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PNAS 2006;103;14367-14372; originally published online Sep 15, 2006; doi:10.1073/pnas.0602348103

This information is current as of December 2006.
Normal-repeat-length polyglutamine peptides accelerate aggregation nucleation and cytotoxicity of expanded polyglutamine proteins

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Edited by Sue Hengren Wickner, National Institutes of Health, Bethesda, MD, and approved August 1, 2006 (received for review March 22, 2006)

The dependence of disease risk and age-of-onset on expanded CAG repeat length in diseases like Huntington’s disease (HD) is well established and correlates with the repeat-length-dependent nucleation kinetics of polyglutamine (polyGln) aggregation. The wide variation in ages of onset among patients with the same repeat length, however, suggests a role for modifying factors. Here we describe the ability of normal-length polyGln repeat sequences to greatly accelerate the nucleation kinetics of an expanded polyGln peptide. We find that normal-length polyGln peptides enhance the in vitro nucleation kinetics of a Q20 peptide in a concentration-dependent and repeat-length-dependent manner. In vivo, we show that coexpression of a Q20 sequence in a Drosophila model of HD expressing Htt exon 1 protein with an Q93 repeat accelerates both aggregate formation and neurotoxicity. The accelerating effect of short polyGln peptides is attributable to the promiscuity of polyGln aggregate elongation and reflects the intimate relationship between nucleus formation and early elongation events in establishing nucleation kinetics. The results suggest that the overall rate of the polyGln protein network in a cellular environment may have a profound effect on the toxic consequences of polyGln expansion and thus may serve as a genetic modifier of age of onset in HD.

Drosophila | Huntington’s disease | in vitro | elongation kinetics | amyloid

A hallmark feature of neurodegenerative disorders such as Huntington’s disease and other expanded polyglutamine (polyGln) repeat diseases (1) is the appearance, in subsets of neurons, of visible aggregates made up of the mutant disease protein and other cellular proteins, including other polyGln repeat proteins. A role for aggregation in disease also is suggested by the close agreement between the repeat-length dependence of aggregation (2, 3) and the repeat-length dependence of disease risk for most polyGln repeat diseases (1). Tracing the role of aggregates in disease is complicated, however, by the variety of aggregate sizes, morphologies, and functionalities observed in cells and tissue (4). For example, depending on conditions, polyGln-containing molecules can make either mature amyloid fibrils (5–7) or oligomeric structures (8–10). This variety of aggregate types may help explain the lack of an apparent relationship between visible aggregates and physiological consequences in some cell and animal studies (11).

Most protein aggregation reactions occur through either colloidal coagulation (12, 13) or nucleated growth (14) pathways. Spontaneous aggregation of simple polyGln peptides occurs via a classic nucleation-dependent polymerization pathway (15). The nucleus for polyGln amyloid formation appears to be an alternatively folded state of the monomer (15) that exists in a highly unfavorable preequilibrium with the bulk-phase, disordered monomer (16). Once polyGln amyloid fibrils have formed, they grow by means of a highly efficient elongation reaction (15, 16). Although elongation of amyloid fibrils is generally highly selective, so that incorporation of other sequences into growing aggregates is very inefficient (17), polyGln amyloid growth is robustly promiscuous. Thus, qualitative studies with polyGln-containing proteins (18, 19) and quantitative studies with simple polyGln sequences (3) indicate that any protein with a sterically accessible polyGln segment can serve to elongate a polyGln-based aggregate. This finding is important in expanded CAG repeat diseases, because, in addition to the nine known polyGln disease proteins (20), the human genome contains a substantial number of other proteins featuring polyGln repeats and Gln-rich sequences (21–23).

We report here the finding that normal-length polyGln sequences can greatly enhance the nucleation phase of the polyGln aggregation reaction. We show that the overall efficiency of nucleation is controlled not only by the thermodynamics of nucleus formation but also by kinetics by which transient nuclei become committed to the aggregation process. Commitment of nuclei to aggregate formation is favored by normal-length polyGln peptides by virtue of the promiscuity of polyGln elongation. We extend these observations to in vivo experiments in which coexpression of a short polyGln peptide in a Drosophila model of Huntington’s disease leads to an acceleration of Htt aggregation that correlates with an acceleration of polyGln toxicity. The results suggest that normal-length, endogenous polyGln proteins in the cellular milieu can significantly influence amyloid formation by an expanded repeat-length polyGln protein, potentially influencing the age of onset for disease. The results also support the thermodynamic model of nucleation in which aggregation nuclei are treated as being formed in a rapid and reversible preequilibrium with bulk-phase monomer (14, 15).

