Remodeling of the interdomain allosteric linker upon membrane binding of CCTα pulls its active site close to the membrane surface

Received for publication, June 18, 2019; in revised form, August 18, 2019. Published Papers in Press, September 4, 2019. DOI 10.1074/jbc.RA119.009850

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Edited by George M. Carman

The rate-limiting step in the biosynthesis of the major membrane phospholipid, phosphatidylcholine, is catalyzed by CTP:phosphocholine cytidylyltransferase (CCT), which is regulated by reversible membrane binding of a long amphipathic helix (domain M). The M domain communicates with the catalytic domain via a conserved ~20-residue linker, essential for lipid activation of CCT. Previous analysis of this region (denoted as the αEc/J) using MD simulations, cross-linking, mutagenesis, and solvent accessibility suggested that membrane binding of domain M promotes remodeling of the αEc/J into a more compact structure that is required for enzyme activation. Here, using tryptophan fluorescence quenching, we show that the allosteric linker lies superficially on the membrane surface. Analyses with truncated CCTs show that the αEc/J can interact with lipids independently of the M domain. We observed strong FRET between engineered tryptophans in the αEc/J and vesicles containing dansyl-phosphatidylethanolamine that depended on the native J sequence. These data are incompatible with the extended conformation of the αE helix observed in the previously determined crystal structure of inactive CCT but support a bent αE helix conformation stabilized by J segment interactions. Our results suggest that the membrane-adsorbed, folded allosteric linker may partially cover the active site cleft and pull it close to the membrane surface, where cytidyl transfer can occur efficiently in a relatively anhydrous environment.

This work was supported by a grant from the Canadian Institutes for Health Research (to R. B. C.). The authors declare that they have no conflicts of interest with the contents of this article.

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3The abbreviations used are: CCT, CTP:phosphocholine cytidylyltransferase; domains C and M, catalytic and membrane-binding domains, respectively; AI, auto-inhibitory; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PG, phosphatidyglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; LPC, 1-palmitoyl lysophosphatidylcholine; Br-PC, dibrominated phosphatidylcholine; SUV, small unilamellar vesicle; PDB, Protein Data Bank; FPLC, fast protein liquid chromatography; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; nSMase2, neutral sphingomyelinase; NSL, nuclear localization signal.
Recent molecular dynamics simulations also revealed the plasticity of the αE hinge. In the simulations of CCT-236, residues of the αE_C and J segment showed a high degree of malleability and divergence from the starting structure (15). In simulations of residues 40–223 missing the J segment and M domain, the αE_C was externally restrained to remain helical, but the rest of the αE was unrestrained. The αE_C pair retained helical structure and interhelical contacts throughout the 1-μs simulations, but the hinge at residues 212–215 sampled many conformations (14). The most long-lived conformers (up to 700 ns) displayed a prolonged sharp bend at the hinge that brought the C terminus of the αE_C proximal to the active site and loop L2. The bent αE helix conformations showcased a new interdomain aromatic cluster involving two tyrosines within or proximal to the αE hinge and a phenylalanine in loop L2 of the active site.

In vitro support for αE malleability as essential to drive CCT activation was provided by “straight-jacketing” the αE helices with chemical cross-links (14). When the helices were constrained from bending, activation of the membrane-bound enzyme (CCT_{mem}) was impaired. The activity-promoting impact of mutations in the αE hinge that destabilize interactions between the αE helix pair underscored the importance of helix αE malleability (13). The solvent accessibility of residues in the αE hinge in CCT_{mem} was consistent with a hinge that exists within a folded ensemble (13). The working hypothesis is that the membrane-triggered displacement of the Al helices frees the αE_C to sample other conformations, including ones more productive for catalysis. Based on the conserved and amphipathic nature of the αE_C/J segment and its proximity to domain M, we further hypothesize that the fully active conformation of this sequence includes an association with membranes as a compact, folded unit. Such an association could stabilize a rearrangement of the αE helices that would dock the catalytic domain close to the membrane surface.

In the present work, we show that the αE_C and J segment interact with the membrane surface in CCT_{mem} and both can do so independently of the adjacent M domain. FRET analysis between Trp donor sites in CCT and a lipid acceptor shows that the active site is pulled closer to the membrane surface than would be predicted in a model with the αE helices fully extended. These data provide evidence for a compact structure of the αE and J segment that together comprise an allosteric linker whose conformational ensemble is constrained and directed by membrane association. Last, we address how catalysis could be enhanced by drawing the active site close to the membrane surface.

Results

Brominated PC (Br-PC) quenching of single-Trp variants reveals a superficial association of the allosteric linker with membranes

As there is no solved structure of lipid-bound CCT, the orientation of the αE_C/J relative to the membrane surface is not known. To assess whether sites in this segment interact with the membrane surface, we explored the fluorescence quenching of
These data support a stable interaction was monitored in the presence of vesicles composed of POPC/egg PG (1:1) (unquenched; \( F_0 \)) or dibrominated PC/egg PG (1:1) (quenched; \( F_q \)). The [CCT] was 3 \( \mu \text{M} \), and the (lipid) was 450 \( \mu \text{M} \). Spectra were corrected for lipid blanks, and the peak fluorescence intensities were obtained. Individual representative spectra are found in Fig. 5A. Data are means \( \pm \) average deviation (error bars) of 2–5 independent determinations. The *large and small asterisks* indicate significant \( p \) values (\( \leq 0.01 \) and 0.05, respectively) compared with quenching of F55W by the respective Br-PC. Single-Trp constructs are derived from a CCT-312-His \( \Delta \text{12–16} \) background with native Trps removed (W151F; W278F). “W151” and “W278” retain the indicated single native Trp.

The preparation and analysis of the impact of many of the \( \alpha \text{E/J} \)-engineered Trps on CCT activity and thermal stability were presented in the companion paper (13). In addition, we analyzed single Trps engineered into the catalytic domain at F121W and F124W that were characterized previously (14). The Trp substitutions did not affect the stability of the catalytic domain fold (Table S1). The Trps engineered into \( \alpha \text{E/J} \) did not impair the interaction of the M domain with membrane vesicles. All active \( \alpha \text{E/J} \) Trp variants had approximately the same affinity as the WT CCT for the highly curved vesicles used in the fluorescence experiments (13).

