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Backbone engineering within a latent β -hairpin structure to design inhibitors of polyglutamine amyloid formation

Karunakar Kar^{a,c}, Matthew A. Baker^{b,d}, George A. Lengyel^{b,e}, Cody L. Hoop^{a,f}, Ravindra Kodali^a, In-Ja Byeon^a, W. Seth Horne^b, Patrick C.A. van der Wel^a, and Ronald Wetzel^a

^aDepartment of Structural Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15260, USA

^bDepartment of Chemistry, Kenneth P. Dietrich School of Arts and Sciences, University of Pittsburgh, Pittsburgh PA 15260.

Abstract

Candidates for the toxic molecular species in the expanded polyglutamine (polyQ) repeat diseases range from various types of aggregates to “misfolded” monomers. One way to vet these candidates is to develop mutants that restrict conformational landscapes. Previously we inserted two self-complementary β -hairpin enhancing motifs into a short polyQ sequence to generate a mutant, here called “ β HP”, that exhibits greatly improved amyloid nucleation without measurably enhancing β -structure in the monomer ensemble. We extend these studies here by introducing single backbone H-bond impairing modifications ^{α} N-methyl Gln or L-Pro at key positions within β HP. Modifications predicted to allow formation of a fully H-bonded β -hairpin at the fibril edge while interfering with H-bonding to the next incoming monomer exhibit poor amyloid formation and act as potent inhibitors in *trans* of simple polyQ peptide aggregation. In contrast, a modification that disrupts *intra*- β -hairpin H-bonding within β HP, while also aggregating poorly, is ineffective at inhibiting amyloid formation in *trans*. The inhibitors constitute a dynamic version of the edge-protection negative design strategy used in protein evolution to limit unwanted protein aggregation. Our data support a model in which polyQ peptides containing strong β -hairpin encouraging motifs only rarely form β -hairpin conformations in the monomer ensemble, but nonetheless take on such conformations at key steps during amyloid formation. The results provide insights into polyQ solution structure and fibril formation while also suggesting an approach to the design of inhibitors of polyQ amyloid growth that focuses on conformational requirements for fibril and nucleus elongation.

Corresponding author: Ronald Wetzel, Department of Structural Biology, University of Pittsburgh School of Medicine, Rm. 2046, Biomolecular Sciences Tower 3, 3501 Fifth Avenue, Pittsburgh PA 15260, USA. rwetzel@pitt.edu. Phone: 412-383-5271. Fax: 412-648-9008.

^cPresent address: School of Life Sciences, Jawaharlal Nehru University, New Delhi -110067, India

^dPresent address: Department of Chemistry, Carnegie Mellon University, Pittsburgh PA 15213, USA

^ePresent address: Department of Chemistry, Slippery Rock University, Slippery Rock PA 16057, USA

^fPresent address: Department of Chemistry and Chemical Biology, Rutgers University, Piscataway NJ 08854, USA

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Graphical abstract

Keywords

NMR; MAS-NMR; elongation; nucleation; dock-and-lock; trpzip; D-Pro-Gly; expanded polyglutamine repeat diseases

INTRODUCTION

The expanded CAG repeat diseases consist of a family of at least nine ^{1, 2} disorders, the most prevalent of which is Huntington's disease ³. These conditions are associated with inheritance of enlarged CAG repeats in protein-encoding sequences that result in the expression of disease proteins containing expanded polyglutamine (polyQ) sequences. The molecular and cellular mechanisms of these diseases continue to be investigated and debated ³⁻⁷. In particular, it is important to sort out, at the molecular level, how repeat length expansion creates the unique polyQ physical properties that trigger downstream neurodegenerative pathology. Identifying these fundamental connections is the key to developing structure and mechanism based therapeutics. Although there is a consensus that these diseases are related to defects in protein folding, the manner by which expansion of the intrinsically disordered polyQ sequence alters folding and induces toxicity has not been worked out and remains controversial.

One group of mechanisms hypothesizes a repeat length dependent conformational change within the polyQ sequence ⁸ to generate a toxic conformation of the monomer that is often represented as containing one or more β -hairpins ^{9, 10}. Another group of mechanisms hypothesizes a repeat length dependent increase in the efficiency of formation of toxic aggregates ¹¹ with candidate structures ranging from α -helix rich multimers ¹²⁻¹⁴ and oligomeric intermediates ^{13, 15} to β -rich microfibrils ^{14, 16} and on to large amyloid fibrils ¹⁷ and fibril-rich inclusions ¹⁸. The wealth of energetically similar structures populating both the monomer ensembles and the aggregation pathways of polyQ disease proteins introduces particular challenges to identifying candidates for the toxic species through *in vitro* studies. Likewise, correlative studies in cells and animals tend to be inconclusive due to significant overlap in the respective time lines for the evolution of structure and pathology ¹⁴.

One strategy with the potential to cut through these structural ambiguities is to develop research tools consisting of mutated forms of polyQ proteins exhibiting dramatically altered conformational preferences. Thus, introduction of β -hairpin enhancing motifs within polyQ sequences can dramatically enhance aggregation kinetics ¹⁹⁻²¹. This effect cannot be attributed to any detectable increase in β -structure in the monomer ensemble ²¹, but rather to a combination of enhanced nucleation ²¹, elongation ²², and thermodynamic stability of the fibrils ²¹ consistent with the compatibility of β -hairpins with the polyQ amyloid core structure ^{19, 23, 24}. Furthermore, introduction of a " β -breaker" Pro residue into a polyQ

stretch within such a β -hairpin motif modified polyQ restricts the protein's ability to spontaneously aggregate¹⁹ while bestowing upon it an ability to inhibit the aggregation of other polyQ proteins²⁵. Optimization of such modified polyQ sequences and their installation into polyQ disease proteins has the potential to create powerful probes of folding and disease mechanism.

Recently we found that it is possible to obtain additive effects of two complementary β -hairpin encouraging motifs placed within the same mutated polyQ sequence. Thus, the peptide AcWQ₁₁pGQ₁₁WTGK₂ (here called “ β HP”; Fig. 1a), containing both a D-Pro-Gly and a “trpzip”²⁶ motif, undergoes spontaneous amyloid formation more rapidly than similar peptides containing either the D-Pro-Gly or trpzip motif alone²¹. Here, we test the hypothesis that the two complementary β -hairpin encouraging mutations in β HP provide substantial constraints on the conformational changes that can take place during amyloid nucleation and elongation, thus providing a framework for optimizing the effects of additional point mutations. The results provide new data on the energetics controlling the conformational ensemble of polyQ monomers, and on the design of powerful inhibitors of spontaneous amyloid formation that hold promise both as diagnostics of molecular mechanisms of cytotoxicity and as lead structures for pharmaceutical design.

RESULTS

Theoretical background

The design strategies utilized here rest largely on some fundamental features of anti-parallel β -sheet and β -hairpin structure. Figure 1a schematically illustrates how various mutations are expected to either tolerate or greatly disfavor a hypothetical β -hairpin structure, and Figure 1b illustrates some fundamental features of canonical anti-parallel β -sheet structure that are critical to our design strategies. Thus, across any two adjacent β -strands of an anti-parallel β -sheet, aligned residues are either “H-bonded” (Fig. 1b, red box) or “non-H-bonded” (Fig. 1b, green box)²⁷. In the H-bonded pairs, the N-H and C=O groups of both residues engage in two cross-strand contacts. In the non-H-bonded pairs, the N-H and C=O of each residue are directed outward. If the two reference β -strands lie within a wider β -sheet, as shown in Figure 1b, then these latter groups will be H-bonded to the next neighboring strands. For β -strands that are part of an isolated β -hairpin, however, or are an edge strand in a β -sheet, then these outward-projecting groups are solvent-exposed. Moving across a pair of strands in a canonical anti-parallel β -sheet in the extended chain direction (arrows), H-bonded and non-H-bonded residue pairs alternate (Fig. 1b).

These features of anti-parallel β -sheets and β -hairpins have several consequences relevant to the peptide designs and data presented here. In particular, it is important to consider the structural preferences of mutations with respect to these H-bonding relationships. β -hairpin motifs, for example, have preferences and consequences for H-bonding within the β -hairpins, and it is important to keep these in mind when designing multiply mutated peptides. In addition, the β -breaking modifications of Pro insertion and backbone N-methylation utilized here are known to have very strong preferences for being located in the non-H-bonding position in edge strands of β -sheets. Thus, in an analysis of amino acid preferences, Wouters and Curmi found that Pro residues are reasonably well-tolerated in

anti-parallel β -sheets, but only in edge strands and then only at non-H-bonding positions²⁷ (For examples, see Fig. 2 and Supplemental Fig. 1). Likewise, N-methylation of a single backbone amide group in the edge strand of a β -sheet can effectively block extension of the sheet *via* dimerization²⁸ but may have little or no effect on folded protein stability²⁹, while a single backbone N-Me group in a non-H-bonding position does not preclude formation of β -hairpins³⁰⁻³². The above notwithstanding, Pro and N-Me groups at non-H-bonding positions in β -strands often impose non-ideal geometries on the extended chain that can be expected to have some destabilizing effects^{27-29, 33}. Thus, although the edge strand in an anti-parallel β -sheet of satellite tobacco mosaic virus coat protein contains a Pro residue at a non-H-bonding position while exhibiting normal backbone H-bonding (Fig. 2 a,b), the corresponding edge strand in an anti-parallel β -sheet of UDP-N-acetylglucosamine acyltransferase places the Pro in a non-H-bonding position but also features some non-canonical backbone H-bonding (Fig. 2, c,d). Whether or not the structure of the Pro-containing edge strand features a distorted H-bonding pattern, these segments nonetheless are incorporated stably into the edge strands, and at the same time can be considered to be “protected” from extension of the β -sheet and therefore examples of negative design features that protect evolved proteins from unwanted aggregation³⁴.

