Biochemical Characterization of Mutants in Chaperonin Proteins CCT4 and CCT5 Associated with Hereditary Sensory Neuropathy*

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*Running title: Defects in CCT4 and CCT5 Mutants Associated with Neuropathy

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The abbreviations used are: CCT: Chaperonin Containing TCP-1; TCP-1: Tailless Complex Polypeptide, TRiC: TCP-1 Ring Complex

Background: Point mutations in two genes encoding chaperonin subunits have been implicated in neuropathies.

Results: CCT4 and CCT5 proteins carrying these mutations were expressed in bacteria and investigated for their biochemical defects.

Conclusion: H147R CCT5 is faulty in chaperoning function, while C450Y CCT4 may be defective in protein stability.

Significance: These biochemical defects may be the source of these neuropathies in patients.

ABSTRACT

Hereditary sensory neuropathies are a class of disorders marked by degeneration of the nerve fibers in the sensory periphery neurons. Recently, two mutations were identified in the subunits of the eukaryotic cytosolic chaperonin, TRiC, a protein machine responsible for folding actin and tubulin the cell. C450Y CCT4 was identified in a stock of Sprague-Dawley rats, while H147R CCT5 was found in a human Moroccan family. As with many genetically identified mutations associated with neuropathies, the underlying molecular basis of the mutants was not defined. We investigated the biochemical properties of these mutants using an expression system in E. coli that produces homo-oligomeric rings of CCT4 and CCT5.

Full-length versions of both mutant protein chains were expressed in E. coli at levels approaching that of the wild-type (WT) chains. Sucrose gradient centrifugation revealed chaperonin-sized complexes of both WT and mutant chaperonins, but with reduced recovery of C450Y CCT4 soluble subunits. Electron microscopy of negatively stained samples of C450Y CCT4 revealed few ring-shaped species, while WT CCT4, H147R CCT5, and WT CCT5 revealed similar ring structures. CCT5 complexes were assayed for their ability to suppress aggregation of and refold the model substrate γD-crystallin, suppress aggregation of mutant huntingtin, and refold the physiological substrate β-actin in vitro. H147R CCT5 was not as efficient in chaperoning these substrates as WT CCT5. The subtle effects of these mutations is consistent with the homozygous disease phenotype, in which most functions are carried out during development and adulthood, but some selective function is lost or reduced.

Sensory neurons are nerve cells that convert external stimuli from the environment into internal stimuli. A rare group of disorders, hereditary sensory neuropathies (HSNs), affect sensory neurons resulting in a range of clinical symptoms (1). These disorders are marked by the degeneration of the myelinated nerve fibers in the peripheral sensory neurons and the autonomic neurons that control the involuntary nervous
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These defects may manifest as ulceration of the feet, inability to feel pain (especially in the lower limbs), and severe pains in the distal limbs (1,2). Genetic screening of many neuropathy families has led to the discovery of several mutated genes associated with HSNs and other related neuropathy diseases. These neuropathies may be inherited through autosomal dominant or autosomal recessive forms, and are heterogeneous in their pathological and behavioral symptoms (2-4). While age of onset is variable, severe instances of this disease can have both onset and death within childhood (2).

Point mutations in three chaperonin genes have been implicated in this class of neuropathies (Table 1) (5-7). While only two are true HSNs, the other, hereditary spastic paraplegia (HSP), has some important phenotypic overlaps with HSNs (8). One of these HSNs is actually characterized as being a HSN with spastic paraplegia, even further showing the phenotypic heterogeneity of these disorders (5). Two of these have been found in human populations, making their study potentially valuable for understanding and eventually treating human neuropathy diseases. How these mutations lead to the disease phenotypes is still unknown (1).

Chaperonins are ATP-dependent chaperones that assist in folding substrate proteins inside a cavity. They are made of back-to-back rings of 7-9 subunits each (9). Chaperonins are divided into two classes: type I, found in bacteria, chloroplasts, and mitochondria; and type II, found in archaeal and eukaryotic cytosols (9). While there are structural and functional differences between the two classes, they share the same domain architecture: an equatorial domain making subunit-subunit contacts and forming the ATP binding and hydrolysis site; an apical domain recognizing substrate to be brought into the cavity; and an intermediate domain acting as a hinge-like region between the other two domains (9). The eukaryotic cytosolic chaperonin, TRiC, is involved in the folding and assembly of dozens of essential eukaryotic proteins (9,10). The most important proteins it folds are tubulin and actin, which are especially crucial in neurons (11). Unlike most of the type I and some of the archaeal type II chaperonins, which contain identical subunits in both rings, TRiC contains 8 different subunits (termed CCT1-8) in each of its two rings (10).

Two of the identified HSNs have mutations in two of the CCT genes: CCT4 and CCT5. A point mutation in the CCT5 gene, A492G, has been associated with human hereditary sensory neuropathy in a Moroccan family (5). These patients are homozygous recessive for this mutation in exon 4 of the CCT5, which translates to H147R in the protein (5). Hereditary sensory neuropathy has also been identified in a Sprague-Dawley rat strain, associated with a single point mutation in the CCT4 gene: G1349A (7). The affected rats are homozygous recessive for this mutation in CCT4, resulting in the mutant C450Y in the protein (7). Both H147 in CCT5 and C450 in CCT4 are well conserved in a variety of species (5,7). Both mutant amino acid replacements are in the equatorial domain of the CCT subunit, therefore possibly affecting intra- or inter-ring formation in the chaperonin complex, or ATP hydrolysis activity. However, the actual molecular basis has not been investigated.

