugated dye was complete. The same gels were stained for protein by silver to confirm that NF-L was the only component of the preparation and that the fluorescence co-migrates with the protein (Fig. 1). Since these are SDS gels run under denaturing and reducing conditions, the results indicate that all of the fluorophore is covalently bound to the protein.

Spectroscopic Properties

The labeled proteins display a number of spectroscopic properties that are useful for in vitro assembly, disassembly, and exchange experiments and for molecular cytochemistry in living cells. Both the absorption and emission maxima of the derivatives are at long wavelengths: FM-labeled NF-L has an absorption maximum at 495 nm and a fluorescence emission maximum at 520 nm; CPM-labeled NF-L has a broad absorption spectrum extending to 450 nm with a maximum at 395 nm, and fluorescence emission at 495 nm. Both derivatives have high molar extinction coefficients $\epsilon_{495 \text{ nm}} = 75,000 \text{ M}^{-1}\text{cm}^{-1}$ for FM-labeled NF-L, and $\epsilon_{395 \text{ nm}} = 30,200 \text{ M}^{-1}\text{cm}^{-1}$ for CPM-labeled NF-L, with quantum yields of 0.33 and 0.24, respectively. The low emission anisotropies, 0.14 for FM-labeled NF-L and 0.26 for CPM-labeled NF-L, indicate that the probes have unrestricted mobility.

One of the more important qualities of the derivatives is the spectral overlap between the fluorescence emission of CPM-labeled NF-L and the absorption spectrum of FM-labeled NF-L and between the emission and absorption of FM-labeled NF-L itself. The extensive spectral overlap shown by the derivatives indicates that fluorescence resonance energy transfer can be used to follow the assembly. From the overlap integral and the orientation of the probes ($K^2 = 2/3$), we have calculated Forster critical distances, (R_0), of 48 Å for the CPM-FM donor-acceptor pair and 49 Å for fluorescein to fluorescein.

The energy transfer measurements also provided some structural information. The distance of closest approach between donor- and acceptor-labeled subunits assembled into the filament was determined by integrating over all possible donor and acceptor pairs, using a modified population analy-

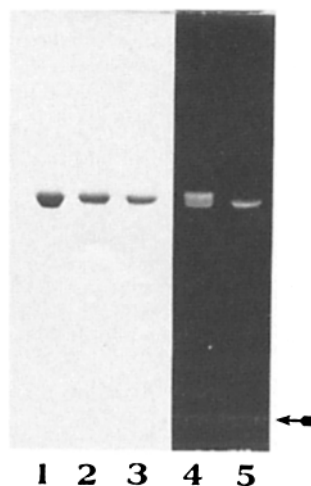


Figure 1. SDS 7.5% PAGE of fluorescently labeled NF-L. Fluorescent image of 10 μg of FM-labeled NF3 (lane 4) and 10 μg of CPM-labeled NF-L (lane 5) photographed with 300-nm illumination and a filter (model KV-470; Schott Glass Technologies Inc., Duryea, PA). Corresponding silver staining pattern of FM-labeled NF-L (lane 2), CPM-labeled NF-L (lane 3), and 20 μg unlabeled NF-L (lane 1). Arrow indicates the dye front.