Conformation and aggregation of polyQ with multiple β -hairpin motifs

The β HP peptide (Fig. 1a) was designed to contain two separate β -hairpin enhancing motifs: (a) D-Pro-Gly in the middle of the sequence, and (b) a “trpzip” motif, centered on Trp residues near the peptide N- and C-termini, that can encourage peptide closure non-covalently²¹. We showed previously for this peptide that, while a $\sim 100 \mu\text{M}$ K₂Q₂₃K₂ sequence requires about 200 hrs to aggregate to 50% completion, a much lower concentration (14 μM) solution of a similar polyQ length sequence β HP containing these two β -hairpin motifs requires only ~ 3 hrs to achieve 50% aggregation²¹ (Fig. 3, $\blacklozenge$). The amyloid product of the β HP peptide consists of bundles of thin filaments (Fig. 4b) with a somewhat less rigid appearance than the small blocks of aligned filaments typically seen for K₂Q₂₃K₂ fibrils (Fig. 4a).

The large rate enhancement seen for the β HP peptide is achieved in part because the effects of the two individual β -hairpin mutations are additive²¹. To achieve this additivity, we had to ensure that the placement of the two β -hairpin motifs was complementary, in the context of the organizing principles for anti-parallel β -sheet and β -hairpins outlined above (Fig. 1b). Thus, trpzip motifs are known to place the Trp residue pair at non-H-bonded positions in β -hairpins²⁶. Likewise, both previous literature and our own structural studies suggest that the Gln residues flanking the D-Pro-Gly sequence in β HP and related peptides will be found in H-bonded positions in the β -hairpin. For example, previous studies in a non-polyQ context have shown that D-Pro-Gly can adopt a two-residue ‘mirror image’ turn that is not accessible to its L-Pro counterpart³⁵⁻³⁷. To probe whether this is the case for polyQ sequences, we applied magic angle spinning (MAS) solid-state NMR (ssNMR) spectroscopy to fibrils of polyQ peptides featuring stable-isotope labeled glutamines surrounding a central D-Pro-Gly insertion (Fig. 5a). The labeled sites were assigned using 1D and 2D ssNMR experiments (Supplemental Fig. 2). We previously identified the NMR chemical shifts of the β -strands forming the polyQ amyloid core^{24, 38}. The ¹³C chemical shifts of the Gln residues flanking

the D-Pro-Gly motif (Fig. 5b, bottom) reproduce those characteristic and unusual β -strand shift patterns (Fig. 5b, Supplemental Fig. 2). Thus, the flanking Gln adopt two distinct conformations that allow for backbone- and side-chain hydrogen bonding in the antiparallel β -sheet²⁴. Interestingly, this contrasts with L-Pro-Gly insertions in polyQ, which were previously shown to prevent the directly flanking Gln residues from taking part in these β -strands²⁴. Given these structural preferences of the trpzip and D-Pro-Gly motifs, the number of intervening Gln residues in β HP were chosen to allow a complementary, self-reinforcing alternating pattern of H-bonded and non-H-bonded amino acid pairs up and down the β -hairpin (Fig. 1a).

Importantly, in spite of the presence of two complementary β -hairpin motifs that greatly enhance spontaneous aggregation, the β HP monomer exhibits a CD spectrum essentially unchanged from the random coil-like spectrum of unbroken polyQ²¹. This finding speaks against a mechanism for aggregation enhancement involving a substantial increase in the steady state concentration of β -hairpin monomers. The absence of a measurable increase in β -structure in the β HP monomer ensemble is not, however, inconsistent with a role of the β -hairpin motifs in facilitating nucleation, elongation, and fibril stabilization (see Discussion).

NMeGln modifications within β HP

Previously we utilized single L-Pro residues placed midway within polyQ segments between two L-Pro-Gly motifs to produce mutated polyQ peptides exhibiting impaired ability to undergo spontaneous aggregation¹⁹ but significant aggregation inhibitor activity²⁵. These peptides were hypothesized to work as inhibitors by being able to attach to the growing polyQ fibril but, while resident on the fibril end, to inhibit new additions of polyQ²⁵ due to the poor ability of Pro residues to be located at H-bonding, interior positions in β -sheets²⁷. Here we extend this design concept by preparing mutations within the more tightly constrained β HP peptide that allows much better control over the structural roles of each residue position. We first prepared a β HP variant (β HP-NMeQ²⁰; Fig. 1a, Table 1) containing a single N-Me group on the backbone amide (for structure, see the model in Fig. 5c) of a predicted *non-H-bonding* Gln residue centrally located in the C-terminal Q₁₁ arm in the latent β -hairpin of β HP.

We found that β HP-NMeQ²⁰ exhibits no detectible aggregation after 300 hrs when incubated at 18 μ M (Fig. 3, ●), while at a similar concentration β HP itself aggregates to completion within 20 hrs (Fig. 3, ◆). The β HP-NMeQ²⁰ peptide is not completely incapable of spontaneous aggregation, however, forming typical polyQ-like amyloid (Fig. 4c) upon incubation for long periods of time at relative high, millimolar concentrations. The slow kinetics of this residual aggregation behavior, in analogy to the well-known dependence of aggregation rates on polyQ repeat length^{39, 40}, is consistent with a model in which only the short, unbroken Q₁₁ arm of this peptide (Fig. 1a) is available for amyloid formation.

The very low aggregation tendency of β HP-NMeQ²⁰ allowed us to contemplate a more thorough examination of its solution structure than is possible with a highly aggregation-prone derivative. In far UV CD spectroscopy, this peptide exhibits a random coil spectrum essentially identical to those of β HP itself²¹ as well as K₂Q₂₃K₂ (Fig. 6a). The absence of a substantial amount of β -hairpin structure within the monomer suggested by the CD spectrum

is also supported by proton NMR analysis. Although this analysis was complicated by the time-dependent fibril formation of the sample described above, we were able to acquire good data from a freshly disaggregated monomer sample before any substantial aggregation occurred (Fig. 6b). From these data, we estimated the potential maximal β -structural content in this peptide using the spectral analysis method of Wishart et al.⁴¹. Integration of the spectral region from 4.85 to 5.90 ppm yields a relative integrated area of 0.078, normalized relative to the Trp proton resonances at ~ 10.1 ppm (Fig. 6b). Given that there are 12 amide protons with the potential to be H-bonded in the β -hairpin (Fig. 1a), this corresponds to an estimate of $\sim 0.6\%$ of the maximum possible polyQ β -sheet content. Although we cannot exclude the possibility that some of this amplitude may be due to a small level of impurities, it is interesting to note that several of the small, individual peaks in this spectral region each give integrated areas corresponding to 0.6% of one proton. If, nonetheless, some or all of the peaks in this region are due to small impurities, then the β -content of $\beta\text{HP-NMeQ}^{20}$ would be even lower than 0.6% .

Based on the above described structural model for the poor spontaneous aggregation ability of the $\beta\text{HP-NMeQ}^{20}$ peptide, it seemed possible that it might be capable of inhibiting the aggregation of other polyQ peptides. In fact, we found that $\beta\text{HP-NMeQ}^{20}$ is an excellent inhibitor of spontaneous aggregation of $\text{K}_2\text{Q}_{23}\text{K}_2$ (Fig. 7 a,b). Thus, while a $150\text{ }\mu\text{M}$ concentration of $\text{K}_2\text{Q}_{23}\text{K}_2$ begins to aggregate within 50 hrs and goes to 20% completion in ~ 70 hrs (Fig. 7, ■), when this level of $\text{K}_2\text{Q}_{23}\text{K}_2$ is incubated with $40\text{ }\mu\text{M}$ of $\beta\text{HP-NMeQ}^{20}$ (an approximate 1:4 ratio), no aggregation is observed after 350 hrs (Fig. 7a, ●). When, in a separate experiment, the ratio of $\beta\text{HP-NMeQ}^{20}$ to $\text{K}_2\text{Q}_{23}\text{K}_2$ is reduced from 1:4 to 1:8, the time to 20% aggregation of $\text{K}_2\text{Q}_{23}\text{K}_2$ is almost doubled, being extended from ~ 70 hrs to 135 hrs (Fig. 7b, ●). These results are noteworthy, since most previously described peptide-based inhibitors of amyloid formation, including polyQ amyloid formation, require stoichiometric amounts, or even molar excesses, to achieve measurable inhibition (see Discussion).

