An auto-inhibitory helix in CTP:phosphocholine cytidylyltransferase hijacks the catalytic residue and constrains a pliable, domain-bridging helix pair

The activity of CTP:phosphocholine cytidylyltransferase (CCT), a key enzyme in phosphatidylcholine synthesis, is regulated by reversible interactions of a lipid-inducible amphipathic helix (domain M) with membrane phospholipids. When dissociated from membranes, a portion of the M domain functions as an auto-inhibitory (AI) element to suppress catalysis. The AI helix from each subunit binds to a pair of α helices (αE) that extend from the base of the catalytic dimer to create a four-helix bundle. The bound AI helices make intimate contact with loop L2, housing a key catalytic residue, Lys122. The impacts of the AI helix on active-site dynamics and positioning of Lys122 are unknown. Extensive MD simulations with and without the AI helix revealed that backbone carbonyl oxygens at the point of contact between the AI helix and loop L2 can entrap the Lys122 side chain, effectively competing with the substrate, CTP. In silico, removal of the AI helices dramatically increased αE dynamics at a predicted break in the middle of these helices, enabling them to splay apart and forge new contacts with loop L2. In vitro cross-linking confirmed the reorganization of the αE element upon membrane binding of the AI helix. Moreover, when αE bending was prevented by disulfide engineering, CCT activation by membrane binding was thwarted. These findings suggest a novel two-part auto-inhibitory mechanism for CCT involving capture of Lys122 and restraint of the pliable αE helices. We propose that membrane binding enables bending of the αE helices, bringing the active site closer to the membrane surface.

Many regulatory enzymes are silenced by interdomain interactions that are broken by activating ligands. The inhibitory interactions can involve a direct steric block of the active site by occupation of a pseudosubstrate or ligand-binding domain in its apo form (1, 2) or by binding of the unoccupied ligand-binding domain to an allosteric site that shifts the equilibrium toward unproductive configurations of an element in the active site (3–5). An X-ray structure of the catalytically silenced form of CCT4 as well as molecular dynamics simulations hinted at a novel allosteric silencing mechanism involving electrostatic redirection of a key catalytic residue (6). We have probed the silencing mechanism in depth in this publication.

The enzyme CCT catalyzes a rate-limiting step in PC synthesis and controls PC homeostasis by being active only when the membrane PC content is low. Its conserved catalytic domain is linked to a weakly conserved regulatory domain via a highly conserved short linker segment (Fig. 1A). CCT’s regulatory domain (domain M) is an inducible 60–70-residue membrane-binding amphipathic helix (7–10), and the membrane is its “ligand.” The enzyme can thus interconvert between a soluble, inactive form and a membrane-bound, active form by transformation of domain M from mostly disordered into a long α helix (Fig. 1B). CCT membrane binding and hence its catalytic power are regulated by physical properties of the membrane (11). PC-rich membranes have low net surface charge and tight packing between neighboring lipid molecules of the bilayer. PC-deficient membranes have a higher surface negative charge and increased packing stress (12). CCT’s domain M responds to the latter properties, binds and inserts partway into the membrane as an amphipathic helix, and this binding event is communicated to the active site to enhance catalysis more than 2 orders of magnitude by effects on both $k_{cat}$ and $K_m$ for CTP (13, 14). This process accelerates PC synthesis to restore PC compositional homeostasis. CCTα mutations are linked causatively to three human diseases that impair development of bone, cartilage, and/or retinal tissue (15–17) or lipid metabolism (18).

The catalytic domain of CCT derives from an ancient domain designed to catalyze nucleotide transfers to a variety of

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1. Both authors contributed equally to this work.
2. Holder of the Alberta Innovates Technology Futures Strategic Chair in (Bio) Molecular Simulation. To whom correspondence may be addressed: Dept. of Biological Sciences and Centre for Molecular Simulation, University of Calgary, 2500 University Dr. NW, Calgary, Alberta T2N 1N4, Canada. Tel.: 403-220-2966; E-mail: tieleman@ucalgary.ca.
3. To whom correspondence may be addressed: Dept. of Moleular Biology and Biochemistry, Simon Fraser University, 8888 University Dr., Burnaby, British Columbia V5A 1S6, Canada. Tel.: 778-782-3709; E-mail: cornell@sfu.ca.

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The abbreviations used are: CCT, CTP:phosphocholine cytidylyltransferase; GCT, CTP:glycerol phosphate cytidylyltransferase; ECT, CTP:phosphoethanolamine cytidylyltransferase; AI, auto-inhibitory; PC, egg phosphatidylcholine; PG, egg phosphatidylglycerol; PDB, Protein Data Bank; MD, molecular dynamics; RMSF, root mean square fluctuation; PR, positional restraint; TCEP, tris(2-carboxyethyl)phosphine; H-bond and H-binding; hydrogen bond and hydrogen-binding, respectively.
metabolites, such as the transfer of AMP from ATP to amino acids and CMP from CTP to phosphoalcohols, such as phosphoglycerol, ethanolamine, or choline (19, 20). In the cytidylyltransferases (glycerol phosphate (GCT), choline phosphate (CCT), and ethanolamine phosphate (ECT)), catalysis is accomplished by charge stabilization of the highly charged CTP with basic residues residing in loops L1, L2, and L6 and in the junction of β4/L5. Residues at the N terminus of helix αE make contacts with CTP to promote a contorted U-shape (21). This enables an in-line attack of the nucleophilic phosphate at the α-phosphate of CTP to displace diphosphate (20). The crystal structure of the catalytically silenced form of CCT (6) as well as fluorescence anisotropy analyses (22) showed that when not engaging membranes, the M domain is composed of a disordered leash segment of ~40 residues followed by a ~22-residue auto-inhibitory (AI) helix that docks onto two elements of the catalytic domain: the αE helices and loop L2 (Fig. 1C). These two elements are special in that their sequence differs between lipid-regulated CCTs and nonregulated cytidylyltransferases, such as GCT and ECT. The αE helices are much longer in the CCTs (~22 residues versus 10–12 residues in GCT or ECT) and are predicted to be interrupted by a short disordered segment in the middle (Fig. 1A) (23). Thus, the long uninterrupted αE helices observed in the structure of the silenced form may be stabilized by the AI helices, which, together with the two αE helices, form a four-helix bundle and may be destabilized once the AI helices are dissociated by membrane binding. The L2 sequence in all cytidylyltransferases contains one or more lysines that participate in catalysis based on mutagenesis (24, 25) and solved structures with substrate and product (6, 20, 21, 25). In rat CCT, a substitution with arginine at Lys122 in L2 results in a ~5-order of magnitude decrease in catalytic efficiency (24), the strongest mutagenic effect for any single residue tested in CCT. Any impact on Lys122 dynamics or alignment could have severe consequences on enzyme activity.