We probed Trp quenching by PCs brominated at the 6,7-, 9,10-, or 11,12- positions of the acyl chain. Quenching by Br-PC requires van der Waals contact of the excited Trp and bromine on the acyl chain (16). In keeping with published data using domain M peptides (17), Trp-278 was strongly quenched by all three depths of bromine quenchers in the context of CCT-312, with the strongest impact using 9,10-Br-PC (Fig. 2). These data support a stable interaction between the vesicles and domain M. In contrast, Trp-55 at the N terminus of the catalytic domain was not significantly quenched by any Br-PC vesicles. Trp-151 near the active site was also not quenched. These results provide evidence that Br-PC quenching monitors the persistence of Trp residue–membrane interactions and that random collision with vesicles does not contribute significantly to quenching.

Trps at the N terminus (Trp-225), middle (Trp-230), and C terminus (Trp-234) of the J segment were moderately quenched by Br-PC, most prominently at the shallower 6,7- and 9,10-positions (Fig. 2). Interestingly, Trp-216 and Trp-221 were weakly quenched, despite their location in the \( \alpha \text{E/C} \). Even more surprisingly, Trp-124 in loop L2 of the catalytic domain was quenched beyond background levels by the Br-PC. These data suggest that the conformational ensemble for CCT\(_{\text{mem}}\) includes a population where the allosteric linker situates superficially at the membrane surface and where Phe-124 in loop L2 makes transient excursions toward the membrane surface. The Trps in the \( \alpha \text{E/C} \) remain accessible to acrylamide, except for the Trp at residue 216, near the \( \alpha \text{E} \) hinge, which is buried in CCT\(_{\text{mem}}\) (13).

We attempted to interrupt the membrane interaction of the allosteric linker by mutation to probe whether this association is required for signaling from the bound M domain to the active site. We tested the effects of three mutants on quenching by Br-PC: 3\( \Phi \) (L230A/V232A/F234A) to reduce the hydrophobicity, 3\( \text{R} \) (R219Q/R223Q/K228Q) to reduce the electrostatic drive, and 3\( \text{A} \) (Y216A/Y225A/F234A) to eliminate the aromatic pull toward the bilayer interface (Fig. 3A). The transition temperatures for unfolding of these variants were the same as the parent CCT from which they were derived, indicating proper domain C folding (49.8 \( \pm \) 0.2 °C for the four CCTs). The mutations destabilized the enzyme activity (Fig. 3B), confirming the strong dependence of catalytic function on the native linker sequence (13). We did not assay the activity of the triple aromatic variant, as we knew that the substitution of just one of the sites, Y216A, was strongly inactivating (13). The interaction with the membrane was monitored by Br-PC quenching of either engineered Trp-225 or Trp-221. To our surprise, none of the mutations reduced the quenching of Trp-225 or Trp-221 (Fig. 3C). These results suggest that either the linker interaction with the membrane involves multiple disperse interactions that survive the loss of a few sites or that the method of Br-PC quenching of engineered Trps is not accurately reporting a binding event for the linker. The allosteric linker is in close proximity to the M domain with its known membrane-anchoring properties. Could the bound M domain and the engineered Trps be pulling the linker to the surface at the engineered site? To address this concern, we probed whether the linker has membrane-binding properties independent of the M domain and engineered Trps.
Linker remodeling pulls CCTα active site toward membrane

Lipid interactions with CCT-236 implicate the αE/J segment as a membrane-binding region

CCT-236 is truncated at the boundary between the allosteric linker and domain M. It has weak constitutive activity, but we have documented that its $V_{\text{max}}$ can be elevated ~2-fold by lipid vesicles (13). By contrast, the $V_{\text{max}}$ for full-length CCT is elevated >30-fold by lipids. In the companion paper (13) we showed that deletion of residues 227–232 of the J segment decreased the activity of CCT-236 both in the absence and presence of lipids, but the mutant was still activated ~2-fold by lipids (Fig. 3B of Ref. 13). We examined the membrane binding of CCT-236 with a native J sequence or with the J segment deletion, using Br-PC quenching of an engineered Trp-225 at the start of the J segment. We found that Br-PC quenching of Trp-225 was even stronger in CCT-236 than in CCT-312 (Fig. 4). Trp-151 in the active site, a negative control, showed no quenching. Surprisingly, the quenching of Trp-225 in CCT-236 was not reduced by deletion of J segment residues 227–232, but was even enhanced (Fig. 4). This suggests that the J segment, although important for catalytic function, is not required for association of CCT-236 with membranes. Rather, membrane association involves alternative or additional residues (e.g. residues in the αE/J).

We then analyzed CCT-236 lipid association using an alternative method that does not rely on engineering tryptophans, which could impart membrane partitioning bias. We used gel filtration of CCTs containing only native Trps in the presence or absence of activating micelles (lyso-PC 16:0). These micelles activate CCT-236 and the 236 ΔJ deletion mutant as effectively as sonicated lipid vesicles (Fig. 5A). They are also effective but weaker activators of full-length CCT and CCT-312. To probe whether Superdex-200 chromatography can distinguish CCT that is free versus micelle-bound, we first examined a CCT with domain M. We used CCT-312Δ32 with a shortened domain M as it retained better solubility than the CCT-312 construct with a full-length M domain during concentration prior to gel filtration. This construct retains the AI helix but is missing the leash portion of domain M, residues 238–269. In the absence of lipid, CCT-312Δ32 eluted as two populations, one with a mass approximating a dimer, slightly larger than the albumin stan-
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The data in support of direct lipid interactions of the αE<sub>C</sub>/J challenge the model shown in Fig. 6E with an extended structure for this region. If the αE helices bend to allow membrane interactions during the conversion from inactive to active CCT, the distance between the membrane and the catalytic domain would be shorter compared with the fully extended model. We estimate, using the solved structure of CCT<sub>sol</sub> and estimating an average of 155 Å for the span of the 12 amino acids of the J segment in a condensed form, that the distance between, for example, Phe-124 in loop L2 and the membrane surface in the fully extended αE, would be >30 Å. We used our set of single Trp variants to monitor FRET signals between the Trp donors and a lipid fluorophore. The Förster length (<em>R</em><sub>0</sub>) for this FRET pair has been reported in the range of ~20–24 Å (45). A donor–acceptor separation of 30 Å would yield a FRET value of ~0.15 when <em>R</em><sub>0</sub> = 22 Å.

