Structure of neurofilaments studied with scanning tunneling microscopy

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Abstract Neurofilaments (NFs) were isolated from bovine spinal cord. The structure of purified NFs was studied by scanning tunneling microscopy (STM). The STM images showed that NF was composed of a long core filament and numerous sidearms flanking the rod regularly. The diameter of the rod was about 10 nm (10.2 ± 0.8 nm). Most of the sidearms were short and the distance between two adjacent sidearms was approximately 10 nm. There were some long sidearms between two proximal core filaments. The distance between two adjacent long sidearms was 21 nm. A three-quarter-staggered fashion of native NF structure was put forward.

Keywords: neurofilament, intermediate filament, scanning tunneling microscopy.

NEUROFILAMENTS (NFs) are intermediate filaments (IFs) present in most populations of neurons in the nervous system. NFs are typical intermediate-sized filaments 10 nm in diameter. In the axons, NFs are parallel. The space between adjacent NFs varies from 20 to 50 nm in width. Crosslinking thin filaments (4—6 nm in diameter) connect adjacent NFs[1]. They are composed of three kinds of proteins: NF-L (apparent M.W. 68 ku), NF-M (apparent M.W. 160 ku) and NF-H (apparent M.W. 200 ku), which are called NF triplet proteins (NFTPs)[2]. Like other members of intermediate-filament family, NFTPs all have α-helix-rich rod domain of approximately 310 amino acids, as well as amino-terminal head domain and carboxy-terminal tail domain. NFs present a test-tube-brush-like structure with 2—4 projections extending from the core filaments 22 nm apart[3]. NF-L is the most abundant subunit of native NFs, which forms core filament with the coassembling of NF-M and NF-H. The tail domains of NF-M and NF-H project from the core filaments to form sidearms, just like the crosslinking bridges *in vivo*[3]. *In vitro* NF-L can reconstitute the filaments by itself, which appear relatively featureless without the sidearms protrusions. NF-M and NF-H cannot reassemble into IF structure on their own.

In this note, STM was used to study the structure of native NFs. STM images of NFs with relatively high resolution were obtained for the first time, which helped to reveal the structure of NFs.

1 Materials and methods

(ⅰ) Purification of NFs. NFs were prepared from bovine spinal cord according to the method of Hisanaga and Hirokawa[3]. Bovine spinal cord was obtained from Dahongmen slaughterhouse and transferred on ice. After meninges were removed, the spinal cord was homogenized in an equal volume of PEM buffer (100 mmol/L pipes, 1 mmol/L EGTA, 1 mmol/L $MgCl_2$, 5 μg/mL Leupeptin and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), pH 6.8). Five full-speed 5-s bursts were given at 0—4℃. The homogenate was then centrifuged at 28 000 × g for 50 min at 2℃. The supernatant was isolated and mixed with 20% Glycerin (v/v), then incubated at 37℃ for 30 min. After the mixture was centrifuged at 150 000 × g for 90 min at room temperature, the precipitate was resuspended in PEM buffer at a protein concentration of about 1.0 mg/mL. It was the crude NFs.

The isolated NFs were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was stained with Coomassie brilliant blue R-250.

(ⅱ) Observation of NFs by scanning tunneling microscopy. The sample was diluted to adjust the protein concentration to about 100 μg/mL with distilled water. 50 μL of the diluted solution was sprayed onto the surface of newly cleaved highly oriented pyrolytic graphite (HOPG). The sample was kept stationary for 5 min while the molecules were allowed to adsorb freely. Then the excess liquid was removed by rotating, and at the same time the molecules could distribute on the substrate evenly. The sample was dried in air at room temperature.

STM experiments were carried out in ambient environment with a domestic STM setup CSPM-930a (manufactured by Institute of Chemistry, Chinese Academy of Sciences). STM measurement was performed at the constant current mode, using tungsten tips made by electrochemical etching. Tunneling current and bias voltage were indicated in the relevant legends of photographs. All STM images presented here were raw data images without any smoothing or filtering. Measurement of the distances was carried out with the image processor. The contrast of the images was increased somewhat photographically.

2 Results

NFs were isolated through a series of purification. When the proteins ran on 7.5% gel in the SDS-PAGE, three main bands were detected by staining with Coomassie brilliant blue R-250. Their molecular weights were 68, 160 and 200 ku, which indicated NF-L, NF-M and NF-H respectively. As typical structure of IF in the electron microscope, NFs presented a convoluted ribbon-like structure after negative staining. There were regular globular structures studded on NFs. It was difficult to resolve more detailed structures.

