Interdomain and Membrane Interactions of CTP:Phosphocholine Cytidylyltransferase Revealed via Limited Proteolysis and Mass Spectrometry

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CTP:phosphocholine cytidylyltransferase (CCT) is a multi-domain enzyme that regulates phosphatidylcholine synthesis. It converts to an active form upon binding cell membranes, and interdomain dissociations have been hypothesized to accompany this process. To identify these interdomain and membrane interactions, the tertiary structures of three forms of CCTα were probed by monitoring accessibility to proteases. Time-limited digestion with chymotrypsin or arginine C of soluble CCTα (CCTsol), phospholipid vesicle-bound CCT (CCTmem), and a soluble constitutively active CCT truncated at amino acid 236 generated complex mixtures of peptides that were resolved and identified by gel electrophoresis/immunoblotting and by matrix-assisted laser desorption/ionization-mass spectrometry, with or without coupling to capillary liquid chromatography. Identification of cleavage sites enabled assembly of peptide bond accessibility maps for each CCT form. Our results reveal a ~80-residue core within the catalytic domain (domain C) as the most inaccessible region in all three forms and the C-terminal phosphorylation domain as the most accessible. Membrane binding has little effect on the protease accessibility of these domains. To map the protease sites onto the catalytic domain, its three-dimensional structure was modeled from the atomic coordinates of glycerol-phosphate cytidylyltransferase (Protein Data Bank code 1COZ). The protease inaccessibility of most sites in domain C could be explained by burial or location within secondary structural elements. The accessibility of the N-terminal domain (domain N) was enhanced upon membrane binding. Residues Phe234-Leu303 were inaccessible in CCTmem, suggesting burial in the membrane. Surprisingly, residues Leu274-Leu303 of this domain were also inaccessible in CCTsol. We propose that this region is buried by interdomain contacts with domain N in CCTsol. Membrane binding and burial of domain M in the lipid bilayer may disrupt this interaction, leading to increased exposure of sites in domain N.

Metabolically active cells are continually undergoing membrane phospholipid turnover, membrane expansion, and trafficking of components between organelles via vesicular transport. The ratio of various phospholipid classes is maintained in the face of this constant flux (1, 2). CTP:phosphocholine cytidylyltransferase (CCT) is a key player in maintaining phosphatidylcholine (PC) homeostasis, acting as a key regulatory enzyme in PC biosynthesis (3). CCT catalyzes the formation of the headgroup donor, CDP choline, from CTP and phosphocholine.

CCT can be rapidly activated by increases in its membrane affinity. This can be accomplished by modulation of the membrane lipid composition or by dephosphorylation of the C-terminal domain of the enzyme (3–5). CCT binding to membranes is promoted by anionic lipids and by lipids that generate negative curvature strain (6). This mechanism built into CCT for sensing the membrane phospholipid composition contributes to PC homeostasis. When there is a drop in the relative PC content, CCT inserts into that membrane, activating the enzyme and accelerating the rate of PC synthesis and restoring the relative PC content. Because of the prominence of its regulation by amphitropism, the lipid-protein interaction mechanism and the consequences of membrane binding on the conformation of the enzyme are questions worthy of scrutiny.

The principle isoforms of mammalian CCT have similar domain structures (4). The present analysis was done on CCTα, the major ubiquitous isoform (7). CCTα can be subdivided into four functional domains designated N, C, C, and P (Fig. 1A). The catalytic domain (domain C; amino acids Arg75–Leu236) is the region of high homology with all other cytidylyltransferases (8). Two crystal structures of this domain have been solved from the bacterial enzyme glycerol-phosphate cytidylyltransferase (GCT) (8, 9). It is a variation of a mononucleotide fold, a five-membered parallel β sheet flanked by five α-helices and a 3–10 helix. The membrane-binding domain (domain M; amino acids Leu235–Lys300) is the other well characterized domain. The NMR-derived structures of overlapping peptides from this domain were supported by grants from the Canadian Institutes for Health Research (to R. B. C.), the Natural Sciences and Engineering Research Council (to G. R. A. and to F. P.), and Waters Technologies Inc. (to G. R. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.‡§ The on-line version of this article (available at http://www.jbc.org) contains supplementary Fig. 1.