We proposed, based on the structure of the silenced enzyme (6), that CCT’s mode of auto-inhibition involves a partial occlusion of the active site entrance by the AI helix and clamping of the active site in a nonproductive configuration by the AI helix. Limited molecular dynamics simulations suggested that the docking of the AI helix could restrict the dynamics of the αE and L2 loops. Most intriguingly, where the end of the AI helix contacts loop L2 (the AI turn), the backbone carbonyls of the turn steered the key catalytic residue, Lys122, away from its orientation into the active site. This electrostatic steering involved a carbonyl on loop L2 at Phe124 and a carbonyl at Phe293 on the AI turn, and we refer to this interaction as the “backbone trap” (6). These single-replicate 200-ns simulations were carried out in the absence of substrate.

A key unanswered question arising from the previous work was whether the backbone trap can operate when the active site is occupied with substrate. Can the backbone carbonyls compete with substrate for the key catalytic residue? In the present work, we determined the impact of the AI helix on active site dynamics and positioning of Lys122 in the presence and absence of the substrate CTP. The new data, involving a total of 40 separate atomistic 1-μs simulations, suggest that the backbone trap can effectively compete with CTP. We also tested loop L2 flexibility in vitro and its role in catalysis by mutation of a conserved glycine, Gly123. Furthermore, simulations in the absence of the AI helices revealed a remarkable plasticity for the αE helices once the AI helices are displaced, generating bends in the αE helices at the predicted helix break (Fig. 1A). Cross-linking measurements in vitro confirmed a reorganization of the αE helices upon membrane binding. Straightjacketing the helices with a disulfide prevented lipid activation, suggesting that the conformational changes observed upon removal of the AI helices may reflect the active membrane-bound configuration of the αE. We discuss various hypotheses for how the bent αE helices might accelerate the CCT-catalyzed reaction.

**Results**

**Optimal catalysis requires a flexible loop L2 at Lys122, enabled by Gly123**

Loop L2 has been identified as a key loop in catalysis based on studies with CCT and a related bacterial cytidylyltransferase, GCT (24, 25), which is involved in cell-wall biosynthesis and is not regulated by lipids. The loop L2 sequence of CCT differs from that of the lipid-regulated eukaryotic CCTs. Whereas the latter contain a single lysine (Lys122) followed by an invariant glycine, L2 in GCT has two conserved lysines and no glycine (Fig. 2A). Mutagenesis of *B. subtilis* CCT showed that both Lys44 and Lys46 are required for catalysis (25). In the crystal structure of *B. subtilis* CCT in complex with CTP (PDB code 1COZ), Lys46 contacts the ψ-phosphate of CTP. In the structure of the same enzyme in complex with the product CDP-glycerol, Lys46 contacts the α- and β-phosphates of CDP-glycerol, and Lys44 contacts the β-phosphate (PDB code 1N1D) (Fig. 2B). Thus, it appears that two lysines participate in multiple alternative interactions with substrate and product during a catalytic cycle. The backbone configurations of L2 at these lysines are similar in both crystal structures, and movement of the lysines to engage the two phosphates of the product are rigid body translocations of ≤3 Å (21).

In CCT, however, loop L2 (residues 122–127) is 3 residues shorter and likely to be less mobile, and Lys122 is the sole lysine. In the crystal structures of CCT in complex with CDP-choline (PDB codes 3HL4 and 4MVC), Lys122 occupies a position between that of Lys44 and Lys46 of GCT and contacts both the α- and β-phosphate of CDP-choline (Fig. 2B). The backbone at Lys122 in CCT occupies a configuration in the α-helix portion of a Ramachandran plot, whereas the ψ,φ angles at the lysines in the crystal structures of GCT occupy a configurational space within or near the β portion. We hypothesized that the single lysine in L2 of CCT must be agile during a reaction cycle so that its ε-amino group can partner with either the α-, β-, or ψ-phosphate oxygens of CTP or with phosphocholine and that this requires a very flexible backbone, enabled by Gly123. This hypothesis was explored by mutagenesis to alanine or proline, which would restrict the ψ,φ angles and present a larger barrier opposing backbone conformational sampling at Lys122. The data in Fig. 2C show that neither Ala nor Pro can effectively substitute for the glycine at residue 123. The Pro substitution was completely inactivating in the context of CCT236, which lacks the membrane-binding domain. In a construct with
**CCT silencing and activation mechanisms**

**Figure 1. Structure of mammalian CCT and its active site.** A, domain map. The N region is nonconserved and disordered, and the catalytic (C) domain is a conserved dinucleotide fold encompassing ~150 residues. The locations of the two elements in domain C where the Al helices dock (L2 and αE) are indicated. In lipid-regulated CTs, the αE helix is predicted to be two helices (αEα and αEγ) with a helical break at residues ~212–214. When not membrane-bound, the M domain consists of a disordered leash followed by the Al helix. The phosphorylation (P) region is poorly conserved and disordered. B, schematic of the CCT fold for silenced and active forms. Left, residues 40–223 of the catalytic dimer (cyan and green chains) with the Al helices docked onto the αE helix pair to create a four-helix bundle. Elements in the crystallized protein that are not visible in the solved structure (PDB code 4MVC) are represented as dotted lines. The box encompasses one active site, enlarged in C. Right, a proposed membrane-bound active form using the catalytic dimer from the solved structure of CCT236 (PDB code 3HL4) and the M domain modeled as a simple unbroken helix (not to scale). The αEα and linker are of unknown configuration in the active form and are represented as dotted lines. CDP-choline occupies both active sites. C, close-up of the CCT active site showing conserved residues that influence catalysis (stick representation of Lys122, Tyr173, Arg196, and Thr202), the backbone-to-backbone stick representation (gray bars) of 0.2 mM PC/PG (1:1) vesicles. Data are means ± average deviation (error bars) of 4–6 independent determinations.

**Figure 2. CCT relies on a single lysine followed by glycine in loop L2 for catalysis.** A, sequence comparison of the L2 loop. B, overlay of GCT–CTP (1COZ), GCT–CDP-glycerol (1N1D), and CCT–CDP-choline (3HL4) showing H-bonds between the lysines in loop L2 and CTP, CDP-glycerol, or CDP-choline. C, impact of mutations at Gly123 in L2 on the activity of CCT. Purified CCTs were assayed for activity under conditions optimal for the CCT312 or CCT236 constructs in the absence (gray bars) or presence (green bars) of 0.2 mM PC/PG (1:1) vesicles. Data are means ± average deviation (error bars) of 4–6 independent determinations.
domain M (CCT312), the effect of the proline substitution on the lipid-activated enzyme was curiously less severe, suggesting that when membrane-bound, additional factors may mitigate the detrimental loss of flexibility. Further experiments will be needed to establish the basis for the less stringent L2 flexibility requirement of membrane-bound CCT. These data do support the hypothesis that configurational lability of loop L2 promotes catalysis. In support of Lys\(^{122}\) as a primary target of regulation in lipid-dependent CCTs, the positions of four other functional active-site residues, Arg\(^{196}\), Thr\(^{202}\), His\(^{89}\), and His\(^{92}\), superimpose within \(<1.5 \text{ Å}\) with the analogous residues in the GCT structures with substrate or product.