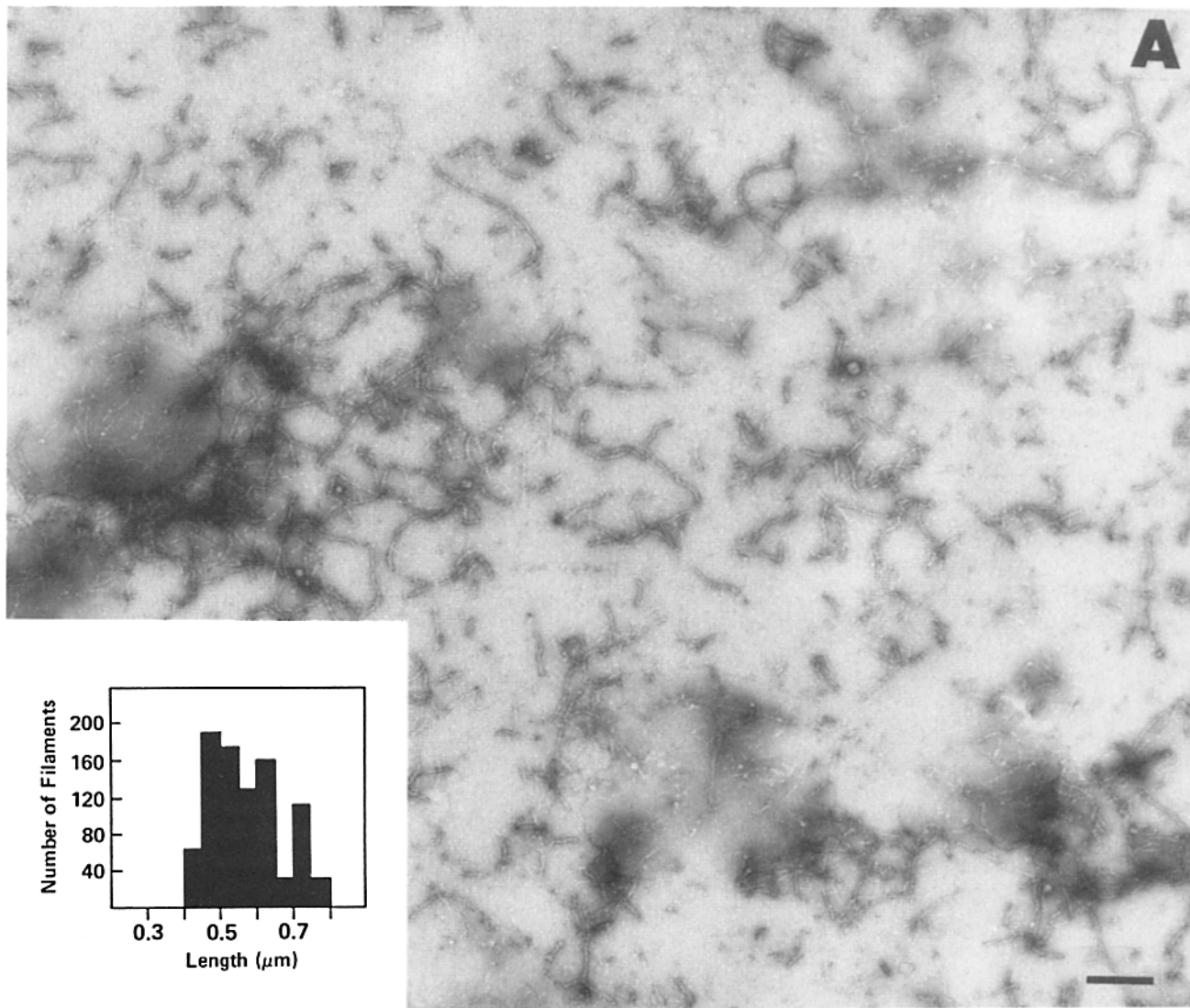


Figure 2. Electron micrograph of fluorescently labeled and unlabeled NF-L filaments. (A) Unlabeled control NF-L filaments (1.5 mg/ml). (B) FM-labeled NF-L filaments (1.1 mg/ml). (Inset) Histograms of length distribution. Bar, 250 nm.

sis of a parallel infinite plane model (3). Using this model and treatment, we have determined that the distance between sulfhydryls on adjacent subunits are within 15 Å. (The model we used was the infinite plane model [3] with one dimension removed, and the individual surface function was expanded and integrated after replacing dP [where dP is the probability that a donor and acceptor are separated by a distance between r and $r + dr$]. A copy of this treatment is available from the authors upon request. The most probable range for the distance of closest approach were determined from the probability function for R'/R and polarization values, and is 13.7–16.7 Å.)

Assembly Properties of Derivatized NF-L

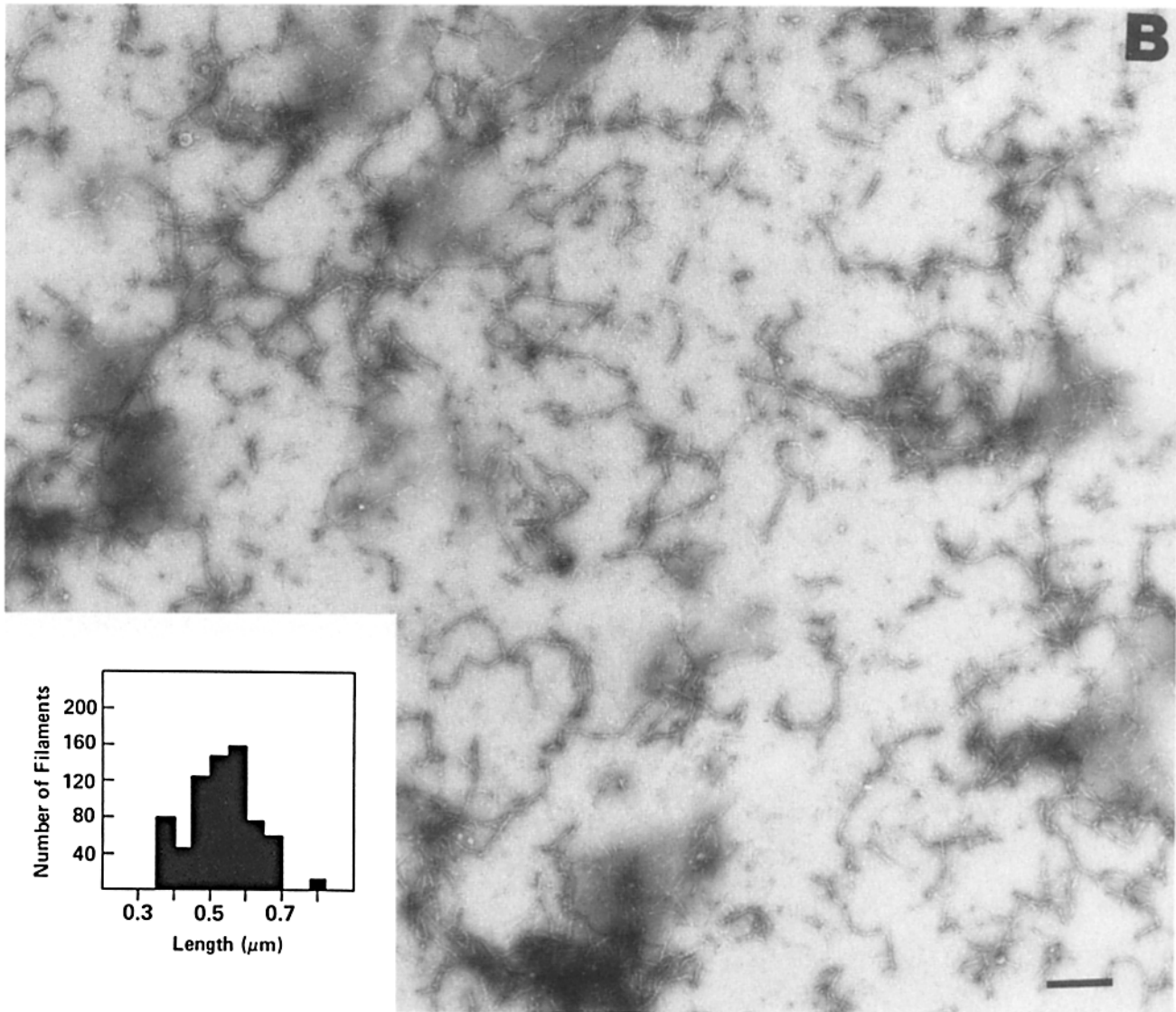
Several experiments were performed to demonstrate that the labeled proteins retain their native properties.