1 The abbreviations used are: CCT, CTP:phosphocholine cytidylyltransferase; anti-N, antibody against Met1–Arg15; anti-cat, antibody against Asp164–Ala176; anti-M, antibody against Val256–Ser288; Arg-C, endoproteinase arginine C, cleaves C-terminal to arginine; CapLC, capillary liquid chromatography; CCTsol, full-length CCT in buffered saline; CCTmem, CCTα, mixed with sonicated PG vesicles; CCT236, CCTα truncated at amino acid 236; CHCA, α-cyano-4-hydroxycinnamic acid; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; PBAM, peptide bond accessibility map; LC, capillary liquid chromatography.
region showed that it is a continuous amphipathic α-helix in a membrane-mimetic environment (10). By comparing the secondary structure content of soluble and membrane-bound forms of CCT derived from deconvoluted circular dichroism spectra, Taneva et al. (11) showed that this domain is a mixture of conformers in the soluble form of the enzyme and is transformed into an α-helix in the presence of membranes containing anionic lipids. The structure of the N-terminal domain (N) is by contrast a black box. It bears no significant sequence homology with any other sequence in the NCBI protein structure database. The C-terminal domain (domain P), which is known to house up to 16 phospho-serine sites (12), is also structurally uncharacterized, as are the linkers between domains C and M and between domains M and P.

CCTα is a noncovalently associated homodimer of a 367-amino acid subunit. The dimer interface involves both domains N and C (13). Binding to anionic lipid membranes causes a repositioning of the dimer interface (13), but whether this change is instrumental in the activation process is not known.

Previous analysis by limited proteolysis with chymotrypsin and mapping the major SDS-PAGE-resolved fragments by epitope-specific antibodies gave rise to the proposal that CCT consists of a protease-resistant compact head (up to residue 225) and a protease-sensitive, flexible, exposed tail (14). This approach suffered the limitation of inaccurate estimation of the specific cleavage sites. Moreover, the effects of membrane binding on the tertiary structure assessed by protease accessibility had not been examined. In this study we compare the protease accessibility of three forms of CCT: the soluble form, the membrane-bound form, and the form truncated at the end of domain C. The identification by MALDI-MS of >100 peptide fragments generated by time-limited digestion with chymotrypsin or Arg-C resolved the uncertainty associated with fragment identification based only on electrophoretic mobility and epitope-specific antibody reactivity and enabled the construction of a peptide bond accessibility map for each CCT form.

According to a prominent model for CCT regulation (15), the soluble form of CCT is inhibited by interdomain interactions between domain M and the catalytic domain (Fig. 1B). Membrane binding relieves inhibition by dissociation of this domain interaction. That domain M is autoinhibitory is supported by the findings that deletion of domains M and P, but not P alone, results in a constitutively active enzyme (15, 16). Our data support this general model and provide a clue as to the origins of the interdomain regulatory contacts. The accessibility of the interdomain contact points involved in the regulatory conformational switch should be low in the soluble form and high in the membrane-bound form. Based on this premise the present work identifies domain N rather than domain C as the best candidate for a site of contact with the C-terminal region of domain M.

EXPERIMENTAL PROCEDURES

Protein Purification and Preparation of CCTmem—Recombinant rat CCT α-isofrom was purified from a baculovirus expression system using the method of Friesen et al. (15) as modified by Davies et al. (17). CCT236 was constructed, expressed, and purified as described (11). To prepare CCTmem, sonicated egg phosphatidylglycerol (PG) vesicles were prepared (18) and mixed at ~100-fold molar excess over CCT 5 min prior to the initiation of proteolysis reactions.