**The AI helices selectively repress the dynamics of helix \(\alpha E\)**

To explore the impact of the AI helix on the dynamics of loop L2 and other active site loops and catalytic residues, we performed 1-\(\mu\)s MD simulations of the CCT dimer, residues 40–223, comprising the complete catalytic domain, with and without the AI helices. A 3.0 Å structure of this segment with bound AI helices (6) provided the starting coordinates for the simulations. The effect of the substrate CTP was also probed in each of these sets. The final 8 residues of the \(\alpha E\) helices were restrained by either of two methods (referred to as PR or NOE) to prevent their unwinding and migration deep into the active site, which was observed in a preliminary 200-ns simulation. Five replicates of each of the four conditions (protein alone; + AI; + CTP; + CTP and + AI) were simulated for each restraining method, for a total of 40 independent simulations (40 \(\mu\)s total). We discovered early on that the mobility and interactions of key loops and residues were frequently dissimilar for the two active sites. For example, when Lys\(^{122}\) interacted with CTP in chain A, it might be contacting a carbonyl of the backbone trap in chain B. Thus, effectively, the simulations span 80 \(\mu s\) of CCT monomer dynamics.

In the 40 chains containing CTP in the active site, CTP was maintained in the pocket except for the last 150 ns of just one replicate, where it diffused away from most of its partners in the starting structure (e.g. Arg\(^{196}\) and Thr\(^{202}\)). More common was a somewhat fixed position of the cytosine and ribose while a rotation about the ribose C1 oxygen–\(\alpha\) phosphorus bond redirected the \(\beta\)- and \(\gamma\)-phosphates away from the starting contact with Thr\(^{202}\) in \(\alpha E\) and toward Lys\(^{122}\) and loop L2. This transformed the CTP from its U-shaped starting conformation to a more stretched conformation.

The loops contributing to the active site are L1, L2, L5, and L6 (Fig. 1). The N terminus of \(\alpha E\) (\(\alpha E_N\)) also contributes Thr\(^{202}\) and Ser\(^{203}\) to the active site. The docking site for each AI helix is formed from the \(\alpha E\) helices of both chains and loop L2 of one chain (Fig. 1). This prompted an investigation of the impact of the AI helices on L2 and \(\alpha E\) dynamics by computing the RMSF for backbone atoms and key catalytic residues housed in these loops. Loops L5 and L6, which are longer and more dynamic, were also investigated. From a visual inspection of the many simulations, it was obvious that L1 is not mobile, and neither CTP nor the AI reduced its mobility further. Fig. 3 shows that the dynamics of L2 was reduced a small and variable degree by the AI and by CTP in isolation, but the combination of CTP and the AI helix reduced dynamics significantly by 20%. The same effect was observed on the dynamics of Lys\(^{122}\) in L2, where the combination of CTP and the AI resulted in a significantly depressed RMSF (24%), but neither CTP nor the AI alone showed a significant effect on mobility. The immobilization of \(\alpha E_N\) and its resident active-site residue, Thr\(^{202}\), reflected the combined impact of active-site residues bonding with the ligand and the AI. The AI helix dampened the dynamics of the \(\alpha E_N\) backbone and Thr\(^{202}\) side chain by ~30%. The dynamics at Thr\(^{202}\) was reduced \(\geq 40\%\) by CTP, in keeping with a direct H-bonding contact. Together, the AI and CTP reduced Thr\(^{202}\) RMSF by \(>2\)-fold (Fig. 3A and Fig. S1).

The dynamics of loop L5 and its resident active site residue Tyr\(^{173}\) were high and were not suppressed by either CTP or the AI helix (Fig. 3). The dynamics of loop L6 and its active site residue, Arg\(^{196}\), were not impacted by the AI helix but were reduced 40–50% by CTP, with which it formed direct H-bonds. The dynamics of the loop/hinge region linking helix \(\alpha E_N\) and helix \(\alpha E_C\) was profoundly reduced by the AI helix in the NOE-restrained simulations (Fig. S5B). The high mobility of this region when the AI is not present is discussed below. In summary, the docking of the AI helices to form the four-helix bundle has a strong immobilizing effect on the \(\alpha E\) helices and only weakly constrains loop L2, which is ordered even with no AI present.

To investigate L2 dynamics in vitro, we examined the effect of a docked AI helix on the mobility of a tryptophan substituted for Phe\(^{124}\) in loop L2. The F124W substitution did not affect the activity in the presence or absence of lipid. We also monitored an engineered F121W in the \(\alpha B\) helix adjacent to loop L2 and a native Trp (Trp\(^{151}\)) located in the active site (20). The location of these Trp residues is shown in Fig. S2A. The fluorescence anisotropy of Trp\(^{124}\) was compared for this CCT construct in its soluble form and when bound to a lipid micelle that induces dissociation of the AI helices from the catalytic domain (22) and fully activates the enzyme (10). The anisotropy measurements are sensitive to motions in the 1–10–\(\text{ns}\) regime. The anisotropy values for each of the three Trp residues were 0.15–0.17, typical values for a Trp that is constrained in a folded element of a protein (26). There was only a marginal decrease in the anisotropy of Trp\(^{124}\) upon binding lipids (12 \(\pm 3\%\) decrease) and a slightly stronger effect on Trp\(^{121}\) (20 \(\pm 8\%\) decrease; Fig. S2B).

In keeping with these decreases in dynamics, the RMSF value for Phe\(^{124}\) computed from a set of 10 NOE simulations was reduced 18% by the AI (\(p = 0.01\)). There was no change induced by lipids when Trp\(^{124}\) was evaluated in the context of a CCT lacking an M domain (CCT236; Fig. S2B), confirming that the effect on dynamics is due to lipid binding. Thus, in keeping with the simulation results, docking of the AI onto loop L2 has only a small restraining effect on the dynamics of a hydrophobic side chain at residue 124 in L2. Phe\(^{124}\) rotations are sustained within a hydrophobic pocket that is created from side chains of L2 (Val\(^{126}\)) and the AI helix (Met\(^{292}\), Phe\(^{293}\), and Phe\(^{289}\)) (Fig. S2A).