First, EM of the labeled preparations reveal filaments that are indistinguishable from unlabeled NF-L filaments in both diameter and length. NF-L length distribution between fluorescently labeled and unlabeled filaments was analyzed sta-

tistically from electron micrographs. In Fig. 2, representative fields of labeled and unlabeled filaments are shown. Histograms of the length distribution compiled from both labeled and unlabeled filaments are very similar with average lengths of $0.5 \pm 0.1 \mu\text{m}$. At least at the morphological level and by comparison of the polymer length distributions, formation of the NF-L core is not adversely affected by labeling.

Energy transfer measurements also demonstrated that fluorescently labeled protein coassembles with unlabeled protein. This is shown by the linear increase in percent fluorescence quench of donor-labeled NF-L with increasing mole fraction of acceptor-labeled NF-L in coassembled NF-L filaments (Fig. 3). Approximately 60% quench of the CPM fluorescence at 495 nm occurs during NF-L filament assembly when the mole fraction of FM-labeled NF-L in filaments is 0.66. The results indicate that the labeled derivatives assemble with the same efficiency as their unlabeled counterparts and that the quenching in donor fluorescence is proportional to the mole fraction of acceptor. Thus, energy transfer can be used as a sensitive and linear index of the assembly process.

Sedimentation also confirmed that the critical concentra-



tion of fluorescently derivatized NF-L was the same as the unlabeled NF-L protein. At each concentration measured, the protein concentration in the supernatant remained constant at $\sim 40 \mu\text{g}$ and was identical between fluorescently labeled and unlabeled protein ($\sim 40 \mu\text{g}/\text{ml}$, see below).

Self-Assembly of the Neurofilament Core

As donor- and acceptor-labeled subunits assemble into filaments, there is a decrease in the fluorescence of the donor resulting from energy transfer to an adjacent acceptor. The percent fluorescence quench of CPM-labeled NF-L increased by 30–40% over several minutes when CPM- and FM-labeled NF-L subunits were assembled by the addition of NaCl (Fig. 4 A). The degree of percent fluorescence quench observed at equilibrium depended on the mole fraction of acceptor FM-labeled NF-L that is coassembled with CPM-labeled NF-L donor subunits. EM confirms that the fluorescence change corresponds to the assembly of NF-L filaments (Fig. 4 B).

As shown in Fig. 4 A both the rate and extent of assembly are dependent on the total protein concentration. At 1.08 mg/ml, the observed rate for filament assembly is 0.69 min^{-1} ($\tau_{1/2} = 4 \text{ min}$), while at a lower concentration of 0.58 mg/ml, the observed rate constant is 0.31 min^{-1} ($t_{1/2} = 9 \text{ min}$). The extent of assembly also increases as the total protein concentration increases (Fig. 5 A) and saturates at 1.2 mg/ml. The critical concentration of NF-L, the concentration that remains unassembled at equilibrium with fully formed filament under the conditions used for the assembly assay, was obtained from this data. After complete assembly, the percent fluorescence quench was measured and the extent of assembly at each concentration was calculated (Fig. 5 A). We assumed that assembly was complete at 1.2 mg/ml, as determined by sedimentation and EM, and the concentration of NF-L that remained unassembled was calculated at each NF-L concentration. The concentration of unassembled NF-L was $38 \mu\text{g}/\text{ml}$ or $0.6 \mu\text{M}$ at all concentrations of NF-L we examined (Fig. 5 B).

The assembly can be also characterized by its high temper-

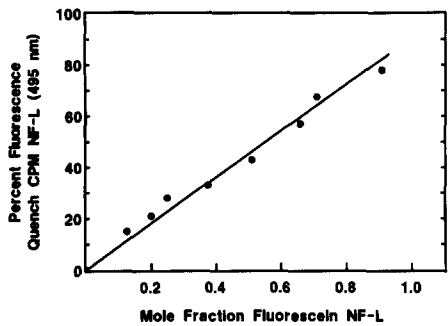


Figure 3. Competition for assembly of fluorescent NF-L with unlabeled NF-L. The energy transfer efficiency between CPM-labeled NF-L (donor) and FM-labeled NF-L (acceptor) was determined as a function of the mole fraction of acceptor-labeled NF-L in NF-L filaments. Mixtures of CPM-labeled, FM-labeled, and unlabeled NF-L were coassembled. All samples contained a constant amount of CPM-labeled NF-L (0.15 mg/ml) and a mixture (0.9 mg/ml) of FM-labeled and unlabeled NF-L containing increasing proportions of FM-labeled NF-L. The percent quench was calculated from the final fluorescence after a 3-h assembly from eq. 1.

ature and ionic strength dependencies. For example, at 1.08 mg/ml at 37°C, the observed rate for assembly is 0.69 min⁻¹; at 30°C, under the same conditions, the rate is 0.18 min⁻¹; and at 22°C the rate is ≥ 0.03 min⁻¹. The calculated energy of activation from an Arrhenius plot is 30 kcal/mol, and the plot is linear over the temperature range 4°C to 37°C, indicating no change in the rate-limiting step.

At pHs lower than 6.0 or at concentrations of NaCl lower than 30 mM, assembly cannot be detected. Increasing the NaCl concentration to >100 mM yields an immediate increase in the fluorescence quenching indicative of polymerization.

On the other hand, assembly of NF-L subunits into filaments does not require nucleotides or NF accessory proteins. Addition of 1 mM ATP, AMPNP, GTP, or GMPPNP neither alters the kinetics nor the extent of assembly. When either NF-H or NF-M are added to assembly mixtures at ratios equal to or exceeding those found in vivo (4:2:1 H/M/L) (32), no change in the rate or extent of NF-L filament assembly are observed.

Intermediate Filament Subunit Exchange

One of the more important properties for these derivatives is whether labeled molecules compete and exchange into unlabeled filaments. Such a situation would be encountered, for example, in an experiment where labeled NF-L is microinjected into a cell that contains an endogenous pool of NF-L. In addition, such experiments are important in providing information on whether under conditions presumably appropriate to the cytoplasm NF-L subunits could be in an exchange equilibrium with filaments. Therefore, we examined the exchange of NF-L subunits using two independent methods. If subunit exchange occurs between filaments, then mixing CPM-FM-labeled copolymer filaments with unlabeled filaments or separately prepared CPM-labeled NF-Ls and FM-labeled filaments should result in a redistribution of the labeled protein. In the former an increase in the CPM fluorescence (or decrease in fluorescence quench) would occur,

while in the latter a decrease in CPM fluorescence (or increase in fluorescence quench) would occur as FM-labeled acceptor subunits exchange with CPM-labeled subunits.