Proteolysis and Gel Electrophoresis/Immunoblot Analysis—The chymotrypsin digestion method has been described previously (14, 10). Briefly, the reactions were at 37 °C and contained ~12 μM CCT (without or with ~1.2 mM PG vesicles) and a 1:250 weight ratio of chymotrypsin to CCT in 10 mM Tris, pH 7.4, 2 mM dithiothreitol, 0.15 mM NaCl, 1 mM EDTA. Aliquots of the reaction mixture were transferred after various times to vials containing the protease quencher, phenylmethysulfonyl fluoride (final concentration, 10 mM). Arg-C reactions contained ~15 μM CCT (without or with ~1.2 mM PG vesicles) and a 1:175 weight ratio of Arg-C:CCT in 50 mM Tris, pH 7.8, 8 mM CaCl₂, 0.5 mM EDTA, 5 mM dithiothreitol, 120 mM NaCl. The aliquots were transferred at various times to tubes containing the quencher, EGTA (final concentration, 20 mM). A portion of each quenched sample was aliquoted for MALDI-MS analyses, and the remainder was analyzed by SDS-PAGE. The samples were boiled for 3 min in Laemmli sample buffer containing 4% SDS and 2% β-mercaptoethanol (19). Protein fragments were separated on 12% polyacrylamide gels (19). The fragments were visualized by silver staining (20) or by electrophoretic transfer to polyvinylidene difluoride and reaction with antibody against amino acids 1–15 (anti-N), 164–176 (anti-cat), or 256–288 (anti-M) as described (13).

Delipidation of CCTmem Samples Prior to MS Analysis—The presence of PG vesicles in the CCTmem samples inhibited the production of ionized peptides by interfering with the requisite MALDI matrix peptide co-crystallization and desorption processes. To alleviate this interference, we extracted the phospholipids from the CCTmem samples prior to MALDI analysis by the dried droplet method. While vortexing, a 20-μl sample was injected into 0.25 ml of ethanol/diethyl ether/H₂O (74.6/24.9/0.6) (21) at ~20 °C. The vortexing continued for 3 min at ~20 °C. After sedimentation at 13,000 rpm for 3 min at 0 °C, a minute white pellet was visible. The solvent in the supernatant was removed, and the pellet was dried under N₂ and redissolved in 10 mM Tris, pH 7.4, 1 mM EDTA, 2 mM dithiothreitol, 0.15 mM NaCl. This protocol removed 98% of the phospholipid as assessed by tracking radiolabeled tracer phospholipid but retained each of the CCT fragments with masses >6000 Da, as assessed by SDS-PAGE/silver staining of the sample before and after extraction.

Capillary Liquid Chromatography—Capillary liquid chromatographic separation of the peptides was performed using a CapLC System (Waters Technologies, Milford, MA) with a Symmetry 300 C₁₈.
5-µm packing diameter, 0.32-mm-inner diameter × 150-mm-long column. Proteolytic CCT digests were analyzed by CapLC without prior sample delipidation. For the CapLC separation, we used an injection volume of 5.0 µl and a flow rate of 5.0 µl/min. A gradient of solution A (0.1% trifluoroacetic acid in acetonitrile) and solution B (H2O) was created as follows: 10% A and 90% B at 0 min; 43% A and 57% B at 80 min; 60% A and 40% B at 100 min. No peptides were eluted with additional eluent. The flow exiting the CapLC column was directed into a device (LC-MALDIPrep module; Waters Technologies) that automatically concentrates and deposits the eluent onto a MALDI target (22). In this device eluent was directed through a small orifice where it was aerosolized in a 40 °C chamber and delivered by a 15-p.s.i. N2 flow onto the MALDI target plate precoated with the matrix α-cyano-4-hydroxycinnamic acid (CHCA). The instrument was synchronized with the CapLC to deposit and concentrate 25 µl of the eluent onto discrete ~1-mm spots on the MALDI plate at 5-min intervals, creating an archived library of LC-separated peptides on different positions on the MALDI target.