The other chaperonin mutation leading to neuropathy was V98I in the mitochondrial Hsp60 (HSPD1 gene), identified in a French family with HSP (6). While this is in a type I chaperonin, unlike the type II chaperonin CCT mutations, the two chaperonins have similar functions, and may therefore share a molecular defect in order to manifest similar disease phenotypes. This mutant protein was studied biochemically and within bacterial cells. In vitro studies showed that this substitution affected both ATP hydrolysis and chaperoning (aggregation suppression and refolding) ability as a homo-oligomer (12). In vivo studies showed that the ATP hydrolysis defect was ameliorated when only a few of the mutated subunits were in the chaperonin rings. However, the chaperoning defect, while slight, was enough to cause problems with protein folding (12). Having a subtle defect in these diseases is not too surprising because these patients do live to adulthood, so the chaperonins have to be functional, albeit slightly suppressed, through their lifetimes.

Human TRiC expressed in HeLa cells is assembled from eight different protein subunits (13) and has not been amenable to efficient genetic manipulation. However, CCT4 subunits and CCT5 subunits form homo-oligomeric TRiC-like rings when expressed in E. coli (14). These rings have eight subunits per ring and are active in
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hydrolyzing ATP, suppressing aggregation, and refolding a variety of substrates (14). Therefore, we have used expression of the single CCT4 and CCT5 subunits as an experimental system to study the biochemical basis of the CCT4 and CCT5 mutants associated with hereditary sensory neuropathies.

Materials and Methods

Mutagenesis and Expression

Wild-type plasmids were previously constructed by modifying the pET21b vector to contain a TEV protease cleavage site between the end of the inserted gene (CCT4 or CCT5) and the C-terminal 6x-His-tag (14). Site-directed mutagenesis was used to introduce the neuropathy mutations (G1349A to make C450Y in CCT4; A440G to make H147R in CCT5) into the plasmids. Mutations were confirmed by sequencing (Genewiz). Plasmids were transformed into E. coli BL21 (DE3) RIL cells. Proteins were expressed as before (14). Briefly, the cells were grown in Super Broth to OD 5.0 at 37 °C and then shifted to 18 °C and induced with 0.5 mM IPTG. After the overnight induction, cultures were pelleted by centrifugation for 15 min, and the cells were resuspended in CCT-A (20 mM HEPES/ KOH pH 7.4, 300 mM NaCl, 10 mM MgCl2, 10% glycerol, 1 mM DTT, 1 mM ATP) with addition of one EDTA-free Complete protease inhibitor (Roche) per L of culture.

Long-term Lysate Supernatant/Pellet Separation

E. coli expressing WT and mutant CCT4 and CCT5 were grown and expressed as above but without the addition of protease inhibitors. The cells were lysed via French Press and incubated at 4 °C. At specified time points (0, 4, 7, 11, and 14 days), 200 µL aliquots were taken from the lysates and spun down at 11,500 x g for 30 minutes. The supernatant was extracted and the pellets were resuspended in CCT-A. SDS-PAGE loading dye (see below) was added to both the supernatant and pellet, and samples were boiled for 10 minutes, and then frozen at -20 °C until all samples were collected.

Sucrose Gradient Sedimentation

Using CCT-A buffer, 5-40% sucrose gradients were prepared by the gradient master (BioComp Instruments). Lysates (100 µL) were added carefully to the top and gradients were ultracentrifuged at 4 °C for 18 h at 37,000 rpm using a SW41 rotor (Beckman). Twenty fractions were collected using a gradient fractionator (BioComp Instruments), and one bottom fraction was collected from the remaining gradient.

SDS-PAGE and Immunoblots

Proteins were separated by SDS-PAGE (10%, 12%, or 14%) at 165 V for 1 h after boiling in reducing buffer (60 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, bromophenol blue for color) for 5 min. The gels were stained with Coomassie blue. Transfer was conducted for 1.5 h at 300 mA in transfer buffer (10% methanol, 25 mM Tris, 192 mM glycine) onto 0.45 µm polyvinylidene difluoride (PVDF) membranes (Millipore). The primary antibodies for the CCT subunits were from Santa Cruz Biotechnology: CCT4, sc-48865; and CCT5, sc-13886. The secondary antibodies were Alkaline Phosphatase (AP)-conjugated (Millipore) and the membranes were visualized using the AP-conjugate substrate kit (BioRad). Band quantification was done using ImageJ.

CCT Subunit Purification

Purification was carried out as previously published (14) with a few slight differences outlined below. Briefly, after lysis via French Press, the lysate was centrifuged, and the supernatant was removed by pipetting. The supernatant was passed through a 0.45 µm filter and loaded over a Co-NTA column (Pierce). After loading, the column was first washed with 100% CCT-A, then 5% CCT-B (CCT-A but with 250 mM imidazole), the CCT single subunit was eluted off of the column in a linear gradient from 5 to 100% CCT-B. CCT4 protein was washed with more column volumes of 5% CCT-B than CCT5 protein, due to the presence of a 53-kDa fragment that could be decreased by more thorough washing at that percentage of imidazole. The fractions containing the CCT single subunit were combined and concentrated, and then diluted with CCT-A down to 25 mM imidazole. The His-tag was cleaved by TEV protease and the protein was applied again to the Co-NTA column, to which it no longer bound. The fractions containing the CCT single subunit were combined, further concentrated, and passed over a Superose 6 10/300
GL size exclusion column (GE Healthcare). CCT single subunits were eluted by CCT-SEC (CCT-A but with 5% glycerol and no ATP) off of the size exclusion column. These fractions were pooled, concentrated, and the protein concentration was measured using the Bradford assay (BioRad) with BSA as the standard.

Electron Microscopy and Circular Dichroism

Negative stain transmission electron microscopy was carried out as published previously (14). The secondary structure of the chaperonins was assayed by far-UV circular dichroism at 100 µg/mL of protein in filtered and degassed 10 mM Tris, 20 mM KCl. Spectra from 260 nm to 195 nm were obtained for each chaperonin and the buffer using an AVIV Model 202 CD spectrophotometer. Thermal denaturation was carried out by increasing the temperature in 5 °C increments from 25 °C to 100 °C, with a 5 min incubation before each spectra was measured. Mean molar ellipticity at 227 nm was used as the metric for protein folded percentage. Points were fit to a two-state denaturation curve in Prism.