The AI helix supplies H-bonding partners for Lys\(^{122}\) that compete with CTP

Previously published simulations without substrate suggested that the AI interactions could steer the Lys\(^{122}\) eN into an H-bonding trap with backbone carbonyls contributed from the
AI and the L2 loop (6). When the active site is occupied by ligand, can the AI compete with the phosphates of the ligand for contact with Lys122 to inhibit catalysis? To further explore the likelihood of the backbone trap for Lys122, we mined the full set of 40 simulations with and without CTP and the AI helix for the frequency of Lys122 H-bonding with one or more oxygen atoms from 5 residues that were frequently found in shared H-bonds with Lys122. These are the Phe124 carboxyl in loop L2, Asp196 carbonyl and backbone carbonyl in loop L1, and, in the presence of the AI helix, three backbone carbonyls at the C-terminal turn of the AI at Phe293, Gly294, and Pro295. Fig. 4A shows a snapshot of a common three-way interaction with Lys122. In some of the simulations in the presence of the AI, contact between the AI C terminus and loop L2 was lost for prolonged time intervals during the simulations. The contact between AI and L2 was monitored by the formation of an H-bonding interaction between Met292 carbonyl and Phe124 backbone NH, a contact that was also observed in the crystal structure of the silenced CCT (PDB code 4MVC). For analysis of the impact of the AI helix on Lys122 H-bonding partners, we deleted time intervals where the Met292–Phe124 contact was disengaged for more than 50 ns. This step reduced total simulation time associated with the production runs from 38.4 to 32.4 μs.

In both the PR and NOE-restrained simulations, there was a significant effect of the AI helix on Lys122 contact with the trapping atoms (Fig. 4B). The frequency of contact with these atoms due to the AI alone increased 2–4-fold, from 32 to 83% (PR simulations) and from 31 to 72% (NOE simulations). When CTP occupied the active site, the AI helix increased the frequency of Lys122 contact with the trap from 32 to 83% (PR) and from 8 to 36% (NOE). At the same time, the H-bonding frequency with CTP decreased from 67.5 to 43% (PR) and from 67 to 39% (NOE). This analysis strongly supports the hypothesis that the interaction of the AI helix with the L2 loop steers Lys122 away from CTP by forging direct contacts with backbone atoms. Fig. 4C shows an image of the electrostatic surface surrounding the active site, with and without the docked AI helix. The C terminus of the AI helix bends at its juncture with loop L2, and that bend is highly electronegative, thus exerting an electrostatic pull on the Lys122 amino group. With no side chain, Gly123 enables close docking of the AI-turn on loop L2, enhancing this pull. The AI helix did not affect the H-bonding of two other nearby active site residues that are significant partners with CTP: Arg196 in loop L6 and Thr202 in the αE N (data not shown). These residues have no or little interaction with the atoms of the backbone trap, and their stable
also shows that CTP and/or the AI helix reduce the AI configuration could deter contact of Lys122 with the substrate, with CTP for Lys122 for the duration of the 1-μs simulation. B, effects of AI and CTP on Lys122 H-bonding partners. The simulation conditions are indicated in the matrix at the top. Lys122 H-bonding frequency with the indicated residues or CTP was analyzed using the GROMACS tool, g_hbond. A contact involving 1–3 H-bonds to a partner was scored as one H-bond. Lys122 H-bonded with the prominent partners for Lys122 in the absence of CTP or the AI. Asp86 and the carbonyl of residues 124, 293, 294, and/or 295. Without AI With AI

Fig. 4. Backbone trap for Lys122. A, left, active site of chain A with CTP in its productive U-shape, interacting with Ser203 in αE and Lys122 H-bonding with CTP. Right, 10 ns later in the same simulation, Lys122 is in a split H-bond with Asp286 in loop L1, Phe293 in loop L2, and Phe295 in the AI. These interactions compete with CTP for Lys122 for the duration of the 1-μs simulation. B, effects of AI and CTP on Lys122 H-bonding partners. The simulation conditions are indicated in the top row. C, electrostatic surface of active site with and without the AI helix. PDB images shown are of 4MVC with the AI removed (left) and AI present (right). The electrostatic surface was calculated using default settings in PyMOL.

Interactions with CTP were not disturbed by the AI helix. Thus, it appears that the backbone trap device is selective for Lys122.

Removal of the constraining AI helices allows bending of the αE helices

In 17 of 20 simulations carried out in the presence of the AI helices, the four-helix bundle remained intact for the large majority of the simulation time, with occasional unwinding of the N-terminal portion of one AI helix at the base of the four-helix bundle. The contacts between the four helices are mostly hydrophobic, and the AI-helix-turn/loop L2 interaction involves an aromatic cluster (Fig. S4). In these simulations, the αE segments remained unbroken α helices with minimal backbone fluctuation, even in the hinge region. The αE–αE contact was maintained by the hydrophobic side chains of Ile206, Ile209, and Val210 in the N-terminal segment of the αE (Fig. S4). This hydrophobic interaction is also seen in the crystal structure (6). The two turns at the C terminus of the αE helices have fewer interactions but were fixed by restraints. In 3 of the 20 simulations, one of the AI helices disengaged for hundreds of ns at the N terminus (Fig. S4, image on right) and rotated on its axis to become nearly perpendicular to the αE helices. Despite this rotation, hydrophobic contacts between the middle of the AI (Phe285, Ile286, and Phe289) and the αE helices (Val210, Tyr213, and Tyr216) were maintained. The two αE helices in all simulations
remained stable in their conformation and interactions with each other (Fig. S4).

One of the most striking observations, which occurred in all 10 NOE-restrained simulations without the AI helices, was the bending of helix αE at the hinge (residues 211–215). We refer to this helical break as a hinge following the definition of Wolfson and colleagues as a region about which protein domains rotate (27). In the equilibrated starting structures, the two αE helices were unbroken (Fig. 5A), and their interaction was mediated by the hydrophobic cluster described above. The two helices cross over at the Ile209–Val210 contact site. In all 10 simulations, the helices drifted together toward one active site for 10–200 ns, and then the segment between residues Arg211 and Val215 unwound and/or bent in both helices, enabling contact of at least one αE C with loop L2. In 6 of 10 dimer simulations, the bend at this loop accompanied the complete dissociation of the αE C portions from each other (αE C splay; configurations 4, 5, and 6; Fig. 5). The αE C in the splayed conformation formed contacts with loop L2, principally via an aromatic cluster composed of Phe124 in L2 and Tyr213 and Tyr216 in the αE C of the opposite chain (Fig. 5, C–E). In 15 of 20 simulated chains, this aromatic contact (with a minimum distance of ~3 Å) persisted for more than 550 ns. The αE N retained its tight interchain hydrophobic interaction for the duration of the simulations. There were at least five common configurations of the CCT dimer with bent αE helices (Fig. 5), and these were observed in the absence or presence of CTP, but never in the presence of the AI helices. These bent configurations could interconvert (i.e. 2 ↔ 3 and 4 ↔ 5 ↔ 6) but did not return to the starting configuration. Once the two αE C helices had engaged separate L2 loops, they did not break away to reforge contacts with each other. In six replicates, the splayed configurations were maintained for more than 500 ns, for a cumulative total of 4 μs of a total of 10 μs. In the splayed configurations 4–6, the frequency of H-bonding of Tyr213 or Tyr216 hydroxyl with Phe124 carbonyl in L2 increased, especially when assuming a configuration with the αE C helix at an approximate right angle beneath L2 (Fig. 5E).

