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Probing the interaction of huntingtin exon-1 polypeptides with the chaperonin nanomachine GroEL

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Abstract: Huntington’s disease arises from polyQ expansion within the exon-1 region of huntingtin (htt[ex1]), resulting in an aggregation prone protein that accumulates in neuronal inclusion bodies. We investigate the interaction of various htt[ex1] constructs with the bacterial analog (GroEL) of the human chaperonin Hsp60. Using fluorescence spectroscopy and electron and atomic force microscopy we show that GroEL inhibits fibril formation. The binding kinetics of htt[ex1] constructs with intact GroEL and a mini-chaperone comprising the apical domain is characterized by relaxation-based NMR measurements. The lifetimes of the complexes range from 100-400 µs with equilibrium dissociation constants (Kd) of ~1-2 mM. The binding interface is formed by the N-terminal amphiphilic region of htt[ex1] (which adopts a partially helical conformation) and the H and I helices of the GroEL apical domain. Sequestration of monomeric htt[ex1] by GroEL likely increases the critical concentration required for fibrillization.

Introduction

CAG expansion resulting in abnormally long polyglutamine (polyQ) tracts, is responsible for a number of protein misfolding diseases,[1] including Huntington’s disease, a fatal, autosomal dominant, neurodegenerative condition.[1,2] Huntington’s disease arises when the CAG (polyQ) region within exon-1 of the huntingtin ( htt ) gene expands beyond 36 glutamine repeats. Fragments of huntingtin exon-1 ( htt[ex1] ) are generated through proteolysis or incomplete mRNA splicing, yielding aggregation-prone polypeptides that form fibrillar neuronal tangles.[3] A key component in the very earliest stages of htt[ex1] fibrillation is the N-terminal, 16-residue amphiphilic region (htt[ex1]-H/I) which, with as few as 7 glutamines C-terminal to the NT region ( htt[ex1]-Q7 ), undergoes branched oligomerization on the micro to sub-millisecond timescale.[4] The latter involves transient, sparsely-populated species, with the productive pathway leading to helical coiled-coil tetramer via a coiled-coiled helical dimer, and the non-productive pathway to an amorphous ensemble of partially helical dimers.[5,6]

A number of chaperones, including the class II chaperonin TRiC,[7,8] and Hsp70, Hsp110 and Hsp40 either alone or in combination[9] inhibit htt[ex1] aggregation into oligomers and fibrils. Mitochondrial deformation and dysfunction are hallmarks of Huntington’s disease.[10,11] The class I chaperonin Hsp60, as well as the chaperone Hsp70, constitute the major components of the protein quality control machinery in the mitochondrion.[12] Hsp60 comprises two heptameric rings stacked upon one another, each

Results and Discussion

A summary of the htt[ex1] constructs used in the current work, together with a depiction of GroEL and the mini-chaperone, is shown in Figs. 1A and B, respectively. All htt[ex1] constructs eventually form fibrils as can be seen from EM and AFM microscopy (Fig. S1). Details of expression and purification are provided in the Supporting Information. Assignments of the 1H-13N correlation spectra of various htt[ex1] constructs and of the mini-chaperone are provided in the Supplementary Figs. S2 and S3, respectively; and NMR characterization of very weak (Kd ~ 10 mM) mini-chaperone dimerization is shown in Fig. S4.

The effect of GroEL on fibril formation was studied using the htt[ex1]-Q10-(S15C) construct with a cysteine residue substituted in place of a serine at position 15 to allow for the covalent attachment of the Alexa Fluor 647 (AL647) fluorophore. In the absence of GroEL, fluorescence of 15 µM htt[ex1]-Q10-(S15C-AL647) is quenched within 6 days at 37 °C without shaking (Fig. 2A, blue trace), and negative stain electron microscopy (EM) clearly shows the presence of fibrils (Fig. 2B, left panel). In the presence of 5 µM (in subunits) GroEL, however, fluorescence is not quenched and no fibrils are observed by EM (Fig. 2B, right panel).

The effect of the mini-chaperone, which contains the two helices H and I involved in substrate binding (Fig. 1B),[20] on the aggregation of htt[ex1] was monitored using a thioflavin T (ThT)
For 75 \( \mu M \) htt\(^{NTQ10} \) an increase in ThT fluorescence emission, indicative of the formation of amyloid-like fibrils containing cross-\( \beta \) structure.[28] is seen after about 1 day (Fig. 2C, blue trace), with oligomers and fibrils observed by EM and AFM (Fig. 2D). When 500 \( \mu M \) mini-chaperone is added to the 75 \( \mu M \) htt\(^{NTQ10} \) solution, however, no increase in ThT fluorescence emission is observed over the same time period (Fig. 2C, red trace), and no fibrils, protofibrils or large oligomers are observed by EM or AFM.

