



Monitoring fibrillation of the pathogenic huntingtin protein using NMR

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Abstract Huntington's disease (HD) is an inherited neurodegenerative disease caused by abnormal polyglutamine (polyQ) expansion in the huntingtin protein (Htt). There is no cure for HD so far. Although exact molecular mechanism of HD pathogenesis is still elusive, fibril formation of the expanded Htt is linked to the toxicity. In this study, we prepared the expanded Htt containing 46 glutamines, and induced the fibrillation by proteolytic cleavage. Fibrillation of the pathogenic Htt has been monitored by time course NMR experiment. The NMR-based monitoring method could be widely used to screen the candidates to inhibit the fibrillation of the pathogenic Htt.

Keywords Huntington's disease, huntingtin protein, polyQ, fibrillation, NMR

Introduction

Huntington's disease (HD) is an inherited neurodegenerative disorder caused by an abnormal expansion of CAG repeat (i.e., coding polyglutamine) in the first exon of *Htt* gene.^{1,2} Wild-type huntingtin protein (Htt) contains less than 35 polyglutamine (polyQ), whereas HD patients carry a dominant mutation in *Htt* gene that encodes the expanded Htt containing 40 or more glutamine repeats.³ It has been reported that polyglutamine

region can be changed to β -hairpin loop, and the region may provide the nucleation sites for the formation of β -sheets that promote aggregate formation.⁴

There have been many reports regarding a correlation between the aggregation process and toxicity to neuronal cells. For instance, expanded Htt is oligomerized and accumulated in neurons, which form intracellular aggregates.⁵ Another proposed mechanism is that normal and abnormal Htt interact with other cellular proteins, altering their function and ultimately leading to neuronal dysfunction and cell death.⁶ However, the exact molecular mechanism of HD pathogenesis is still unclear and controversial.⁷ Here, we have prepared the expanded Htt containing 46 glutamines (hereafter referred to as Htt46Q) as a pathogenic Htt model, and demonstrated the initiation of fibril formation by proteolytic cleavage. Fibrillation of Htt46Q has been monitored by time course NMR experiment.

Experimental Methods

Gene cloning – The plasmid DNA coding the expanded huntingtin protein containing 46 glutamines (Htt46Q) was kindly provided from Prof. Meewhi Kim (University of Texas Southwestern Medical Center at Dallas). The gene encoding Htt46Q was amplified by polymerase chain reaction

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(PCR), and the amplified products were inserted between the *Bam*HI and *Eco*RI sites in PVFT4S vector (Novagen, Madison, WI, USA) for N-terminal MBP (maltose-binding protein) fusion (Figure 1A). The recombinant plasmid verified by DNA sequencing was transformed into *Escherichia coli* strain BL21 (DE3) Codon plus competent cells for expression.

Purification of MBP-Htt46Q – The MBP-fused Htt46Q proteins (hereafter referred to as MBP-Htt46Q) were grown in LB medium at 37°C. When absorbance at 600 nm reached 0.8, the protein expression was induced by 0.5 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) at 15°C. After 16 h induction, cells were harvested by centrifugation. Harvested cells were disrupted by sonication in lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.4). The supernatants were loaded onto a HisTrap FF column (GE Healthcare, Little Chalfont, UK), equilibrated with lysis buffer. MBP-Htt46Q proteins were eluted by increasing imidazole concentration in the range from 20 mM to 500 mM, and eluted fractions were dialyzed with final buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.5). Uniformly [15 N]-labeled MBP-Htt46Q was prepared by growing the cells in M9 minimal medium. NMR samples were prepared as described above, and labeled protein was dissolved in final buffer containing 10% D₂O.

Thioflavin T (ThT) assay – Measurement of ThT fluorescence was performed on 96-microwell black plates (Corning, New York, USA) with a microplate reader (Biotek Instruments Inc., Winooski, VT, USA). The 20 μ M ThT fluorescence kinetics was recorded at 310 K for 6 h reading every 10 min. The wavelengths of excitation and emission were 450 nm and 490 nm, respectively. ThT fluorescence emission curves associated with β -sheet-structured Htt46Q aggregates were recorded after the addition of 5 U of TEV (5 units of TEV per 1 mg of MBP-Htt46Q).