The simulations with CTP present revealed a frequent H-bond between Arg223 at the C terminus of αE C and the γ-phosphate of CTP (Fig. S5A). This occurred in 5 of 10 chains for a total of ~2.7 μs of 5.0 μs. This contact was specifically associated with splayed αE C configurations that featured close packing of αE C under and perpendicular to loop L2 (Fig. 5, D

\[\text{Figure 5. } \alpha E \text{ conformational change triggered by removal of the AI helices. The central panel shows a time plot of the interchain distance between Tyr}^{213} \text{ and Phe}^{124} \text{ (top), and the interchain distance between the two Tyr}^{213} \text{ hydroxyls of the dimer (bottom) for one replicate of the NOE simulations. The abrupt change after 180 ns correlates with the loss of } \alpha E-\alpha E \text{ contact. A–E configuration 1 is the equilibrated starting structure. Configuration 2 occurs after a short period of drift. The } \alpha E \text{ helices remain in contact with each other, and one } \alpha E \text{ forges contact with L2 via the aromatic cluster composed of Tyr}^{213} \text{ and Tyr}^{216} \text{ in the } \alpha E \text{ and Phe}^{124} \text{ in L2 (gray arrow). Configuration 3 is shown in Fig. S6. Configuration 4 has both } \alpha E \text{ helices splayed apart and interacting with L2 via the aromatic cluster. In configuration 5, a sharp bending at the hinge in one of the } \alpha E \text{ helices positions the } \alpha E \text{ at right angles to loop L2; this orientation is stabilized by aromatic cluster and Arg}^{223} \text{–CTP contacts. Configuration 6 features both } \alpha E \text{ helices adopting a close orientation under L2, with } \alpha E \text{ helices anti-parallel to each other.} \]
The CTP contact with Arg223 did not preclude an interaction of Lys122 with CTP (Fig. S5A). The impact of the AI on the flexibility of the αE hinge is shown in Fig. S5B. The RMSF of residues 211–215 was reduced by 2-fold, both in the presence and absence of CTP. For the most part, the AI helices constrain the helices of the four-helix bundle to diffuse as a unit.

Membrane binding reorganizes the αE helices, a prerequisite for activation

Membrane binding dissociates the AI helices, which incorporate into the long M helices. If the αE helices are malleable and splay apart upon dissociation of the AI helices, as suggested by the MD simulations, then their average interchain distances should increase upon membrane binding (Fig. 6A). We substituted cysteine in place of a nonconserved, native alanine at residue 217 in the αEC helix in a cysteine-free CCT312 background where five native cysteines were changed to serines (22, 28). The activity was lowered ~2-fold by the substitution from Ala to Cys at position 217, relative to the Cys-free enzyme. We probed interchain cross-linking efficiency using a set of sulfhydryl-reacting bis-maleimides. Inter-αE helix cross-bridging will generate a covalent CCT dimer that can be detected on gels (Fig. 6B). Because cross-bridging is in competition with reac-
CCT silencing and activation mechanisms

![Figure 7](image)

**Figure 7. A disulfide bond linking the αE helices at Cys217 inhibits CCT activity.** CCT312 (Cys-free) and CCT312 (A217C) with a single cysteine in the αE were purified under oxidizing conditions. A, DTT reduction of oxidized CCT. Oxidized CCT preparations were incubated for 10 min at 37 °C in the presence of the indicated DTT concentration before analysis by nonreducing SDS-PAGE of monomeric (36-kDa) and dimeric (72-kDa) species. B, reduction of the disulfide between αE helices is required for lipid activation. In a separate experiment, enzyme activities were determined for A217C (■), and Cys-free (○) using standard conditions, 0.2 mM egg PC/egg PG (1:1) sonicated vesicles, and the indicated concentration of DTT. The data are means of two independent experiments with ranges (error bars) and are expressed as units/mg of CCT normalized to peak activity at 10 mM DTT (10,378 ± 1,654 units/mg for the Cys-free CCT and 4460 ± 780 for CCT-A217C).

positive impact on the activity of the Cys-free control, suggesting a minor oxidation effect unrelated to disulfide bond formation. However, the activity of oxidized CCT-A217C was only 12 ± 5% of the activity of the reduced A217C, assayed in the presence of 10 mM DTT, an ~8-fold effect of reduction. To ensure that the low activity of the disulfide-bridged CCT was not due to an impairment of the membrane binding of domain M, we compared oxidized and reduced CCT in an assay that monitors FRET between Trp778 in the M domain and dansylphosphatidylethanolamine in lipid vesicles. The FRET signal was the same for both oxidized and reduced CCT-A217C and was the same as FRET observed with a Cys-free CCT resistant to disulfide formation (Fig. S8). These data suggest that preventing restructuring of the αE by disulfide bonds (or alternatively by formation of a stable four-helix bundle with the αE helices) blocks lipid activation.

**Discussion**

In the MD simulations, the docked AI helices did not cause any major rearrangements of the active site. The AI helices reduced access to the active site from the underside of the catalytic domain, although the lateral opening remained sufficiently wide for entry and escape of CTP and CDP-choline (16 × 10 Å, larger than the length of CDP-choline at 13.5 Å; Fig. 4C). The sequence of the AI helix-turn is acidic and glycine-rich across species that show lipid regulation. The placement of the electronegative AI helix-turn at the mouth of the active site might also deter an approaching CTP or phosphocholine molecule from entering the active site. In addition to the restricted access of substrates and release of products, we have considered three possible mechanisms for auto-inhibition by the AI helices: (i) restricting the dynamics of active site loops and participating residues; (ii) hijacking the catalytic lysine; and (iii) preventing a conformational transition in the αE helices that enables an αE-Ec interaction with the active site. The AI helices dock onto the αE helices and loop L2. Thus, we focused on these elements, but in doing so, we are not excluding other indirect impacts of the AI.

**AI restriction of L2 dynamics**

Our previous single 200-ns simulation showed that the AI helix reduced the root mean square deviation from the starting structure for all backbone atoms in loop L2 (residues 121–128) by ~1 Å and the RMSFs by ~60% (6). The more extensive analysis here suggests that the AI has specific effects on the dynamics of L2 (modest) and the αE helices (strong). The RMSF analysis we performed reports on the nanoscale fluctuations of active-site loops and residue side chains. A 20–30% reduction in fluctuations, which we observed over 20 × 1 μs, could influence catalysis by limiting the range of conformational sampling, and this may reduce the frequency of productive orientations for groups participating in catalysis (29). However, given a kcat of 50 ms for fully active CCT, the dampening of nanosecond fluctuations may be irrelevant.

