Disease-linked mutations in the phosphatidylcholine regulatory enzyme CCTα impair enzymatic activity and fold stability

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CTP:phosphocholine cytidylyltransferase (CCT) is the key regulatory enzyme in phosphatidylcholine (PC) synthesis and is activated by binding to PC-deficient membranes. Mutations in the gene encoding CCTα (PCYT1A) cause three distinct pathologies in humans: lipodystrophy, spondylometaphyseal dysplasia with cone-rod dystrophy (SMD-CRD), and isolated retinal dysfunctions in humans: lipodystrophy, spondylometaphyseal dysplasia with cone-rod dystrophy. Previous analyses showed that for some disease-linked PCYT1A variants steady state levels of CCTα and PC synthesis were reduced in patient fibroblasts, but other variants impaired PC synthesis with little effect on CCT levels. To explore the impact on CCT stability and function we expressed WT and mutant CCTs in COS-1 cells, which have very low endogenous CCT. Over-expression of two missense variants in the catalytic domain (V142M and P150A) generated aggregated enzymes that could not be refolded after solubilization by denaturation. Other mutations in the catalytic core that generated CCTs with reduced solubility could be purified. Five variants destabilized the catalytic domain-fold as assessed by lower transition temperatures for unfolding, and three of these manifested defects in substrate Km values. A mutation (R223S) in a signal-transducing linker between the catalytic and membrane-binding domains also impaired enzyme kinetics. E280del, a single amino acid deletion in the autoinhibitory helix increased the constitutive (lipid-independent) enzyme activity ~4-fold. This helix also participates in membrane binding, and surprisingly E280del enhanced the enzyme’s response to anionic lipid vesicles ~4-fold. These in vitro analyses on purified mutant CCTs will complement future measurements of their impact on PC synthesis in cultured cells and in tissues with a stringent requirement for CCTα.

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3 The abbreviations used are: PC, egg phosphatidylcholine; CCT, CTP:phosphocholine cytidylyltransferase; AI, autoinhibitory; PG, egg phosphatidylglycerol; PE, phosphatidylethanolamine; PDB, Protein Data Bank; LUV, large unilamellar vesicles; LD, lipid droplet; DMEM, Dulbecco’s modified Eagle’s medium; TBS, Tris-buffered saline; SMD-CRD, spondylometaphyseal dysplasia with cone-rod dystrophy.
ing defects and/or high negative surface charge, features of membranes with low PC content (17, 18). This regulatory mechanism ensures that CCT will only be fully active when the cell is deficient in PC, the pathway end product.

Recently three autosomal recessive rare human disorders with different phenotypic manifestations have been linked by exhaustive sequencing to PCYT1A (19–22). The mutations in these alleles are distributed throughout the catalytic and regulatory regions of the protein. These mutations affect the enzyme's activity and lead to the disease phenotypes.

Figure 1. CCTα domain structure and location of mutations and regulatory states. A, domain structure of CCTα. The regions with solved structures are represented as boxes; unsolved regions are shown as dashed lines. The N-cap is common to all CCTs, but is missing in some members of the greater cytidylyltransferase family. The αE helix is shown with the potential to form two helices with a hinge at residues 212–214. Domain M contains the AI helix. The P region is the site of up to 16 phosphoserines. The sites of all disease-linked mutations are mapped.

B, model of regulation by reversible membrane binding. The image at the top was from PDB 4MVC, and highlights the sites of mutations in domain C and 4-helix complex (magenta spheres). The image at the bottom is captured from a 1-μs MD simulation of the structure at the top with the AI helices removed (15), showing the Arg-223 contact with the active site (marked by the substrate CTP, in stick representation). The domain M helix is from PDB codes 1PEI and 1PEH, with the three mutations highlighted.
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Table 1
Mutations in CCTα linked to human disease

| Disease | Mutation | Location in CCT | Paired with (other allele) | Predicted effects of mutation in column 2 | Effect on PCYT1A expression | Effect on PC synthesis | Ref. |
|---------|----------|-----------------|---------------------------|------------------------------------------|-----------------------------|-----------------------|------|
| Retinal dystrophy | A93T | Domain C αA | R283* or L299*17 | Impair enzyme kinetics; weaken dimer interface | ND | ND | 21 |
| | R283-STOP (R283*) | | | | | | |
| | L299*17 | P region | A93T | Partial silencing defect; weaker membrane binding | ND | ND | 20 |
| Spondylometaphyseal dysplasia with cone-rod dystrophy (SMD-CRD) | A99V | Domain C αA | A99V or R283* | Perturb packing of hydrophobic core and ligand contacts | ↓15–30% (A99V +/+ ) | ↓2-fold (A99V +/+ ) | 19, 24 |
| | A99T | | | | | | |
| | S114T | Domain C β2/αB | S323R fs.38 | Perturb 3α contacts between αB, αC, and L4 | ↓~70% | ↓3-fold | 24 |
| | E129K | Domain C αC | E129K or F191L | Destabilize αB and αB - αC contacts | ↓~70% (E129K +/+ ) | ↓5-fold | 19, 22, 24 |
| | P150A | Domain C L4 | A99V | Destabilize L4; impair substrate binding | ND | ND | 19 |
| | F191L | Domain C β5 | E129K | Weaken 3α interactions near active site | ND | ND | 19 |
| | R22SS | Inter-domain, αE | S331P fs.? | Impair transition to full activity | ND | ND | 19 |
| | Y240H | Domain M | Y240H | Weaken membrane binding | ND | ND | —a |
| | R283* | Domain M (Al) | A99T | Silencing defect; Weaker membrane binding | ND | ND | 19 |
| | S323R fs.38 | P region | S323R fs.38 | Replaces 44 C-terminal amino acids with 38 foreign amino acids | ↓>85% | ↓5-fold | 22, 24 |
| Lipodystrophy | S331P fs.? | P region | R223S | Frame shift extends coding region to polyA | ND | ND | 19 |
| | V142M | Domain C | E280 del | Perturb packing of hydrophobic core | ↓↓ | ↓≥4-fold | 20 |
| | E280del | Domain M (AI) | V142 M or S333L fs.164 | Partial silencing defect; Weaker membrane binding | ↓↓ | ↓≥4-fold | 20 |
| | R333L fs.164 | P region | E280 | Replaces 35 C-terminal amino acids with 164 foreign amino acids | ↓↓ | ↓≥4-fold | 20 |