NMR experiments – The 2D 1 H- 15 N HSQC spectra of 15 N-labeled MBP-Htt46Q were measured at 288 K on

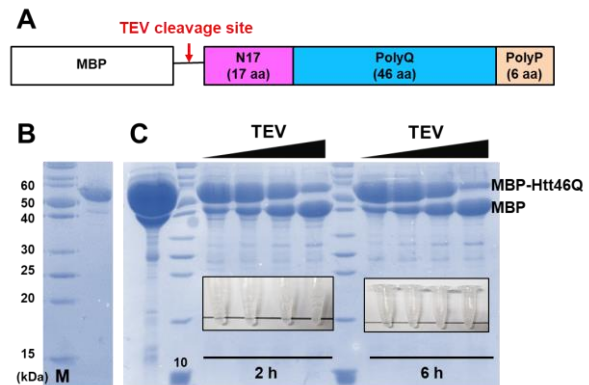


Figure 1. Purification and TEV-induced cleavage of Htt46Q. (A) Schematic representation of MBP-Htt46Q construct. (B) SDS-PAGE image for the purified MBP-Htt46Q. (C) TEV-induced cleavage of MBP-Htt46Q with various TEV concentrations for 2 h (lanes 3-6) and 6 h (lanes 8-11), respectively. Insets show the pictures of tubes containing the corresponding TEV-treated MBP-Htt46Q samples. The black lines behind tubes are gradually blurry as TEV concentrations increase, and the samples with 6 h incubation are more cloudy than those with 2 h incubation.

a Bruker AVANCE 600 spectrometer equipped with a cryoprobe. The concentration of 15 N-labeled MBP-Htt46Q was 648 μ M. The NMR measurements were started after the addition of 5 U of TEV, and total 48 HSQC spectra were measured sequentially for 60 h. All NMR spectra were processed using NMRPipe/NMRDraw software⁸ and analyzed with NMRView J program.⁹

Results

Initiation of fibril formation by proteolytic cleavage – The MBP-Htt46Q could be purified (Figure 1B). Fibrillation of the Htt46Q was initiated by TEV-induced proteolytic cleavage. When TEV was added to MBP-Htt46Q for cleavage, severe aggregation was observed after short lag time. We monitored the fibrillation of Htt46Q with various TEV concentrations and incubation time. As TEV concentrations increase, the amounts of MBP-Htt46Q decrease (*i.e.* increases of MBP alone) in SDS-PAGE (Figure 1C). However, the intact Htt46Q without

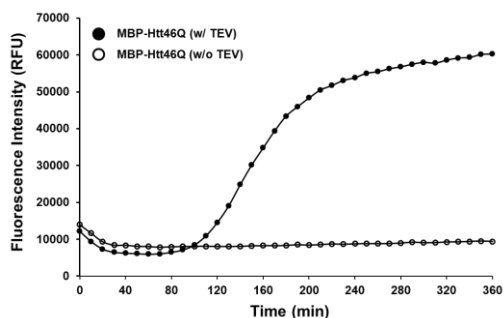


Figure 2. Time-dependent ThT fluorescence of MBP-Htt46Q in the presence (filled circles) and absence (empty circles) of TEV.

MBP tag was not observed, which is probably due to the fast fibrillation of Htt46Q after cleavage. This is also supported by the observation of the tubes containing the cleaved MBP-Htt46Q. The solutions containing the MBP-Htt46Q samples with higher concentrations of TEV and longer incubation time were more cloudy (insets in Figure 1C). Collectively, fibril formation of Htt46Q could be initiated by proteolytic cleavage, and the extent of fibrillation depended on TEV concentration and incubation time.

Thioflavin T (ThT) fluorescence assay for Htt46Q – To confirm the fibril formation of Htt46Q and determine the kinetics of fibrillation, the time-course

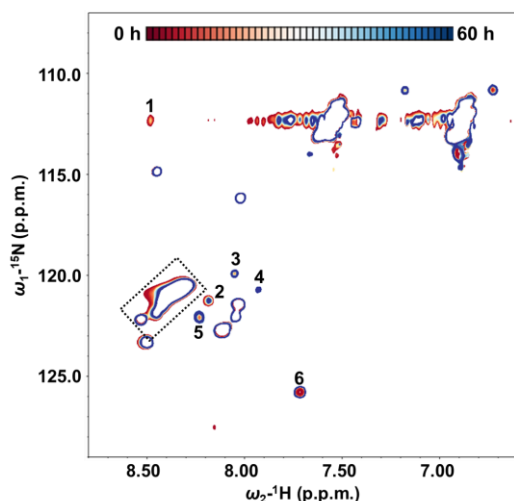


Figure 3. A series of 2D-[^1H - ^{15}N] HSQC spectra of MBP-Htt46Q in the presence of TEV for 60 h.