In the first set of experiments, we monitored the increase in dansyl-PE fluorescence with increasing concentration of CCT marker positions are indicated at the top of B with arrows. These constructs have N-terminal His tags and, with the exception of CCT-236 ΔJ, retain the NLS sequence.

Figure 5. Lipid affinity of αE/J can be independent of domain M interaction with membranes. A, activation of CCTs by LPC (160). CCTs were incubated with buffer, 10 μM LPC, or 100 μM egg PC/egg PG (1:1) SUVs for 5 min prior to assay under conditions optimal for the indicated CCT. Data are means ± average deviation (error bars) for 2–3 independent determinations. *<em>p</em> < 0.01, with reference to the samples lacking lipid. B–E, elution profiles for the indicated CCTs concentrated without (gray lines) or with LPC (green lines) prior to application to a Superdex 200 FPLC column. The panels monitor the continuous A<sub>280</sub> in milliabsorbance units (B–E) and the LPC concentration in a subset of 1-ml elution fractions (F). The lipid profiles shown in F are also plotted alongside their respective CCT elution profiles in B–D. Molecular mass shift to a larger size, broadened, and co-eluted with lipid, confirming that the J segment is not solely responsible for the lipid interaction (Fig. 5D). However, CCT-212, lacking all of the α<em>E</em> and J segment, eluted as a 70-kDa dimer both in the absence and presence of micelles (Fig. 5E), suggesting that the α<em>E</em> contributes to the lipid interaction. The mass shifts in both the protein and lipid components (Fig. 5F) argue strongly for lyso-PC complex formation with CCTs containing the α<em>E</em>/J, and provide evidence that a combination of the α<em>E</em> and J segment can form an independent, catalytically relevant membrane interaction. The lipid/protein molar ratios we measured in the higher mass-shifted peaks were ~70 for CCT-236 and CCT-236ΔJ, and ~115 for CCT-312Δ32. Using the mass shifts indicated in Fig. 5, we estimate an average of ~2 CCT dimers bound to a single lyso-PC particle. Although the interaction shown here is with a micellar lipid particle, it demonstrates that residues in the α<em>E</em>/J are lipophilic and thus would seek to embed in an accessible membrane surface.

Trp–dansyl-PE FRET supports a compact allosteric linker structure

In the absence of lipid, CCT-236 eluted as a single, broad peak approximating a dimer, but upon the addition of micelles, the eluted complex size increased to 220 kDa and co-eluted with phospholipid (Fig. 5C). The J-segment deletion mutant, CCT-236 Δ227–232, eluted as a ~70-kDa dimer in the absence of lipids. With the introduction of micelles, the elution profile shifted to a larger size, broadened, and co-eluted with lipid, confirming that the J segment is not solely responsible for the lipid interaction (Fig. 5B). Whereas CCT-212, lacking all of the α<em>E</em> and J segment, eluted as a 70-kDa dimer both in the absence and presence of micelles (Fig. 5E), suggesting that the α<em>E</em> contributes to the lipid interaction. The mass shifts in both the protein and lipid components (Fig. 5F) argue strongly for lyso-PC complex formation with CCTs containing the α<em>E</em>/J, and provide evidence that a combination of the α<em>E</em> and J segment can form an independent, catalytically relevant membrane interaction. The lipid/protein molar ratios we measured in the higher mass-shifted peaks were ~70 for CCT-236 and CCT-236ΔJ, and ~115 for CCT-312Δ32. Using the mass shifts indicated in Fig. 5, we estimate an average of ~2 CCT dimers bound to a single lyso-PC particle. Although the interaction shown here is with a micellar lipid particle, it demonstrates that residues in the α<em>E</em>/J are lipophilic and thus would seek to embed in an accessible membrane surface.

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and its Trp donor (Fig. 6A). The FRET curves were hyperbolic and saturated because as CCT was titrated into a fixed concentration of vesicles (100 μM), binding to the vesicles likely became restricted due to molecular crowding on the membrane surface. The lipid/protein ratio was only 25 at the highest donor/acceptor ratio, barely sufficient to solvate domain M (18). Despite this potential limitation, it is clear that Trp-278 in domain M, along with Trp-234, produced the highest FRET signals (Fig. 6A). The negative control, Trp-55, at the N-terminal of the catalytic domain, generated a very low FRET signal (0.05) that could reflect random collisions. Trp-151 near the active site and Trp-121/Trp-124 in loop L2 produced measurable FRET signals, suggesting an approach of these residues to the membrane interface that would not be possible with an extended allosteric linker conformation. The FRET signal produced by Trp-124 was especially intriguing, as it was similar in strength to the signals produced by Trp-221 in αEc and Trp-225 in the J segment. FRET from Trp-216 was also surprisingly