*a J. Jurgens, and D. Valle, personal communication.

b ND, not determined.

dulatory domains (Table 1). They include single nucleotide variants (encoding A93T, A99V, A99T, S114T, E129K, V142M, P150A, F191L, R223S, Y240H, and R283-STOP); a codon deletion in the autoinhibitory region of the regulatory domain (encoding E280del); three frameshift mutations (S323R fs.38, S333L fs.164, and S331P fs.?); and one splice site mutation (L299*17). The latter four variants alter the nucleotide sequence of the last exon, encoding the phosphorylation region of CCT. Three of these alleles (V142M, E280del, and S333L fs.164) are linked to lipodystrophy, characterized by dyslipidemia, low high density lipoprotein cholesterol levels, absence of peripheral adipose tissue, fatty liver disease, diabetes, insulin resistance, and short stature (20). Three alleles were found in patients with isolated retinal dystrophy (A93T, R283*, and L299*17) (21). R283* and the other nine alleles are linked to spondylometaphyseal dysplasia with cone-rod dystrophy (SMD-CRD), characterized by dwarfism, skeletal malformation, and vision impairment due to degeneration of rods and cones (19, 22). For all three disorders, manifestation of disease requires biallelic mutations, as the heterozygous parents and siblings are unaffected. As CCT is a homodimer, this suggests that both subunits must be mutated to generate disease. A key question related to these discoveries is why certain tissues are more susceptible to small sequence changes in CCT than others. Do the mutations impair PC synthesis and if so, do the affected tissues have a higher demand for PC synthesis during development? Do they lack the ability to up-regulate the CCTβ isoform? Recently a correlation was unveiled between the amount of active CCT, assessed indirectly by membrane association in situ, and the proliferative status of cells affected by the CCTα mutations, adipose and photoreceptor cells (23). The paradigm is that CCTα is required for generating PC (and other phospholipids derived from PC) for membrane expansion.

The question we are tackling is how could such small changes, for example, a switch from alanine to valine involving addition of only two methyl groups, affect the enzyme structure and function to trigger impaired growth and development in sensitive tissues. Analysis of the expression of these disease alleles in patient cells and measurement of PC synthesis rates have been completed for only a few alleles. Although the protein levels of mutant CCTs associated with lipodystrophy (V142M, E280del, and S333L fs.164) are barely detectable in patient fibroblasts (20), the SMD-CRD–linked alleles A99V and E129K result in only modestly reduced CCT levels (24) (Table 1). Thus the manifestations of the disease in these and other unexplored alleles may be due to defects in enzymatic
function and/or regulation. Because CCT levels can be reduced 95% in a fibroblast cell line without impacting PC synthesis (25), CCT may be expressed in excess, and only very large reductions in protein levels and/or enzymatic activity will translate into PC deficiency. That threshold was achieved with the lipodystrophy mutants, where a significant 30% reduction in the PC/PE ratio in patient fibroblasts was measured (20).

Each of the catalytic domain mutations could destabilize the packing of the catalytic-fold, leading to impairment of enzyme function. To explore this hypothesis we purified CCT enzymes with mutations associated with all three conditions. We monitored the stability of the soluble form of the mutant enzymes by thermal denaturation analysis. We explored the impacts of the mutations on kinetic constants of the lipid-bound enzymes. For mutations in the M domain we explored the impact on membrane affinity. Most of the mutants analyzed had defects in one or more of these properties.