measurements of ThT fluorescence were performed. ThT is a small molecule that binds to β -sheet rich structures, such as amyloid fibril, resulting in a strong fluorescence signal.¹⁰ The expanded polyQ region of Htt undergoes a conformational transition to β -sheet structure during fibrillation.⁴ Fluorescence intensities of MBP-Htt46Q increased 1.5 h after TEV treatment, and were fully saturated in about 5 h (filled circles in Figure 2). In contrast, there were no significant changes in fluorescence signals of MBP-Htt46Q in the absence of TEV (empty circles in Figure 2).

Monitoring the fibrillation of Htt46Q – The time-course ^1H - ^{15}N HSQC spectra of MBP-Htt46Q in the presence of TEV were measured for 60 h. Although resonances from MBP were not visible in the HSQC spectra due to the large size (~50 kDa),

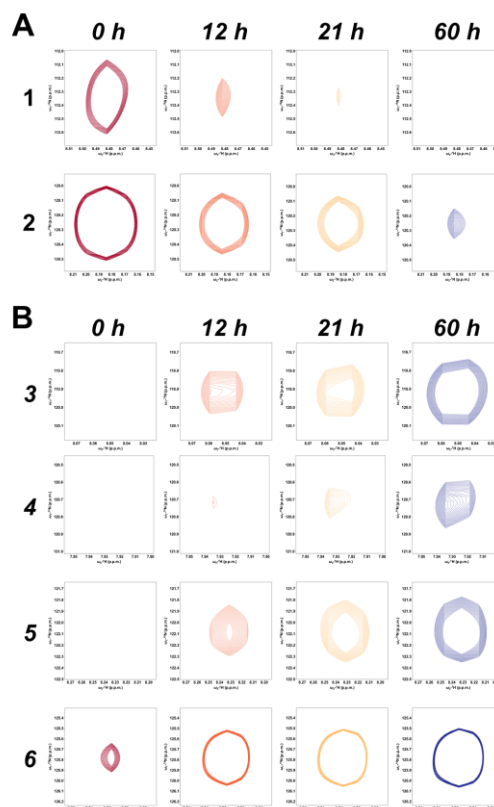


Figure 4. Time-dependent spectral changes of group 1 (A) and group 2 (B). Each peak at the selected time points (0 h, 12 h, 21 h, and 60 h) is shown. Peak numbering and color codes of the peaks are the same as in Figure 3.

several crosspeaks of Htt46Q, including some part of N17 and polyQ regions, were clearly shown (Figure 3). Therefore, we could monitor the fibrillation of Htt46Q in a time-dependent manner.

The resonances of Htt46Q were categorized into three groups based on the time-dependent intensity changes. The first group comprises the residues showing decreased peak intensities during fibrillation. The intensities of polyQ region (rectangle with dotted line in Figure 3), including glutamine side chains, gradually decreased as fibrillation progressed. Also, two resonances (marked as 1 and 2 in Figure 3) were shown the similar changing pattern (Figure 4A). The second group includes four residues (marked as 3-6 in Figure 3), and the intensities of those peaks increased during fibrillation (Figure 4B). The resonances experiencing no spectral changes during fibrillation were classified as the third group.

Discussion

In our study, intact form of the pathogenic Htt containing 46 glutamines could not be obtained, because the expanded Htt was rapidly fibrillated after cleavage of MBP tag. Instead, we measured a series of ^1H - ^{15}N HSQC spectra continuously, immediately after inducing proteolytic cleavage reaction, and we could monitor the several resonances during fibrillation process of Htt46Q. Although further studies should be performed, such as backbone assignment of Htt46Q, our results would contribute to understanding the complicated process of fibrillation at atomic level. In addition, time course NMR experiment based on the proteolytic cleavage could be used to screen the chemicals (or proteins) to inhibit the fibrillation of the pathogenic Htt.

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