Results

A general depiction of the thermodynamic model for nucleated growth polymerization (14) is shown in Fig. 1. Formation of the nucleus Nn from bulk-phase monomer M0 is modeled as a rapid and reversible, but highly unfavorable, process (15, 16). Once formed, the metastable Nn can either disintegrate back to the monomer pool or elongate, via reaction with additional M0 molecules, to generate lower free-energy reaction mixtures containing Nn+1, Nn+2, etc. The more efficient these elongation reactions occur through either colloidal coagulation or nucleated growth pathways. Spontaneous aggregation of simple polyGln peptides occurs via a classic nucleation-dependent polymerization pathway (15). The nucleus for polyGln amyloid formation appears to be an alternatively folded state of the monomer (15) that exists in a highly unfavorable preequilibrium with the bulk-phase, disordered monomer (16). Once polyGln amyloid fibrils have formed, they grow by means of a highly efficient elongation reaction (15, 16). Although elongation of amyloid fibrils is generally highly selective, so that incorporation of other sequences into growing aggregates is very inefficient (17), polyGln amyloid growth is robustly promiscuous. Thus, qualitative studies with polyGln-containing proteins (18, 19) and quantitative studies with simple polyGln sequences (3) indicate that any protein with a sterically accessible polyGln segment can serve to elongate a polyGln-based aggregate. This finding is important in expanded CAG repeat diseases, because, in addition to the nine known polyGln disease proteins (20), the human genome contains a substantial number of other proteins featuring polyGln repeats and Gln-rich sequences (21–23).

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steps, the more likely a transiently formed nucleus will be channeled essentially irreversibly into aggregate formation. In principle, any $M_b$ capable of elongation of $N^*$ promotes nucleation, by increasing the effectiveness with which elongation competes with disintegration of $N^*$. This trend is implicit in the simplified equation describing nucleation, in which the concentration of monomers converted to aggregates at time $t$ depends on the square of the elongation rate constant $k_+$ (14, 15). Thus, although the nucleation phase of nucleated growth is normally considered as being separate from the elongation phase, the thermodynamic model of nucleation suggests that the kinetics of the elongation reactions operating on the nucleus and early aggregates will contribute to the overall kinetics of nucleation.

Because the polyGln amyloid system exhibits promiscuity of aggregate elongation by different polyGln peptides, the thermodynamic model of nucleation predicts that relatively short polyGln molecules, even those too short to effectively undergo nucleation themselves, should be effective promoters of the nucleation of expanded polyGln sequences. Previously, we showed that the number of molecules of polyGln comprising $N^*$ (called critical nucleus, or $n^*$) is equal to 1 for nucleation of polyGln aggregation (15); the general aspects of theory and interpretation discussed in this work, however, are independent of the molecularity of $N^*$.

**Normal-Length polyGln Peptides Enhance Aggregation Nucleation of an Expanded polyGln Peptide in Vitro.** To test the above hypothesis, we followed the early aggregation kinetics of the simple polyGln peptide Q$_{47}$ in the presence of different concentrations of the normal-repeat-length peptide Q$_{20}$. At 1.8 $\mu$M and 37°C in PBS, this Q$_{47}$ peptide alone does not aggregate for up to 6 days (Fig. 2a; only data up to 6 h are shown). However, in the presence of low micromolar concentrations of the Q$_{20}$ peptide, aggregation of a 1.8 $\mu$M solution of Q$_{47}$ is observable within a few hours, and
higher concentrations of Q20 further increase Q47 aggregation (Fig. 2a). In comparison, incubation of neither Q20 alone at 100 μM or Q47 alone at 7 μM produces any aggregation at 6 hours; to obtain the degree of aggregation of Q47 observed after 6 h with 1.8 μM Q47/36 μM Q20 (Fig. 2a, ○), it is necessary to incubate 15 μM Q47 (data not shown).