When CPM-FM filaments are rapidly mixed with unlabeled filaments, a time-dependent increase in fluorescence intensity or decrease in fluorescence quench, corresponding to the redistribution of donor- and acceptor-labeled NF-L subunits in the filament, is observed (Fig. 6). These observations are consistent with subunit exchange between labeled and unlabeled filaments. As with filament assembly, the extent of exchange is dependent on the protein concentration. When the total protein concentration is lowered, the extent of exchange increases (Figs. 6 and 7). At protein concentrations >0.2 mg/ml, <20% exchange is observed. Extrapolation of the curve in Fig. 7 to the x-axis yields an apparent critical concentration of exchange of 40 μ g/ml, below which the subunit/filament ratio increases dramatically. The results are similar to those found for subunit exchange between actin filaments (26). At subcritical concentrations (e.g., 24 μ g/ml), subunit exchange is no longer between subunits and filaments but most likely between subunits and small oligomers. Sedimentation and cross-linking studies (our unpublished observations) as well as published studies of vimentin (34) suggest that the predominant species at subcritical concentrations is a tetrameric form of NF-L or vimentin.

We also observed that the rate of subunit exchange was highly temperature dependent. At 37°C the observed rate of exchange is 1.38 min⁻¹, while at 30°C, under the same conditions, the rate is 0.23 min⁻¹. At 22°C, the half-time for filament exchange is ≥ 200 min; analysis by an Arrhenius plot yields an energy of activation of 36 kcal/mol.

The kinetics and the extent of NF-L exchange is also independent of the two other NF complex components, NF-H or NF-M. When NF-M and NF-H are added to preformed filaments at ratios of 4:1 (NF-H/NF-L) and 2:1 (NF-M/NF-L), which is the stoichiometry found in the isolated NF complex (32), or even at 10-fold excess, neither the kinetics nor the extent of NF-L subunit exchange are affected. To determine whether specific buffer conditions, nucleotide binding, or coupling to nucleotide hydrolysis were required, NF-L exchange was measured under several different buffer conditions and in the presence of effectors. Nucleotides such as ATP, GTP, AMP-PNP, GMP-PNP, cAMP, or cGMP did not affect the extent of subunit exchange. Exchange of NF-L in buffers containing *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (pH 7.2), imidazole (pH 7.5), or Pipes (pH 7.0) also did not affect NF-L subunit exchange. However, the extent and kinetics of exchange decreased threefold at pH 6.5.

Experiments where subunit exchange was monitored at two wavelengths (e.g., between CPM-labeled filaments and FM-labeled filaments) provided additional insights into the mechanism of subunit exchange. When subunit exchange occurs between the CPM- and FM-labeled filaments, the fluorescence emission from FM-labeled filaments increases (or decreased fluorescence quench) upon subunit dissociation (Fig. 8, ●) while the CPM fluorescence is quenched as fluorescein acceptor subunits incorporate into coumarin filaments (Fig. 8, ○). The increased quench of CPM-labeled filaments in the mixed polymer experiment in Fig. 8 must be due to exchange, thus extending and confirming the subunit exchange results of the copolymer experiments shown in