Figure 6. Trp to dansyl-PE FRET suggests a close membrane approach of the αEc and active site. A and B, increases in dansyl-PE acceptor fluorescence with increasing [CCT]; C and D, decreases in Trp donor fluorescence with increasing [dansyl-PE]. Data in all panels are means ± range (error bars) of two independent determinations. Representative spectra are found in Figs. S4 and S5. Single-Trp constructs are derived from a CCT-312-His Δ12–16 background with native Trps removed (W151F; W278F). “W151” and “W278” retain the indicated single native Trp. A, samples contained CCT (0–4 μM) with a single Trp at the indicated residue and 100 μM vesicles (POPC/egg PG/dansyl-PE (50/48/1.8)). Trp was excited, and dansyl fluorescence was monitored at 520 nm. F, fluorescence at 520 nm when [CCT] = 0 μM; Fmax, intensity at 520 nm with increasing [CCT]. B, FRET from Trp-216 in αEc or Trp-124 in L2 to dansyl-PE is reduced upon substitution of the WT linker with a glycine-rich sequence (GG-J; 227GGGSGG232). C, samples contained CCT (1 μM) with the indicated single Trp and 100 μM vesicles containing 50% POPC and the indicated mol % dansyl-PE balanced by egg PG. Trp was excited, and fluorescence was monitored at the peak wavelength for the Trp. F, FRET peak fluorescence in the presence of vesicles lacking dansyl-PE; Fmax, Trp fluorescence with increasing [dansyl-PE]. D, FRET between dansyl-PE and Trp-216 in the αEc is reduced upon substitution of the WT J segment with a glycine-rich sequence (GG-J, 227GGGSGG232). E and F, two models for the allosteric linker structure in CCTmem. E, fully extended αEc/J. The model shows the positions of the Trp residues we monitored (sphere representation), color-coded to illustrate the degree of FRET expected in the fully extended form. Black, no FRET; red, maximum FRET. The pink background reflects the FRET gradient diminishing with distance from the membrane surface. No FRET is expected for residue 124 in loop L2 in this model of the allosteric linker. PDB entry 3HL4 was used for residues 40–216, and PDB entries 1PEI and 1PEH were used for the M helix (residues 236–288). The Trps missing from these structures (221, 225, 230, and 234) were placed manually. F, condensed αEc/J. The model shows the positions of the Trp residues color-coded to reflect the degree of FRET determined by experiment. Trp-124 in loop L2 is within FRET distance in this model, and the Trps in αEc/J occupy a nearly equivalent distance from the membrane. The CCT catalytic domain with bent α helices is a snapshot obtained from an MD simulation of residues 40–223, without the constraining AI helices (14). The J segment is modeled as a short lavender cylinder. Trps not contained in the input structures were placed manually (Trp-225, -230, and -234).
strong, given its position near the start of the $\alpha_E$, stronger than Trp-221 at the C terminus of $\alpha_E$ and similar to Trp-230 of the J segment. Therefore, the $\alpha_E$ and J segment appear to lie along a similar plane with respect to the membrane surface.

To probe whether the strong FRET can be weakened by preventing $\alpha_E$-hinge bending, we attempted to straighten it by engineering a disulfide at nonconserved Ala-217 just C-terminal to the $\alpha_E$ hinge. Recently, we showed that cysteines engineered at Ala-217 could be oxidized to produce $\sim 50\%$ disulfide-linked dimers, with accompanying inhibition of lipid activation (14). However, the Trp engineered at residue 216 to monitor FRET prevented the formation of a disulfide ($<10\%$), so this approach was sidelined. Next, we tested the role of the native J segment sequence versus a nonspecific flexible sequence (227GGGSGG232) in stabilizing the structure of the allosteric linker that enables FRET between Trp at residue 216 ($\alpha_E$) or 124 (loop L2) and dansyl-PE in the membrane. With the glycine-rich J segment substitution, FRET from either Trp position was substantially decreased (Fig. 6B). Thus, the WT J sequence appears to facilitate the close membrane approach of the $\alpha_E$ hinge and loop L2 of the catalytic domain.

We also measured FRET efficiency by monitoring the decrease in donor (Trp) fluorescence with increasing dansyl-PE concentration in the lipid vesicles (Fig. 6C). In this regime, the total lipid/protein ratio was constant (100 M/M; 2-fold in excess of that needed for complete solvation of domain M (18)), and this prevented FRET signal saturation due to vesicle surface crowding by CCT. These data illustrate a similar trend to that obtained when monitoring dansyl-PE, except Trp-216 produced the strongest FRET signal, even stronger than FRET from Trp-234 and Trp-278. Trp-124 in loop L2 and Trp-225 in the J segment produced similar FRET signals, suggesting that loop L2 hovers close to the membrane surface. The glycine-rich J segment substitution, FRET from either Trp position was substantially decreased (Fig. 6B). Thus, the WT J sequence appears to facilitate the close membrane approach of the $\alpha_E$ hinge and loop L2 of the catalytic domain.

We postulated that the bent $\alpha_E$ would close off the opening to the active site and that this might enhance catalysis by reducing the water content of the active site. Fig. 7 (A and B) provides views of the active site opening of a CCT captured from one MD simulation (14). In Fig. 7A, the $\alpha_E$ helices are both extended, revealing a large pocket at the base of the active site that becomes occupied by the $\alpha_E$ after bending of the $\alpha_E$ helices (Fig. 7B). Guca et al. (19) had found evidence for phosphocholine-independent PPi production by a catalytically compromised K122A analog in the CCT from P. falciparum, suggesting that CTP hydrolysis might in some cases be a competing reaction for the cytidyl transfer reaction. A hydrolysis potential has never been established for CCT, but it could explain why, for example, the soluble form and CCT-236 have such high apparent $K_v$ values for CTP. To determine whether these CCT forms can also catalyze CTP hydrolysis, we developed an assay where we compared the production of PPi in the presence of phosphocholine (enabling the cytidyl transfer reaction) and in the absence of phosphocholine (no transfer). Details of the assay are provided in the supporting information and Fig. S7. PPi generation in the latter condition can originate only from CTP hydrolysis (Fig. S8A). As expected, in the presence of lipids, we observed negligible PPi generation in the absence of phosphocholine in full-length CCT and also CCT-236 (Fig. S8, B and C), indicating that hydrolysis was minimal. But in the absence of lipids, PPi generation was also very low without phosphocholine addition and was elevated in the presence of phosphocholine (Fig. S8, D and E). The rate of PPi generation by hydrolysis was only $\sim 1\%$ of the PPi generation in the fully active enzyme via cytidyl transfer and was the same for reactions with and without lipid (0.22 nmol of PPi/min/µg of CCT). Thus, hydrolysis was very inefficient in both CCTsol and CCTmem. We conclude that membrane binding does not stimulate cytidyl transfer by preventing CTP hydrolysis.

An alternative hypothesis for enhanced catalytic function is that a relatively anhydrous active site would strengthen the charge–charge interactions between enzyme and substrates. The CCT active site provides multiple amine-containing residues that overcome the repulsive negative charges between the substrates, CTP and phosphocholine (19, 20) (Fig. 7A). To probe this mechanism, we compared the ionic strength sensitivity of the activity of CCTmem with that of CCT-236. According to our model, the former would have a solvent-shielded active site and would be less sensitive to ionic strength. We used lipid vesicles composed of zwitterionic phospholipids (DOPC/DOPA (2:3)) to circumvent an impact of ionic strength on membrane binding. In support of the solvent-shielding mechanism, the activity of CCT-236 was highly sensitive to increases in ionic strength, showing $\sim 50\%$ inhibition at 0.3 M, whereas the activity of CCTmem was $<10\%$ inhibited at 0.3 M ionic strength (Fig. 7C).