Analysis (14, 15) of the aggregation kinetics shows that Q47 in the presence of different amplifying concentrations of Q20 exhibits the defining features of a nucleated growth polymerization pathway: (i) disappearance of monomeric Q47 from solution in the early phases of aggregation follows t2 kinetics (Fig. 2b) and (ii) the logarithms of the slope of the t2 plots trending linearly with the logarithms of starting Q47 concentrations (Fig. 2c). As previously found for aggregation of homogeneous repeat-length polyGln solutions (15), the slope of the logarithm–logarithm plot corresponds to a critical nucleus of 1 (see legend to Fig. 2). Thus, the presence of Q20 does not cause a change in mechanism but rather changes the overall nucleation kinetics.

Previously we showed that polyGln repeat length influences the rates of elongation reactions (3). If normal-length polyGln peptides accelerate Q47 nucleation by increasing the rate of nucleus elongation, we would therefore expect that, for any polyGln concentration, acceleration should increase as the repeat length of the added peptide increases. We incubated Q47 (1.5–2.0 μM) plus short polyGln of various repeat lengths (~17 μM). As expected, the nucleation kinetics of Q47 aggregation are enhanced as the repeat length of the added polyGln increases (Fig. 3). Short (10–15 aa) repeat length polyGln peptides give modest but measurable acceleration. Thereafter, acceleration increases as repeat length increases. The mechanism of the length-dependent effect also appears to be an enhancement of the normal nucleation mechanism, because linear t2 plots were obtained for Q47 incorporation into aggregates in the presence of all of these short polyGln peptides (data not shown).

To further probe whether our results are consistent with the thermodynamic nucleation model (Fig. 1), we determined the pseudofirst order (see legend to Fig. 4) rate constants for the elongation of preformed Q47 aggregate seeds by polyGln peptides of different repeat lengths (see, for example, Fig. 4a Inset). In accord with previous results (3), we found that the elongation rate constants for these reactions increase as repeat length increases (Fig. 4a). We then plotted the nucleation rate parameter (the t2 slopes from Fig. 3) for each accelerating peptide against the square of the corresponding repeat-length-dependent elongation rate constant (from Fig. 4a). The resulting plot (Fig. 4b) shows a linear dependence throughout the repeat-length range, consistent with the hypothesis that the accelerating effect of short polyGln sequences on nucleation kinetics of expanded polyGln peptides simply is due to the influence that repeat length exerts over the elongation rate. This finding supports the thermodynamic model (Fig. 1).

Expression of a Short polyGln Peptide Accelerates Aggregation in a Drosophila Model of polyGln Toxicity. These in vitro kinetics results suggest that a given concentration of an expanded polyGln repeat protein would be more likely to form aggregates in a cell that is rich in normal-repeat-length polyGln sequences. To test this hypothesis, we coexpressed a Q20 version of Htt exon 1 (Httex1p-Q20) together with a Q93 form (Httex1p-Q93) (24, 25)
in *Drosophila*. A rhodopsin driver (Rh–GAL4) that drives transgene expression in six (R1–R6) of eight photoreceptor neurons of each ommatidium in adult flies was used. Five days after onset of transgene expression of Httex1p-Q93 alone, the protein is found primarily in the cytosol in multiple, small aggregates (Fig. 5a). Twenty-four hours later, at day 6, nuclear aggregates began to appear. By day 10, most nuclei contained nuclear inclusions (Fig. 5b). In contrast, no visible aggregates were observed at any time in flies expressing Httex1p-Q20 alone (Fig. 5a and data not shown). However, when the Q20 protein was coexpressed with the Q93 form, an increase in the number of visible inclusions is observed. No aggregates are observed with Q20 alone. (b) The number of photoreceptor nuclei with visible inclusions in 5-, 6-, and 10-day-old flies (d5, d6, and d10, respectively). The fraction of nuclei with inclusions at days 5 and 6 is increased when Q20 is coexpressed; however, the percentage of nuclei with inclusions with and without Q20 coexpression plateaus by day 10. The percentage of nuclear accumulation was calculated as Htt-positive nuclei per all nuclei per field [*P < 0.025 (0.0239); **P < 0.015 (0.0145)]. (c) Pathology is evaluated by comparing the number of rhabdomeres per ommatidium in 8-day-old (d8) and 12-day-old (d12) flies [***P < 0.0015 (0.0014)]. No neurotoxicity is observed up to day 8, but toxicity is evident by day 12 and is increased by the presence of Q20.