As an extension of the above model, and consistent with the thermodynamic model for nucleation⁴², the $\beta\text{HP-NMeQ}^{20}$ peptide is incapable of efficient spontaneous aggregation, not because it cannot form monomeric β -hairpin quasi-nuclei (as suggested by the solution NMR experiment above), but because these transiently formed β -hairpins cannot elongate and therefore soon disassemble and return to the compact coil monomer ensemble. To test this model, we examined the ability of $\beta\text{HP-NMeQ}^{20}$ to inhibit seeded elongation. For the uninhibited arm of the experiment, we found that 12% by weight of $\text{K}_2\text{Q}_{23}\text{K}_2$ amyloid fibrils added to $150\text{ }\mu\text{M}$ monomeric $\text{K}_2\text{Q}_{23}\text{K}_2$ dramatically enhances the amyloid formation rate (Fig. 7c, ●) compared to $\text{K}_2\text{Q}_{23}\text{K}_2$ alone (■). With the addition of $17\text{ }\mu\text{M}$ $\beta\text{HP-NMeQ}^{20}$ to an identical seeded reaction, the rate of elongation is significantly reduced (●). This supports the conclusion that $\beta\text{HP-NMeQ}^{20}$ is fundamentally an elongation inhibitor and shows that it is quite effective at slowing down seeded elongation even when only present at a ratio of about 1:9 with respect to $\text{K}_2\text{Q}_{23}\text{K}_2$ monomers.

To test the validity of the structural thinking that went into the design of the $\beta\text{HP-NMeQ}^{20}$ peptide, we prepared an analog, $\beta\text{HP-NMeQ}^{19}$, in which the *N*-methyl group is moved to a directly adjacent backbone amide projected to be in an H-bonding position in any β -hairpins

formed (Figs. 1a, 5c). Unlike the *N*-methylation at Q²⁰, this modification is expected to strongly discourage β -hairpin formation within the highly constrained β HP peptide, allowing us to test our model that β -hairpin formation is required for effective inhibition of polyQ aggregation *in trans*. In analogy to β HP-^{NMe}Q²⁰ and β HP itself, we found that this β HP-^{NMe}Q¹⁹ peptide exhibits a random coil CD spectrum as a freshly disaggregated monomer, consistent with negligible β -hairpin formation in the monomer ensemble (Fig. 6a). In addition, the β HP-^{NMe}Q¹⁹ peptide exhibits no detectible spontaneous aggregation (Fig. 3, ●) under conditions where β HP itself aggregates very efficiently. However, in contrast to β HP-^{NMe}Q²⁰, β HP-^{NMe}Q¹⁹ is completely incapable of inhibiting spontaneous K₂Q₂₃K₂ aggregation at both a 1:4 (Fig. 7a, ●) and a 1:8 (Fig. 7b, ●) ratio. Thus, the data support our hypothesis that the inhibitory activity of the β HP-^{NMe}Q²⁰ peptide is linked to its ability to access the β -hairpin conformation when transiently bound to fibril ends (Fig. 1a) (see Discussion).

An L-Pro substitution within β HP

The weakness of the β HP-^{NMe}Q²⁰ peptide in spontaneous amyloid formation and its potent ability to inhibit amyloid formation by other polyQ peptides suggest the prospect of using such peptides in cell and animal models to test mechanisms of polyQ toxicity. Unfortunately, the D-Pro and ^{NMe}Gln modifications found in β HP-^{NMe}Q²⁰ cannot be produced biosynthetically *in vivo*, limiting their potential use. While there are a number of alternative options to D-Pro-Gly for β -hairpin enhancing motifs²¹, the only one of the 20 amino acids standardly used in ribosomal protein synthesis with an alkylated backbone amide nitrogen that might emulate the effects of backbone N-methylation is L-Pro. As discussed above, Pro residues are often found in non-H-bonding positions on the edge strands of anti-parallel β -sheets²⁷, an environment structurally analogous to the β -strands of a β -hairpin. We therefore prepared the analog β HP-P²⁰ (Table 1, Fig. 1a). As with all β HP sequences, and indeed as with all polyQ sequences³⁹, we found that β HP-P²⁰ exhibits a random coil like CD spectrum (Fig. 6a). In addition, like both *N*-methyl derivatives of β HP, we found that β HP-P²⁰ exhibits no detectible aggregation (Fig. 3, ▲) at a concentration where the parent β HP peptide aggregates very rapidly. Most importantly, in analogy to the activity of β HP-^{NMe}Q²⁰, we found that the β HP-P²⁰ peptide is a very good inhibitor of K₂Q₂₃K₂ spontaneous aggregation. Thus, when incubated at 1:4 inhibitor to the K₂Q₂₃K₂ peptide, no aggregation is observed (Fig. 7a, ●) up to 350 hrs, and even at a 1:8 ratio with K₂Q₂₃K₂ the time to reach 20% aggregation is shifted from 70 hrs to ~ 195 hrs (Fig. 7b, ●), a level of inhibition somewhat better than that seen for β HP-^{NMe}Q²⁰. As expected for a reaction where normal nucleation is delayed rather than eliminated, product fibrils in both of the inhibited reactions entirely resemble K₂Q₂₃K₂ fibrils in the EM (Fig. 4 d,e).

DISCUSSION

Although intrinsically disordered proteins are defined by their inability to achieve a folded state under normal solution conditions, some disordered sequences can be persuaded to adopt an ordered state by the introduction of β -hairpin inducing mutations^{26, 43-47}. In contrast, CD spectra of polyQ peptides containing such mutations show no evidence for the significant acquisition of β -structure, or any regular structure, in the monomer ensemble²¹.

The same mutated polyQs, however, exhibit greatly enhanced spontaneous amyloid formation, which is thought to depend on the formation of a β -hairpin-like critical nucleus^{21, 40}. These seemingly disparate observations can be reconciled if one considers the energetics of nucleation. Since the apparent monomeric nucleation equilibrium constant K_{N^*} for polyQ amyloid formation is typically in the range of 10^{-10} to 10^{-14} ^{21, 48}, the 10-100 fold improvement in the equilibrium constant for β -hairpin formation expected from β -hairpin inducing mutations^{21, 26} would be insufficient to allow detection of these β -hairpin forms by conventional spectroscopy. The special resistance of polyQ to acquiring regular folded monomeric states may be a consequence of a conformational ensemble dominated by robust, fluctuating side chain-main chain H-bonding⁴⁹, a factor that also may be responsible for the compact coil structures reported for polyQ sequences longer than about Q₁₅⁵⁰⁻⁵². In spite of the resistance of polyQ peptides to accessing stable β -hairpin conformations in the monomer ensemble, these peptides – mutated or not – readily access such conformations when incorporated into the amyloid fibril core²⁴.

The reluctance of polyQ to form β -hairpins in the monomeric state is highly relevant given suggestions that the toxic species generated in expanded polyQ repeat diseases is a monomeric hairpin-like structure^{9, 10}. It was therefore seen as important to go beyond CD spectroscopy to probe the resistance of polyQ to β -hairpin encouraging mutations. Unfortunately, the greatly increased ability of β HP to aggregate rules out solution NMR spectroscopy on this molecule. The β HP-NMeQ²⁰ design potentially addresses this problem. Based on the ability of edge strands of β -sheet^{28, 29} and β -hairpins³⁰⁻³² to accommodate backbone N-Me groups in non-H-bonding positions without substantial destabilization, we hypothesized that an *N*-methyl group installed at one of the non-H-bonding positions of the predicted β HP β -hairpin should not greatly interfere with β -hairpin stability, but should interfere with amyloid elongation. In fact, the single N-Me group in β HP-NMeQ²⁰ renders this peptide highly resistant to spontaneous amyloid formation (Fig. 3). Given the peptide's single unbroken Q₁₁ arm, however, the peptide is not completely blocked from slowly forming fibrils at mM concentrations (Fig. 4c), and this limits the amount of time available for NMR data collection. In spite of this challenge, a good NMR spectrum of β HP-NMeQ²⁰ in water was obtained (Fig. 6b), which confirms a very low upper limit to β -hairpin content of less than 1% (Results). These data underscore a central problem for the "toxic monomer" hypothesis for polyQ toxicity: the compact coil nature of polyQ, which is the favored form of the monomer for sequences from Q₁₆^{51, 52} to Q₅₃⁵⁰, is highly resistant to engaging any stable, regular secondary structure, including a β -hairpin. Previous solution NMR studies already demonstrated the absence of detectible β -structure in simple monomeric polyQ peptides before they become incorporated into amyloid⁵³⁻⁵⁵. The NMR results reported here confirm our previous CD results²¹ in showing that β -hairpin-like conformations are not only poorly populated in β HP and similar sequences, they are highly energetically discriminated against compared with other possible monomer conformations.

Given the apparent stabilities of both the compact coil monomer and the β -hairpin-based amyloid fibril, how do we imagine the elongation process, which accounts for the incorporation of a new monomer into the growing fibril, to proceed? A hypothesis that elongation depends on the pool of intact β -hairpins present within the monomer ensemble is theoretically possible, but seems unlikely given the very low steady state concentration of β -

hairpins (see Results). This very low concentration of monomers with β -structure was the primary motivation for the proposed⁵⁶ alternative elongation scenario of a templated “dock-and-lock” mechanism, as originally proposed⁵⁷ and kinetically validated⁵⁸ for A β amyloid growth. As shown in the schematic mechanism in Figure 8a, the incoming compact coil polyQ monomer, under the influence of the unsatisfied H-bonding donor and acceptor array on the edge strand of the fibril, docks and aligns to form a metastable adduct held in place by a limited number of newly formed H-bonds involving main chain and, potentially, side chain groups²⁴. This newly added peptide can either dissociate, or consolidate fibril structure in one or more subsequent folding (“locking”) steps on the edge of the fibril. For the β HP peptide, it is anticipated that the β -hairpin enhancing motifs, which are insufficient to populate a β -hairpin conformation in the native monomer ensemble, contribute to the downstream folding steps in the dock-and-lock mechanism to enhance elongation efficiency in analogy to their substantial stabilizing effects on fibrils²¹. In fact, Walters et al. report a 3-fold rate increase for elongation of a polyQ amyloid by a short polyQ outfitted with a central D-Pro-Gly²².