Discussion

Recent molecular dynamics simulations indicated that without the constraining Al helices or membrane tether, the CCT allosteric linker ($\alpha_E$) samples many conformations (14, 15). In this work, we examined the hypotheses that the CCT allosteric linker ($\alpha_E$) interacts with the membrane surface, which could then guide its array of conformers into a preferred configuration for efficient catalysis. Two separate approaches provided evidence that the linker does indeed associate with membrane surfaces and that this association, at least with model membranes, can be independent of the M domain. In the process of trying to disrupt the membrane association of the J segment using mutagenesis, we came to the conclusion that both the $\alpha_E$ and J segment contribute to membrane interaction. We then probed the distance between the membrane surface and selective sites within the allosteric linker and catalytic domain by FRET. The distances were not compatible with an extended conformation for the linker, but rather suggested a condensed structure for $\alpha_E$. Mutations in the J segment decreased FRET between the membrane and sites near the $\alpha_E$ hinge or active site.

These data combined with previous work suggest a sequence of steps accompanying the membrane binding and activation of

**Probing a mechanism for catalytic enhancement by a membrane-localized active site**

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An alternative hypothesis for enhanced catalytic function is that a relatively anhydrous active site would strengthen the charge–charge interactions between enzyme and substrates. The CCT active site provides multiple amine-containing residues that overcome the repulsive negative charges between the substrates, CTP and phosphocholine (19, 20) (Fig. 7A). To probe this mechanism, we compared the ionic strength sensitivity of the activity of CCTmem with that of CCT-236. According to our model, the former would have a solvent-shielded active site and would be less sensitive to ionic strength. We used lipid vesicles composed of zwitterionic phospholipids (DOPC/DOPA (2:3)) to circumvent an impact of ionic strength on membrane binding. In support of the solvent-shielding mechanism, the activity of CCT-236 was highly sensitive to increases in ionic strength, showing $\sim 50\%$ inhibition at 0.3 M, whereas the activity of CCTmem was $<10\%$ inhibited at 0.3 M ionic strength (Fig. 7C).

**Discussion**

Recent molecular dynamics simulations indicated that without the constraining Al helices or membrane tether, the CCT allosteric linker ($\alpha_E$) samples many conformations (14, 15). In this work, we examined the hypotheses that the CCT allosteric linker ($\alpha_E$) interacts with the membrane surface, which could then guide its array of conformers into a preferred configuration for efficient catalysis. Two separate approaches provided evidence that the linker does indeed associate with membrane surfaces and that this association, at least with model membranes, can be independent of the M domain. In the process of trying to disrupt the membrane association of the J segment using mutagenesis, we came to the conclusion that both the $\alpha_E$ and J segment contribute to membrane interaction. We then probed the distance between the membrane surface and selective sites within the allosteric linker and catalytic domain by FRET. The distances were not compatible with an extended conformation for the linker, but rather suggested a condensed structure for $\alpha_E$. Mutations in the J segment decreased FRET between the membrane and sites near the $\alpha_E$ hinge or active site.

These data combined with previous work suggest a sequence of steps accompanying the membrane binding and activation of
CCT that begins with surface attraction of the positively charged leash region (residues 236–271) of the M domain, dissociation of the AI helix from the four-helix complex, and membrane partitioning of the AI and leash as a folded helix. AI dissociation is followed by bending of the E helix at its hinge and conformational sampling of the EC and J segment guided by a fully bound M-helix. The acquisition of a bent, compact structure for the allosteric linker creates a lid that partly covers the opening to the active site and draws it close to the membrane surface. The result is generation of a more anhydrous environment for efficient catalysis.

The αEC/J is more than an extension of the M domain or an unstructured tether

No structural studies of lipid-regulated CCTs have provided the favored conformation of the αEC/J in the active, membrane-bound form. An initial possibility, suggested by the Br-PC quenching, was that the αEC/J segment is simply an amphipathic helical extension of domain M. This is not likely for several reasons. First, the helical hydrophobic moment (21, 22) of the αEC/J is only 0.08 compared with a value of 0.36 for the N-terminal 18 residues of domain M (residues 234–251). Second, Br-PC quenching of Trps in the αEC/J was modest compared with Trps within the M domain and was more effective with the surface-proximal bromine (6,7-Br-PC) rather than deeper bromo positions. This suggests that the J segment is not embedded to the extent of domain M. The small vesicles we used have a highly disordered surface that enables contact between acyl chains and superficially situated peptides. Third, the high sensitivity of CCT activity to mutation in the J segment is not expected if the linker is solely a membrane-binding domain. For example, the L230W or V232W mutations in the J segment should benefit CCT activation as they would enhance membrane partitioning, but they devastated CCT activity (13).

Our data also rule out the αEC/J as either an unstructured tether or an unbroken extended α-helix between domains. The complete loss of activity upon substitution with the glycine-rich J segment argues against a mere tethering function. That the
sequence conservation is as high as the catalytic domain is also hard to rationalize with an unstructured tether, but it fits with a folded structure making intramolecular contacts that are regulated by membrane binding. FRET between Trps within the \( \alpha E/C/J \) and dansyl-PE in the membrane did not abate in relation to their sequence distance toward the N terminus. Rather, the FRET signals were similar, suggesting that they lie roughly within a single plane parallel to the membrane surface, perhaps folding into a condensed structure, with the J segment more penetrating of the surface, based on Br-PC quenching. The FRET data also position Trp-124 in loop L2 of the active site at a similar distance from the membrane surface as Trp-221 (\( \alpha E/C/J \)) and Trp-225 (J segment), which is completely at odds with an extended helical or unstructured \( \alpha E/C/J \) segment (Fig. 6, E and F).