Results

Expression and solubility of mutant CCTs in COS-1 cells

Several of the CCT mutations were expressed at levels similar to WT CCT in this system, or within a factor of two: V142M, P150A, Y240H, A99V, A99T, E129K, R223S, and E280del (Fig. 2). Several mutations (S333L.fs, S114T, F191L, and R283*) were expressed very poorly, making purification...
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Table 2

| Effect of disease mutations on CCT unfolding transition |
|---------------------------------------------------------|
| The fluorescence of samples with the indicated purified CCTs and Sypro Orange was monitored from 25 to 70 °C. The Tm value was determined from the peak of a first-derivative plot. WTm refers to WT CCT purified in native form from the soluble fraction of COS cells, and WT, refers to WT CCT isolated from the particulate fraction after denaturation and renaturation. |

| CCT | Tm (°C) | n |
|-----|---------|---|
| WTm | 55 | 1 |
| WT | 54.5 ± 0.5 | 2 |
| A93T | 49.5 | 1 |
| A99T | 47.5 ± 0.5 | 2 |
| A99V | 50.3 ± 1.7 | 2 |
| E129K | 48.7 ± 0.3 | 2 |
| R223S | 57.1 ± 0.6 | 2 |
| Y240H | 56 | 1 |
| E280del | 50.7 ± 0.3 | 2 |

Impact of mutations on the catalytic domain-fold

Representative thermal scans of several CCT mutants in their soluble forms are shown in Fig. S2 and the Tm values are reported in Table 2. Analysis of the thermal stability of the membrane-bound form is not possible due to partitioning of SYPRO Orange into the lipid bilayer. The Tm value we observed for the full-length WT CCT (55 °C) reflects the unfolding of the catalytic domain and the 4-helix complex at the base. The regulatory domain is known to be largely unstructured (except for the AI helix) in the CCT-soluble form (12, 13). We measured Tm reductions of 4–7 °C for the mutations in the catalytic domain that we predicted would perturb packing of the hydrophobic core (A99V, A99T) or polar contacts between structural elements in the catalytic domain (A93T, E129K). Mutations in the interdomain segment (R223S) or in domain M (Y240H) did not depress Tm values. E280del, a mutation that we predicted would destabilize the 4-helix bundle at the base of the active site showed a 4 °C Tm depression, suggesting that the 4-helix complex imparts stability to the catalytic domain-fold.

Impact of mutations on enzyme kinetic parameters

The specific activities of the purified CCTs were examined under conditions optimal for the WT enzyme. The activity was assessed in the absence and presence of saturating lipid vesicles composed of PC/PG (1:1), a standard system for generating membrane-bound and fully active enzyme in vitro (28–30). These analyses indicated that the lipid-stimulated activities of E129K, Y240H, and E280del were roughly comparable with the WT CCT, but the activities of the catalytic core mutations (A93T, A99T, and A99V) as well as the linker mutation R223S were impaired (Fig. 3A). In addition, the activities of these mutant CCTs in the absence of lipid were significantly lower than that of the WT enzyme. This could be explained by an impact on the substrate Km values, which could be severely impacted for the enzymes in their nonmembrane-bound states. One noticeable exception was the mutation in the AI helix of domain M, E280del. The lipid-independent activity of this enzyme was ~4-fold higher than WT (Fig. 3B), suggesting a destabilization of the 4-helix complex at the base of the catalytic domain.

We assessed the effect of mutations in the catalytic domain on enzyme kinetic parameters. With the exception of A93T, these mutations elevated the Km values for both substrates: CTP by 3–6-fold and phosphocholine by 2.5–4-fold (Fig. 3C). The effects on Vmax were more variable, with the strongest impact linked to the A99T mutant (4-fold). Somewhat surprisingly, the Vmax for the linker mutation R223S was depressed 4-fold, and the Km values for either substrate were elevated 3–4–fold. These results imply a role for the linker in catalysis or coupling membrane binding to catalysis.

Because we were unable to purify a soluble form of CCT-P150A or -V142M we assayed their activities in COS lysates from transfected cells compared with lysates from transfected CCT (vector only) or transfected WT CCT. CCT-V142M activity in the lysates was barely detectable above the vector control, and P150A activity was reduced ~4-fold (Fig. 4). These data are in keeping with the complete misfolding of V142M and partial misfolding of P150A (Fig. 2, B and C, and Fig. S1). We also monitored problematic. We did not attempt to purify these enzymes, nor did we try to express and purify CCTs with the other splicing mutations affecting the C-terminal sequence: L299.17, S323R.fs, or S331P.fs.

To assess the solubility of expressed CCT’s lysates from the COS-1, cells were separated into soluble and particulate fractions by sedimentation and the distribution of CCT particle variants was analyzed (Fig. 2C). WT CCT was recovered almost exclusively from the soluble fraction, which contains cytosolic and membrane material, as were mutants A99T, R223S, and R283*. The mutants A93T, A99V, E129K, F191L, Y240H, and E280del were less soluble than WT, and two mutants, V142M and P150A, were found almost exclusively in the particulate fraction. This fraction represents proteins insoluble in 1% Triton X-100, largely cytoskeleton and misfolded proteins in aggresomes. CCTs V142M and P150A were expressed at levels similar to or lower than WT CCT, thus the appearance in the particulate fraction is not likely because chaperone systems were overwhelmed. These results indicate that many of the mutations may impair the stability of the enzyme’s fold.