The effects of the various mutants of the β HP peptide on amyloid formation described here can be rationalized within this templated dock-and-lock scheme. Since the β HP peptide itself has been shown to initiate aggregation *via* a monomeric critical nucleus (i.e., $n^* = 1$) that is most likely a β -hairpin²¹, both β HP-NMeQ²⁰ and β HP-P²⁰ should also be able to support the formation of a nucleus-like β -hairpin (Fig. 1a) with minimal destabilization (see Results). However, in the thermodynamic model of nucleated growth polymerization⁴², the overall nucleation process requires both nucleus formation and nucleus elongation⁵⁹. This is why a strong elongation inhibitor can be highly effective at blocking nucleation (Fig. 7), and why a nucleus-like structure that can’t elongate is not an effective nucleus (Fig. 3). Nonetheless, low efficiency aggregation is still observed with β HP-NMeQ²⁰, presumably since the unbroken Q₁₁ arm is long enough to support spontaneous aggregation *via* a larger and less efficient critical nucleus that excludes the N-methylated Gln from the H-bond network⁴⁰. In fact, polyQ sequences as short as Q₈ are able to make amyloid fibrils exhibiting a typical polyQ amyloid core structure, if they are able to overcome the highly stringent nucleation barrier¹³.

The inhibition activity of the mutants is also consistent with the dock and lock mechanism, and a model positing that consolidation of the β -hairpin structure within the newly added monomer on the fibril edge greatly enhances the inhibitor's association constant. This is certainly true of the β HP peptide itself, whose fibril formation reaction reaches a point of dynamic equilibrium balancing monomer association and dissociation that is 30-fold more favorable for fibril formation than a simple, unbroken polyQ of similar length²¹. In this model, the mutants β HP-NMeQ²⁰ and β HP-P²⁰, with their largely unencumbered internal H-bonding patterns within the β -hairpin, are able to finish the dock-and-lock cycle, leaving in place a newly added β -hairpin on the fibril edge with an exposed edge strand containing a blockage at a non-H-bonding (Fig.1b) amide that prevents addition of another polyQ monomer (Figs. 5c, 8b). So long as this peptide remains in place – with a binding constant aided by its energetically favorable consolidated β -hairpin structure – no additional polyQ peptides can productively add to this site. Addition of the β HP-NMeQ²⁰ or β HP-P²⁰ molecule is nonetheless reversible, however, and whenever it dissociates there is a renewed

opportunity for productive addition of normal, elongation-competent polyQs. Inhibition is therefore leaky, and fibril elongation (Fig. 7c), and therefore the ability to complete the nucleation process⁴² (Fig. 7 a,b), are slowed, but not eliminated, by the presence of the mutated β HP molecule. Given this requirement for strong inhibitor binding, inhibition by these two mutants is remarkably efficient, for example in essentially completely shutting down spontaneous $K_2Q_{23}K_2$ aggregation at a ratio of one molecule of inhibitor to four molecules of $K_2Q_{23}K_2$ for extended periods (Fig. 7a). In comparison, small, L-Pro mutated fragments of A β , for example, achieved good inhibition of amyloid formation by a target A β peptide only at stoichiometric or higher ratios⁶⁰. The somewhat better inhibition observed for β HP-P²⁰, compared with β HP-NMeQ²⁰, is intriguing, and may indicate that the β HP-P²⁰ peptide has a slightly better association constant for the fibril edge that may be related to a less disruptive influence on β -hairpin formation by the β -breaking modification.

In contrast to these successful inhibition results, under the conditions of our experiments, the β HP-NMeQ¹⁹ peptide is completely incapable of inhibiting $K_2Q_{23}K_2$ aggregation (Fig. 7 a,b). This lack of inhibition probably derives from some combination of the following expected effects, all visualized in the context of the dock-and-lock mechanism (Fig. 8c). While β HP-NMeQ¹⁹ peptide may be capable of transiently adding to the growth edge of the $K_2Q_{23}K_2$ nucleus or fibril, it is not expected to be able to efficiently close to the β -hairpin form due to the disruption of the H-bonded position within the hairpin (Fig. 5c). This failure to close has two consequences. First, it is expected to decrease the association constant of β HP-NMeQ¹⁹ for the β -sheet edge, creating a situation where the target fibril/nucleus is more often exposed to another incoming $K_2Q_{23}K_2$ molecule. Second, should the added β HP-NMeQ¹⁹ peptide remain associated, it will do so only by virtue of its unbroken Q₁₁ arm, which of course should be capable of supporting further additions of $K_2Q_{23}K_2$ (Fig. 8c).

Previous attempts to develop polyQ aggregation inhibitors by modifications of a latent β -hairpin structure have been only modestly successful. Thakur and Wetzel showed that long polyQ mutants featuring alternating segments of Q₉ and L-Pro-Gly exhibit excellent amyloid formation kinetics¹⁹, while the addition of mid-strand L-Pro substitutions for Gln not only abrogated spontaneous aggregation¹⁹ but also bestowed the ability to inhibit, in *trans*, the aggregation of a $K_2Q_{50}K_2$ peptide²⁵. Inhibitory activity was modest, however, requiring an approximate 4:1 ratio of inhibitor to $K_2Q_{50}K_2$ for good inhibition. Lanning et al. prepared a Q₅-D-Pro-Gly-Q₅ derivative in which both Q₅ arms contained multiple backbone N-Me-Gln modifications; this peptide partially inhibited aggregation of a Q₁₂ peptide when present in a 40:1 excess over Q₁₂⁶¹. The vastly improved inhibitory activities of the latent β -hairpin derivatives β HP-NMeQ²⁰ or β HP-P²⁰ described here (Fig. 7) are presumably due to a combination of design features. First, we used complementary β -hairpin motifs²¹ as well as a polyQ strand length sufficient to optimize β -hairpin potential^{62, 63}. Second, we used a design featuring one unmodified polyQ arm to effectively engage the growth face of the fibril/nucleus, and one modified arm – with the single Gln mutation implanted at a non-H-bonding position (Fig. 1) of the β -hairpin – to compromise further additions of polyQ to the growing aggregate.

In addition to the polyQ work discussed above, *N*-methylation of selected backbone amide groups in modified peptides has been shown previously to inhibit fibril formation both by

the modified peptide itself and in *trans* with the unmodified version, both in a fragment of the A β peptide⁶⁴ and in full-length islet amyloid polypeptide (IAPP)⁶⁵. As inhibitors of amyloid formation by the wild type peptides, the efficacies of these N-Me versions, when tested at 1:1 stoichiometry, ranged from good to excellent. Results from tests at lower stoichiometries were not reported. Other peptides, featuring sequences non-homologous with amyloid targets and outfitted with β -hairpin encouraging motifs, have also been shown to be moderately effective, non-specific inhibitors of amyloid assembly⁶⁶⁻⁶⁹.

We hypothesize that our observations of improved inhibitory activity of β HP-NMeQ²⁰ and – perhaps more surprisingly – the stark absence of inhibition by β HP-NMeQ¹⁹ both stem from their carefully designed locations within a mutant, β HP, possessing highly constrained conformational mobility. Although similar strategies may be applicable for other amyloid systems, it should be noted that, while the polyQ amyloid coil consists of anti-parallel β -sheets congruent with β -hairpins, most biological amyloid fibrils characterized so far (including A β and IAPP) are found in *parallel* β -sheets in which intramolecular turns within monomer units in the amyloid core – if they occur at all – are not β -turns but rather what have been termed “ β -arches”⁷⁰. Thus, for this class of amyloid structural motif the strategy of introducing N-Me or L-Pro modifications within a sequence constrained to adopt a β -hairpin structure would not be predicted to be productive, at least not for inhibitory mechanisms fashioned around the dock-and-lock elongation mechanism. In this context, it is interesting that the previously described (see above) β -hairpin-related inhibitors of A β and IAPP are postulated to work by attacking monomers or pre-amyloid aggregated states⁶⁶⁻⁶⁹ rather than the fibril elongation process.