On the other hand, the conversion of the L2 backbone at Lys122 between α and β configurations does occur on the nanosecond timescale. The α and β Lys122 configurations favor inward (into the active site) and outward orientation, respec-
tively. The loss of activity when the conserved Gly in loop L2 next to Lys$^{122}$ was substituted with Ala or Pro supports a requirement for L2 backbone flexibility, spurring the question whether the docking of the AI helix could impede transitions between the $\alpha$ and $\beta$ configurations of the backbone and in this way impede contact with substrates in the active site. However, our analysis showed that although the $\beta$ configuration was highly correlated with the backbone trap, Lys$^{122}$ contact with CTP was compatible with either configuration (Fig. S3). Notably, the $\beta$ configuration did not preclude an interaction of Lys$^{122}$ with CTP when the AI was present. The side chain of Lys$^{122}$ can effectively contort to make contact with CTP. We cannot exclude a requirement for the $\alpha$ configuration (which is enabled by glycine at residue 123) for Lys$^{122}$ interaction with phosphocholine or the transition state. Our simulations did not test this. Thus, the data do not provide support for a lock-in of a particular L2 backbone conformation as a means for AI suppression of catalytic function; however, a role for backbone flexibility of loop L2 to facilitate Lys$^{122}$ sequential access to different partners during a catalytic cycle remains a viable hypothesis.

**Novel backbone competition for the catalytic lysine**

Another more obvious mechanism to reduce the frequency of productive conformations is to capture alternative nonproductive conformations. In CCT, the AI helix-turn forged frequent contacts with the Lys$^{122}$ $\epsilon$-amino group. Even with the active site occupied by CTP, the backbone carbonyls of the AI-turn were effective at steering the catalytic lysine away from the substrate (the backbone trap increased from $8-14\%$ frequency to $\sim40\%$ frequency). The reaction rate will scale inversely with the cumulative time spent in nonproductive alignments of a key catalytic residue. There are other enzymes that use electrostatic steering to forge inhibitory contacts with an active-site residue, but utilizing side-chain rather than backbone carbonyls as the interloper. For example, Src kinases employ one or more basic residues in the regulatory A loop to trap the glutamate in helix C that is a component of the KDE triad involved in ATP binding. Activation by phosphorylation of certain tyrosines steers the arginines away from the glutamate (5). By analogy, in CCT, the catalytic residue Lys$^{122}$ is competitively pulled away from an optimal orientation for substrate contact by the auto-inhibitory helix-turn, which presents a strongly electronegative pull. Another example of electrostatic steering is found in PGE$_2$ synthase, where an oxidation step requiring Arg$^{126}$ is blocked by an electrostatic interaction with Asp$^{49}$ (30). In PGE$_2$ synthase, the cofactor GSH can overcome this inhibitory interaction, whereas in CCT, effective desilencing requires larger domain movements (i.e. the dissociation of the AI helix-turn away from loop L2 and Lys$^{122}$).

**Silencing by constraining the $\alpha E$ helices**

In addition to influencing the positioning of Lys$^{122}$, we suggest that the AI helices inhibit the CCT reaction by preventing conformational change in the $\alpha E$ helices. The simulations in the absence of the AI helices with NOE-type restraints on helices $\alpha E_C$ show that the $\alpha E$ helices are highly dynamic in a hinge segment between residues $\sim210$ and 215. The $\alpha E_N$ region is much less so. The hinge undergoes many different contortions, enabling splaying apart of $\alpha E$ helices to contact loop L2 at the base of the active site. The contact with L2 can be maintained for hundreds of ns, and in one replicate, the splayed conformation was acquired during the equilibration and persisted in both chains for the entire production run (960 ns). The principle stabilizing force for this interaction is an aromatic cluster between Phe$^{214}$ in L2 and the two tyrosines of the $\alpha E$ hinge. This aromatic cluster was observed in 17 of 20 chains in the NOE simulations. In the other three chains, the $\alpha E_C$ of that chain did not migrate toward L2, but stayed linked to its partner $\alpha E_C$. In addition, a hydrogen bond between the Tyr$^{213}$ hydroxyl in the $\alpha E$ hinge and the Phe$^{214}$ backbone carbonyl in L2 was observed for hundreds of ns in splayed conformations. We note that Phe$^{214}$ of the aromatic cluster is not universally conserved. However, the residues that can substitute for it are leucine (hydrophobic), arginine, and glutamine. The latter two may replace a purely aromatic interaction with a $\pi$-cation/amino interaction (31). It is intriguing that the aromatic clusters that mediate the loop L2–AI helix interaction may be replaced with an alternative aromatic cluster between L2 and the $\alpha E$ hinge upon dissociation of the AI helices.

The entire C terminus of $\alpha E$ may be intrinsically malleable in the absence of the AI helices. There was no electron density for these residues in the crystal structure of CCT236, which contains all of the catalytic domain, the $\alpha E$, and the linker to domain M (but no AI helix). However, we were forced to put some constraints on the C terminus of the $\alpha E$ helices, because without them, the tail end entered into the unoccupied active site.

**In vitro cross-linking also provided evidence that lipid-induced dissociation of the AI resulted in increased distance between the two $\alpha E_C$ helices, consistent with the splaying observed in silico with the AI helices removed. An alternative hypothesis, that lipid vesicles reduce the accessibility and/or reactivity of the cross-linkers, is unlikely for two reasons: (i) the disassembly of the four-helix bundle should increase the exposure of the reactive cysteines, leading to elevated cross-linking; and (ii) in separate experiments, we found that the $\alpha E_C$ does not bury in the membrane in the CCT$_{mem}$ form.**

Most importantly, straightjacketing the $\alpha E$ helices by disulfide engineering prevented activation by lipid vesicles. The disulfide was located just C-terminal to the $\alpha E$ hinge segment. This suggests that reorganization and/or dissociation of the $\alpha E_C$ helices is required for activation of the membrane-bound CCT and that the four-helix bundle silences CCT by virtue of the constraining action on the $\alpha E$ helices.

What is the potential impact of the $\alpha E$ plasticity on catalysis? We know that membrane binding displaces the AI helices, which would allow a large increase in conformational sampling of the $\alpha E$ helices. But perhaps only a few of those conformers are productive for speeding up catalysis. We hypothesize that membrane binding of the M domain (and perhaps the short amphipathic linker segment intervening between the $\alpha E$ and domain M) promotes that productive conformation. As illus-

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5 D.G. Knowles and R.B. Cornell, unpublished results.
**CCT silencing and activation mechanisms**

Figure 8. New model for CCT activation. In its soluble form, the two Al helices silence CCT by (i) capturing the catalytic lysine (Lys122) with backbone carbonyls and (ii) restraining helix αE dynamics. In response to increased anionic charge on the membrane surface, the positively charged leash segment is attracted to the membrane, and its binding facilitates dissociation of the Al helices. The αE helices gain conformational freedom, but the binding of the linker segment to the membrane creates a platform to stabilize αE conformations that are productive for catalysis. We propose that the bend at the αE hinge is featured in productive conformations. The linker is represented as a simple trapezoid because its structure is unknown. The linker-membrane interaction may be dynamic, enabling sampling of different αE (and linker) conformations during a catalytic cycle. The bent αE helices and the folding of the linker brings the catalytic domain closer to the membrane surface. Images are from NOE-restrained simulations. The disordered N and P regions are not displayed.

trated in the captured images (Fig. 5) and in the model shown in Fig. 8, the bent helix αE configurations could enable close approach of the catalytic domain to the membrane surface. Whether this is important for catalysis or for efficiency of PC synthesis remains to be probed in future studies. Delivery of product (CDP-choline) at the membrane would facilitate its utilization in the last step of the CDP-choline pathway, which is catalyzed by an integral membrane protein, choline phosphotransferase. Pioneering studies by George et al. (32, 33) showed that [3H]choline flux into PC in cultured glioma cells was not reduced by excess CDP-choline delivered by cell permeabilization, providing evidence for metabolite channeling within the CDP-choline pathway. Although an intriguing idea, CCTα and the phosphotransferase often localize to different membrane systems in cells (34).