Most of the CCTs were purified from the soluble fraction, exploiting the His tag at the N terminus. We attempted to isolate four insoluble mutants from the 20,000 × g pellet by urea denaturation and slow renaturation (E129K, V142M, P150A, and E280del). The reliability of this procedure in refolding CCT into its native structure was tested using the WT enzyme, which partitioned partly into the 20,000 × g pellet when we reduced the lysis buffer volume. The unfolding midpoints were the same for WT-CCT purified from the 20,000 × g supernatant by affinity chromatography or from the pellet by denaturation and refolding (Table 2). Unfolding was measured using SYPRO Orange fluorescence, which is environmentally sensitive and increases as the dye binds to hydrophobic pockets of proteins as they unfold during a thermal scan (26, 27). Moreover, the specific activities of the two WT preparations were very similar (Fig. 3). E129K and E280del, like the WT enzyme, were successfully isolated and refolded by this method, as they retained solubility after removal of the urea by dialysis. However, V142M and P150A precipitated during the final dialysis step for urea removal (Fig. S1). When we attempted to purify E280del from the soluble fraction it was not recovered from the nickel-agarose column, presumably due to aggregation at this step.
the activity of E280del in lysates and found that, when normalized for expression levels (60% of WT by quantitative immunoblot), the activity was reduced almost 5-fold relative to WT activity (Fig. 4).

Similarly, the Y240H mutant showed a 40% reduced activity in cell lysates. Because we measured negligible effects on the activities of the two mutants after purification, the E280del and Y240H mutations may promote aggregation and reduction in the amount of activation-competent enzyme in cells. The soluble versus particulate distributions of E280del and Y240H were 60:40 (Fig. 2C), in support of in situ misfolding.

Impact of domain M mutations on lipid activation

The concentration of lipid required for enzyme activation was monitored for WT CCT and three mutants: Y240H in the N-terminal polybasic subregion of domain M, E280del in the AI region of domain M, and R223S in the linker between domains M and C. We used LUVs composed of 35 mol % anionic lipid as the model membrane, in keeping with other published studies (11). We have shown that CCT activity faithfully reflects membrane binding (11, 28).

We found that the R223S and Y240H mutations had no effect on the lipid EC₅₀ for enzyme activation (Fig. 5A). The EC₅₀ for E280del activation was shifted to ~5-fold lower lipid concentration, implying an enhancement of membrane affinity (Fig. 5B). Because the E280del mutant was prepared by denaturation and refolding we probed whether this process alone can give rise to enhanced lipid responsiveness. However, when we compared the WT enzyme and E280del prepared by the same refolding method, the difference between the two CCTs persisted, with ~3-fold lower EC₅₀ for E280del. Moreover, the activation curves for E280del were almost identical with two independently prepared LUVs (Fig. 5B). The loss of a negatively charged glutamate might make the M domain helix more hydrophobic or less repulsive for contact with an anionic membrane surface. In support of the electrostatic repulsion hypothesis, we found that E280del and WT CCT responded similarly to LUVs composed of zwitterionic dioleoyl-phosphatidylcholine/dioleoyl-phosphatidylethanolamine (2:3) (Fig. S3).

Discussion

Mutations in the catalytic domain impair fold stability

We characterized four SMD-CRD mutations, A93T, A99T, A99V, and E129K, which had diminished fold-stability and impaired enzyme kinetics. The fold-instability manifested as decreased solubility of these expressed proteins and 4–7 °C depressions in the unfolding transition temperature of purified proteins. Two other mutations fall into the destabilized class: P150A, linked to SMD-CRD, and V142M, linked to lipodystrophy. Both mutations caused such severe insolubility that we could not purify the proteins, even after denaturation and refolding attempts. Analysis of the proximal environments of the mutated amino acids in CCT crystal structures can provide explanations for their destabilizing influences. The crystal structures are of rat CCTα (13, 31), but the entire catalytic and M domains are identical with human CCTα.

Both Ala-93 and Ala-99 reside in the αA helix that contributes to the dimer interface. In the crystal structures the methyl
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Figure 4. Impact of selected mutations on CCT activity in cell lysates. CCT activity was monitored in the presence of saturating concentrations of sonicated PC/PG (1:1) vesicles (1.2 mM). Triton X-100 in the assay was maintained at 1.2 mM. The volume on the x axis was normalized to the relative concentration of the CCTs in the lysates, based on quantitative immunoblots that accompanied these analyses. The data are mean ± the range of 2 independent analyses. The values in the box are the slopes of each data set. R2 for the linear fits ranged from 0.92–0.99.

of Ala-93 is directed into the dimer interface and mutation to threonine would cause a clash with His-138 in the opposite subunit (Fig. 6A). His-138 partners with Tyr-141 across the interface and the A93T mutation may strain this H-bond, leading to the 6 °C lowering of the unfolding transition temperature. A web of polar contacts across the dimer interface (αA–αC) is shown in Fig. 6A that could be strained by the mutation to threonine. The Ala-99 side chain does not orient toward the dimer interface; rather it packs into a very hydrophobic pocket formed by residues in the β1, β2, and β4 strands of the β-sheet scaffold (Fig. 6B). Mutation to threonine or valine would likely create a steric clash between residue 99 and Leu-108 in β2 and Val-79 in β1 (Fig. 6B). The second methyl of valine could also clash with Ala-167 in β4. The hydroxyl of a threonine at residue 99 would have no H-bonding partners in this pocket. Like the A93T mutation the steric clash at Thr-99 and Val-99 was associated with 5–7 °C depressions in Tm. It may be surprising then that methionine is tolerated at residue 93 in the CCT from Saccharomyces cerevisiae and Plasmodium falciparum. Both CCTs have a histidine at the site analogous to His-138. The dimer interface in the vicinity of Ala-93 has room for a few water molecules in the solved structures, thus it may accommodate the flexible methionine better than the V-shaped threonine. Bulkier valine and cysteine are also tolerated in some species at residue 99; however, there are no obvious pocket sequence alterations that could explain how the bulk is accommodated. CCTα sequence alignments are found in Refs. 12 and 14.