The STM image in fig. 1 shows a single NF polymer, whose diameter was about 10 nm. There were many protrusions at 10-nm intervals along the core filament. In fig. 2, 3 long fila-

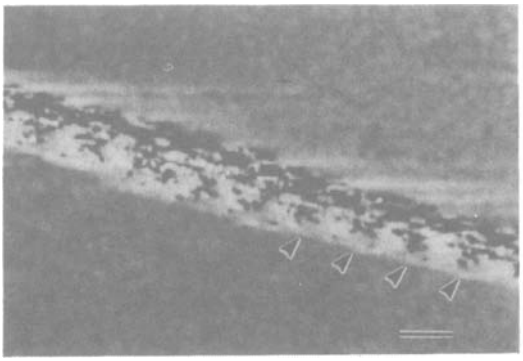

Fig. 1. STM image of purified native NFs. I_{ref} = −0.50 nA; V_{bias} = 192 mV; bar, 10 nm. The arrows indicate the totally shrunk sidearms.

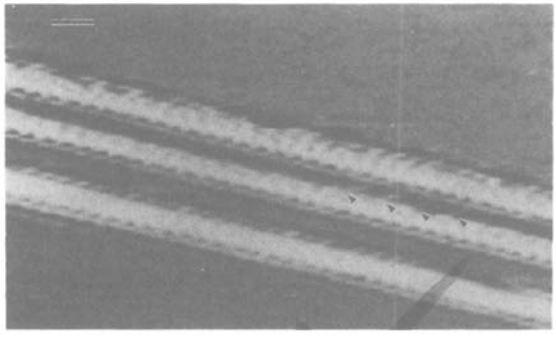

Fig. 2. STM image of purified native NFs. I_{ref} = −0.10 nA; V_{bias} = 479 mV; bar, 20 nm. The arrows indicate the long sidearms.

ments with arm-like projections paralleling each other could be seen. Their lengths were far from the scanning scale. Their lateral diameters were about 10 nm (10.2 ± 0.8 nm), which coincided with the size of NFs obtained from other experiments. The heights of the filaments were also 10 nm or so, indicating the cylindrical surface. Each filament had a core filament with numerous "sidearms" flanking the rod along their entire lengths and it was inferred that such structures were NFs from their sizes and their shapes. In fig. 1, the protrusions might be the totally shrunk sidearms.

The distance between two adjacent sidearms was about 10—11 nm, equal to the diameter. In fig. 2, the lengths of sidearms varied. Most sidearms were short, but some sidearms between the two proximal filaments were longer. The space between each two adjacent longer sidearms was approximately 21 nm, which coincided with the regular distribution of projections observed by a rotary shadowing electron microscope. It was difficult to determine the lengths of the sidearms because they had shrunk under the dry condition. The reason why the longer ones were visible in some regions might be the interaction between the two adjacent core filaments. That is to say, the ends of longer sidearms might stick on the other filament so as to prevent it from shrinking. Such sidearms were just like the cross-bridges between NFs in vivo. Moreover, the longer ones and the shorter ones might be formed from different polypeptides, which could be identified by their lengths. According to the previous studies, these projections should represent the peripheral tail domain of either NF-M or NF-H or both. But it was impossible to explain 10-nm distance between two sidearms according to the existing half-staggered model.

3 Discussion

How the NFTPs assemble into NFs has not been known clearly by now. According to the previous researches, NF-L is able to form the core filament on its own. NF-M and NF-H are only able to assemble into 10 nm-wide filaments along with NF-L. The carboxy-terminal tail domains of NF-M and NF-H mainly contribute to the peripheral projection of NFs[3,4]. There are some pro-

jections 22 nm apart along the native NFs or filaments assembled by NF-L and NF-M/NF-H. The axial interval of about 20 nm also exists in the filaments assembled by NF-L alone. On the basis of the 22 nm-periodicity and the results from NF-L assembly studies, some molecular models for NFs architecture have been proposed[5, 6]. According to these models, the first step of the architecture is the formation of dimer. The dimer is formed by a parallel, unstaggered two-stranded α-helical coiled-coil with a molecular length of about 47 nm. Two dimers form a tetramer. Then two tetramers form an octamer, and the octamers form the intermediate-sized filaments. Whether these subunits align in parallel or antiparallel and whether they align in register or in half-staggered fashion are different in different models. In spite of the difference among different models, all existing models are half-staggered models. That is to say, the α-helix coiled-coil rod domains align in half-staggered fashion within the intermediate-sized filaments, which explains the 22 nm-axial-periodicity of the filaments composed of NF-L and periodicity of sidearms of native NFs. The models above mainly result from the studies of the assembly of NF-L. It has not been determined whether the architecture of NF-L is the same as that of native NFs, because native NFs are composed of NF-L, NF-M and NF-H.