Expression of a Short polyGln Peptide Accelerates Neurodegeneration in a *Drosophila* Model of polyGln Toxicity. To determine whether the increased rate of aggregation correlates with an increase in pathology, we compared the number of surviving photoreceptor neurons in flies expressing Httex1p-Q93 vs. Httex1p-Q20 plus Httex1p-Q93. By as late as 8 days after eclosion, flies expressing Httex1p-Q93 alone had seven intact rhabdomeres (the light gathering organ of the photoreceptors) visible by pseudopupil analysis (26), similar to wild-type flies (Fig. 5a and data not shown). By day 12, the average number of rhabdomeres per ommatidium decreased to 5.8 (Fig. 5c), indicating mild degeneration. When Httex1p-Q93 was coexpressed with Httex1p-Q20,
neuronal degeneration at 12 days was increased, with an average of only 4.7 rhabdomeres per ommatidium remaining (Fig. 5c). Thus, the accelerated rate of aggregate formation observed in response to coexpression with Httex1p-Q25 correlates with an increase in neuronal degeneration.

**Discussion**

The results presented here show that short polyGln peptides, without changing the nucleation mechanism, exert a strong accelerating effect on the nucleation kinetics of an expanded polyGln, an effect that depends on repeat length and concentration. This rate enhancement is reflected in an accelerated appearance of visible aggregates in vivo in a Drosophila model of Huntington’s disease, which in turn is correlated with enhanced degeneration of photoreceptor neurons in adult flies.

The accelerating effect of nonpathogenic polyGln peptides on the nucleation kinetics of an expanded pathogenic polyGln derives from the ability of these short peptides to elongate aggregation nuclei and nascent aggregates. Because the nature of the aggregation nucleus is itself unchanged, acceleration of nucleation by short polyGln sequences might best be considered a kind of assisted homogenous nucleation. Our studies (15, 16, 27) of polyGln aggregation nucleation kinetics are based on a nucleated growth polymerization model in which the nucleus exists in a rapid preequilibrium with the bulk-phase pool of monomers (Fig. 1) and is defined as the least thermodynamically stable species on the aggregation pathway (14). The observations reported here provide strong support for this model. If formation of a nucleus from bulk-phase monomer were kinetically rate-limiting (that is, a very small $k_1$ in Fig. 1), then enhancing the rates of subsequent elongation steps would not be expected to increase the overall nucleation rate. Because we show here that enhancing the early elongation steps enhances the efficiency of nucleation, we conclude that formation of the nucleus itself is a rapid preequilibrium in which (in the absence of added, short-repeat-length polyGln) $[M]_k \gg [N^+][N^+]k_2$.

These results are strictly valid only for spontaneous aggregation reactions that proceed through a nucleated growth polymerization pathway. Although this mechanism holds for simple polyGln peptides, the growth of most other amyloid fibrils appears to involve oligomeric and protofibrillar species whose mechanisms of formation, growth, and conversion into mature amyloid are still being worked out. Besides the nine proteins currently implicated in different polyGln expansion diseases (1), the human genome encodes many other proteins containing polyGln sequences of various repeat lengths (21–23). The analysis described here suggests that genetic modifiers of adult age-of-onset, predicted by human studies (28–30), may include variation in either the concentrations and/or repeat lengths of some of these sequences to directly influence the aggressiveness of expanded repeat polyGln aggregation and disease. For example, the accelerating effect of normal-length polyGln sequences on polyGln aggregation nucleation and toxicity may help explain how overexpression of the polyGln-containing CREB-binding protein can in some animal and cellular models exacerbate expanded polyGln toxicity (Pedro Fernandez-Funez and Juan Botas, personal communication; and N.S., J.S.S., G.R.J., J.L.M. and L.M.T., unpublished data) while being protective in other cases (31, 32). Likewise, the aggressiveness of an expanded polyGln disease might be stimulated by increases in concentration or repeat length of other polyGln disease proteins within the subcellular environment, as has recently been suggested by a genetics analysis of spinocerebellar ataxia 2 (33).

If the hypothesis developed in this paper has merit, it will be especially important to understand the steady-state concentrations, state of proteolytic fragmentation, and cellular and subcellular distributions of all members of this polyGln protein network, not just of molecules with repeat lengths near or above the pathological threshold. More broadly, it will be important to understand all of the factors influencing the formation of various polyGln aggregates if we are to fully describe the molecular mechanisms of these diseases and confidently devise treatment strategies.