Val-142 is located at the L3/β3 junction and inserts into a hydrophobic pocket formed by contributions from multiple residues in αA, αC, β2, and β3. A mutation to the longer methionine in V142M would disrupt the snugly packed interface between these elements, specifically clashing with Val-110 in β2 (Fig. 6E). Val-142 is strictly conserved across all CCTs.

Figure 5. Impact of mutations on the CCT response to lipids. The indicated CCTs (~60 nM) were assayed in the presence of the indicated concentrations of PC/PG (65:35) LUVs. The accessible lipid is 0.5 times the total lipid, because only the outer leaflet is accessible for binding. In A, all CCTs were purified from the soluble fraction. In B, both the WT and E280del CCTs were purified from the particulate fraction by denaturation and refolding. The data represented by dashed lines utilized a batch of LUVs separate from the one used for the data represented by the solid line. Each data set represents duplicate determinations and was fit to a sigmoidal, variable slope model. R2 values for the fits were 0.95–0.99. The EC50 values and 95% confidence interval for each data set are provided in the legend.

The Fersht group (32) showed that small changes in core hydrophobic packing of barnase lowered the unfolding transition temperature by ~3 °C per methylene. This translates to a free energy loss of 1–1.5 kcal/mol per methyl group (32, 33). The destabilization of the barnase mutants was generated by engineering gaps in packing (loss of methyls, e.g. valine → alanine), whereas the CCT disease mutations change alanine to bulkier substituents. However, the ensuing clash may strain contacts between complementary surfaces by creating small packing gaps, for example, between the dimer interface in A93T. Alternatively, the clash may force nonoptimal adjustments within a densely packed interior, for example, between αA, β1, and β2 in A99T(V). As noted in the original publication linking the V142M mutation to lipodystrophy (20), the packing perturbation due to the methionine substitution may cause a strain in the entire hydrophobic core leading to the extreme consequences on the catalytic domain folding and function that we observed (complete insolubility, recalcitrance to refolding, and no catalytic function).

The E129K mutation, on the other hand, does not generate a hydrophobic clash. Rather, it results in loss of an important
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hydrogen bond between the αB and αC helices that is expected to destabilize both helices. The side chain of Glu-129 at the N terminus of αC forms one H-bond with the side chain of Asp-115 at the N terminus of αB and another with the backbone NH at Asp-115 (Fig. 6C). Mutation of Glu-129 to lysine will break this latter H-bond that functions as the “N-cap” for αB. Tertiary contacts with αB are also impacted by another SMD-CRD mutation, S114T. Its side chain hydroxyl H-bonds with a backbone NH in αB at Leu-117 (Fig. 6C). Ser-114 also forms a contact with the Asn-148 side chain in loop L4, so that Ser-114 creates a bridge between αB and loop L4. Mutation to threonine would cause a clash of the methyl with Asn-148 and compensatory rotations could disrupt the H-bonding bridge. The S114T construct failed to express in COS cells, suggesting severe folding issues. We note, however, that neither Glu-129 nor Ser-114 are well-conserved. They can be replaced with a variety of small residues, including glycine that lacks the H-bonding capacity of serine or glutamate. That several disease-causing mutated residues in human CCTα are at nonconserved sites suggests that they can be accommodated within the constraints of the nucleotidyltransferase-fold, but that in the human sequence context the accommodation is nonoptimal. We note that none of the alleles with mutations at strictly conserved sites are found in homozygous individuals, whereas several mutations at nonconserved sites are.

Pro-150 is strictly conserved. It is located in the middle of loop L4 where it makes contact with Phe-157 in helix αB (Fig. 6C). The Pro-150–Phe-157 interaction provides the only tertiary contact between the αD helix and L4. This contact may also stabilize loop L4 positioning. The mutation to alanine may reduce the stability of the short αD helix leading to its unfolding and enzyme aggregation. Although we were not able to measure the $T_m$ values for V142M or P150A, their insolubility is an important indicator of folding defects.

F191L alters another strictly conserved residue, thus we tentatively classify this CCT as destabilized due to clashes between secondary folding elements. Phe-191 is one of four aromatic amino acids (Phe-191, Tyr-182, Tyr-173, and Trp-151) that stabilize contacts between L5, αL, and β5 found on the periphery of the C domain (Fig. 6D). Mutation to leucine creates a potential clash with Ile-185 and Lys-186 in helix αL. This mutant expressed very poorly in COS-1 cells and had reduced solubility.