**Multiple conserved residues throughout the \( \alpha E/C/J \) likely contribute to membrane interactions**

To assess whether the allosteric linker–membrane interaction is required to transduce signals from domain M to the catalytic domain, we tried to disrupt the interaction by mutagenesis. Surprisingly, none of the mutations designed to disrupt potential hydrophobic, electrostatic, or aromatic interactions were effective in dislodging the J segment from the membrane, as monitored by Br-PC quenching potential toward a Trp in the J segment. A limitation of these experiments was that the engineered tryptophans could have a stronger interfacial driving force than the native tyrosine or leucine that they replace (23, 24) and could artificially forge linker–membrane interactions not available to the WT CCT. However, gel filtration analysis of lipid interaction, which did not rely on engineered tryptophans, also revealed that deletion of the J segment from CCT-236 was not sufficient to abolish the lipid interaction, whereas deletion of the entire allosteric linker (CCT-212) destroyed the lipid interaction. One explanation consistent with these results is that the membrane association of the linker relies on multiple sites dispersed throughout the entire \( \alpha E/C/J \) sequence including, but not exclusive to, the sites targeted for mutation. The lipid interaction of CCT-236, observed by the two independent methods, demonstrates the intrinsic affinity of the \( \alpha E/C/J \) segment independent of domain M. The CCT-236 membrane affinity is clearly weaker than that of CCT with the M domain intact. For example, we can isolate by ultracentrifugation a CCT-312 vesicle complex, but not a CCT-236 vesicle complex (25, 26). In cells, oleic acid induces translocation of full-length CCT, but not CCT-236, to intranuclear membranes (11). Moreover, the lipid interaction of the \( \alpha E/C/J \) in CCT-236 is much less effective in promoting conformations and interactions productive for catalysis than when the \( \alpha E/C/J \) is linked to a membrane-embedded M domain, hence the \( \sim 20\)-fold lower catalytic efficiency (13).

**Direct interactions between the \( \alpha E/C/J \) segment, and lipid may direct the folding of the allosteric linker on the membrane surface**

Whereas the allosteric linker mutations we created did not abrogate membrane interaction, they did abolish activation, suggesting that these residues may participate in creating a tertiary structure for the allosteric linker compatible with high catalytic activity. The mutations may have disrupted direct interactions between the \( \alpha E/C/J \) and J segment, even though the allosteric linker was still adsorbed onto the membrane surface. In our model, the \( \alpha E/C/J \) forges a network of protein and lipid interactions that are promoted by the membrane partitioning of the M domain, and this network underlies signal transduction between the M and C domains. Substitution with a glycine-rich J segment reduced FRET between Trp-216 and dansyl-PE, and this was observed when we monitored either dansyl or Trp fluorescence. This supports a role for the J segment in stabilizing the compact structure of the allosteric linker. Bent helix conformations generated during simulations expose a hydrophobic groove between the two \( \alpha E \) helices that could accommodate the J segment or, alternatively, dock onto the membrane.

Reduced solvent exposure of residues Tyr-213 and Tyr-216 in CCT\(_{\text{mem}}\) (13) suggested a folded structure for the \( \alpha E/C \). In the bent \( \alpha E \) visualized in the MD simulations, the \( \alpha E/C \) interacts with loop L2, but the \( \alpha E/C \) could also forge interactions with the J segment on the membrane surface. This idea is supported by the obliteration of lipid-induced activity upon introducing bulky hydrophobic residues in the allosteric linker (L221W, L230W, and V232W) that could prevent close association of the two segments (13). We analyzed CCT sequence alignments from a wide range of eukaryotic phyla for coupled residue substitutions in the \( \alpha E/C \) and J segment that might imply an interaction between the two residues. One striking substitution occurred in Ascomycota fungus sequences. Position 221 in the \( \alpha E/C \), a leucine in most species, is mutated to a phenylalanine, and in concert, position 225 in the J segment is changed from tyrosine to alanine (Fig. S6). The co-evolution of the L221F/Y225A switch suggests that a mutation to the bulky Phe at residue 221 is not compatible with the bulky Tyr at residue 225, but a Y225A mutation would alleviate steric clash between two putative interacting sites. In support of this interaction, an L221W mutation severely inhibited CCT activity, whereas a Y225W mutation did not.

Tyr-216 appears to have an outsized role in the allosteric transitions. It can be replaced by Phe or Trp without impairing activity, but not alanine or cysteine (13). Of all the residues we probed, it shows the largest protection from solvent and a large dequenching upon membrane binding and is located very close to the membrane surface, based on the FRET analyses.

Attempts to solve the crystal structure of CCT bound to micelles have been unsuccessful. However, a previously described 8 Å structure of a CCT tetramer provides a clue for the conformational preference of the J segment. In this structure, the two M helices of one dimer extend from the \( \alpha E \) and interact with two M helices of another dimer to form a >75-Å four-helix bundle (PDB entry 4MVD) (15). The electron density defines the residues comprising the \( \alpha E/C \) and J segment as helical, linked by a \( \sim 75\)° turn at 223RGY225 in two of the four chains (15). We used the rat CCT\(_{\alpha}\) sequence of the \( \alpha E/C/J \) as a query to search the PDB for sequence matches. We obtained just six hits that contained exact matches of 4–7 contiguous residues of the J segment sequence (Fig. S9). The preferred conformation of the J segment from this limited set is a helix or a turn-helix that

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**Linker remodeling pulls CCT\(_{\alpha}\) active site toward membrane**
Linker remodeling pulls CCTα active site toward membrane

docks into clefts formed by other helices. Such a conformation fits well with the FRET data supporting a compact, lipid-associating structure, and the helix-turn-helix preference is reflected in our model for the allosteric linker in Fig. 6F.

How could a compact allosteric linker aid catalysis?