![Figure 1: Summary of constructs used in the current work. Htt\(^{ex1} \) is composed of a N-terminal amphiphilic domain (NT), a polyQ stretch and a polyproline rich domain (PRD) comprising two polyproline sequences. The PDB codes for the GroEL and GroEL mini-chaperone are 1XCK[29, 30] and 1FYA, [31] respectively.](/images/chembiochem/2021/00055/fig1.png)

Figure 1. Summary of constructs used in the current work. Htt\(^{ex1} \) is composed of a N-terminal amphiphilic domain (NT), a polyQ stretch and a polyproline rich domain (PRD) comprising two polyproline sequences. The PDB codes for the GroEL and GroEL mini-chaperone are 1XCK[29, 30] and 1FYA, [31] respectively.

Given the above results showing that htt\(^{ex1} \) fibril formation is inhibited by both GroEL and the mini-chaperone, we proceeded to investigate the interaction of various htt\(^{ex1} \) constructs with GroEL and the mini-chaperone by NMR spectroscopy. \(^{15}N\) lifetime line-broadening (\( \Delta R_2 \)), predominantly within the NT region of htt\(^{NTQ} \), htt\(^{NTQ10} \), htt\(^{NTQ14-P11} \) and htt\(^{NTQ14-P11} \) is observed in the presence of GroEL (Figs 3A and B), as expected for exchange between the low molecular weight, NMR visible monomeric species of the htt\(^{ex1} \) constructs and an NMR-invisible ‘dark’ state bound to a slowly tumbling large macromolecule such as GroEL (~800 kDa).[31] Addition of acid-denatured Rubisco, an unfolded protein substrate that binds with nanomolar affinity to GroEL, (~800 kDa) bound to a slowly tumbling large macromolecule such as GroEL (~800 kDa).[31] Addition of acid-denatured Rubisco, an unfolded protein substrate that binds with nanomolar affinity to GroEL, (~800 kDa) bound to a slowly tumbling large macromolecule such as GroEL (~800 kDa).[31] Addition of acid-denatured Rubisco, an unfolded protein substrate that binds with nanomolar affinity to GroEL, (~800 kDa) bound to a slowly tumbling large macromolecule such as GroEL (~800 kDa).[31] Addition of acid-denatured Rubisco, an unfolded protein substrate that binds with nanomolar affinity to GroEL, (~800 kDa) bound to a slowly tumbling large macromolecule such as GroEL (~800 kDa).[31] Addition of acid-denatured Rubisco, an unfolded protein substrate that binds with nanomolar affinity to GroEL, (~800 kDa) bound to a slowly tumbling large macromolecule such as GroEL (~800 kDa).[31] Addition of acid-denatured Rubisco, an unfolded protein substrate that binds with nanomolar affinity to GroEL, (~800 kDa) bound to a slowly tumbling large macromolecule such as GroEL (~800 kDa).[31] Addition of acid-denatured Rubisco, an unfolded protein substrate that binds with nanomolar affinity to GroEL, (~800 kDa) bound to a slowly tumbling large macromolecule such as GroEL (~800 kDa).[31] Addition of acid-denatured Rubisco, an unfolded protein substrate that binds with nanomolar affinity to GroEL, (~800 kDa) bound to a slowly tumbling large macromolecule such as GroEL (~800 kDa).[31] Addition of acid-denatured Rubisco, an unfolded protein substrate that binds with nanomolar affinity to GroEL, (~800 kDa) bound to a slowly tumbling large macromolecule such as GroEL (~800 kDa).\(^{31}N\) lifetime line-broadening (\( \Delta R_2 \)) of htt\(^{ex1} \) constructs in the presence of GroEL. \(^{15}N\)-\( R_2 \) values are given as the difference in \(^{15}N\)-\( R_2 \) values measured for the htt\(^{ex1} \) constructs in the presence and absence of 25 \( \mu M \) (in subunits) GroEL at a spectrometer frequency of 600 MHz. Experiments were carried out at pH 6.5 and 10 °C in 20 mM sodium phosphate and 50 mM NaCl. fusion; Fig. S5) glutamine repeats shows that the largest \(^{1}H\)/\(^{15}N\) chemical shift perturbations occur between Leu3 and Ser15 with minimal or no perturbations beyond Gin19. These data confirm that binding occurs via the NT region of htt\(^{ex1} \) and does not involve the polyQ repeat beyond the first few glutamines. The converse experiments with \(^{15}N\)-labeled mini-chaperone and unlabeled htt\(^{NTQ} \) (Fig. 5A) or htt\(^{NTQ14-P11} \) (Fig. 5B) indicate that the predominant interaction surface on the mini-chaperone comprises helices H and I, as well as a small helical turn region close to the N-terminus. This is further confirmed from intermolecular paramagnetic relaxation enhancement (PRE) measurements[32] in which a nitroxide label (R1) is covalently attached to cysteine in htt\(^{NTQ} \)-(S15C) resulting in an increase in \(^{15}N\) transverse...
To characterize the kinetics of the interaction of hntTOQ with the GroEL mini-chaperone, we simultaneously fit 1H and 15N chemical shift perturbation data recorded on 15N/13C-labeled hntTOQ in the presence of the mini-chaperone, combined with 1H and 15N chemical shift titration data (Fig. 6). The complete relaxation dispersion (Figs. S6 and S7) and chemical shift titration (Figs. S8 and S9) data used in the fits are provided in the Supplementary Information. At the concentration of 300 µM hntTOQ employed, no relaxation dispersion is observed as a result of pre-nucleation transient oligomerization, and hence the data in the presence of the mini-chaperone can be fully accounted for by a two-state exchange model with a lifetime of the bound state of ~140 µs, association (k+a) and dissociation (k+d) rate constants of ~4 × 10^6 M^-1 s^-1 and 6900 s^-1, respectively, and an equilibrium dissociation constant, K_d, of ~1.7 mM.