Mutations in the catalytic domain impair enzyme kinetics

All of the catalytic domain mutants that we characterized had impacts on enzyme kinetics. For the two mutants that we could not purify due to their instability, the activities in the cell lysates were reduced 4-fold (P150A) and 1000-fold (V142M). P150A is the disease-linked mutant that has the most obvious direct links to catalysis. The proline serves to create a loop configuration that

Figure 6. Structural analysis of disease-related amino acid substitutions. Chain A is gray and chain B is wheat. Images were obtained from the PDB entry 4MVC, except for C, which was from PDB 3HL4. Mutated amino acids are colored magenta. A, pocket for Ala-93. The web of H-bonding interactions across the dimer interface is shown as well as the close contact between Ala-93 in chain A and His-138 in chain B. B, pocket for Ala-99. Ala-99 is not on the nonpolar surface of helix αA, which interfaces with a wall of hydrophobicity contributed from β1, β2, and β4. C, Pro-150 is adjacent to active site residue Trp-151 and makes tertiary contacts between loop L4 and αD. Glu-129 and Ser-114 participate in the H-bonding web mediating tertiary contacts between αB, αC, and L4. D, pocket for Phe-191. Phe-191, along with Tyr-182 and Tyr-173, form an aromatic cluster that mediates contacts between the peripheral αL helix and the interior.
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positions Trp-151 for contact with the choline group of the substrate phosphocholine and the product CDP-choline (Fig. 6C). Mutation of Trp-151 to Phe is strongly inactivating. Substitution of Pro-150 with alanine would increase the conformational dynamics of L4 and could misorient Trp-151 for ligand engagement. The other mutations may impact enzyme kinetics via more indirect routes, such as destabilizing loops L1, L2, or L4, which participate in catalysis leading to misorientation of catalytic residues within these loops. With the exception of A93T, we observed that the enzymatic defect impacted both $V_{\text{max}}$ and the $K_m$ values for both substrates. A93T was linked to a lower $V_{\text{max}}$ but had no significant effect on substrate $K_m$ values.

**Mutation of a residue in the linker between catalytic and membrane domains depresses enzymatic function**

Arg-223 is strictly conserved across all CCTs. It is the terminal residue in the catalytic domain visible in the structure of the silenced form of CCTa, residing at the terminus of the αE helix (Fig. 1) (13). This helix appears continuous from Thr-202–Arg-223 in this crystal structure, stabilized by contacts with two inhibitory helices from the M domain that form a quasi-stable 4-helix complex (13). Arg-223 is 20 Å from the base of the active site, yet its substitution with serine leads to $V_{\text{max}}$ and $K_m$ defects as severe as the mutations in the catalytic fold. The defects are not due to destabilization of the folding elements of domain C. In fact, the $T_m$ for this mutant was 2 °C higher than the WT enzyme.

Evidence is accumulating that when the stabilizing AI helices are displaced from the αE helices (as in membrane binding), the latter break in the middle bringing the C-terminal tail closer to the active site. In MD simulations with the AI helices removed and with CTP docked in the active site, the long basic side chain of Arg-223 makes contact with the γ-phosphate of CTP (Fig. 1, lower panel). When the bend was prevented by an engineered disulfide bridge, activation of the enzyme by lipids was thwarted (15), suggesting that αE dynamics are implicit in conformational changes leading to a lipid-activated CCT. Serine at residue 223 would provide neither the charge nor sufficient length to engage CTP. An R223S mutation in a CCT truncated at residue 236 (deleting all of the regulatory M domain including the AI helix) also led to inactivation of catalysis (data not shown), arguing that helix αE bending and contact with the active site may be required for the constitutive activity in that construct, as well. Although the evidence that Arg-223 can engage CTP in the active site is solely from in silico analysis, that the R223S mutation causes lower catalytic function in vitro and defects in the development of cells of the retina, cartilage and bone, supports a requirement for a dynamic αE helix in catalysis.

**Mutations in domain M do not impair the response to membrane lipids**

CCT-Y240H differed from WT CCT only in manifesting a reduced partitioning into the soluble cell fraction and a lower activity in cell lysates, as the particulate (aggregated) CCT is inactive. There was no effect on the soluble, purified enzyme’s specific activity or its $T_m$ for unfolding. The EC$_{50}$ for activation by anionic lipid vesicles was the same as the WT enzyme. The lack of effect on lipid responsiveness is not surprising. Tyr-240 resides in the N-terminal polybasic portion of the M domain that is disordered in the CCT-soluble form and is located at the membrane surface in the membrane-bound form. Across species Tyr-240 can be substituted with other hydrophobic residues. Although loss of a tyrosine in Y240H removes a residue that has high preference for membrane interfaces (34), it is replaced by a residue that is easily protonated at the surface of an anionic membrane (35, 36) and this portion of the M domain would then gain positive charge to enhance electrostatic interactions.

The effect of the mutation E280del was more surprising. The membrane association of E280del was previously examined in cells by fluorescence recovery after photobleaching (20). In Drosophila S2 cells fed oleic acid to induce lipid droplets (LDs), CCTo localizes with the surface of these droplets (20, 37), and this localization was not affected by the Glu–280 deletion (20). However, fluorescence recovery was very slow for the WT enzyme and much faster for E280del. The authors suggested that this change reflected the faster dissociation of the mutant CCT from the droplet surface. Our analysis in vitro showed the opposite; deletion of Glu–280 created an enzyme with enhanced membrane binding affinity to anionic membranes and no change in affinity for membranes rich in unsaturated PE.