Native Gel Electrophoresis

CCT5 and its mutant were diluted to 0.5 mg/mL and mixed 2:1 with Bio-Rad Native Sample Buffer (161-0738). Samples were loaded on Bio-Rad Criterion XT 3-8% Tris-Acetate gels (345-0131) with 100 mM tricine and 100 mM Tris base running buffer (the cathode buffer contained 0.02% Coomassie blue G 250). Gels were run at 4 °C either for 3 hours at 150 V or overnight at 10 mA, and stained with Coomassie blue.

ATP Hydrolysis and Human γD-Crystallin Refolding Assays

The ATP hydrolysis assay was first described in Reissmann et al. (15) and repeated with slight modifications in Sergeeva et al. (14). The human γD-crystallin aggregation suppression assay is described in detail in Acosta-Sampson & King (16) and Knee et al. (17) and was modified in Sergeeva et al. (14) to the conditions used in this study. Refolding percentages were calculated as in Sergeeva et al. (18) with the same mutant (Y92A/Y97A) human γD-crystallin protein purification outlined there.

Mutant Huntingtin Aggregation Suppression Assay

Mutant huntingtin (mHtt) aggregation suppression assay was modified from Tam et al. (19). Briefly, GST-, His-, and S-tagged exon 1 of Htt with 53 poly glutamines, and containing a TEV protease cleavage site between the GST-tag and the rest of construct, was purified using a Co-NTA column, followed by a glutathione agarose column (Pierce). To initiate an aggregation suppression reaction, 5 µM of the mHtt protein in a buffer (20 mM Tris, 50 mM KCl, 5 mM MgCl₂, 5 mM DTT, and 1 mM ATP) containing various concentrations of chaperonin was cleaved with 0.1 mM TEV protease. This reaction was left at 30 °C for 16 hours. The reaction was stopped by equal volume addition of 4% SDS, boiled for 10 minutes, and filtered through 0.22 µm cellulose acetate membrane (GE Healthcare). The membrane was washed and blocked using 5% milk in TBS. An AP-conjugated antibody against the S-tag (EMD Millipore) was used to detect amount of mHtt trapped in the membrane. Ovalbumin was used as a control and concentration of CCT5 was calculated as in the HyD-Crys assay. Quantification was done in ImageJ where suppression was calculated as decrease from the ovalbumin control.

Actin Refolding Assay

Actin refolding assay was modified from Machida et al. (20). Briefly, pET28a containing T7- and His-tagged β-actin was translated using New England Biolabs PURExpress In Vitro Synthesis kit (E6800S) for 2 h at 37 °C. The translated actin was diluted by half into an equal mix of buffer (100 mM HEPES/KOH pH 7.5, 300 mM KCl, 10 mM MgCl₂, and 1 mM ATP) and 4 mg/mL chaperonin or BSA, and actin was allowed to be refolded for 2 h at 37 °C. Variations of ionic strength (changing KCl concentration to 100 and 500 mM) and concentration (changing chaperonin concentration to 1 and 2 mg/mL) were also carried out. Trypsin was added to a final concentration of 20 ng/µL for 15 min at 32 °C to degrade all non-native actin. SDS-PAGE loading dye (see above) was added to the samples and samples were boiled for 10 minutes. Samples were run on 12% SDS-PAGE, transferred to PVDF, and probed with an anti-T7 antibody (Novagen 69522-3). Quantification was done using ImageJ, with ratios taken for each in vitro actin experiment and normalized to 1000 for WT CCT5.
Results

Mutant Protein Expression and Stability

Each neuropathy mutation was introduced into the plasmid constructs containing the CCT4 or CCT5 WT sequences using site-directed mutagenesis. Both full-length mutant proteins were expressed in E. coli at, at sufficiently high levels to be directly identifiable using Coomassie stain (Fig. 1). The mutant expression level was divided by the WT expression levels for each dilution to quantify how much less of the mutant was expressed. For C450Y CCT4, the levels monitored by Coomassie stain were reduced to about 80% as compared to WT CCT4 in both the supernatant and pellet. By Coomassie stain, CCT5 expression levels were comparable for both the WT and the H147R mutant in the supernatant. In the pellet, the levels of the mutant were slightly lower than those of the WT, at about 80% of the WT accumulation.

To increase the sensitivity of detection of the truncated chains, the same gels were probed with a CCT4 and CCT5 antibodies, respectively. With the increased sensitivity of immunoblotting, a shorter fragment of 53 kDa was clearly detected, for both WT and C450Y CCT4 chains. This truncated product was previously shown to be missing the first 60 amino acids of the protein either due to a delayed translation start or a specific protease in the E. coli lysate (14). The shortened mutant chain was present at higher levels in the pellet than the supernatant. This suggests association into an inclusion body, common for misfolded or incomplete polypeptide chains.

A more significant difference was seen in the recovery of C450Y CCT4 as compared to WT CCT4. The mutant chains accumulated to about 30% of the level of the WT in the supernatant, and 60% of the level of the WT in the pellet. This presumably represents reduced efficiency in the partial refolding of the chains during the transfer out of SDS to the membrane in the immunoblot procedure. This is consistent with increased fractionation of the mutant chains into the pellet fractions. In the immunoblot assays using the CCT5 antibody, H147R CCT5 was not significantly decreased from WT CCT5 in either the supernatant or pellet. Therefore, the expression and recovery of the H147R CCT5 mutant in E. coli did not differ from expression and recovery of WT CCT5.