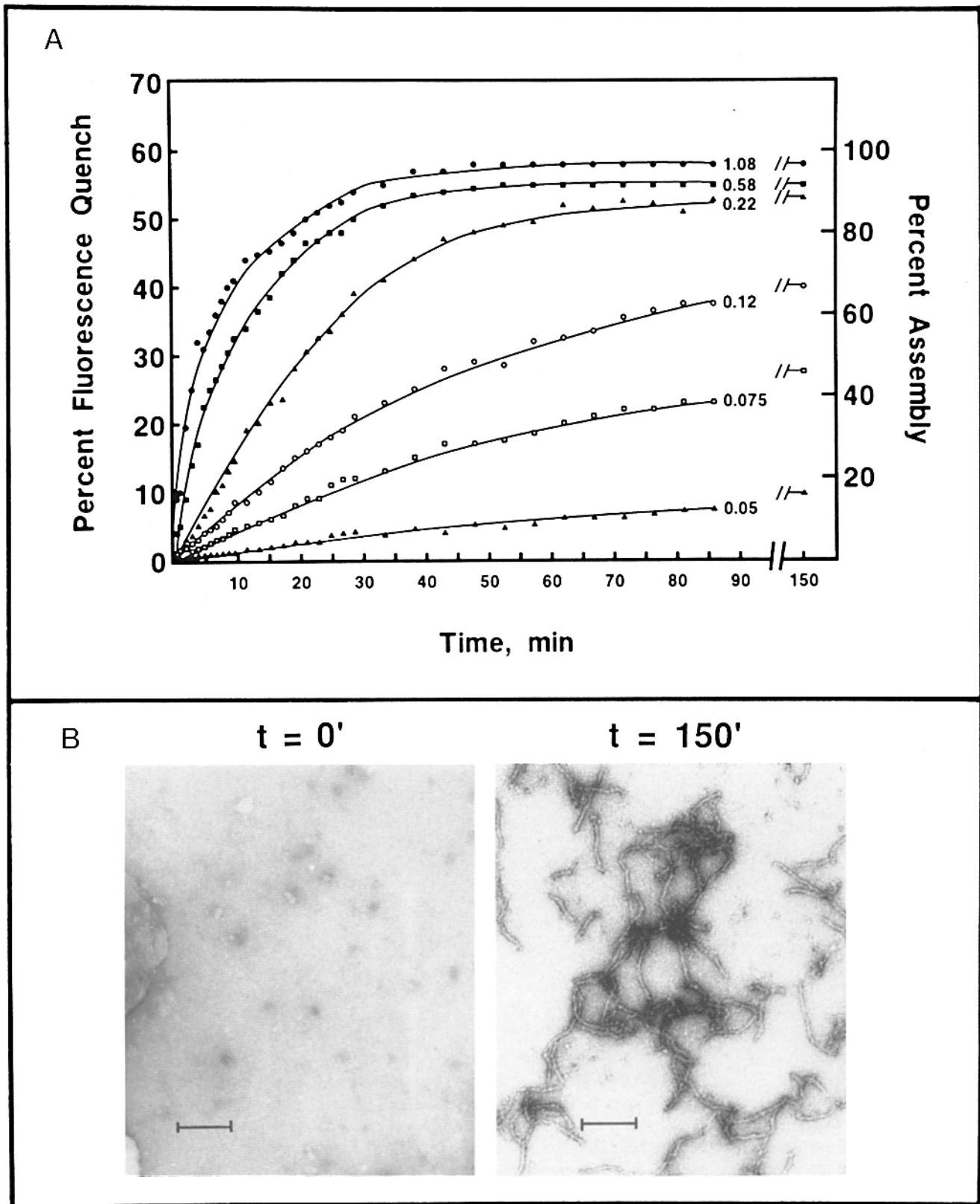


Figure 4. Assembly of NF-L filaments by fluorescence energy transfer. **(A)** CPM-labeled NF-L was added to FM-labeled NF-L at a final ratio of 1:2 in assembly buffer at the protein concentrations indicated. The assembly was initiated by the addition of NaCl to a final concentration of 170 mM NaCl at 37°C. The assembly was monitored by the increase in fluorescence quench at 495 nm. When no further changes in fluorescence quench were observed, a concentrated aliquot of SDS was added to a final concentration of 1%, and the fluorescence was redetermined. In some samples, the assembly was determined to be complete by centrifugation at 100,000 g for 60 min and by EM. **(B)** Morphology of assembled NF-L filaments by negative stain EM. At the end of the assembly period, when no further changes in fluorescence occurred, aliquots were withdrawn and examined in the electron microscope. Times are indicated on the panels. Bars, 180 nm.

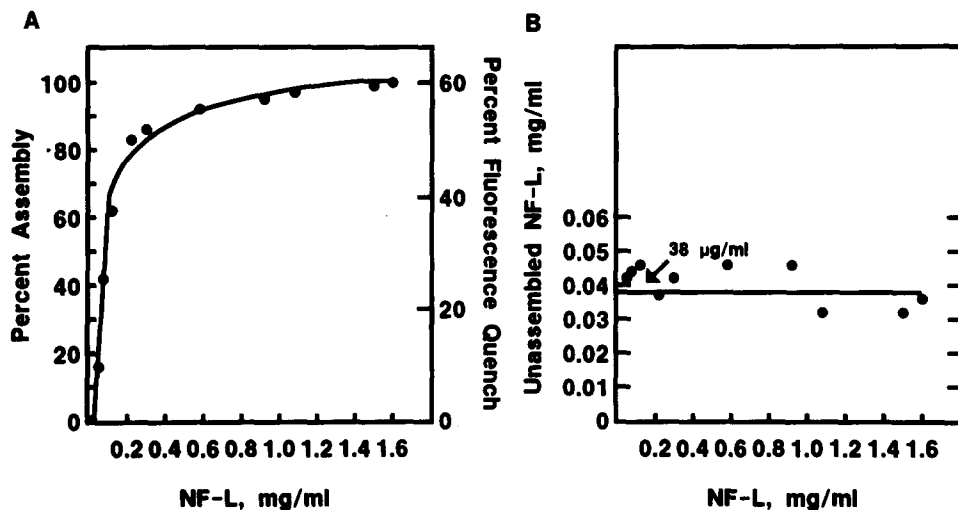


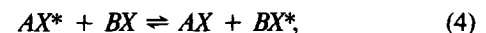
Figure 5. Critical concentration of NF-L assembly. (A) The extent of assembly determined by energy transfer at different NF-L concentrations. CPM- and FM-labeled NF-L were combined in assembly buffer at 37°C at a 1:2 ratio at the total NF-L protein concentrations indicated and assembly was monitored by the increase in donor fluorescence quench after addition of NaCl to 170 mM. When no further increase in quench was observed, the percent assembly was calculated as in eq. 1. (B) Calculation of the critical concentration for NF-L assembly. The amount of NF-L that remained unassembled at each concentration was calculated from the data in A, which assumed that assembly was complete at 1.2 mg/ml.

Figs. 6 and 7. In Fig. 8, the fluorescence quench at equilibrium for CPM reaches 24%, or 32% subunit exchange, while for FM-labeled filaments the change in percent fluorescence quench reaches 30% at equilibrium or a disassembly of 34%. In control experiments, we confirmed that disassembly due to dilution accounted for very little of the fluorescein signal change (<10%). The observed rate of CPM fluorescence quenching is also more rapid than the relief of FM quenching (k_{obs} CPM donor quenching = 2.3 min^{-1} ; k_{obs} relief FM self-quenching = 0.52 min^{-1}) (Fig. 8). These results suggest that the slow step of the exchange reaction is dissociation to soluble subunits that, once generated, are incorporated rapidly into filaments, and that subunit dissociation probably occurs before insertion.

To test this possibility further, we examined two potential kinetic mechanisms for exchange, one which included a prior disassembly step of subunits, and the other where

subunit exchange would occur through the formation of a bimolecular or activated complex (interfilament-interfilament) formed between two filaments. For exchange, at equilibrium the net rate of reaction is zero. These exchange experiments are experiments carried out near equilibrium. All reactions near equilibrium become first order with respect to any variable that indicates the distance from equilibrium. The gross rate of reaction, that is the forward or reverse rate at equilibrium, can be obtained by tracers, either fluorescent or isotopic.