Given the long time course of the developing cytotoxicity in response to expanded polyQ expression, with a programmatic rise and fall of various metastable and stable molecular forms of polyQ and various cell pathologies, it has been extremely difficult to construct a convincing case for a causative role for any one molecular species. Absent this much-desired but highly elusive result, we are developing a dual approach consisting of one arm in which we catalog the stabilities and hence physical realities connected with hypothesized toxic physical states of polyQ¹⁴, and a second arm of developing analogs capable of enhancing or suppressing various states *in vivo* in order to directly probe the activities of hypothesized toxic physical states^{21, 71}. The results described here represent advances in both of these efforts. Regarding the first arm, the proton NMR spectrum (Fig. 6b) of the aggregation resistant β HP-NMeQ²⁰ peptide confirms and extends results of previous CD and NMR studies indicating a very low level of β -structure in the native monomer ensemble of either simple polyQ^{39, 53-55, 72} or polyQ with β -hairpin enhancing motifs²¹. These data, coupled with recent results showing the absence of any monomeric forms of expanded polyQ Huntingtin exon1 *in vitro* and *in vivo*¹⁴, represent strong biophysical arguments against monomeric β -hairpin forms of polyQ as toxic species. For the second arm, the aggregation inhibition data for the β HP-P²⁰ peptide (Fig. 7) suggest the possibility of extending our design approach to develop ribosomal synthesis-competent analogs that can be used, either alone or in co-expression with expanded polyQ disease proteins, to test various hypotheses about the physical nature of the toxic species⁷¹. Such derivatives possessing inhibitory activity that neutralizes polyQ toxicity would then become obvious candidates for lead

structures for a new class of drugs attacking this series of genetic neurodegenerative diseases.

METHODS

Materials and general methods

Peptides β HP-P²⁰, K₂Q₂₃K₂, and K₂Q₁₁pGQ₁₁K₂ (pG-Q₂₂) were obtained as crude reaction products from the small scale custom synthesis of peptides at the Keck Biotechnology Center at Yale University. Peptide K₂Q₁₁pGQ₁₁K₂ was synthesized with U-¹³C, ¹⁵N-labeled residues (Q¹³, G¹⁵, and Q¹⁶) surrounding the D-Pro residue. Peptide β HP was prepared as described²¹. General polyglutamine peptide handling procedures, including reverse phase HPLC purification of crude synthetic products, concentration determination, and disaggregation, were described previously^{73, 74}. Peptides are always disaggregated immediately before any experiment. Far-UV CD measurements were performed on 20 - 30 μ M peptide solutions in 20 mM Tris.HCl, pH 7.4, on a JASCO J-810 spectropolarimeter using a 0.1 cm path length cuvette.

Synthesis of N-Me-Gln containing peptides

Site specifically *N*-methylated peptides were prepared by incorporating a suitably protected N-Me-Gln derivative (Supplemental Material) through Fmoc solid phase synthesis.

Peptide β HP-NMeQ²⁰ was synthesized by microwave-assisted Fmoc solid-phase synthesis (MARS microwave, CEM Corp.) on NovaPEG Lys(Boc) HMPB NovaPEG resin. Couplings were carried out in *N*-methyl-2-pyrrolidone (NMP) with a two minute ramp to 70 °C and a four minute hold at that temperature, using 4 equivalents of Fmoc-protected amino acid, 4 equivalents of O-(6-Chlorobenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HCTU) (or (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) in the case of the Fmoc-NMeQ residue), and 6 equivalents *N,N*-diisopropylethylamine (DIEA). Deprotections were performed with a 2 minute ramp to 80 °C followed by a two minute hold at that temperature, using 20% 4-methylpiperidine in dimethylformamide (DMF). After each coupling or deprotection cycle, the resin was washed three times with DMF.

Peptide β HP-NMeQ¹⁹ was synthesized using a PTI Tribute Automated Peptide Synthesizer on NovaPEG Lys(Boc) HMPB NovaPEG resin. Couplings were carried out in NMP using 45 minute coupling reactions run with 7 equivalents of Fmoc-protected amino and 7 equivalents of HCTU in 0.4 M *N*-Me-morpholine in DMF. For addition of the Fmoc-NMeQ residue in peptide β HP-NMeQ¹⁹, 5 equivalents of Fmoc-protected amino acid were used with 5 equivalents of PyBOP. All deprotection reactions were carried out using an excess of 20% 4-Me-piperidine in DMF and N-terminal acetylation was carried out on resin by treatment with 8:2:1 v/v/v DMF:DIEA:Ac₂O.

Prior to cleavage, resins were washed three times each with DMF, dichloromethane, and methanol and then dried under vacuum. Peptide cleavage was performed using 94% v/v trifluoroacetic acid, 2.5% v/v water, 2.5% v/v ethanedithiol, and 1% v/v triisopropylsilane. After 3-4 h, the cleavage mixture was filtered, precipitated into cold anhydrous ether,

centrifuged, and the supernatant drained to afford the peptide as a crude pellet which was purified by reverse phase HPLC.

Aggregation kinetics analysis

All peptides were disaggregated just prior to use. Aggregation data was determined by sedimentation of reaction aliquots at different aggregation times, followed by analytical HPLC determination of the concentration of monomer in the sedimentation supernatant ⁷³. Since K₂Q₂₃K₂ migrates differently in RP-HPLC under our conditions compared with the various β HP inhibitors, the HPLC sedimentation assay allowed direct measurement of K₂Q₂₃K₂ at different reaction times regardless of the presence of inhibitor. Preparation and concentration determination of K₂Q₂₃K₂ amyloid seed stocks were as described ^{40, 75}.

Solution NMR spectroscopy

One-dimensional ¹H NMR spectrum was recorded at 25 °C for freshly disaggregated 0.75 mM β HP-NMEQ²⁰ in PBS (pH 7.2) using a Bruker pulse sequence (zgpg30) utilizing water suppression with excitation sculpting with gradients ⁷⁶ on a Bruker AVANCE 800 spectrometer, equipped with 5 mm triple-resonance, z-axis gradient cryoprobe. Total acquisition time was 1.2 h (2810 scans).

MAS ssNMR experiments

Fibril formation by labelled K₂Q₁₁pGQ₁₁K₂ was performed in PBS buffer (pH 7.4) at 37 °C and allowed to proceed to completion as determined by the HPLC sedimentation assay. Fibrils were washed with deionized water or PBS buffer and then pelleted into 3.2 mm zirconia MAS rotors (CortecNet, Voisins-le-Bretonneux, France) using a home-built ultracentrifugal sample packing tool. The sample was kept unfrozen and hydrated at all times. MAS ssNMR experiments were performed with a widebore Bruker Avance I NMR spectrometer operating at 600 MHz ¹H Larmor frequency (14.1 T) using a 3.2 mm Efree HCN (Bruker Biospin) MAS NMR probe. ¹³C assignments were based on 2D ¹³C-¹³C experiments obtained with ¹H-¹³C cross polarization (CP) and dipolar assisted rotational resonance (DARR) ¹³C-¹³C transfer ⁷⁷ as well as 2D single quantum - double quantum (SQ-DQ) experiments with the ¹³C-¹³C DQ coherence generated via SPC5₃ mixing ⁷⁸. 1D and 2D ¹⁵N-¹³C double-CP-based experiments, including NCACX, NCOCX, and CONCX measurements ⁷⁹, were used to make intra- and inter-residue assignments of ¹⁵N and ¹³C chemical shifts. Typically, 83 kHz two pulse phase modulation (TPPM) ⁸⁰ ¹H decoupling was applied during acquisition and evolution. The sample temperature was regulated using a constant flow of cooled gas. Spectra were processed and analyzed with NMRPipe ⁸¹, Sparky, and CCPNMR/Analysis software ⁸². External referencing to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was performed based on the ¹³C signals of adamantane ⁸³.

Electron microscopy

An aliquot of 5 μ l from the ongoing aggregation reaction mixture at different time points was placed on freshly glow-discharged carbon coated 400 mesh size copper grids. This was adsorbed for 2 min, and then the grid was washed with a drop of deionized water before staining the sample with freshly filtered 5 μ l of 1% (w/v) uranyl acetate for 2 sec. The excess

of sample, washes and stains was gently blotted off with filter paper. Grids were imaged in the Structural Biology Department's EM facility on a Tecnai T12 microscope (FEI) operating at 120 kV and 30,000x magnification and equipped with an UltraScan 1000 CCD camera (Gatan) with post-column magnification of 1.4x.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CP	cross polarization
DARR	dipolar assisted rotational resonance
MAS	magic angle spinning
polyQ	polyglutamine
ssNMR	solid-state NMR
TPPM	two pulse phase modulation

REFERENCES

1. Bates, GP., Benn, C. The polyglutamine diseases.. In: Bates, GP.,Harper, PS., Jones, L., editors. Huntington's Disease. Oxford University Press; Oxford, U.K.: 2002. p. 429-472.
2. Wilburn B, Rudnicki DD, Zhao J, Weitz TM, Cheng Y, Gu XF, Greiner E, Park CS, Wang N, Sopher BL, La Spada AR, Osmand A, Margolis RL, Sun YE, Yang XW. An antisense CAG repeat transcript at JPH3 locus mediates expanded polyglutamine protein toxicity in Huntington's disease-like 2 mice. *Neuron*. 2011; 70:427–440. [PubMed: 21555070]
3. Bates GP, Dorsey R, Gusella JF, Hayden MR, Kay C, Leavitt BR, Nance M, Ross CA, Scahill RI, Wetzel R, Wild EJ, Tabrizi SJ. Huntington disease. *Nature Revs Dis Primers*. 2015:15005. [PubMed: 27188817]
4. Orr HT. Polyglutamine neurodegeneration: expanded glutamines enhance native functions. *Curr Opin Genet Dev*. 2012; 22:251–5. [PubMed: 22284692]
5. Wetzel R. Physical chemistry of polyglutamine: intriguing tales of a monotonous sequence. *J Mol Biol*. 2012; 421:466–90. [PubMed: 22306404]
6. Labbadia J, Morimoto RI. Huntington's disease: underlying molecular mechanisms and emerging concepts. *Trends Biochem Sci*. 2013; 38:378–385. [PubMed: 23768628]
7. Wetzel, R., Mishra, R. Structural biology: Order, disorder, and conformational flux.. In: Bates, G.,Tabrizi, SJ., Jones, L., editors. Huntington's Disease 4th edit. Oxford University Press; Oxford, U.K.: 2014. p. 274-322.
8. Trotter Y, Lutz Y, Stevanin G, Imbert G, Devys D, Cancel G, Saudou F, Weber C, David G, Tora L, et al. Polyglutamine expansion as a pathological epitope in Huntington's disease and four dominant cerebellar ataxias. *Nature*. 1995; 378:403–6. [PubMed: 7477379]