To understand the fate of both WT and mutant chains, we incubated the lysate at 4 °C without protease inhibitors for up to 2 weeks, taking samples for pellet/supernatant separations every 3-4 days. Over time, both WT and mutant proteins accumulated in the pellet, suggesting that they became aggregated rather than becoming susceptible to proteases and being degraded in the lysates (Fig. 2). This was especially true for C450Y CCT4, which was mostly in the pellet fraction by about day 7 by both Coomassie-stained gel and immunoblot. WT CCT4 had a much higher level in the pellet initially than C450Y CCT4 as seen by immunoblot, especially for the 53-kDa fragment. However, by looking at the Coomassie-stained gel, we see that WT CCT4 also accumulated in the pellet over time, however slower than C450Y CCT4. For CCT5, by Coomassie-stained gel and immunoblot, both WT and mutant levels in the pellet increased from day 0 to day 11, suggesting a fraction of chains aggregated. However, overall, the amount in the pellet and supernatant of CCT5 had much smaller changes over time than those for CCT4. In general, CCT5 was more stable than CCT4 in the lysate over the period assayed, with C450Y CCT4 being the least stable subunit of the four tested. The loss of soluble chains looks to be due to aggregation rather than proteolysis for all four proteins.

Mutant Protein Sedimentation

The supernatants of the E. coli lysates expressing both WT and mutant chaperonins were assayed by sucrose gradient ultracentrifugation, to assess whether they were organized into high molecular weight complexes (Fig. 3). The sedimentation patterns for both WT CCT5 and H147R CCT5 were similar, with a distinct species in the 18S complex region and some presence of soluble subunits at the top of the gradient. For CCT4, WT CCT4 exhibited a distinct 22S complex species composed of both full length and truncated CCT4 chains. For the C450Y CCT4 lysate, recovery of unassembled subunits was sharply reduced compared to the WT control. The majority of C450Y subunits recovered sedimented at the 22S region of the gradient, but the mutant species seemed to sediment slightly faster and
more broadly than the WT species. The rapidly sedimenting chains to the right of the 22S peak may represent aggregated chains, corresponding to the increased recovery in the pellets from Fig. 1. The mutant fragments behaved similarly as the mutant full-length chains. This overall pattern is consistent with misfolding and loss of mutant soluble subunits – either through degradation or inclusion body formation, but with some successful assembly of the remaining subunits. The two CCT oligomer species, identified here as 18S and 22S, sediment similarly to the 20S sedimentation seen for the endogenous WT TRiC isolated from HeLa cells (13).

**Mutant Protein Purification**

The CCT chaperonins and their neuropathy mutants were purified from the lysates by cobalt affinity chromatography (Fig. 4). The elution profiles of WT and H147R CCT5 were similar, both proteins eluting off of the cobalt affinity column in approximately the same amounts. For CCT4, the pattern of elution of full-length WT chains differed from that of the fragment, suggesting that they were not in a complex with each other under these conditions. Note that the fragments also bind to the cobalt column indicating that they carry the C-terminal His-tags. In order to decrease the amount of CCT4 fragment eluting with full-length CCT4 off of the cobalt column, a longer 5% CCT-B wash was used. Therefore, the WT CCT4 protein partitioned between weakly bound chains eluting at low imidazole and tightly bound chains eluting at higher concentrations. C450Y CCT4 – both full-length and fragment - was recovered from the column at significantly lower levels than WT CCT4. This suggested that the conformation and stability of the mutant CCT4 subunits was altered, so that it was either aggregating, or that it no longer efficiently bound to the cobalt column. This may be because the His-tag was buried or otherwise inaccessible for binding.

Both WT and mutant proteins were further purified by TEV protease cleavage to remove the His-tag, followed by size exclusion chromatography. Due to the low concentration off of the cobalt column, the CCT4 C450Y mutant protein was much less pure and at a significantly lower yield than the WT CCT4. However, it did elute off the size-exclusion column at the same place as WT CCT4, suggesting that some proportion of ring-like complexes were assembled, but they were not stable or sufficient enough for a large sample to be purified. This limited our ability to assay its properties compared to WT CCT4. The neuropathy mutant of CCT5, on the other hand, was successfully purified with the His-tag removed to levels similar to those of WT CCT5.

**Mutant Protein Structure**

Final purified samples, off of the size exclusion column, were examined by negative stain transmission electron microscopy (TEM). WT CCT4, WT CCT5, and H147R CCT5 all had similar morphology (Fig. 5) appearing as well formed rings oriented along the beam axis. C450Y CCT4 had few to no rings and for the most part appeared as aggregated species by negative stain TEM. The lack of ring species at the end of the mutant CCT4 purification suggests that the mutant CCT4 protein may be unstable, even in the multimeric state. Fractions of mutant CCT4 off of the cobalt column did show a few rings by negative stain EM, and the size exclusion elution volume and lysate sucrose ultracentrifugation gradients did suggest a chaperonin-sized species. While C450Y CCT4 may be capable of forming rings, they did not persist throughout the purification, possibly succumbing to aggregation or dissociation. The experiments in Fig. 1-5 taken together indicate that the defect in C450Y is one of subunit folding and stability.

For WT and H147R CCT5, purified samples could be obtained and were run on native gel electrophoresis. H147R CCT5 repeatedly ran slightly slower than WT CCT5, suggesting that its charge difference was on the surface of the protein, therefore altering its running properties on a native gel (Fig. 6). Additionally, both WT and mutant CCT5 were well-formed complexes of approximately 1 MDa with no smear of degraded subunits or monomer subunits. This assay also verified that the protein purified was indeed mutant CCT5.

To assess the conformation of the mutant CCT5 subunits, far-UV circular dichroism (CD) scans of WT and H147R CCT5 were obtained, along with thermal melts of both proteins as tracked by CD. They exhibited very similar spectra with minima at 227 nm and a very similar
thermal denaturation midpoint of approximately 60 °C (Fig. 7A). The denaturation of the mutant was less cooperative than the denaturation of the WT, possibly pointing to some difference in subunit contacts within or between the rings (Fig. 7B). However, in general, the H147R mutation in CCT5 did not disrupt subunit structure or complex assembly.