Bending of the αhelices positions conserved residues of one face of the αEC helix within close proximity of the epicenter of CCT catalysis at loop L2. The opposite face of the αEC helix could orient toward the J segment near the membrane interface, forging both protein and lipid interactions (Fig. 6F). Both sets of interactions may be important for stabilizing an αEC helical structure, compensating for contacts lost during dissociation of the AI helices. The folded allosteric linker may play a fundamental role in accelerating catalysis by creating a relatively anhydrous milieu for the cytidyl-transfer reaction as it covers the large active site entrance and pulls the active site close to the membrane. The protection of Trp-151 in the active site from acrylamide quenching upon membrane binding (13) is in agreement with this proposal. We ruled out the possibility that the enzyme can also catalyze CTP hydrolysis in competition with cytidyl transfer, where a water molecule would substitute for the phosphocholine in the reaction. But there is another more basic advantage of water exclusion. In the cytidyl-transfer reaction, both the nucleophilic phosphate and the α-phosphate target have strong repulsive negative charge. The enzyme donates several basic groups to counter that charge (20), and dehydra-
tion of the active site cleft would strengthen the charge–charge interactions between enzyme and substrates. This is a fundamental mechanistic basis for lid closure over active sites and can yield rate enhancements of orders of magnitude (27–29). In support of this mechanism, we found that the activity of the catalytic fragment, CCT-236, with its open active site, was much more sensitive to inhibition by raising the ionic strength of the medium than was membrane-bound CCT. This simple test has been used to show the involvement of basic residues in electrostatic facilitation of superoxide dismutase using superficial sites sensitive to ionic strength and one site deep in the active site that is resistant (30, 31). The sensitivity differences of the two CCT forms cannot be ascribed to structure destabilization of CCT-236 by high salt concentration, as the crystals used to solve its structure were grown in 1.2 M salt. The structure of the CCT containing the AI helix of the M domain was obtained from crystals grown in 0.2 M salt, and both structures provide catalytic domains that superimpose upon each other (15). MD simulations revealed that upon bending of the αE helices, the basic αEc residues, Arg-218, Arg-219, and especially Arg-223, made frequent excursions into the active site and made contact with the CTP γ-phosphate (14) (Fig. 7B). Possibly, one or more of these arginines contribute to charge facilitation in the active site at some step in the catalytic cycle. In support of this, Arg-223 is mutated to a serine in a CCTα allele linked to the rare human skeletal and retinal disease, SMD-CRD (32), with a consequent 16-fold drop in $k_{cat}/K_m$ (33).

Why then would the unregulated GCT have high catalytic efficiency despite lacking the αEC/J? Loop L2 may provide a substitute device in GCT, as it is longer and more flexible and is extremely basic, with 3 lysines and 1 histidine in contrast to just 1 lysine at residue 122 in the shorter mammalian CCT loop L2. Two of the GCT lysines have been probed, showing that they are critical for activity (34). The extra positive charge may compensate for the higher water density in the active site during the reaction.

The AI helices inhibit catalytic function by partly occluding the active site entrance. Is it then incongruous to propose that closing off the active site by the αEC promotes activity? Not if the interactions have different time scales and consequences. The MD simulations showed that the contact between the AI and loop L2 of the active site was relatively stable and steered the catalytic lysine away from substrate. Because the entrance to the active site must be open for substrate entry/product release, we envision that the allosteric linker might sample different conformations during a catalytic cycle, with a bent αE and membrane-proximal active site most prominent after substrates are bound and prior to product release. The present FRET analysis of interdomain distance was done with an empty active site. Future work should explore the interdomain distance with different ligands occupying the active site.

In summary, part of the CCT inactivation mechanism involves immobilization of the allosteric linker using a portion of the membrane-binding domain (the AI helix). Displacement of the AI by membrane engagement frees the linker to sample many new conformations, but the membrane tether is likely to dictate a restricted ensemble. Moreover, the intrinsic affinity of the allosteric linker for the membrane interface will promote a hierarchical folding pathway leading to a condensed linker structure that draws the active site toward the membrane and away from bulk solvent.

Several aspects of the mechanism of CCT activation by its allosteric linker may be shared with other membrane-associated enzymes, most notably another enzyme of phospholipid metabolism, neutral sphingomyelinase (nSMase2). The catalytic domain of nSMase2 is linked to an integral membrane-binding domain by a juxtamembrane region that, like the αEC/J of CCT, is triggered to fold upon interaction with anionic phospholipids, mediates contact between the catalytic domain and the membrane-binding domain, and induces active site restructuring to facilitate substrate (sphingomyelin) access (35, 36). Group IVA iPLA2 has no discrete membrane-binding domain and no linker, but two loops flanking the active site opening for PC interact with the membrane surface to facilitate opening of the cleft in the catalytic domain for substrate (37). More broadly, small GTPases like H-Ras and Arf1 can adopt different orientations with respect to the membrane surface that display different states of GTPase activity. These orientations are dictated by the fluctuating ensemble of conformers for the membrane-interacting linker (HVR) joining the GTPase domain to the lipidated C-terminal anchor (38). Last, membrane-bound kinases such as the EGF receptor (39, 40) also use refolding/reorientation of membrane-interacting linkers bridging two domains to induce substantial shifts in the quaternary structure and activity of the enzyme domain.
Experimental procedures

Construction, expression, and purification of CCTs

The materials and methods for construct preparation were described in the companion paper (13). In addition to the many CCT forms described in that paper that were used in the present work, we prepared additional mutants delineated in Table S1. CCT-312 variants contained a C-terminal His tag for purification \(^{(31)}\) and a deletion of the nuclear localization signal (NLS; residues 12–16) to prevent vesicle aggregation, which interferes with fluorescence analysis (13). CCT-212, CCT-236, and CCT-312(A32) had N-terminal His tags (MGSSHH\(_5\)SSGLVPRGSH) and intact NLS sequences.

CCT enzyme activity

Monitoring \([\text{14C}]\text{CDP}-\text{choline production}\)—The specific activity was determined as described previously (33, 41). For the standard assay, optimal for WT CCT, the concentrations of CTP and phosphocholine in the assay were 8 and 1.6 mM, respectively. Kinetic constants were determined by varying the concentration from 0 to 16 mM using a fixed (2 mM) concentration of phosphocholine. Sonicated lipid vesicles were prepared as described previously (41), except the suspensions were sonicated for 15 min on 50% output and were centrifuged for 4 min at 13,000 \(\times\) g to remove titanium debris introduced by the sonicator probe. The vesicles were stored under argon at 4 °C and used within ~2 days. The vesicle lipid compositions are described in the figure legends. For probing the effects of NaCl concentration on CCT activity, the ionic strength was calculated as follows,

\[
I = 0.5 \left( \sum c_i Z_i^2 \right) \tag{Eq. 1}
\]

for all anions and cations, where \(c_i\) represents molar concentration and \(Z_i\) is charge. The ionic strength was 0.12 M based on buffer, substrate, and cofactor contributions. NaCl was the added variable. The substrate CTP was added to the enzyme either before or after introduction of the salt (NaCl), with no significant differences in the activity outcome.

Monitoring unlabeled PP\(_3\) production—This novel assay is described in the supporting information.