We propose three hypotheses for how the bent αE with its close contacts with loop L2 could facilitate catalysis. (i) The bent αE could eliminate the backbone trap. In simulations without the Al helices, bent αE helices enabled H-bonding between the hydroxyl of Tyr213 with the Phe124 carbonyl, removing this carbonyl oxygen from a trapping interaction with Lys122. A hydrogen bond between these two atoms was observed in one chain of the partially active CCT236 crystal structure (20). Loss of H-bonding potential at Tyr213 by mutation to phenylalanine partially inhibited lipid activation,6 implying a function for the Tyr213 hydroxyl. (ii) Residues in the bent αE helices may participate in one or more steps in the catalytic cycle. Arg223 at the C terminus of the αE made frequent interactions with the γ-phosphate of CTP that lasted for up to 700 ns. Thus, upon membrane binding and acquisition of a bent αE, Arg223 may assist in the binding of the substrate CTP. Alternatively, Arg223, which bonds only to the γ-phosphate, may provoke Lys122 to interact with the α-phosphate of CTP, phosphocholine, or the transition state. A third possibility is that Arg223 may assist in displacement of the product, diphosphocholine. Interestingly CCT-R223S is one of several alleles in humans that suffer from an inherited disorder, SMD-CRD (15). CCT-R223S has a 16-fold lower $k_{cat}/K_m$ for both substrates than the WT enzyme.7 The contact frequency between Ser223 and CTP phosphates would be much lower than that for Arg223. (iii) Residues from the bent αE may seal off the opening of the active site bounded by loop L2 and the αE of both chains (Fig. 4C). This could assist catalysis by trapping the reaction intermediates and perhaps excluding water. It is tempting to speculate that the bent helix conformation might be dominant in one step of the catalytic cycle (e.g. in the lead-up to the chemical step) and that subsequently its reorganization to a more open conformation allows product release.

Signaling helix pairs are emerging as agents of communication between domains of regulatory proteins, such as receptor tyrosine kinases (35), two-component signaling systems (36), and cytokine receptors (37–39). The helix deformations that transduce interdomain signals can occur within microseconds after a trigger is applied in vitro (36), lending validity to our in silico results. We propose that the conformational malleability of the αE helix pair that bridges the membrane binding and catalytic domains of CCT makes it an ideal element adapted by evolution for transducing signals from membrane to active site.

**Materials and methods**

**Molecular dynamics simulations**

The crystal structure of the rat CCTα dimer, CCT1-312(Δ238–269), was used as the starting structure for simulation (PDB code 4MVC) (6). The N termini of each chain were acetylated, and the C termini were amidated to eliminate charged ends. The crystal structure was solved in complex with CDP-choline. To create a CTP-bound model, the crystal structure of GCT (PDB code 1COZ) (21) was first aligned to 4MVC using the superimpose module of Coot software (40). Next, the CTP coordinates from the aligned GCT were combined with the coordinates of the CCT protein from 4MVC. Hydrogens were added to the CTP molecule using the program Avogadro.

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6 J. Lee, S.G. Taneva, and R.B. Cornell, unpublished results.
7 R.B. Cornell, unpublished results.
The CTP acidic oxygens were deprotonated, giving it a charge of $-4$. Hydrogens were added to the protein using the pdb2gmx module of the GROMACS package. Each structure was solvated with TIP3P water molecules in a truncated dodecahedron-shaped simulation box with a minimum distance of 1.2 nm around the protein. $Na^+$ and $Cl^-$ ions were added to neutralize the protein/ligand charge and then to obtain a 0.15 M ionic concentration.

MD simulations were run with the GROMACS version 4.6.7 package (44, 45). The protein was described using the all atom AMBER99SB-ILDN force field (46). The parameters for CTP were taken from Demir and Amaro (47) and were adapted for compatibility with GROMACS using the Python script ACYPYE (48).

The simulations fall into four different groups distinguished by the presence or absence of CTP and the presence or absence of the AI N terminus (MGSSH6SSGLVPRGSH). value of 3 Å was used as the “donor–acceptor” distance cutoff. The RMSF analysis used the coordinates at the end of the 40-ns equilibration as the reference for the change in position of (i) heavy atoms of a specified residue side chain or (ii) the N, Cα, and carbonyl backbone atoms of specified loops using the GROMACS module g_rmsf (with options -res and -nofit). Outputs of the analyses were imported into Excel for averaging and statistical analyses. Unpaired two-tailed t-tests were used to compute the probability of sameness between sample sets.

Construction of CCT mutants

All CCT constructs were derived from Rattus norvegicus pcy11a (α isoform; UniProtKB/Swiss-Prot accession number P19836). All primer designs and codon substitutions were performed using QuikChange site-directed mutagenesis (Agilent). Schematics of all constructs are shown in Fig. S10.

**pET14b-CCT constructs**

The preparation of pET14b-CCT236 and pET14b-CCT312 was described by Lee et al. (6). These were used as the templates for engineering an Ala or Pro substitution at Gly123 by site-directed mutagenesis. pET14b-CCT236 was also used for engineering a Trp replacement at Phe124. All pET14b CCT constructs contained a plasmid-derived His tag and a thrombin cleavage sequence at the N terminus (MGSSHPSPQK).

**pET24a-CCT312 constructs**

C-terminally truncated proteolytic fragments were sometimes found as contaminants in the His-CCT312 preparations. To facilitate the removal of these fragments during purification, we prepared pET24a-CCT312 with the His tag at the C terminus (LEHHHHHHHH). Fragments lacking the C terminus would not be retained on nickel-agarose. To prepare pET24a-CCT312-His, we amplified the ORF (codons 1–312) and engineered NdeI and XhoI restriction sites at 5 primers, respectively, by standard PCR methods using the template pAX142-His-CCTα312, whose construction was described by Dennis et al. (56). The amplicon was inserted into NdeI/XhoI-cut pET24a. This construct was subsequently used to create CCT312-His with two native tryptophans at Trp151 and Trp278 mutated to phenylalanine (CCT312(W151F/W278F)). We then used the Trp-free construct to engineer CCT312-His F124W.