CCT5 Mutant Activity

In order to investigate how the mutation may lead to neuropathy, chaperonin activity assays were performed. Due to the position of the mutation in the equatorial domain, one likely defect might be in ATP hydrolysis of the mutant chaperonin. The purified CCT5 and H147R CCT5 complexes were therefore assayed for their ability to hydrolyze ATP. As shown in Fig. 8, the hydrolysis rates were very similar between WT and mutant CCT5.

The critical functions of group II chaperonins are believed to be suppressing the intracellular aggregation of partially folded intermediates, and assisting the folding to the native state. We therefore assayed CCT5 and H147R CCT5 for suppression of off-pathway aggregation, and refolding in vitro to the native state. The substrate used in these experiments was human γD crystallin (HγD-Crys), whose off-pathway aggregation and productive refolding has been systematically studied (16,21-28). Endogenous human TRiC purified from HeLa cells and WT CCT4 and CCT5 homo-oligomers are active in both assays (13,14).

As can be seen in Fig. 9, the HγD chains aggregated to high molecular weight complexes after dilution out of denaturant (27). When WT CCT5 was added, the aggregation of WT HγD-Crys was suppressed. This is consistent with what was seen previously for CCT5 suppression of WT HγD-Crys aggregation (14). H147R CCT5 was able to suppress mutant aggregation at first, but showed an increase in turbidity that was similar to WT HγD-Crys alone at the end of the reaction (Fig. 9A). Therefore, the mutant protein appears to have an altered reaction with the substrate in this reaction compared to WT CCT5. A potentially more stringent substrate was also assayed with WT and H147R CCT5. In this case, the aggregating protein was HγD-Crys carrying a double alanine substitution of tyrosines, Y92A/Y97A (18,25). Suppression of aggregation by WT CCT5 was similar to that found with WT HγD-Crys (Fig. 9B). The H147R CCT5 protein had an altered interaction compared to WT CCT5, mimicking the results seen for WT HγD-Crys. For both HγD-Crys substrates, H147R CCT5 less efficiently suppressed aggregation than WT CCT5.

Previously, we showed that CCT5 had an increase in turbidity throughout the assay, which we attributed to self-polymerization. However, when WT and H147R CCT5 were added to the assay without HγD-Crys (Fig. 9), we did not see an increase in turbidity, suggesting that it was not self-polymerization but rather polymerization or aggregation of the complex between CCT5 and HγD-Crys that was causing the increase in turbidity throughout the assay. We cannot exclude that the decrease in aggregation suppression of HγD-Crys by H147R CCT5 may be due to increased aggregation of the H147R CCT5/HγD-Crys complex.

Along with aggregation suppression, we can also assay the amount of HγD-Crys refolded by the chaperonin. Residual background refolding is present but is significantly less than the amount of HγD-Crys actively refolded by the chaperonins (Fig. 10A). When we quantified the amount of WT and Y92A/Y97A HγD-Crys refolded by WT and H147R CCT5, we observed a significant decrease in the amount refolded by H147R CCT5 as compared to WT CCT5 (Fig. 10B). This decrease was approximately 30% for WT HγD-Crys and 20% for Y92A/Y97A HγD-Crys, but this amount of refolded HγD-Crys by H147R CCT5 was not significantly different than background refolding in both cases. In general, Y92A/Y97A HγD-Crys was refolded to lower levels than WT HγD-Crys, contrary to what was seen for the archaeal Mm-Cpn chaperonin previously (18).

While HγD-Crys is an authentic substrate of TRiC in the periphery of the eye lens, its value is limited when surveying how H147R CCT5 may lead to neuropathy. Therefore, we also challenged mutant CCT5 to two other human substrates associated with the brain. The first is huntingtin (Htt), a very large, 3144 amino acid (348 kDa), soluble cytoplasmic protein. Although it is ubiquitously expressed, it is found at high levels in the central nervous system and the testes (29). WT Htt has various functions in cells such as acting as a scaffold protein, and playing a role in neuronal
gene transcription, and axonal and vesicular transport (30). Htt in its pathological form contains an expanded repeat of CAG resulting in 36+ polyglutamines (31). Aggregates of mutant Htt (mHtt) have been found in patient brains, consistent with the idea that aggregation of the pathological protein is part of the disease (32,33). These aggregates contain fragments of the mHtt protein, the shortest of which includes only the first exon of Htt wherein the poly glutamine region is located (29). Previous studies have shown that TRiC interacts with mHtt and decreases its aggregation (19,34-36). We assayed both WT and H147R CCT5 for their ability to suppress mHtt. For this assay, we used an mHtt protein that was GST-tagged and contained at TEV protease site. When we added TEV protease to the reaction containing mHtt and either WT or H147R CCT5, the mHtt would aggregate. We were able to see how much aggregation was suppressed by the chaperonins by using a filter trap assay and probing with an antibody against the mHtt construct. While both were able to suppress mHtt, WT CCT5 was more efficient in at least one concentration than H147R CCT5 (Fig. 11).

The second more neuropathy-related substrate we assayed is highly expressed in neurons and is one of TRiC’s major substrates: β-actin (11). For this assay, we synthesized T7-tagged β-actin in vitro and allowed WT or H147R CCT5 to fold it to native state (with BSA as a control). The samples were cleaved with trypsin so only the native β-actin persisted, run on SDS-PAGE, transferred to immunoblot, and probed with an anti-T7 antibody. We found that H147R CCT5 folded significantly less β-actin than WT CCT5 (Fig. 12). However, in this assay, unlike the HγD-Crys refolding assay, the background folding of β-actin, as seen by the BSA negative control, was minimal, so the amount folded by H147R CCT5 was still significant. To further investigate the actin refolding properties, we varied both concentration of chaperonin and ionic strength of the buffer (Fig. 13). For each of these conditions, the mutant CCT5 did not refold as much as WT CCT5. Interestingly, while we saw a concentration dependence when we varied concentration, we were able to confirm that the concentration of KCl in the buffer we used above was the optimal concentration for actin refolding.