If we consider the exchange where the fluorescent subunits exchange with unlabeled subunits, the overall reaction scheme is



where X^* represents an unlabeled subunit of NF-L filament A, that can exchange with a fluorescent subunit of NF-L fila-

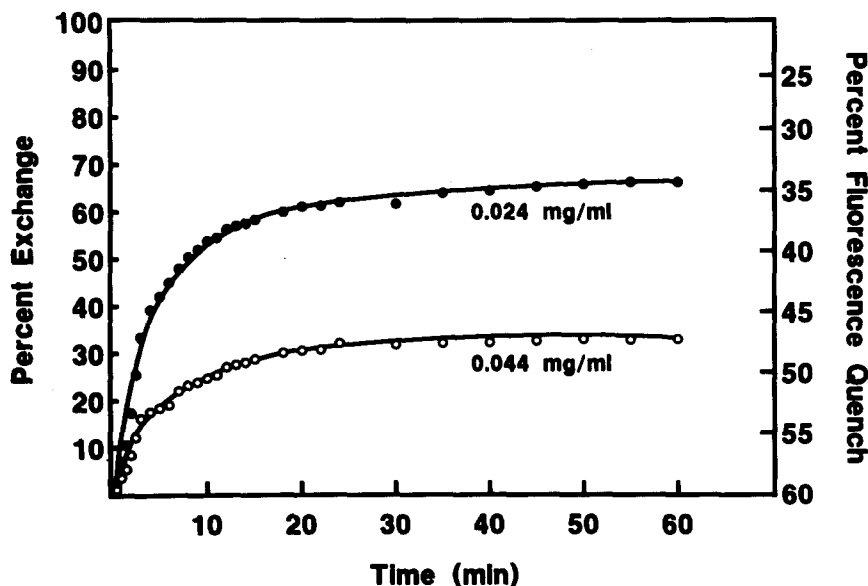


Figure 6. NF-L subunit exchange. Exchange of CPM-FM copolymer NF-L filaments with unlabeled filaments at total protein concentrations of 0.024 (●) or 0.044 mg/ml (○). CPM-FM NF-L filaments were mixed with unlabeled filaments at a ratio of 1:2, and the fluorescence was monitored at 495 nm with 410-nm excitation. After 60 min, 1% SDS was added to the cuvette and the fluorescence endpoint, F^* , was determined. The axes show both the percent subunit exchange and percent fluorescence quench.

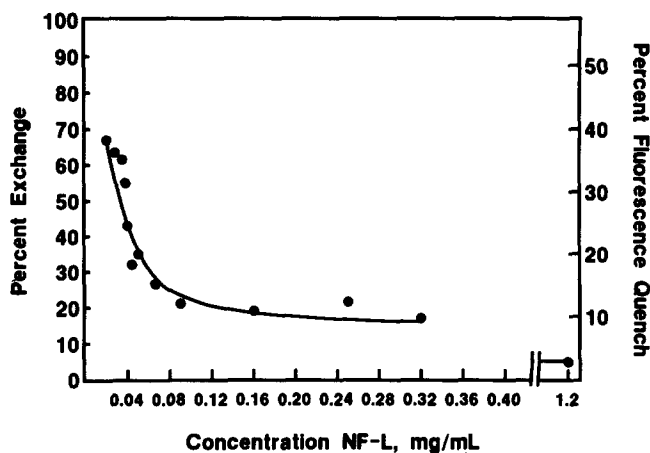
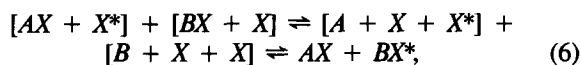


Figure 7. Critical concentration for NF-L subunit exchange. CPM-FM NF-L filament were exchanged at 37°C with unlabeled NF-L filaments at total protein concentrations between 0.012 and 1.2 mg/ml. The percent exchange was determined as in eq. 2.

ment *B*. The exchange could proceed at least through two different mechanisms: exchange by association and exchange by dissociation.



where eq. 5 is a simple bimolecular mechanism with (AX^* , BX) representative of an activated complex. The second scheme (eq. 6) is an exchange that results from an active dissociation of subunits, X , from filaments A and B , and a reassociation of unlabeled subunit, X^* , with fluorescently labeled B filaments.

The rate expression for the bimolecular and activated complex is (reference 7)

$$-\ln(1 - F) = k_1 ab (a + b) t, \quad (7)$$

and for the dissociation and reassociation

$$-\ln(1 - F) = k_1 \cdot \frac{a + b}{ab} t, \quad (8)$$

where F is the fraction of exchange that has occurred and a and b are the concentrations of tracer and unlabeled species, respectively.

If we use these two rate expressions by simply examining the dependence of the rate on the concentration of each species, we can arrive at a mechanism for subunit exchange. Eq. 7 would predict a strong dependence on the rate of exchange with changes in the concentration of unlabeled species, while eq. 8 predicts that the rate of exchange would be largely independent of the ratio of the tracer to unlabeled species, but would increase as the ratio approached 1:1.

Experimentally, we have found that the rate of exchange is invariant over the ratio of 100:1 to 10:1 of unlabeled to tracer and over the protein concentration range of 0.05–0.74 mg/ml (Fig. 9). However, when the ratio of unlabeled to labeled filaments approaches 1:1, the rate of exchange increases significantly (Fig. 9). These results are consistent with the kinetic predictions of eq. 8 for a mechanism where exchange is preceded by dissociation of subunits followed by

incorporation of these dissociated subunits into filaments. These results also explain the rather puzzling observation of a lack of protein concentration dependence on the rate of subunit exchange.

The kinetic analysis, the direct observation of exchange, and the concentration dependence of the rates are consistent with a mechanism in which subunits first dissociate from the core and then incorporate into filaments.

Discussion

Fluorescent and biotinylated derivatives of tubulin and actin have provided important tools to study both the *in vitro* and *in vivo* mechanisms of self-assembly and interactions of these proteins. We have prepared and characterized assembly-competent fluorescent and biotinylated derivatives of NFs as probes for the study of IF dynamics *in vitro* and *in vivo*. To the best of our knowledge, this is the first report of such IF derivatives and their use in assembly studies. NF-L was selected because this protein displays the general structural features of all IF proteins (8), it forms the structural core of the NF complex, interacts with the two higher molecular mass NF polypeptides, and, in nerve cell development, it is the first to appear among the NFs.