The construction of CCT312 with five Cys to Ser mutations (pET24a-CCT312 (Cys-free)) utilized the template pAX142-HisCCTα367-T207C, described by Huang et al. (22), which contained a single cysteine at T207C in the context of a full-length CCTα. The Cys207 mutation was first converted back to Thr by site-directed mutagenesis, and an internal KpnI/SacI restriction site at 5’ and 3’ ends, respectively, by standard PCR methods using the template pAX142-His-CCTα312, whose construction was described by Dennis et al. (56). The amplicon was inserted into NdeI/XhoI-cut pET24a. This construct was subsequently used to create CCT312-His with two native tryptophans at Trp151 and Trp278 mutated to phenylalanine (CCT312(W151F/W278F)). We then used the Trp-free construct to engineer CCT312-His F124W.

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CCT silencing and activation mechanisms

Expression and purification of CCT constructs

All proteins were expressed in *Escherichia coli* (Rosetta-DE3) cells as His$_6$ fusion proteins and were purified using nickel-agarose affinity chromatography as described previously (6, 28, 57) with modifications described below. The His tag was at the N terminus of all CCT236 constructs as well as CCT312 (WT), CCT312 (G123A), and CCT312 (G123P). The tags were not cleaved after protein purification. The His tag was at the C terminus of CCT312 (F124W), CCT312 (F124W), and CCT312 with a single Cys at 217.

His-CCT236 constructs were purified from the 20,000 g x 15 min supernatant fraction of the cell lysates. They were eluted with 350 mM imidazole, 300 mM NaCl, 10 mM Tris, pH 8.0, and 2 mM DTT and subjected to Q-Sepharose ion-exchange chromatography to remove the imidazole. The 312His F121W and 312His F124W proteins were mostly in the 10,000 g pellet and were purified after solubilization of this pellet with 6 M guanidine hydrochloride at room temperature with occasional vortexing over 30–60 min. The denatured proteins were refolded while bound on the nickel-agarose resin using a 6 to 0 M urea gradient in 50 mM NaH$_2$PO$_4$ (pH 8.0), 0.5 mM NaCl, 25 mM imidazole, and 1 mM DTT at a flow rate of ~2 ml/min over ≥1 h. The refolded proteins were eluted and dialyzed against 200 mM NaCl, 10 mM Tris, pH 7.4, and 2 mM DTT to remove the imidazole.

The single-Cys CCT312His (∆NLS) A217C protein used in cross-linking experiments was purified from the 10,000 g pellet as described above; however, the elution and dialysis buffers contained TCEP as a reducing agent instead of DTT. TCEP is compatible with the bis-maleimide cross-linkers (Thermo Fisher Scientific); DTT is not.

Preparation of lipid vesicles or micelles

Lyso-PC (18:1)/egg PG (4:1) micelles used for fluorescence spectroscopy were prepared as described by Taneva et al. (10). Small unilamellar vesicles, egg PC/egg PG (1:1), were prepared by sonication as described (59) and were used for enzyme activity assays and cross-linking reactions.

Tryptophan fluorescence anisotropy

Steady-state fluorescence anisotropy measurements were made using a Horiba Jobin Yvon FluoroLog 3 spectrometer equipped with polarizers. Intensities with excitation polarization either vertical (V) or horizontal (H) and emission polarization either V or H ($I_{VV}$, $I_{VH}$, $I_{HV}$, and $I_{HH}$) were measured, and the anisotropy ($r$) was calculated by the software of the instrument. The temperature was controlled at 20 °C with a thermo-regulated cell holder. The integration time was 1 s. The excitation wavelength was 295 nm, the emission wavelength was 345 nm, and the excitation and emission bandwidths were 4 and 12 nm, respectively. Anisotropy values are the average of 4–6 replicates for each sample. The protein concentration was 5 μM, and the lipid/protein molar ratio was 150.

CCT activity assay

CCT activity was performed as described previously (60). The standard reaction mixture contained 50 nM CCT, 20 mM Tris, pH 7.4, 10 mM DTT, 88 mM NaCl, 12 mM MgCl$_2$, 2 mM [${}^{14}$C]phosphocholine (1 mCi/mmol), and either 10 mM CTP (CCT312 constructs) or 20 mM CTP (CCT236 mutants). Incubations were for 10 min at 37 °C with or without 0.2 mM PC/PG (1/1) SUVs.

Bis-maleimide cross-linking

Thiol-specific homobifunctional cross-linkers were obtained from Sigma (N,N’-o-phenylene-dimaleimide) and TCI America (1,2-(bis-maleimido)ethane and 1,4-(bis-maleimido)butane). Immediately before use, the cross-linkers were dissolved in DMSO at a 25–50 mM concentration.

The single-Cys CCT312His (∆NLS; A217C) was purified and stored in 1 mM TCEP to prevent oxidation of Cys residues. Chemical cross-linking was carried out at 37 °C for 10 min in a volume of 50 μL containing ~2 μM CCT, 0.5 mM TCEP, and variable concentrations of cross-linkers and phospholipid vesicles (egg PC/egg PG (1:1)). The reactions were initiated with the cross-linking agent and were terminated with 5.5 mM DTT. Control reactions in parallel were quenched with 5.5 mM DTT at time 0. Aliquots were mixed with reducing SDS sample buffer, heated to 85 °C, and evaluated using 11% SDS-PAGE. Gels were stained with Coomassie Blue, or proteins were electroblotted onto poly(vinylidene difluoride) membranes (Immuno-Blot PVDF, Bio-Rad) and probed with antibody directed against residues 164–176 of domain C (28).

Disulfide bond formation by air oxidation

CCT312His (∆NLS; A217C) and Cys-free CCT312His (∆NLS) were purified from the 10,000 g pellet under denaturing conditions as described above. Low concentrations of the reducing agent DTT were used throughout the protocol to prevent misfolding of the denatured proteins. The concentrations of DTT in the refolding, elution, and dialysis buffer were 100, 50, and 10 μM, respectively. Gentle oxidation of the single Cys CCT312His (∆NLS; A217C) was achieved by exposure to the atmospheric air during the purification. We avoided copper phenanthroline–catalyzed disulfide induction because copper inhibits CCT activity.

Acknowledgments—Simulations used West-Grid/Compute Canada facilities. We are grateful to Ronnie Tse for construction of the single-Trp variants of CCT.

Author contributions—M. R., J. L., D. P. T., and R. B. C. conceptualization; M. R., J. L., S. G. T., and R. B. C. data curation; M. R., J. L., S. G. T., D. P. T., and R. B. C. formal analysis; M. R., M. R., J. L., S. G. T., D. P. T., and R. B. C. validation; M. R., J. L., S. G. T., D. P. T., and R. B. C. investigation; M. R., J. L., S. G. T., D. P. T., and R. B. C. methodology; M. R., J. L., S. G. T., and R. B. C. writing—original draft; M. R., J. L., S. G. T., D. P. T., and R. B. C. writing—review and editing; J. L., S. G. T., and R. B. C. visualization; D. P. T. and R. B. C. supervision; D. P. T. and R. B. C. funding acquisition; D. P. T. and R. B. C. project administration.

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