There are several possible explanations for the discrepancy. First, our LUVs with simple phospholipid compositions are poor models for a cellular membrane, although they have been used extensively to compare intrinsic membrane affinities for many other mutations in domain M, mutations that have also been compared in cells, with good agreement (16, 38, 39). Second, the slow and fast return of fluorescence of WT and E280del, respectively, was interpreted as reflecting slow and fast dissociation from the LDs, which is reasonable. However, it is also possible that the LD surfaces are not saturated with CCT, and that the slow and fast recovery of fluorescence could be reflecting slow and fast association of WT and E280del, respectively, in line with our in vitro analyses. Neither proposal explains why the distribution of CCTs between the nucleoplasm and the LD surfaces was identical, nor pinpoints the source location of the fluorescent CCT responsible for the fluorescence recovery.

**Destabilized and/or catalytically compromised CCT could contribute to reduced PC synthesis in the PCYT1A-linked disorders**

The underlying causes of tissue-specific defects in CCT function and PC synthesis due to mutations in PCYT1A will require further study. Our study has focused on the impacts of each variant on the folding stability and enzymatic function of CCT homodimers, but most patients with mutated PCYT1A are heterozygotes with two distinct mutant alleles, potentially leading to 50% heterodimers. In the future one could examine the function in vitro of heterodimers composed of e.g. A99T/R283*, A99V/P150A, or E129K/F191L, using established methods for creation of functional CCT heterodimers (30). Two variants that we have examined (E129K and A99V) are homozy-
gous in several patients. Western blots of patient tissue revealed that these alleles express enzyme at relatively high levels, but reduce PC synthesis by 2–5-fold (24). The PC synthesis defect could in these cases be caused by enzymes with less stable catalytic folds that impede catalysis, even after binding to a cell membrane.

Although E280del did not show a defect in membrane binding in our assay, it also had a less stable catalytic fold and reduced catalytic activity in cell lysates. The deletion of Glu-280 is likely to cause misalignment of Trp-278, and disrupt the hydrophobic network that stabilizes the 4-helix complex at the base of the catalytic domain of soluble, inactive CCT (Fig. 6F). The influence of Trp-278 on the integrity of the 4-helix complex is revealed in the ~10-fold increase in lipid-independent activity upon substitution to phenylalanine. This instability is supported by analyses in cells showing an accelerated turnover of this CCT (20). Mutations in medium chain acyl-CoA dehydrogenase lead to impaired fatty acid metabolism during fever spikes, and potential fatality. Some of the missense mutations disrupt core hydrophobic packing resulting in fold-destabilization (40, 41) with $T_m$ depressions similar to those we have measured for CCTα. The $T_m$ depressions for unfolding correlated with lower temperature thresholds for enzyme inactivation, thus explaining the functional outcome of the mutations.

In summary, we show that 13 CCTα disease alleles, associated with three distinct clinical phenotypes, display a spectrum of biochemical features that may underlie the manifestation of the disorders. Eleven mutants fall into four classes: (i) low solubility linked to reduced activity in cell lysates, consistent with aberrant folding and aggregation (V142M and P150A); (ii) severely reduced expression levels, suggesting aggregation and potential degradation of misfolded protein (S114T, F191L, E129K); (iii) moderate solubility, but impaired catalytic activity and thermal destabilization (A93T, A99T, A99V, E129K); and (iv) impaired enzymatic activity without fold-destabilization (R223S). Two outliers were mutants in the M domain. E280del had multiple impacts including elevated constitutive activity and enhanced membrane binding of the pure enzyme, but decreased thermal stability and lower activity in cell lysates. Y240H manifested no functional disability other than reduced solubility.

**Experimental procedures**

**Preparation of mutant forms of human PCYT1A**

pET14b plasmids containing human PCYT1A and the lipodystrophy-related PCYT1A alleles were generously donated by Iona Isaac and David Savage, Cambridge University, UK. Expression in *Escherichia coli* was very poor due to proteolysis by endogenous bacterial proteases. The cDNAs were transferred into the COS-cell expression vector pAX142 by first engineering MluI and SalI restriction sites flanking the 5’ and 3’ ends of the cDNAs, and were then cut and inserted into the MluI/SalI sites in the pAX-142 vector. The SMD-CRD and retinal dystrophy mutations were engineered into the WT PCYT1A cDNA using QuikChange (Stratagene). The mutations were confirmed by sequencing. Milligram quantities of the engineered pAX plasmids were prepared using maxikits from Nucleobond, following the manufacturer’s instructions. All cDNAs contained an N-terminal His tag and a linker composed of the sequence SSGLVPGRSG before the PCYT1A coding region.