According to our STM observation, two kinds of sidearms exist along the core filaments. The interval between two adjacent longer sidearms is about 21 nm, which is the same as that observed by metal-shadowing. However, the space between two adjacent shorter sidearms is only 10—11 nm. Considering that the longer sidearms may shrink onto the surface of the core filaments in the course of air drying, some shorter sidearms are formed from the longer ones. Because the space between two adjacent longer sidearms is 21 nm, it is reasonable to predict that the longer sidearms and the shorter ones align along the core filaments alternately. The longer sidearms are composed of the carboxy-terminal tail domains of NF-H and NF-M, while the shorter ones might be composed of those of NF-L[4]. The carboxy-terminal tail domain of NF-L is very short (142 amino acids) and lowly phosphorylated. So it is too short to be detected by metal-shadowing. Only the long sidearms composed of the tail domains of NF-M and NF-H are long enough to be detected. The former half-staggered models cannot explain the 10-nm axial periodicity of the sidearms. Some NFTPs rod domains must align in 3/4(three-quarter)-staggered fashion, which produces 10-nm periodicity.

On the basis of our STM images, we propose a new model of architecture of NFs (fig. 3). The first step is the formation of dimer. As we have known, there are only three kinds of

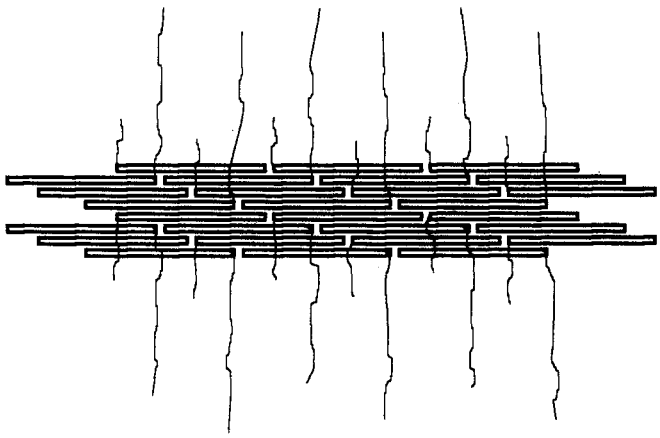

Fig. 3. Schematic representation of the construction of native NFs in the three-quarter-staggered mode. The curves represent the sidearms from tetramers, which are indicated by open bars.

dimers: NF-L/NF-L, NF-L/NF-M and NF-L/NF-H. NF-M and NF-H cannot form dimers on their own[4, 7]. Then two same dimers align in register and parallel to form a tetramer[5]. The third step is that the octamer is formed by two different tetramers aligning in 3/4-staggered and antiparallel fashion. The octamers link up to form protofibril. At last, four protofibrils bind laterally to produce NFs. For sake of brevity, a curve is used in fig. 3 to represent the sidearms from one tetramer. The short curves indicate the sidearms from tetramers formed by NF-L alone while the long ones indicate the sidearms from tetramers formed by NF-L and NF-M/NF-H together. Further work is needed to explain how the four tail domains of a tetramer are arranged.

In this model, the longer sidearms formed by NF-H and NF-M and the shorter sidearms formed by NF-L are arranged alternately along the surface of NFs. The distance between two longer sidearms or two shorter sidearms is about 22 nm. In this model, 3/4-staggering takes place between two different tetramers. In other words, it must take place between tetramers composed of NF-L alone and tetramers composed of NF-L and NF-M or NF-H. There is no 3/4-staggering between the same tetramers. So the molecular model for NF-L assembly is half-staggered fashion, which causes 22 nm periodicity.

According to our model, the stoichiometry of NF triplet proteins in the native NFs will be that the ratio of NF-L : (NF-M + NF-H) is 3 : 1. We estimated the relative amount of each NF subunit from the density of Coomassie brilliant blue-stained bands. The ratio of NF-L : (NF-M + NF-H) is 2.5 : 1, which approaches the ratio of 3 : 1.

STM is a powerful tool to study the surface structure. In our work, many new details of NFs have been revealed by STM, which give us new information of the architecture of NFs. It helps us to reveal the arrangement of the three NF subunits. Further studies are being done.

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