Overall, WT CCT5 was more efficient at suppressing HγD-Crys aggregation, refolding HγD-Crys (by about 30%), suppressing mHtt aggregation (by about 40%), and folding β-actin than H147R CCT5 (by about 20%). This suggests that the defect in H147R CCT5 is that of chaperonin function.

Discussion

Human CCT4 and CCT5 subunits of the TRiC group II chaperonins assemble into double barrel TRiC-like rings in the absence of the other seven CCT subunits (14). We have used this homooligomerization of CCT4 and CCT5 subunits to investigate two neuropathy mutations identified in these chaperonin subunits. Based on the in vitro work on V98I Hsp60, and the fact that these patients survive to adulthood, we were expecting a only subtle differences in the function of these mutated subunits (12).

The H147R CCT5 mutant subunits assembled into oligomeric rings with similar efficiency as WT CCT5 subunits. The melting temperature for the mutant rings was similar to that for the WT CCT5 indicating that the H147R substitution did not cause a major defect in chaperonin structure. These chaperonin-like complexes hydrolyzed ATP with similar efficiency as WT CCT5 complexes. However, when assayed for the ability to suppress in vitro aggregation of HγD-Crys, their efficiency was reduced. The ability of the mutant complexes to chaperone the refolding of HγD-Crys back to the native-like state was also significantly reduced. Additionally, H147R CCT5 folded significantly less β-actin than WT CCT5. Note however, that in most of these assays the mutant complexes exhibited substantial levels of activity, with respect to WT CCT5 and negative controls. Our experiments do not distinguish a reduction in the initial efficiency of recognizing and binding partially folded substrates, from an actual alteration of the chaperoning reaction that proceeds within the lumen of the complex.

The HγD-Crys aggregation suppression and refolding assay used in this study has been used for many other chaperonins (13,14,17,18). Interestingly, the crystallin mutant used herein, Y92A/Y97A, was refolded to higher levels by the archaeal chaperonin Mm-Cpn (18). Here both WT CCT5 and H147R CCT5 refolded the mutant substrate chains to levels of about a third of those of WT HγD-Crys chains. H147R CCT5 refolded less of both WT and Y92A/Y97A HγD-Crys than...
WT CCT5, showing that the refolded amount is even worse when mutant substrates are chaperoned by this mutant chaperonin. The β-actin assay was used before in various iterations (20,37,38), but is novel for these homo-oligomeric complexes. Seeing a significant difference between our WT and mutant CCT5 with this stringent substrate bolstered the theory that this mutant was responsible for neuropathy due to its decreased chaperoning ability.

Another discrepancy worth noting is that of mHtt and CCT5. It was previously shown that only CCT1 and CCT4 could suppress aggregation of mHtt (19). Here we show that CCT5 is capable of also suppressing mHtt aggregation. In that study, CCT subunits were co-overexpressed in yeast with mHtt constructs. The conformation of the CCT subunits in these over-expressed cells is unknown so they could have been misfolded or aggregated, therefore not showing any efficacy. These latest results of CCT5 being able to suppress mHtt aggregation may allow the study of CCT subunits other than CCT1 in modulating mHtt aggregation.

The H147R substitution introduces a charge change into the CCT5 subunits. The guanido group of arginine is generally found at the surface of soluble proteins. Direct evidence of this change was seen by native gel electrophoresis. Such increased charge density might reduce chaperonin activity both in suppressing aggregation and refolding, due to electrostatic effects.

The defect in C450Y CCT4 was in the folding and stability of the mutant subunit itself, which may affect the complex formation ability. Compared to WT CCT4 chains, a larger fraction accumulated aggregated in the pellet fraction of cells, accumulation of soluble subunits was reduced, and formation of organized rings was sharply reduced.

Due to the homo-oligomer nature of our system, it is hard to assess whether a normal TRiC ring with seven other CCT subunits would be equally disrupted. That may depend on how TRiC is assembled in the cell. If CCT4 is one of the last subunits added, even an unstable CCT4 subunit may be incorporated and function sufficiently as part of the full ring. However, if CCT4 needs to form homo-oligomeric rings on the way to the mature TRiC complex, the mutation may result in a more defective phenotype. In either case, if C450Y CCT4 mutant folds less efficiently in the cytoplasm, or is subject to increased aggregation, it could reduce levels of functional TRiC, thus affecting folding of any of the numerous TRiC substrates. These are likely to have differential importance in different cell types.

Another feature of C450Y CCT4 mutant subunit is that the amino acid change itself is from a cysteine to a tyrosine, which may be easily post-translationally modified by a kinase. Either the loss of the cysteine or the gain of the tyrosine could affect post-translational modifications for downstream signaling (39). If C450Y CCT4 does incorporate itself as part of TRiC, it may be modified with respect to WT subunits. Unfortunately, very little is known of the control of chaperonin activity by post-translational modifications of TRiC.

It was encouraging to see a similar chaperoning defect in H147R CCT5 as seen for V98I Hsp60 (12). While in our system, the defect was exaggerated due to the homo-oligomer nature of the chaperonins, any decrease in protein folding function of TRiC will negatively affect many essential substrate proteins, including tubulin and actin. Since neurons contain a high abundance of microtubules, tubulin is a good candidate for a substrate that may be most affected (11). There have been reports of sensory neuropathy induced by taxanes (paclitaxel and docetaxel; anti-cancer drugs used in chemotherapy), where it is postulated that the taxanes promote microtubule aggregation, specifically in neurons (40). Therefore, the H147R CCT5 hereditary sensory neuropathy may very well be working through the same mechanism.