**Mass Spectrometry**—Crude mixtures of the peptides were prepared for MALDI analysis without prior CapLC separation, using the dried droplet method. 1.0 µl of crude digest was mixed with 1.0 µl of sinapinic acid matrix (10 mg/ml in acetonitrile with 0.25% trifluoroacetic acid in distilled deionized water, 60:40) or 1.0 µl of CHCA (10 mg/ml in 0.1% trifluoroacetic acid in acetonitrile methanol 50:50) for 10 min on ice. An aliquot of 1.0 µl was delivered to the target plate and dried at room temperature. Mass standards (MS-CALI; Sigma) were used for external calibration. Two mass spectrometers were used in this study. A linear MALDI-time of flight-MS (Perseptive Biosystems Voyager-DE, Framingham, MA) was used exclusively to analyze dried droplet preparations. Mass spectra of CCT fragments <10 kDa were collected with an accelerating voltage of 25 kV, a grid voltage of 89.0%, a guide wire voltage of 0.303%, and a delay time of 350 ns, and the low mass gate was set at 10 kDa. Mass spectra of CCT fragments <10 kDa were collected with an accelerating voltage of 25 kV, a grid voltage of 89.7%, a guide wire voltage of 0.010%, and a delay time of 50 ns, and the low mass gate was set at 800 Da. Mass spectra for both the dried droplet and CapLC sample preparations were collected on a MALDI -time of flight-MS equipped with delayed ion extraction and operated in linear and reflectron mode (MALDI-LR; Waters Technologies). For all of the samples the source voltage was 15 kV, the detector voltage was 1.8 kV, and the delay time was 500 ns. To accommodate the wide mass range of peptides from 1 to 42 kDa for full-length CCTs and from 1 to 27 kDa for CCT236, each sample was analyzed using separate pulse and/or reflectron voltage conditions to optimize the sensitivity and resolution for peptides detected in each mass range, typically 1–7, 2–15, and 10–50 kDa. The reflectron operation mode offers higher resolution but lower sensitivity; thus it was used only for peptide ions with m/z <7000. In addition each sample was prepared in three modes: dried droplet with CHCA matrix, dried droplet with MALDI matrix sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), and CapLC fractionation deposited each sample was prepared in three modes: dried droplet with CHCA matrix and anti-cat). This indicates that these fragments arise from progressive digestion of CCT from the C terminus. Bands 7–11 did not react with antibody directed against amino acids Val197–Ser206 in the core of domain M (anti-M; not shown). Bands 7–11 were also generated upon digestion of CCT236 (Fig. 2, A–C, right-hand panels) and reacted with both anti-N and anti-cat antibodies. Thus bands 7–11 are produced by cleavage in domain C, proceeding from the C terminus of that domain. Bands 13–17 migrating ahead of the 21-kDa marker were not produced upon digestion of CCT236 and did not react with either the anti-N or anti-cat domain antibody (Fig. 2, B and C), indicating that they derive from the C-terminal domains, M and/or P. In agreement with previous antibody mapping of chymotrypsin fragments (29, 30), bands 13–17 reacted with anti-M (Fig. 2D, lanes 2 and 3). Previous work also showed that bands in the 11–17-kDa range react with a lipid photolabel (29). No fragments of CCT236 reacted with anti-M (Fig. 2D, lane 1). Upon dephosphorylation of the digest of both CCT subtypes, we did not detect any CCT fragments that corresponded to the phosphopeptide bands obtained with anti-M (Fig. 2D, lane 1). The results indicate that the phosphopeptide bands obtained with anti-M represent posttranslational modifications that are not observed in the native CCT proteins.
forms with PP1α, the electrophoretic mobility of bands 13–15 increased, indicating that these fragments contain domain P (Fig. 2D, lanes 4 and 5). Dephosphorylation did not affect the mobility of band 17, selective to CCTmem, indicating that it does not contain domain P. Together these data suggest the following assignments: bands 1–4 span the N terminus to cleavage sites in domain P, bands 5–6b span the N terminus to cleavage sites in domain M, and bands 7–11 span the N terminus to sites in domain C; bands 13–16 derive from fragments containing both domains M and P; band 17 derives from domain M.

The electrophoretic profiles of CCTsol and CCTmem differ as follows: bands 5, 6, 6b, and 15 are missing from CCTmem, band 12 in CCTsol is replaced by 12b in CCTmem, band 13 is more prominent in CCTmem, and band 17 is generated only in CCTmem (Fig. 2). Other bands appear to be produced and processed at similar rates in both CCT forms. These data suggest
that the rates of proteolysis of domain P and domain C are similar for both the soluble and membrane-bound forms, that cleavage sites in domain M to generate bands 5, 6, 6b, and possibly 15 are bypassed in CCT_mem, and that the cleavage pattern of fragments containing domains M and P is altered in membrane-bound CCT.