9. Zhang QC, Yeh TL, Leyva A, Frank LG, Miller J, Kim YE, Langen R, Finkbeiner S, Amzel ML, Ross CA, Poirier MA. A compact beta model of huntingtin toxicity. *J Biol Chem.* 2011; 286:8188–96. [PubMed: 21209075]
10. Peters-Libeu C, Miller J, Rutenber E, Newhouse Y, Krishnan P, Cheung K, Hatters D, Brooks E, Widjaja K, Tran T, Mitra S, Arrasate M, Mosquera LA, Taylor D, Weisgraber KH, Finkbeiner S. Disease-associated polyglutamine stretches in monomeric huntingtin adopt a compact structure. *J Mol Biol.* 2012; 421:587–600. [PubMed: 22306738]
11. Davies SW, Turmaine M, Cozens BA, DiFiglia M, Sharp AH, Ross CA, Scherzinger E, Wanker EE, Mangiarini L, Bates GP. Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. *Cell.* 1997; 90:537–48. [PubMed: 9267033]
12. Ossato G, Digman MA, Aiken C, Lukacsovich T, Marsh JL, Gratton E. A two-step path to inclusion formation of huntingtin peptides revealed by number and brightness analysis. *Biophys J.* 2010; 98:3078–85. [PubMed: 20550921]
13. Jayaraman M, Kodali R, Sahoo B, Thakur AK, Mayasundari A, Mishra R, Peterson CB, Wetzel R. Slow amyloid nucleation via alpha-helix-rich oligomeric intermediates in short polyglutamine-containing huntingtin fragments. *J Mol Biol.* 2012; 415:881–99. [PubMed: 22178474]
14. Sahoo B, Arduini I, Drombosky KW, Kodali R, Sanders LH, Greenamyre JT, Wetzel R. Folding Landscape of Mutant Huntingtin Exon1: Diffusible Multimers, Oligomers and Fibrils, and No Detectable Monomer. *PLoS One.* 2016; 11:e0155747. [PubMed: 27271685]
15. Poirier MA, Li H, Macosko J, Cai S, Amzel M, Ross CA. Huntingtin spheroids and protofibrils as precursors in polyglutamine fibrilization. *J Biol Chem.* 2002; 277:41032–7. [PubMed: 12171927]
16. Sahl SJ, Lau L, Vonk WI, Weiss LE, Frydman J, Moerner WE. Delayed emergence of subdiffraction-sized mutant huntingtin fibrils following inclusion body formation. *Q Rev Biophys.* 2015:1–13. [PubMed: 25420508]
17. Scherzinger E, Lurz R, Turmaine M, Mangiarini L, Hollenbach B, Hasenbank R, Bates GP, Davies SW, Lehrach H, Wanker EE. Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. *Cell.* 1997; 90:549–58. [PubMed: 9267034]
18. DiFiglia M, Sapp E, Chase KO, Davies SW, Bates GP, Vonsattel JP, Aronin N. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science.* 1997; 277:1990–3. [PubMed: 9302293]
19. Thakur A, Wetzel R. Mutational analysis of the structural organization of polyglutamine aggregates. *Proc Natl Acad Sci U S A.* 2002; 99:17014–17019. [PubMed: 12444250]
20. Walters RH, Murphy RM. Aggregation kinetics of interrupted polyglutamine peptides. *J Mol Biol.* 2011; 412:505–19. [PubMed: 21821045]
21. Kar K, Hoop CL, Drombosky KW, Baker MA, Kodali R, Arduini I, van der Wel PC, Horne WS, Wetzel R. beta-hairpin-mediated nucleation of polyglutamine amyloid formation. *J Mol Biol.* 2013; 425:1183–97. [PubMed: 23353826]
22. Walters RH, Jacobson KH, Pedersen JA, Murphy RM. Elongation kinetics of polyglutamine Peptide fibrils: a quartz crystal microbalance with dissipation study. *J Mol Biol.* 2012; 421:329–47. [PubMed: 22459263]
23. Buchanan LE, Carr JK, Fluit AM, Hoganson AJ, Moran SD, de Pablo JJ, Skinner JL, Zanni MT. Structural motif of polyglutamine amyloid fibrils discerned with mixed-isotope infrared spectroscopy. *Proc Natl Acad Sci U S A.* 2014; 111:5796–5801. [PubMed: 24550484]
24. Hoop CL, Lin HK, Kar K, Magyarfalvi G, Lamley JM, Boatz JC, Mandal A, Lewandowski JR, Wetzel R, van der Wel PCA. Huntingtin exon 1 fibrils feature an interdigitated beta-hairpin-based polyglutamine core. *Proc Natl Acad Sci U S A.* 2016; 113:1546–1551. [PubMed: 26831073]
25. Thakur AK, Yang W, Wetzel R. Inhibition of polyglutamine aggregate cytotoxicity by a structure-based elongation inhibitor. *FASEB J.* 2004; 18:923–5. [PubMed: 15001566]
26. Kier BL, Shu I, Eidenschink LA, Andersen NH. Stabilizing capping motif for beta-hairpins and sheets. *Proc Natl Acad Sci U S A.* 2010; 107:10466–71. [PubMed: 20484672]
27. Wouters MA, Curmi PM. An analysis of side chain interactions and pair correlations within antiparallel beta-sheets: the differences between backbone hydrogen-bonded and non-hydrogen-bonded residue pairs. *Proteins.* 1995; 22:119–31. [PubMed: 7567960]

28. Rajarathnam K, Sykes BD, Kay CM, Dewald B, Geiser T, Baggiolini M, Clark-Lewis I. Neutrophil activation by monomeric interleukin-8. *Science*. 1994; 264:90–2. [PubMed: 8140420]
29. Lengyel GA, Reinert ZE, Griffith BD, Horne WS. Comparison of backbone modification in protein beta-sheets by alpha->gamma residue replacement and alpha-residue methylation. *Org Biomol Chem*. 2014; 12:5375–81. [PubMed: 24909436]
30. Kreutzer AG, Hamza IL, Spencer RK, Nowick JS. X-ray Crystallographic Structures of a Trimer, Dodecamer, and Annular Pore Formed by an Abeta17-36 beta-Hairpin. *J Am Chem Soc*. 2016; 138:4634–42. [PubMed: 26967810]
31. Salveson PJ, Spencer RK, Nowick JS. X-ray Crystallographic Structure of Oligomers Formed by a Toxic beta-Hairpin Derived from alpha-Synuclein: Trimers and Higher-Order Oligomers. *J Am Chem Soc*. 2016; 138:4458–67. [PubMed: 26926877]
32. Spencer RK, Li H, Nowick JS. X-ray crystallographic structures of trimers and higher-order oligomeric assemblies of a peptide derived from Abeta(17-36). *J Am Chem Soc*. 2014; 136:5595–8. [PubMed: 24669800]
33. Reinert ZE, Lengyel GA, Horne WS. Protein-like tertiary folding behavior from heterogeneous backbones. *J Am Chem Soc*. 2013; 135:12528–31. [PubMed: 23937097]
34. Richardson JS, Richardson DC. Natural beta-sheet proteins use negative design to avoid edge-to-edge aggregation. *Proc Natl Acad Sci U S A*. 2002; 99:2754–9. [PubMed: 11880627]
35. Karle IL, Awasthi SK, Balaram P. A designed beta-hairpin peptide in crystals. *Proc Natl Acad Sci U S A*. 1996; 93:8189–93. [PubMed: 8710845]
36. Stanger HE, Gellman SH. Rules for antiparallel beta-sheet design: D-Pro-Gly Is superior to L-Asn-Gly for beta-hairpin nucleation. *J Am Chem Soc*. 1998; 120:4236–37.
37. Stanger HE, Syud FA, Espinosa JF, Giriat I, Muir T, Gellman SH. Length-dependent stability and strand length limits in antiparallel beta-sheet secondary structure. *Proc Natl Acad Sci U S A*. 2001; 98:12015–20. [PubMed: 11593011]
38. Sivanandam VN, Jayaraman M, Hoop CL, Kodali R, Wetzel R, van der Wel PC. The aggregation-enhancing huntingtin N-terminus is helical in amyloid fibrils. *J Am Chem Soc*. 2011; 133:4558–66. [PubMed: 21381744]
39. Chen S, Berthelie V, Yang W, Wetzel R. Polyglutamine aggregation behavior in vitro supports a recruitment mechanism of cytotoxicity. *J Mol Biol*. 2001; 311:173–82. [PubMed: 11469866]
40. Kar K, Jayaraman M, Sahoo B, Kodali R, Wetzel R. Critical nucleus size for disease-related polyglutamine aggregation is repeat-length dependent. *Nat Struct Mol Biol*. 2011; 18:328–36. [PubMed: 21317897]
41. Wishart DS, Sykes BD, Richards FM. Simple techniques for the quantification of protein secondary structure by ¹H NMR spectroscopy. *FEBS Lett*. 1991; 293:72–80. [PubMed: 1959674]
42. Ferrone F. Analysis of protein aggregation kinetics. *Meths. Enzymol*. 1999; 309:256–274.
43. Blanco FJ, Jimenez A, Herranz J, Rico M, Santoro J, Nieto JL. NMR evidence of a short linear peptide that folds into a beta-hairpin in aqueous solution. *J Amer Chem Soc*. 1993; 115:5887–5888.
44. Blanco F, Ramirez-Alvarado M, Serrano L. Formation and stability of beta-hairpin structures in polypeptides. *Curr Opin Struct Biol*. 1998; 8:107–11. [PubMed: 9519303]
45. Lacroix E, Kortemme T, Lopez de la Paz M, Serrano L. The design of linear peptides that fold as monomeric beta-sheet structures. *Curr Opin Struct Biol*. 1999; 9:487–93. [PubMed: 10449370]
46. Espinosa JF, Syud FA, Gellman SH. Analysis of the factors that stabilize a designed two-stranded antiparallel beta-sheet. *Protein Sci*. 2002; 11:1492–505. [PubMed: 12021448]
47. Santiveri CM, Jimenez MA. Tryptophan residues: scarce in proteins but strong stabilizers of beta-hairpin peptides. *Biopolymers*. 2010; 94:779–90. [PubMed: 20564027]
48. Bhattacharyya AM, Thakur AK, Wetzel R. polyglutamine aggregation nucleation: thermodynamics of a highly unfavorable protein folding reaction. *Proc Natl Acad Sci U S A*. 2005; 102:15400–5. [PubMed: 16230628]
49. Wang X, Vitalis A, Wyczalkowski MA, Pappu RV. Characterizing the conformational ensemble of monomeric polyglutamine. *Proteins*. 2006; 63:297–311. [PubMed: 16299774]