By studying purified human C450Y CCT4 and H147R CCT5 expressed in E. coli, we have found very subtle biochemical defects in these neuropathy-associated mutants as compared to WT. Whether these defects are exactly the issues contributing to neuropathy within the Moroccan family or the Sprague-Dawley rat strain remains to be seen. To further investigate chaperonin activity of the CCT5 neuropathy mutant, it will be crucial to use more physiological neuronal substrates in these aggregation and refolding assays. β-Actin is a good first candidate, but others will need to be tested. However, sorting out which substrates are predominantly affected by the CCT mutant substitutions will require characterizing the
substrates associated with TRiC within human neuronal cells expressing the neuropathy mutations. The use of patient or rat cell lines of these neuropathies would be ideal in being able to investigate these mutants in the disease context.

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Figure Legends

Figure 1: Expression levels of CCT4, CCT5, and their neuropathy mutants
Supernatant (left) and pellet (right) of E.coli cells expressing CCT4 or CCT5 were diluted by two from 1/25 to 1/800; solid arrows point to full-length CCT protein, dashed arrows point to CCT4 fragment of 53 kDa. The expression levels were quantified via ImageJ and calculated for the mutants as mutant level divided by WT level for each dilution. For the Coomassie-stained gels (top half), the expression levels were almost the same in the WT and mutant. The immunoblots (against CCT4 or CCT5, respectively) shows a decreased recovery of antigenic C450Y CCT4 as compared to WT CCT4 in the supernatant.

Figure 2: Long-term lysate incubation of CCT4, CCT5, and their neuropathy mutants
Lysates of CCT4 (A) and CCT5 (B) and their neuropathy mutant were incubated for 0, 4, 7, or 11 days and then underwent pellet/supernatant separations. Both coomassie and immunoblot SDS-PAGE is shown with full-length CCT4 or CCT5 in between dotted lines, respectively. Two E.coli fragments that accumulate in the pellet are indicated with asterisks, while two CCT4 fragments that accumulate in the pellet are indicated with + -signs. The full-length proteins are quantified to the right of each gel with WT in blue and Mutant in magenta, and pellet in solid-lined circles and supernatant in dashed-lined squares. Both CCT4 and CCT5 and their neuropathy mutants (especially C450Y CCT4) accumulate in the pellet over time, suggesting aggregation of the full-length species.

Figure 3: Sucrose ultracentrifugation gradients of CCT4, CCT5, and their neuropathy mutants
Centrifuged lysates were immunoblotted for CCT4 (top) and CCT5 (bottom), respectively; solid arrows point to full-length CCT protein, dashed arrows point to CCT4 fragment of 53 kDa. C450Y CCT4 showed a distinctly different sedimentation pattern (no soluble monomer species and a more broad 22S species, possibly slightly faster sedimenting) as compared to WT CCT4. H147R CCT5 and WT CCT5 had very similar sedimentation patterns. The WT sedimentation patterns shown here are consistent with those published in Sergeeva et al. (14), but have been more specifically labeled as 22S and 18S for CCT4 and CCT5, respectively.

Figure 4: CCT4 and CCT5 purification off of the Co-NTA column
Fractions of CCT4 (left; WT, top and C450Y, bottom) and CCT5 (right; WT, top and H147R, bottom) from the 5% wash (5%) and elution (arrow to 50%) off of the Co-NTA column were run on 10% Coomassie-stained SDS-PAGE; solid arrows point to full-length CCT protein, dashed arrows point to CCT4 fragment of 53 kDa. WT CCT4 had significantly more protein eluting off of the column than C450Y CCT4, even with the difference in expression levels taken into account. There was no significant difference between the elution of WT CCT5 and H147R CCT5.

Figure 5: Negative stain transmission electron micrographs of CCT4, CCT5, and their neuropathy mutants
WT CCT4 (top, left), WT CCT5 (bottom, left) and H147R CCT5 (bottom, right) formed TRiC-like rings of approximately the same size that were visualized here after a full purification and elution off of the size exclusion column. At the end of the purification, C450Y CCT4 (top, right) contained more aggregates and did not display rings by TEM. Scale bars, 100 nm. WT CCT4 and WT CCT5 rings are consistent with those published in Sergeeva et al. (14).

Figure 6: Native gel electrophoresis of CCT5 and its neuropathy mutant
Mm-Cpn (control), WT CCT5, and H147R CCT5 were run on native gel electrophoresis. Vertical lines for visual comparison designate the chaperonin complexes in each lane. The H147R CCT5 mutant runs slightly slower than WT CCT5, suggesting that the mutation alters the outer charge of the mutant chaperonin.
Figure 7: *Far-UV circular dichroism scans and thermal denaturation of CCT5 and its neuropathy mutant*
A. CD scans of WT CCT5 (blue) and H147R CCT5 (magenta) showed similar spectra from 260 nm to 195 nm; the minima are approximately 227 nm. B. Thermal denaturation of WT CCT5 (blue) and H147R CCT5 (magenta) by CD had approximately the same midpoint of 60 °C, although the profiles were slightly different in terms of cooperativity. The mean molar ellipticity at 227 was used as the proxy for protein folding percentage. The WT CCT5 scan and thermal melt are consistent with those published in Sergeeva et al. (14).

Figure 8: *ATP hydrolysis of CCT5 and its neuropathy mutant*
WT CCT5 (blue) and H147R CCT5 (magenta) showed similar rates of ATP hydrolysis as measured by quantified generation of [α-32P]ADP over time. The values shown for WT CCT5 were previously published in Sergeeva et al. (14).

Figure 9: *Aggregation suppression of HγD-Crys by CCT5 and its neuropathy mutant*
Aggregation of WT (orange, A) or Y92A/Y97A (orange, B) HγD-Crys was suppressed more efficiently by WT CCT5 (blue) than H147R CCT5 (magenta). Without HγD-Crys, WT CCT5 (purple) and H147R CCT5 (green) did not show any self-polymerization. The curves are representative; the assays were repeated three to five times and showed the same trends.