**Transfection of pAX-142–PCYT1A cDNA**

The transfection method used polyethyleneimine (PEI)–DNA complexes for uptake (42). COS-1 cells were seeded onto 10- or 15-cm dishes at a density of ~8 x 10⁴ cells/cm² and cultured for 24 h in DMEM, 5% FBS. We prepared PEI–DNA complexes using a mass ratio of 10. For a 15-cm dish we used 125 μg of PEI and 12.5 μg of DNA. These were mixed in 2.0 ml of DMEM for 20 min and then added to dishes of cells that had been washed once with PBS and were overlaid with 10 ml of DMEM, 5% FBS. After 3 h incubation at 37 °C in a 5% CO₂ incubator the medium was replaced with fresh DMEM, 5% FBS and 100 units/ml of penicillin/streptomycin. After ~42 h the cells were harvested as described (30).

**Partitioning of expressed CCTs**

The cell pellet from a 10-cm dish was resuspended in 0.5 ml of lysis buffer (20 mM sodium phosphate, pH 7.4, 1% Triton X-100, 2 mM DTT, and a battery of protease inhibitors (43)). We sonicated the cell pellet on ice twice for 20 s each for complete cell breakage. NaCl was added to a 0.5 M concentration with vortexing. The suspension was centrifuged at 20,000 × g for 10 min at 4 °C. The supernatant (soluble fraction) was transferred and the pellet was resuspended in 0.5 ml of lysis buffer. Equivalent volumes (15 μl) were analyzed by SDS-PAGE and stained with Coomassie or processed for immunodetection (44) using an antibody against residues 164–176 of the catalytic domain (45), which are not subject to a disease-related mutation. This epitope localizes to loop L5, a highly disordered loop on the periphery of the catalytic domain (15, 31). Reactivity with the antibody would likely not be altered among the various disease-linked CCT variants. CCT signals on immunoblots were captured using a Fuji LAS-4000 chemiluminescence imager. We quantified several exposures using ImageQuant 5.2 or ImageQuant-TL software. For analysis of expression in lysates the CCT signals in each lane were first normalized to signal from β-tubulin, which was probed by washing the blot in TBS, 0.1% Tween 20 and incubating with mouse antibody against β-tubulin (Applied Biological Materials), followed goat anti-mouse horseradish peroxidase conjugate. Both antibodies were diluted 1:2000 in in TBS-Tween, 5% milk powder. The blots were developed with Super Signal West Pico Plus from Thermo Sciences. WT CCT was run on the same gel and blot with the mutant CCTs.

**Purification of CCTs**

The cell pellet from 10, 15-cm dishes transfected for 42 h with CCT cDNA was lysed and fractionated into soluble and particulate fractions as described above, with a 20-fold scale up of lysis buffer volume. CCTs that were expressed primarily in the soluble fraction were isolated as described exploiting the His tag for nickel-agarose affinity chromatography (11, 30, 44). Imidazole was removed from the purified fractions by dialysis.
15-cm dishes was washed twice with 20 mM Na2HPO4, pH 7.4, dialyzed against 100 volumes of dialysis buffer (10 mM Tris, pH 7.4, 0.1 mM dodecyl maltoside) by sonication for 10 s and further gentle end over end mixing for 30 min at 20 °C. The sample was centrifuged at 20,000 × g for 10 min at 4 °C. The supernatant (S4) was dialyzed against 190 volumes of dialysis buffer (10 mM Tris, pH 7.4, 0.15 mM NaCl, 2 mM DTT, 0.1 mM dodecyl maltoside) containing successively less urea: 4, 2, 0.5, and 0 M for 2, 2, 1, and 0.5 h, respectively. The final sample in 0 M urea was centrifuged for 10 min at 20,000 × g at 20 °C. The supernatant (S5) was transferred and the pellet (P5) was resonicated in the spent dialysis buffer.

**CCT activity analysis**

Enzyme activity was determined as described previously (46) using conditions optimal for WT CCT (8.5 mM CTP and 1.5 mM phosphocholine). The CCT concentration in the assay was 60 nM, assessed by a micro Bradford assay. To measure the kinetic constants for phosphocholine, the CTP concentration was held at 10 mM and the phosphocholine concentration varied over a 0–7.5 mM range. To measure the CTP $K_m$, the phosphocholine concentration was held at 1.5 mM and the CTP concentration was varied over a 0–14 mM range. Sonicated lipid vesicles or extruded large 100- and 200-nm LUVs were prepared as previously described (47). The CCT was incubated for 10 min on ice with the lipid vesicles prior to transfer to 37 °C and initiating the reaction with [14C]phosphocholine.

**Thermal denaturation**

Protein unfolding was measured using the hydrophobic dye SYPRO Orange (Invitrogen, Molecular Probes). Protein samples were prepared by mixing the protein (1 or 2 μM) with 1:1667 dilution of SYPRO Orange in 0.2 mM NaCl, 10 mM Tris, pH 7.4, 2 mM DTT. The absolute concentration of SYPRO Orange is not disclosed; it is provided by the supplier as a 1:5000 concentrate in DMSO. The samples (300 μL) were placed in a 10-mm quartz cuvette that was sealed with a Teflon cap, and heated from 25 to 70 °C at 0.5 °C/min increments in a Varian Cary Eclipse Fluorescence Spectrophotometer. The fluorescence intensity of SYPRO Orange was measured using excitation and emission wavelengths of 492 and 590 nm, respectively. The excitation and emission slits were 5 nm. The apparent melting temperature, $T_m$, was obtained by first derivative analysis of the melting curves (26, 27), using the instrument software.

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