Chemical and Molecular Properties of Derivatized NF Proteins

We have shown that NF-L can be selectively and stoichiometrically modified at cysteine 321. The modification does not perturb the structural integrity or the kinetics or extent of NF-L assembly or exchange. On the other hand, when we modified lysines by acylation the ability of NFs to assemble or exchange was abolished. The sulfhydryl-labeled NF-L protein forms filaments that are indistinguishable from unlabeled filaments with similar diameters and lengths and com-

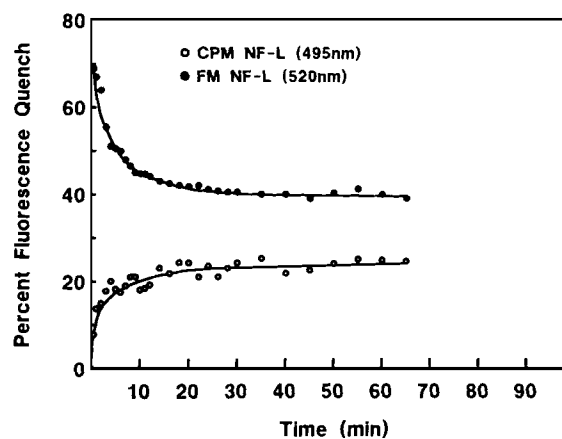


Figure 8. Kinetics of subunit dissociation (●) and subunit incorporation (○). FM-labeled NF-L filaments were mixed 1:2 with CPM-labeled NF-L filaments at a total protein concentration of 0.05 mg/ml and the quenching of CPM fluorescence was monitored simultaneously with the increase in FM fluorescence. Quenching of CPM fluorescence (○) with 410-nm excitation and emission at 495 nm and relief of fluorescein self-quenching with 495-nm excitation and 520-nm emission (●). There was no contribution of sensitized emission of the acceptor (FM-labeled NF-L) because excitation was at 495 nm.

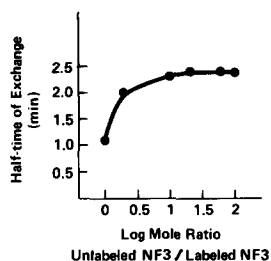


Figure 9. Dependence of exchange rate on unlabeled/labelled mole ratio.

petes quantitatively with unlabeled protein in co-assembly experiments. Proteins labeled at ratios between 0.1 and 1.0 show the same kinetics and extent of assembly and exchange indicating that assembly and exchange are independent of the labeling ratio.

Dynamics of NF Assembly and Exchange

The significant changes observed in the fluorescence quench indicate NF-L subunits can exchange extensively among filaments. The phenomenon cannot be explained if subunit exchange (under these conditions) is limited only to filament ends. On the other hand, subunit exchange could arise from extensive breakage-reannealing coupled to end-on exchange, dynamic instability (20), or an equilibrium exchange between soluble kinetically active subunits and the filament.

Under certain conditions, both microfilaments and microtubules have been shown to anneal *in vitro* from preformed filaments (22, 29, 40). The experimental data with IFs, however, are inconsistent with breakage and reannealing of filaments because in mixed polymer experiments, the magnitude of the changes in donor (CPM) fluorescence quench and relief of FM fluorescence quench are comparable. First, with breaking and reannealing the unequal distribution of exchanged subunits would lead to significant differences between the percent fluorescence quench of the donor and the relief of fluorescence quench of FM-labeled subunits particularly in the early phases of the mixed polymer experiment (28). As broken fragments reanneal, a large increase in fluorescence quench would be observed when CPM-labeled subunits with no neighboring acceptors become associated with acceptor fluorophores (FM-labeled subunits) during the early phases of the mixed polymer experiment. Secondly, the kinetic analysis indicates that the subunits dissociate before incorporation, and the rate of exchange is limited by subunit dissociation.

The results also argue against the possibility of treadmill-type mechanisms because at steady state the rate of incorporation should equal the rate of dissociation, where each subunit should undergo a complete replacement of neighboring subunits as it becomes dissociated from the original filament and reassociated with a new filament. Accordingly, the rate of change in fluorescence quench in the copolymer experiment should match that in the mixed polymer experiment.

In addition, in preliminary experiments at the single filament level, we have found that during exchange colloidal gold-decorated biotinylated subunits uniformly distribute within filaments, with no corresponding change in filament length during the exchange period. Although further experiments at the EM level are clearly needed, both the EM ex-

periments and the kinetic analyses do not support a mechanism where filaments are rapidly breaking and reannealing.

For dynamic instability, an energy source is required. The catastrophic depolymerization of microtubules, for example, is catalyzed by the hydrolysis of GTP bound to tubulin subunits at the ends of microtubules (20). Although *in vivo*, an energy source for IF assembly and exchange could be derived from coupling to other catalytic processes, no energy source is needed for IF assembly or exchange *in vitro*. Furthermore, although dynamic instability could account for the location of gold-decorated biotinylated subunits at internal locations of the filament at steady-state exchange, this mechanism would not account for the maintenance of the constant filament length during the exchange period.

The mechanism that is most consistent with the results is that subunit exchange occurs between soluble kinetically active NF-L subunits and the filament. Both experimental and kinetic analysis indicate that subunits dissociate before incorporation and that the rate of exchange is limited by subunit dissociation.

The critical concentration suggests the existence of a dynamic equilibrium between a small but constant pool of unassembled NF-L molecules and subunits within the NF-L filament. This critical concentration for both NF-L assembly and exchange is 40 $\mu\text{g/ml}$. Although the concept of a critical concentration for NF assembly and exchange should seem reasonable, this is the first report of a NF critical concentration. In comparison, at 40 $\mu\text{g/ml}$ or 0.6 μM , the critical concentration for NF-L is lower than the critical concentration of 0.3 mg/ml for ADP-actin filaments at infinite number of filaments (25) or 1.1 mg/ml for microtubule polymerization (14), but higher than the value of 17 $\mu\text{g/ml}$ or 40 nM reported for the polymerization of myosin into thick filaments (29) or ATP-actin (28). At NF-L concentrations estimated in axons (0.5 mg/ml) (21), $\sim 10\%$ of the total NF-L protein would be found in the soluble pool. However, there are several ways in which the concentration of this soluble pool could be altered. For example, the concentration of this soluble pool could be increased through regulatory modifications or local ionic changes. Recent evidence suggests that phosphorylation/dephosphorylation could regulate this pool (12, 13).