Figure 10: *SDS-PAGE and quantification of HγD-Crys refolded by CCT5 and its neuropathy mutant*
A. 14% Coomassie-stained SDS-PAGE of either WT (left) or Y92A/Y97A (right) refolded HγD-Crys alone (---), with WT (WT), or with H147R (H147R) CCT5 is shown. Some residual background refolding can be seen, but there is significantly more refolding by the chaperonins. B. WT CCT5 (blue) refolded significantly more WT (left) or Y92A/Y97A (right) HγD-Crys than H147R CCT5 (magenta). Both chaperonins refolded more WT than Y92A/Y97A HγD-Crys. Error bars are SEM from 3 independent quantifications; single asterisks denote significance at p < 0.05 by t-test, double asterisks denote significance at p < 0.01 by t-test.

Figure 11: *Mutant huntingtin aggregation suppression by CCT5 and its neuropathy mutant*
A. Representative filter trap samples probed with an antibody to mHtt; ratios are mHtt: CCT5. B. Quantifications of multiple experiments as in A. WT CCT5 suppressed mHtt more efficiently than H147R CCT5 at all ratios, but only significantly at the 1:1 ratio. Data normalized to ovalbumin control (1.0); Error bars are SEM from 2 independent quantification; double asterisks denote significance at p < 0.01 by t-test.

Figure 12: *Quantification of β-actin refolded by CCT5 and its neuropathy mutant*
A. Representative 12% SDS-PAGE immunoblot probed with anti-T7 antibody of refolded actin in the presence of BSA, WT CCT5, or H147R CCT5 is shown. The arrow points to β-actin. B. Quantification of multiple experiments as in A. H147R CCT5 refolded significantly less actin than WT CCT5. WT CCT5 refolded intensity was normalized to 1000; Error bars are SEM from 4 independent quantifications; single asterisks denote significance at p < 0.05 by t-test, triple asterisks denote significance at p < 0.001 by t-test.

Figure 13: *Variations in protein concentration and ionic strength of β-actin refolded by CCT5 and its neuropathy mutant*
Same assay as in Fig. 12 but with variations in protein concentration (A) and ionic strength of the buffer (B). H147R CCT5 refolded significantly less actin than WT CCT5 in all variations. There was a protein concentration dependence (A) and the optimal ionic strength was 300 mM KCl (B). Conditions used in Fig. 12 were normalized to 1000; Error bars are SEM from 3 independent quantifications; double asterisks denote significance at p < 0.01 by t-test, triple asterisks denote significance at p < 0.001 by t-test.
Table 1: Mutations in chaperonin genes leading to neuropathy diseases

| Protein | Mutation | Domain | Inheritance | Identified          | Disease                                      |
|---------|----------|--------|-------------|---------------------|----------------------------------------------|
| CCT4    | C450Y    | Equatorial | Recessive   | Sprague-Dawley rats | Hereditary sensory neuropathy<sup>a</sup>     |
| CCT5    | H147R    | Equatorial | Recessive   | Moroccan family     | Mutilating sensory neuropathy<sup>b</sup>     |
| HSPD1<sup>a</sup> | V98I    | Equatorial | Dominant    | French family       | Hereditary spastic paraplegia<sup>c</sup>    |

<sup>a</sup>HSPD1: human mitochondrial Hsp60  
<sup>b</sup>(7)  
<sup>c</sup>(5)  
<sup>d</sup>(6)
Defects in CCT4 and CCT5 Mutants Associated with Neuropathy

Figure 1

![Supernatant and Pellet Coomassie and Immunoblot analysis of WT and Mutant samples showing expression levels of CCT4 and CCT5.](http://www.jbc.org/)
Figure 2

Defects in CCT4 and CCT5 Mutants Associated with Neuropathy
Figure 3

Defects in CCT4 and CCT5 Mutants Associated with Neuropathy
Figure 4
Figure 5
Figure 7

A

B

Mean Molar Ellipticity (rad cm² dmol⁻¹) x 10³

WT CCT5
H147R CCT5

Wavelength

Protein Folded (%)

WT CCT5
H147R CCT5

Temperature (°C)
Defects in CCT4 and CCT5 Mutants Associated with Neuropathy

Figure 8

![Graph showing ADP intensity (Fold Over Background) over time (min) for WT CCT5 and H147R CCT5.]
Figure 9

Defects in CCT4 and CCT5 Mutants Associated with Neuropathy
Defects in CCT4 and CCT5 Mutants Associated with Neuropathy

Figure 10

A

WT HγD  |  Y92A/Y97 HγD
--- | ---
WT H147R  |  WT H147R

B

Refolded HγD (μg)

WT HγD  |  Y92A/Y97A HγD
--- | ---
HγD Only  |  WT CCT5  |  H147R CCT5

ns  |  *  |  *

*  |  **  |  *

ns  |  **  |  *
Defects in CCT4 and CCT5 Mutants Associated with Neuropathy

Figure 11

A

WT CCT5

H147R CCT5

B

Quantified Htt Suppression (scaled to ovalbumin control)

Htt:CCT5 Ratio

1:0.5

1:1

1:2

ns

**

ns
Defects in CCT4 and CCT5 Mutants Associated with Neuropathy

Figure 12
Defects in CCT4 and CCT5 Mutants Associated with Neuropathy

Figure 13

A

B

Quantified Refolded Actin (Arbitrary Units)

WT CCT5  H147R CCT5

CCT5 Concentration (mg/mL)

1  2  4

Quantified Refolded Actin (Arbitrary Units)

WT CCT5  H147R CCT5

Ionic Strength (mM KCl)

100  300  500

***  **  ***  ***  ***