To confirm that hntTOQ binds to the GroEL mini-chaperone as a monomer we carried out intermolecular 1H-PRE measurements (Fig. S10). In the absence of the GroEL mini-chaperone, the intermolecular 1H-PRE profile observed on 15N-labeled hntTOQ in the presence of a small amount of amino-terminal nitroxide-labeled R1-hntTOQ (see Supplementary) is characteristic of transient, sparsely-populated, pre-nucleation helical coiled-coil dimers and tetramers, as described previously. The 1H-PRE profile remains unaltered upon addition of GroEL mini-chaperone, allowing one to conclude that the bound state of hntTOQ is a monomer.

CPMG relaxation dispersion and chemical shift titration experiments were also performed with hntTOQ-P11 and the GroEL mini-chaperone (Fig. 7, and Figs. S11-S16). Analysis is a little more complex since relaxation dispersion, owing to sub-millisecond pre-nucleation oligomerization, is observed and therefore has to be taken into account. Although pre-nucleation relaxation for the same regions on the mini-chaperone that exhibit 1H/15N chemical shift perturbations (Fig. 5C).
The CPMG relaxation dispersion and chemical shift titration data also yield the changes in $^{13}C\alpha$, $^{15}N$ and $^1H$ shifts of htt$^{11}Q_7P_1$ (Table S2) and htt$^{11}Q_7P_1$ (Table S3) upon binding the mini-chaperone. In both cases, the shift changes within the NT region are positive for $^{13}C\alpha$, negative for $^{15}N$, and negative for $^1H$ with the exception of Leu3 and Glu4. These shifts are largely impacted by the backbone $\psi/\psi$ angles, and the magnitude of the $^{13}C\alpha$ shifts (ranging from ~0.4 to 1 ppm) is suggestive of an ensemble of partially helical conformations in the bound state. It is also worth noting that the observed changes are comparable to those obtained with htt$^{11}Q_7$. The lifetime of htt$^{11}Q_7P_1$ bound to the mini-chaperone is ~170 µs, with $k_{on}$ ~2.7 x 10^{-6} M^{-1}s^{-1}, k_{off} ~5040 s^{-1}$ and $K_o$ ~1.9 mM (Table S1). Thus, the length of the polyQ repeat (beyond the first few glutamines) and the presence or absence of a polyproline stretch has little impact on the binding of htt$^{11}Q_7$ constructs to GroEL.