50. Crick SL, Jayaraman M, Frieden C, Wetzel R, Pappu RV. Fluorescence correlation spectroscopy shows that monomeric polyglutamine molecules form collapsed structures in aqueous solutions. *Proc Natl Acad Sci U S A*. 2006; 103:16764–9. [PubMed: 17075061]
51. Walters RH, Murphy RM. Examining polyglutamine peptide length: a connection between collapsed conformations and increased aggregation. *J Mol Biol*. 2009; 393:978–92. [PubMed: 19699209]
52. Digambaranath JL, Campbell TV, Chung A, McPhail MJ, Stevenson KE, Zohdy MA, Finke JM. An accurate model of polyglutamine. *Proteins*. 2011; 79:1427–1440. [PubMed: 21337625]
53. Bennett MJ, Huey-Tubman KE, Herr AB, West AP, Ross SA, Bjorkman PJ. A linear lattice model for poly-glutamine in CAG expansion diseases. *Proc Natl Acad Sci U S A*. 2002; 99:11634–11639. [PubMed: 12193654]
54. Klein FA, Pastore A, Masino L, Zeder-Lutz G, Nierengarten H, Oulad-Abdelghani M, Altschuh D, Mandel JL, Trotter Y. Pathogenic and non-pathogenic polyglutamine tracts have similar structural properties: towards a length-dependent toxicity gradient. *J Mol Biol*. 2007; 371:235–44. [PubMed: 17560603]
55. Eftekharzadeh B, Piai A, Chiesa G, Mungianu D, Garcia J, Pierattelli R, Felli IC, Salvatella X. Sequence context influences the structure and aggregation behavior of a polyQ tract. *Biophys J*. 2016; 110:2361–2366. [PubMed: 27276254]
56. Chen S, Ferrone F, Wetzel R. Huntington's Disease age-of-onset linked to polyglutamine aggregation nucleation. *Proc Natl Acad Sci U S A*. 2002; 99:11884–11889. [PubMed: 12186976]
57. Esler WP, Stimson ER, Jennings JM, Vinters HV, Ghilardi JR, Lee JP, Mantyh PW, Maggio JE. Alzheimer's disease amyloid propagation by a template-dependent dock-lock mechanism. *Biochem*. 2000; 39:6288–95. [PubMed: 10828941]
58. Cannon MJ, Williams AD, Wetzel R, Myszkowski DG. Kinetic analysis of beta-amyloid fibril elongation. *Anal Biochem*. 2004; 328:67–75. [PubMed: 15081909]
59. Slepko N, Bhattacharyya AM, Jackson GR, Steffan JS, Marsh JL, Thompson LM, Wetzel R. Normal-repeat-length polyglutamine peptides accelerate aggregation nucleation and cytotoxicity of expanded polyglutamine proteins. *Proc Natl Acad Sci U S A*. 2006; 103:14367–72. [PubMed: 16980414]
60. Soto C, Sigurdsson EM, Morelli L, Kumar RA, Castano EM, Frangione B. Beta-sheet breaker peptides inhibit fibrillogenesis in a rat brain model of amyloidosis: implications for Alzheimer's therapy. *Nat Med*. 1998; 4:822–6. [PubMed: 9662374]
61. Lanning JD, Hawk AJ, Derryberry J, Meredith SC. Chaperone-like N-methyl peptide inhibitors of polyglutamine aggregation. *Biochemistry*. 2010; 49:7108–18. [PubMed: 20583779]
62. Landrum E, Wetzel R. Biophysical Underpinnings of the Repeat Length Dependence of Polyglutamine Amyloid Formation. *J Biol Chem*. 2014; 289:10254–10260. [PubMed: 24596088]
63. Chen MC, Zheng WH, Wolynes PG. Energy landscapes of a mechanical prion and their implications for the molecular mechanism of long-term memory. *Proc Natl Acad Sci U S A*. 2016; 113:5006–5011. [PubMed: 27091989]
64. Hughes E, Burke RM, Doig AJ. Inhibition of toxicity in the beta-amyloid peptide fragment beta - (25- 35) using N-methylated derivatives: a general strategy to prevent amyloid formation. *J Biol Chem*. 2000; 275:25109–15. [PubMed: 10825171]
65. Yan LM, Taterek-Nossol M, Velkova A, Kazantzis A, Kapurniotu A. Design of a mimic of nonamyloidogenic and bioactive human islet amyloid polypeptide (IAPP) as nanomolar affinity inhibitor of IAPP cytotoxic fibrillogenesis. *Proc Natl Acad Sci U S A*. 2006; 103:2046–51. [PubMed: 16467158]
66. Huggins KN, Bisaglia M, Bubacco L, Taterek-Nossol M, Kapurniotu A, Andersen NH. Designed hairpin peptides interfere with amyloidogenesis pathways: fibril formation and cytotoxicity inhibition, interception of the preamyloid state. *Biochem*. 2011; 50:8202–12. [PubMed: 21848289]
67. Hopping G, Kellock J, Caghey B, Daggett V. Designed Trpzip-3 beta-Hairpin Inhibits Amyloid Formation in Two Different Amyloid Systems. *ACS Med Chem Lett*. 2013; 4:824–8. [PubMed: 24900756]