Equilibrium polymers, such as MT and MF, respond to changes in the concentration of monomers and decrease in length when the monomer concentration declines. MT or MF polymer length is regulated by GTP or ATP hydrolysis, respectively (16, 22, 33). Without a direct energy source how can NF length distribution (or exchange) be regulated and maintained? It is possible that the assembly or disassembly reactions could be blocked at both ends; if the ends are blocked, the NF-L polymer would be viewed as stable and would not disassemble from the ends when the monomer concentration is reduced. This type of stable polymer would differ markedly from polymers such as MT and MF that have active dissociation reactions at one or both ends. It is known that *in vitro* and *in vivo* NFs are among the most heavily phosphorylated proteins known and can be phosphorylated by cAMP-dependent and effector-independent protein kinase (41). If NFs ends are capped by phosphorylation, analogous to the GTP or ATP caps found on MTs and MFs, respectively, one potential mechanism to regulate the dissociation reaction at NF-L ends is by cycles of dephosphorylation and phosphorylation. Recent experiments with desmin and vimentin

filaments have shown that phosphorylation by cAMP-dependent protein kinase or kinase C, respectively, inhibit IF assembly or induce disassembly of preformed filaments (12, 13). We have found that, like desmin and vimentin, phosphorylated forms of NF-L cannot assemble. However, in contrast to desmin and vimentin, phosphorylation of NF-L filaments appears to stabilize NF-L filaments and to inhibit both the rate of disassembly and subunit exchange. For example, phosphorylated NF-L filaments will disassemble and exchange only at much lower protein concentrations (0.004 mg/ml), and the half-time for subunit exchange between phosphorylated filaments is 80 min (compared to ~ 2 min for dephosphorylated subunits). A provocative possibility is that dephosphorylation could regulate subunit exchange by destabilization of the filament, subunit dissociation, and the creation of a transient soluble pool of dephosphorylated subunits, that are assembly competent, followed by stabilization of the newly exchanged subunits by phosphorylation. Alternatively, because dephosphorylated subunits can assemble, the dephosphorylated subunits generated could be recycled and polymerize off of filament ends. This hypothesis raises interesting new questions for the maintenance and assembly of the NF complex in neurons, and the ability of NFs to reorganize at peripheral sites.

Although the speculation is attractive, is there any evidence to show that in cells NFs or IFs are dynamic structures? Unfortunately, early studies in squid axon using Triton extractability suggested that NF proteins were stably assembled into NFs and there was little, if any, diffusible monomer, and led to the concept that NF proteins exemplified proteins that were stably associated with a transported structure. However, while this may be true for squid axons where NFs must provide a maintained stability for the axon, in mammalian axons, NFs must also be capable of change because these axons exhibit marked plasticity. For example, in long axons, after injury, new polymers in a regenerating sprout are formed from existing pools of cytoskeletal proteins that are present in the axon. Reorganization of the axonal cytoskeleton does not require the participation of newly synthesized proteins from the cell body. In addition, the ability of neurites to rearrange their cytoskeletal matrix in response to environmental cues such as trophic factors requires that the NF structure can be altered *in vivo* (2). Our analyses indicate that NF-L polymerization and exchange can occur under physiological conditions present in the axon, and that with a critical concentration of 40 $\mu\text{g/ml}$, and the possibility of regulating this pool, subunits could be drawn from free monomer pools and used for NF-L polymerization during regeneration. In other cells, there is precedence that a dynamic pool of IFs does exist, and that the regulation of the monomer/polymer equilibrium are set to provide a balance between the requirements for stability and plasticity (4, 5). For example, a soluble pool of tetrameric vimentin subunits is found in cultured cells (34), a pool that can be regulated by phosphorylation (12, 13), and substantial changes in the fibroblast cell IF network occurs during cell movement (4).

One pivotal role for subunit exchange would be in the rapid reorganization of NF-L filaments during early axonal growth, degeneration, or turnover in mature axons. Classically, the role of NFs have been classified as stable structural polymers that maintain the structural pattern within the cytoskeleton as it slowly is transported together with MTs

and MFs from the cell body to the synaptic terminal (17, 21). Recent experimental evidence has challenged this traditional view of transport of the entire cytoskeletal matrix and supports a role for a more dynamic behavior of NF subunits (24). When axons are pulse-labeled, labeled NF proteins do not proceed as a continuous wave down the axon as would be predicted for a transported matrix. Both a spreading of the moving wave and a nonuniform distribution of deposited NF proteins along the axon is observed, and is consistent with an interpretation that turnover at different sites along the axon arises from an exchange between NF proteins and the existing NF lattice. A reservoir of subunits could contribute to the remarkable ability of the axon to undergo morphological changes at any point along its length without direct participation of protein synthesis. Thus, exchange of subunits and any regulation of exchange could play an important role in axonal growth and regeneration.

The preparation of fluorescent and biotinylated derivatives of NF-L and the use of fluorescence and ultrastructural methods now provide convenient and continuous recording assays to examine NF assembly and transport. The fluorescent derivatives fulfill the criteria as molecular cytochemical analogues by retaining the properties of the native proteins in addition to possessing high quantum efficiencies, long wavelength spectra, and chromophore stability. Although the distances between labeled proteins in a cell microinjected with fluorescent NF-L subunits would be too great to take advantage of resonance energy transfer methods to study the assembly, fluorescence photobleach recovery in combination with digital fluorescence microscopy should be able to provide a means where the integration, dynamics, and interactions of the NF proteins can be examined. Using these techniques, the fluorescent and biotinylated NF-L proteins will serve as tracers in studies of the neuronal cytoskeleton in living neurons during axonal growth, degeneration, and regeneration. Finally, our results showing that NF-L subunits assemble and exchange and are dynamic have broader implications for the organization and dynamics of IFs in other cells.

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