Figure 7. Characterization of binding kinetics and equilibrium for the interaction of htt$^{11}Q_7P_1$ with the GroEL mini-chaperone. Examples of (A) $^{15}N$ and $^{13}C\alpha$ CPMG relaxation dispersion profiles in the presence (left) and absence (right) of the mini-chaperone, and (B) $^1H$ (left) and $^{15}N$ (right) chemical shift titration data obtained at 10°C in 20 mM sodium phosphate, pH 6.5, 50 mM NaCl. The experimental data are shown as circles and the best-fit curves to the three-state exchange model shown in (C) are displayed as continuous lines. Data in (A) were acquired at 600 and 800 MHz on 300 µM $^{15}N$-labeled htt$^{11}Q_7P_1$ in the presence of 300 µM unlabeled GroEL; data in (B) were acquired at 600 MHz on 300 µM $^{13}C\alpha$-labeled htt$^{11}Q_7P_1$ with the concentration of unlabeled mini-chaperone ranging from 0 to 1 mM. The full data sets used in the global fit are shown in Figs. S11-S16. The values of the rate constants and $K_o$ are given in (C). The populations of free and bound htt$^{11}Q_7P_1$, as well as the value of $k_{off}$, shown in (C) relate to the conditions of the CPMG relaxation dispersion experiments (right-side). The conformation of htt$^{11}Q_7P_1$ bound to the mini-chaperone is shown as a transparent blue helix to indicate that the bound state samples an ensemble of partially helical conformations; note also that the depiction is purely a cartoon as we have no information on the orientation of bound htt$^{11}Q_7P_1$ on the surface of the mini-chaperone. The pre-nucleation oligomers that exchange with free monomeric htt$^{11}Q_7P_1$ represent an ensemble of helical dimers and trimers. The relative uncertainties in the values of the fitted parameters range from 5-10%. The fitted $\Delta\omega$ values are given in Tables S3 and S4.
The kinetics of htt\textsuperscript{NT-Q7} binding to intact GroEL were investigated by a combination of 15\textsuperscript{N} Dark state Exchange Saturation Transfer (DEST), lifetime line broadening (\(\Delta R_{2}\)) and CPMG relaxation dispersion (\(\Delta R_{2}\)). Examples of these data are shown in Figure 8 and the full data set used for fitting to a two-state exchange model are provided in Figures S17 and S18. The lifetime of the complex is \(~370\) \(\mu\)s, with \(k_{\text{on}} = 2.3 \times 10^{10}\) M\(^{-1}\)s\(^{-1}\), \(k_{\text{off}} = 2650\) s\(^{-1}\), and \(K_D = 1.3\) mM (Table S1). (Note that \(K_D\) and \(K_D\) are calculated under the assumption that the apical domain of all 14 subunits (7 per ring) can potentially bind htt\textsuperscript{NT-Q7}. Under the conditions of the experiment comprising 200 \(\mu\)M htt\textsuperscript{NT-Q7} and 25 \(\mu\)M GroEL (in subunits), one can conclude that each GroEL cavity is occupied by a single htt\textsuperscript{NT-Q7} molecule (i.e. the bound population of htt\textsuperscript{NT-Q7} is \(~1.8\%\), corresponding to a concentration of \(~3.6\) \(\mu\)M bound htt\textsuperscript{NT-Q7}, which is equal to the total concentration of GroEL cavities. The best-fit 15\textsuperscript{N}-R\textsubscript{2bound} profile for htt\textsuperscript{NT-Q7} bound to GroEL indicates that the region most immobilized upon binding extends from residues 3-10 (Fig. 8E). The average <15\textsuperscript{N}-R\textsubscript{2bound}> value for residues 3-10 is \(~800\) s\(^{-1}\) at 900 MHz, consistent with the molecular weight of GroEL.

Although the \(K_D\) values for the binding of htt\textsuperscript{NT-Q7} to GroEL and the mini-chaperone are very similar (\(~1.3\) and \(~1.7\) mM, respectively; Table S1), the approximately two-fold lower values of \(K_{\text{on}}\) and \(K_{\text{off}}\) obtained with intact GroEL (Fig. 8) compared to the mini-chaperone (see Fig. 6) may be due to two potential factors: (a) multiple trials required for htt\textsuperscript{NT-Q7} to penetrate the GroEL cavity and bind to an apical domain; and (b) germination rebinding of htt\textsuperscript{NT-Q7} to the same or another apical domain within the cavity prior to exiting the cavity into bulk solution.

**Conclusions**

In summary we have shown that bacterial Hsp60 (GroEL) inhibits fibril formation of a variety of htt\textsuperscript{NT} constructs comprising differing lengths of the polyQ repeat with and without the polyproline-rich region. Binding predominantly involves residues 3-13 within the NT region of htt\textsuperscript{NT} and a contiguous surface on the apical domain of GroEL formed by helices H and I and a small helical turn. The affinity for both GroEL and the mini-chaperone is in the low millimolar range with complex lifetimes in the 100-400 \(\mu\)s range. Despite the low affinity, the avidity of htt\textsuperscript{NT} for intact GroEL is high on account of the presence of multiple binding sites (7 per ring). Since GroEL binds monomeric htt\textsuperscript{NT}, the population of pre-nucleation, transient oligomers (dimers and tetramer) formed by the self-association of the NT region will necessarily be reduced, thereby effectively increasing the critical concentration required for nucleation and consequent fibril formation.

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Polyglutamine expansion within the region of huntingtin encoded by exon-1 of the \( htt \) gene results in an aggregation prone protein that accumulates in neuronal inclusion bodies. We show that the chaperonin GroEL, the bacterial homolog of human mitochondrial Hsp60, inhibits fibril formation of huntingtin exon-1 peptides and probe the interaction using NMR relaxation-based experiments designed to explore short-lived sparsely-populated states.