**Experimental Procedures**

**In Vitro Kinetics Analysis.** All peptides were obtained by custom synthesis from the Keck Center at Yale University (New Haven, CT). All synthetic polyGln peptides include pairs of Lys residues at the N and C termini for solubility (7). All procedures have been described in detail previously (34, 35). Peptides were purified by reverse-phase HPLC, pooled with the aid of mass spectrometric characterization of chromatography fractions, lyophilized, and stored at −80°C (34). Before use in an aggregation reaction, peptides were disaggregated by using reversible exposure to organic solvents (34–36). Peptide concentrations were determined by using a centrifugation–HPLC sedimentation assay (34, 35); HPLC separation allows independent analysis of Q21 peptides in the presence of shorter peptides. Kinetics experiments were set up by dilution into PBS of freshly disaggregated peptide stocks. Aggregates used as seeds were grown (7), harvested, and characterized as described (35). Nucleation kinetics analysis was as described previously (15, 34, 35).

**Drosophila Stocks and Crosses.** The polyGln-expressing transgenic stocks used in this study were w; P[UAS-Httex1p Q93]4F1 and w; P[UAS-Httex1p Q20]111M1 (24, 25). These flies were mated with an Rh1-Gal4 driver line (37) in which the rhodopsin promoter drives transgene expression in adult photoreceptors R1–R6 (a kind gift from C. Desplan, New York University, New York, NY). Cultures were raised at 29°C.

**Pseudopupil Analysis.** Flies 8 and 12 d old were decapitated and mounted in a drop of nail polish on a microscope slide as described previously (24, 25). The head was then covered with immersion oil and examined under a Nikon (Tokyo, Japan) EFD-3 Optiphot-2 scope with ×50 oil objective. At least 50 ommatidia in 4 flies were examined, and the number of visible rhabdomeres was counted for each.

**Immunohistochemistry and Confocal Analysis.** Heads of adult flies were prefixed in 4% formaldehyde at room temperature for 1 h, and eyes were dissected in PBS. The tissue was fixed for an additional 10 min in formaldehyde at room temperature. After permeabilization and blocking (0.2% Triton X-100 in PBS for 2 h at room temperature and 5% normal goat serum/0.2% Triton X-100 in PBS at 2 h at room temperature), tissues were incubated with primary antibody in blocking solution overnight at 4°C. After washing in blocking buffer, secondary antibody was applied for 2 h at room temperature. The primary antibodies were anti-Htt S830 (1:1,000 dilution; gift from G. Bates, King’s College London School of Medicine, London, U.K.) (38) and anti-elav (1:200 dilution; Iowa Hybridoma Bank, University of Iowa, Iowa City, IA). Rhabdomeres of adult photoreceptors were visualized by staining F-actin with 2 ng/ml TRITC-phalloidin (Sigma, St. Louis, MO). Secondary antibodies at 1:200 dilutions were from Jackson ImmunoResearch (West Grove, PA). The photographs featured in Fig. 5 are from a single confocal (model no. LSM510; Zeiss, Thornwood, NY) section through the eye. The position of section (cross-sections and longitudinal sections) was chosen at the level of photoreceptor nuclei localization.

**Quantitation of Inclusions.** Visual counts of nuclear inclusions and neuronal nuclei were performed by using images obtained by confocal microscopy. Every image was taken from one individual cell.
eye, and five to seven animals were analyzed. For each data point, 500–800 cells were counted. Aggregation is expressed as the percentage of nuclei with inclusions versus the total number of nuclei per field.

We thank Frank Ferrone for reviewing the manuscript, Barbara Apostol for assistance with the figures, C. Desplan for the generous gift of Rhi-Gal4 flies, and G. Bates for the anti-Htt S830 antibody. The anti-elav antibody was obtained from the Developmental Studies Hybridoma Bank. We also gratefully acknowledge Optical Biology Shared Resource of Cancer Centre Support Grant CA-62203 from the University of California. This work was supported by U.S. Public Health Service Grants R01 AG19322 (to R.W.) and NS-45283 (to J.L.M.), a Hereditary Disease Foundation postdoctoral fellowship (to N.S.), the Hereditary Disease Foundation (L.M.T.), the Huntington’s Disease Society of America (L.M.T. and J.L.M.), and the HighQ Foundation (J.L.M. and L.M.T.).