68. Shaykhalishahi H, Mirecka EA, Gauhar A, Gruning CS, Willbold D, Hard T, Stoldt M, Hoyer W. A beta-hairpin-binding protein for three different disease-related amyloidogenic proteins. *Chembiochem*. 2015; 16:411–4. [PubMed: 25557164]
69. Sivanesan K, Shu I, Huggins KN, Tatarek-Nossol M, Kapurniotu A, Andersen NH. Peptide Inhibitors of the amyloidogenesis of IAPP: verification of the hairpin-binding geometry hypothesis. *FEBS Lett*. 2016; 590:2575–83. [PubMed: 27317951]
70. Kajava AV, Baxa U, Steven AC. Beta arcades: recurring motifs in naturally occurring and disease-related amyloid fibrils. *FASEB J*. 2010; 24:1311–9. [PubMed: 20032312]
71. Mishra R, Jayaraman M, Roland BP, Landrum E, Fullam T, Kodali R, Thakur AK, Arduini I, Wetzel R. Inhibiting the nucleation of amyloid structure in a huntingtin fragment by targeting alpha-helix-rich oligomeric intermediates. *J Mol Biol*. 2012; 415:900–17. [PubMed: 22178478]
72. Masino L, Kelly G, Leonard K, Trotter Y, Pastore A. Solution structure of polyglutamine tracts in GST-polyglutamine fusion proteins. *FEBS Lett*. 2002; 513:267–72. [PubMed: 11904162]
73. O'Neill B, Thakur AK, Williams AD, Bhattacharyya AM, Chen S, Thiagarajan G, Wetzel R. Kinetics and thermodynamics of amyloid assembly using a high-performance liquid chromatography-based sedimentation assay. *Meths Enzymol*. 2006; 413:34–74.
74. Jayaraman M, Thakur AK, Kar K, Kodali R, Wetzel R. Assays for studying nucleated aggregation of polyglutamine proteins. *Methods*. 2011; 53:246–54. [PubMed: 21232603]
75. O'Neill B, Shivaprasad S, Kheterpal I, Wetzel R. Thermodynamics of abeta(1-40) amyloid fibril elongation. *Biochem*. 2005; 44:12709–18. [PubMed: 16171385]
76. Hwang TL, Shaka AJ. Multiple-pulse mixing sequences that selectively enhance chemical exchange or cross-relaxation peaks in high-resolution NMR spectra. *J Magn Reson*. 1998; 135:280–7. [PubMed: 9878458]
77. Takegoshi K, Nakamura S, Terao T. C-13-H-1 dipolar-assisted rotational resonance in magic-angle spinning NMR. *Chem Phys Lett*. 2001; 344:631–637.
78. Hohwy M, Rienstra CM, Griffin RG. Band-selective homonuclear dipolar recoupling in rotating solids. *J Chem Phys*. 2002; 117:4973–4987.
79. Shi L, Ladizhansky V. Magic angle spinning solid-state NMR experiments for structural characterization of proteins. *Meths Mol Biol*. 2012; 895:153–65.
80. Bennett AE, Rienstra CM, Auger M, Lakshmi KV, Griffin RG. Heteronuclear decoupling in rotating solids. *J Chem Phys*. 1995; 103:6951–6958.
81. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR*. 1995; 6:277–93. [PubMed: 8520220]
82. Stevens TJ, Fogh RH, Boucher W, Higman VA, Eisenmenger F, Bardiaux B, van Rossum BJ, Oschkinat H, Laue ED. A software framework for analysing solid-state MAS NMR data. *J Biomol NMR*. 2011; 51:437–47. [PubMed: 21953355]
83. Harris RK, Becker ED, De Menezes SM, Granger P, Hoffman RE, Zilm KW. Further conventions for NMR shielding and chemical shifts (IUPAC Recommendations 2008). *Magn Reson Chem*. 2008; 46:582–98. [PubMed: 18407566]
84. Larson SB, Day J, Greenwood A, McPherson A. Refined structure of satellite tobacco mosaic virus at 1.8 Å resolution. *J Mol Biol*. 1998; 277:37–59. [PubMed: 9514737]
85. Raetz CR, Roderick SL. A left-handed parallel beta helix in the structure of UDP-N-acetylglucosamine acyltransferase. *Science*. 1995; 270:997–1000. [PubMed: 7481807]
86. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem*. 2004; 25:1605–12. [PubMed: 15264254]

HIGHLIGHTS

- β -hairpin formation is implicated in polyglutamine amyloid nucleation and structure
- we prepared polyQ peptides combining β -hairpin motifs with backbone modifications
- peptides with $^{\alpha}\text{N-Me-Gln}$ or Pro mutations in the predicted hairpin aggregate poorly
- such mutations at predicted non-H-bonding positions inhibit aggregation in *trans*
- the data clarify assembly mechanisms and provide valuable tools for disease studies

Figure 1.

Positional effects of polyQ β-hairpin structure. a. Design of polyQ peptides containing β-hairpin encouraging mutations, with H-bonding pairs connected by dotted lines joining the two strands and with non-H-bonded amino acids indicated by outwardly projecting arrows.^a N-alkylated amino acids (from the Pro side chain or from an N-Me group) are indicated as green circles at the blocked residue. b. H-bonding patterns within anti-parallel β-sheet. Portion of anti-parallel β-sheet showing how residues in adjacent strands alternate along the width of the β-sheet (arrow) between “H-bonded” (red) and “non-H-bonded” (green) structural roles. Adapted from reference²⁷.

Figure 2.

X-ray structures of edge-strand Pro residues. (a) Anti-parallel β -strands β B and β I, and (b) overall ribbon representation, of the coat protein of satellite tobacco mosaic virus, with the outward-facing Pro-42 indicated (PDB 1A34)⁸⁴. (c) Surface-exposed β -hairpin, and (d) overall ribbon representation, of the β -helical protein UDP-N-acetylglucosamine acyltransferase (PDB 1LXA)⁸⁵. In (c), black arrows indicate carbonyl groups of residues 95 and 96, where the hydrogen bonding pattern is re-arranged but nonetheless continues to close the β -hairpin beyond Pro-94. Graphics prepared using PDB coordinates and UCSF Chimera software⁸⁶.

Figure 3. Spontaneous amyloid growth by polyQ derivatives, expressed as time dependent decrease of monomer remaining after sedimentation of amyloid. Concentrations in key are the starting concentrations.

Figure 4. Electron micrographs of final aggregates. a. $K_2Q_{23}K_2$; b. βHP ; c. $\beta HP-NMeQ^{20}$; d. $K_2Q_{23}K_2 + \beta HP-NMeQ^{20}$; e. $K_2Q_{23}K_2 + \beta HP-P^{20}$. The scale bar is 50 nm.

Figure 5.

ssNMR-based structural model. a. Sequence of the K₂Q₁₁pGQ₁₁K₂ (“pG-Q₂₂”) peptide (¹³C, ¹⁵N-labeled residues bold and underlined) that was studied by MAS ssNMR in the aggregated state. b. 1D ¹³C ssNMR spectra comparing the aliphatic ¹³C signals of Gln residues directly flanking the D-Pro-Gly motif in the pG-Q₂₂ fibrils (bottom) to those of ¹³C, ¹⁵N-labeled Q⁶ within the amyloid core β-strands of K₂Q₃₀K₂ fibrils ³⁸ (top). The D-Pro-Gly motif allows a two-residue turn, with the flanking residues H-bonding to form part of the amyloid core. c. Schematic structural model of the βHP peptide, bound to the exposed β-strand of a pre-existing polyQ aggregate. Red and blue arrows indicate the alternating ‘a’ and ‘b’ type β-strands that make up the polyQ β-sheets ²⁴. Shown are the ssNMR-based core structure of polyQ amyloid ²⁴, the two-residue D-Pro-Gly β-turn, locations of the backbone methylation sites (Q¹⁹ and Q²⁰), and the location of the trpzip tryptophans (W¹ and W²⁶).

Figure 6. Solution structures of polyQ peptides. a. Circular dichroism spectra of freshly disaggregated monomers. PBS, pH 7. b. ^1H NMR spectrum (800 MHz) of a freshly disaggregated 0.75 mM solution of $\beta\text{HP-NMeQ}^{20}$ in pH 7.2 PBS highlighting the 4.85-5.90 ppm H- α proton region.

Figure 7.

Inhibition of spontaneous and seeded amyloid assembly by $\text{K}_2\text{Q}_{23}\text{K}_2$ in PBS, pH 7 as monitored by the HPLC-sedimentation assay. a,b. Spontaneous amyloid assembly by 150 μM $\text{K}_2\text{Q}_{23}\text{K}_2$ alone (■) or with either 1:4 (a) or 1:8 (b) ratios of inhibitor to $\text{K}_2\text{Q}_{23}\text{K}_2$ (●, ●, ●). $\beta\text{HP-NMeQ}_{20}$ (●), $\beta\text{HP-NMeQ}_{19}$ (●), $\beta\text{HP-P}_{20}$ (●). c. Amyloid assembly by $\text{K}_2\text{Q}_{23}\text{K}_2$ incubated alone (■), or with 12% by weight of $\text{K}_2\text{Q}_{23}\text{K}_2$ amyloid fibril seed either with no further additions (●) or with 17 μM $\beta\text{HP-NMeQ}_{20}$ (●).

Figure 8.

Templatd dock-and-lock model for amyloid elongation and its inhibition. a. A successful dock-and-lock elongation cycle. b. Successful inhibition of elongation of a fibril seed or nucleus by a mutant containing a properly configured β -breaker (i.e., β HP-NMeQ²⁰ and β HP-P²⁰). c. Unsuccessful inhibition of elongation by a mutant containing an improperly configured β -breaker that weakens β -hairpin propensity and thereby compromises inhibitory activity. Dark green, existing amyloid or nucleus structure; light green, elongation competent wild type peptide; orange, potential inhibitor; red triangle, β -breaking mutation; red dotted lines, backbone H-bonding.

Table 1

Peptide Sequences

<u>Name</u>	<u>Sequence</u> ^a
βHP	Ac-WQQQQQQQQQ QQpGQQQQQQ QQQQWTGKK
βHP-NMeQ ²⁰	Ac-WQQQQQQQQQ QQpGQQQQQQ Q QQQQWTGKK
βHP-NMeQ ¹⁹	Ac-WQQQQQQQQQ QQpGQQQQQQ Q QQQQWTGKK
βHP-P ²⁰	Ac-WQQQQQQQQQ QQpGQQQQP QQQQWTGKK
K ₂ Q ₂₃ K ₂	KK-QQQQQQQQQQ QQQQQQQQQ QQQKK
K ₂ Q ₁₁ pGQ ₁₁ K ₂	KK-QQQQQQQQQQ QpGQQQQQQ QQQKK

^a Q underscored and bold = NMeQ. Ac = α-N-Ac group. Lower case “p” is the D-enantiomer of Pro.