Thermal denaturation using SYPRO Orange

The transition temperature for unfolding was measured using the hydrophobic dye SYPRO Orange (Invitrogen, Molecular Probes), as described (33).

Quenching of single-tryptophan CCT variants by dibrominated PC

Nonquenching vesicles contained egg PG/POPC (1:1), and quenching vesicles contained egg PG/dibrominated PC (1:1) with bromines conjugated to carbons 6,7, 9,10, or 11,12. The perturbation to the bilayer by dibromo-PC substitution is similar to that of a double bond (42). The lipid concentration dependence for CCT-312 activation and the maximal activity was nearly identical for vesicles composed of 50% egg PG and either 50% 11, 12-dibromo-PC or 50% POPC. Samples (300 \(\mu\)l) were prepared by mixing 3 \(\mu\)M CCT and 450 \(\mu\)M vesicles in 10 mM Tris, pH 7.4, 150 mM NaCl, 2 mM DTT and incubating for 5 min at room temperature. Fluorescence scans were measured at 20 °C on a Cary Eclipse fluorescence spectrophotometer using the parameters for Trp fluorescence described by Taneva et al. (13). Duplicate scans were averaged, and lipid-only blanks were subtracted to generate corrected fluorescence emission spectra. The fluorescence intensity for each spectrum was recorded at the peak emission wavelength average for each set of Trp variants.

Superdex-200 gel filtration of CCT-micelle complexes

The CCT preparation was mixed with a 175–180-fold molar excess of LPC (16:0) in 10 mM Tris, pH 8, 300 mM NaCl, 2 mM DTT, and concentrated using an Amicon Ultra-spin filter (30-kDa cutoff) at 4000 rpm to a protein concentration of 150–300 \(\mu\)M. Samples (0.4-ml volume) were applied to a Superdex 200 10/300 FPLC gel-filtration column (24-ml bead volume) pre-equilibrated with 10 mM Tris, pH 8, 0.1 M NaCl, and 2 mM DTT. The column had been precalibrated with the mass standards: dextran, >2000 kDa; ferritin, 440 kDa; catalase, 220 kDa; BSA, 67 kDa; and RNase, 13.7 kDa. The FPLC-controlled flow rate was 0.4 ml/min, and 1-ml volume fractions were collected. For each elution fraction, lipid phosphorus (43) and protein concentration (44) were measured in duplicate.

Tryptophan–dansyl-PE FRET

Monitoring increases in dansyl-PE fluorescence—Sonicated vesicles were prepared containing POPC/egg PG/dansyl-PE (50:48:1.8). Samples (300 \(\mu\)l) were prepared by mixing 100 \(\mu\)M vesicles with 0–4 \(\mu\)M CCT in 10 mM Tris, pH 7.4, 150 mM NaCl, 2 mM DTT and incubating for 5 min at room temperature. In pilot experiments, we explored the CCT and lipid concentrations and percentage dansyl-PE vesicle compositions that produced optimal FRET signals, with maximal signal/noise ratio, lack of interference by lipid vesicle light scattering, and differentiation among the various Trp sites. The optimal parameters were a donor/acceptor ratio of up to 4:1 and lipid/protein ratios of 100 to 25. The excitation wavelength was 280 nm to maximize donor tryptophan excitation, and the emission was scanned from 290 to 590 nm. The remainder of the fluorimeter settings were identical to the Trp quenching experiments. The FRET efficiency was calculated from the intensity increase at 520 nm (dansyl emission peak) as follows,

\[
\left( \frac{F_{DA}}{F_A} \right) - 1 = \tag{Eq. 2}
\]

where \(F_{DA}\) is the acceptor fluorescence in the presence of donor and \(F_A\) is the acceptor fluorescence in the absence of donor. The plot of the FRET signal versus D/A molar ratio was fit to a one-site binding hyperbola fit using GraphPad Prism 4.

The fluorescence values at 520 nm for the FRET calculation were taken directly from the raw spectra. To assess whether the increases in dansyl fluorescence with increasing [CCT] included a contribution of the CCT Trp fluorescence, we monitored the fluorescence signal at 520 nm of samples containing 0–4 \(\mu\)M CCT but no dansyl-PE vesicles. Signal increases were negligible up to a 4 \(\mu\)M concentration of each single-Trp CCT, and this background signal was not subtracted.
Linker remodeling pulls CCTα active site toward membrane

An important caveat to consider is that dansyl fluorescence increases could be affected by differences in intensity and peak wavelengths of the donor that vary based on the environment of different Trp sites. However, all of the native and engineered Trps emitted near the dansyl absorbance maximum of 336 nm. Moreover, the Förster distance, R0, is not highly dependent on spectral overlap or donor quantum yield (16), and thus FRET efficiency values would not vary significantly among Trps based solely on small blue shifts and intensity differences. The main determinant of FRET in these experiments is the donor–acceptor distance.

Monitoring decreases in tryptophan fluorescence—Sonicated vesicles were prepared containing 0, 2.5, 5, or 10% dansyl-PE in 50% POPC, balanced with egg PG. Samples (300 μl) were prepared by mixing 100 μM vesicles with 1 μM CCT in 10 mM Tris, pH 7.4, 150 mM NaCl, 2 mM DTT and incubating for 5 min at room temperature. The excitation wavelength was 290 nm to minimize tyrosine excitation, and the emission was scanned from 300 to 420 nm. The remainder of the fluorimeter settings were identical to the Trp quenching experiments. Two scans were averaged, and lipid blank scans were subtracted to generate corrected fluorescence spectra. The FRET efficiency was calculated from intensity changes at the peak wavelength for each Trp mutant as follows,

\[
1 - \frac{F_{DA}}{F_D} \quad \text{(Eq. 3)}
\]

where \(F_{DA}\) is the donor fluorescence in the presence of acceptor and \(F_D\) is the donor fluorescence in the absence of acceptor. The plot of \(1 - \frac{F_{DA}}{F_D}\) versus the acceptor/donor molar ratio was fit linearly using GraphPad Prism 4.

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

Acknowledgments—We are grateful to Jillian Smith for the original discovery that CCT-236 is activated by lysolipids. We thank Dr. Tom Claydon for discussions of the FRET data, Dr. Peter Tieleman for valuable comments on the manuscript, and Cameron Proceviat for his contribution early in the project.

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