Comparison of the Chymotrypsin Digestion Pattern of CCT_sol, CCT_mem, and CCT236 by MALDI-MS—To precisely identify the chymotryptic fragments of the three CCT forms, we analyzed the digests by MALDI-MS. A mass spectrum of peptides in the 19–42-kDa range is shown in Fig. 3, and the masses of the observed peaks are compared with their theoretical masses in Table I. Because of a limited number of cleavage options and an average mass accuracy of −100 ppm for peptides in this mass range, this analysis readily identified all of the primary cleavages giving rise to gel bands 1–11 (Table I). Peaks 1, 2, and 3 contain all or portions of the domain P, so they appear very broad because of heterogeneous phosphorylation. In agreement with the gel electrophoretic analysis, species corresponding to 30, 27, and 26.5 kDa (gel bands 5, 6, and 6b) were observed only in the CCT_sol samples. These species correspond to Met1–Phe263, Met1–Tyr240, and Met1–Phe234. Thus membrane association of CCT converts these sites from highly accessible to very inaccessible.

Interestingly, although CCT contains seven chymotrypsin sites with >50% propensity for cleavage between Phe263 and Leu311, we did not observe, on gels or in the mass spectra, fragments corresponding to Met1–Leu274, Met1–Trp278, Met1–Phe285, Met1–Phe289, Met1–Phe293, Met1–Leu299, or Met1–Leu303 in digests of either CCT_sol or CCT_mem. The region of the
high mass spectra where these fragments would appear is shown in Fig. 4 (boxed areas). The lack of cleavage to produce these fragments indicates that the C-terminal end of domain M is inaccessible in both forms of CCT.

The mass spectra in the 10–28 kDa range of CCTsol and CCTmem digests also show some differences (Fig. 5). Many of these species are heterogeneously phosphorylated giving rise to a set of peaks separated by 80 Da, the additional mass associated with each phosphorylation (Fig. 5, boxed areas). This provides additional proof that these species are derived from the C-terminal end and the hinge between domain C and M are the most inaccessible core in all forms of CCT. The lack of cleavage to produce high mass N-terminal fragment data (Fig. 3 and Table I), the high mass spectra of CCT236 digests, where Arg42–Asn236 was generated by cleavage at residues Phe285, Tyr240, and Phe263 (Fig. 5), Thr226–Asp367 does not accumulate in CCTmem because of accelerated processing of this fragment at Leu351, Phe338, and Leu311 compared with CCTsol. In corroboration with the intensity of peptides generated by cleavage of a peptide bond. If a 25-s digest of CCTsol generated five new peptides ending at Tyr216 and only two peptides ending at Phe234, both sites are colored yellow, because both sites were cleaved within the same time frame. The PBAMs in Fig. 6 show that region I is less accessible in CCTmem, region II is more accessible in CCTmem and CCT236, and region III is least accessible in all forms. The lack of cleavage to produce high mass N-terminal fragment data (Fig. 3 and Table I), the high mass spectra of CCT236 digests, where Arg42–Asn236 was generated by cleavage at residues Phe285, Tyr240, and Phe263 (Fig. 5), Thr226–Asp367 does not accumulate in CCTmem because of accelerated processing of this fragment at Leu351, Phe338, and Leu311 compared with CCTsol. In corroboration with the high mass N-terminal fragment data (Fig. 3 and Table I), the identification of these peptides from the C-terminal domains shows that Phe234, Tyr240, and Phe263 are exposed in CCTsol and are buried upon membrane binding.

Peptide Bond Accessibility Maps of the Three CCT Forms—The chymotrypsin-digested samples shown in Fig. 2 were subjected to a complete MALDI-MS analysis of peptides in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range. MALDI-MS cannot be relied upon to quantitatively detect all peptide species in a crude mixture in the 1100–7000-Da range.
at Tyr59 and by C-terminal trimming at Tyr225, Tyr216, and Tyr182. However, the analysis of high mass fragments of CCTmem did not identify peptides derived from cuts at these sites. To verify that domain N accessibility is greater in CCTmem, we utilized another protease, Arg-C. CCT has four potential Arg-C cleavage sites between Met1–Leu41 in domain N, but no chymotrypsin sites in this region. Arg-C also has one potential site, Arg283 near the chymotrypsin site, Phe285, within the domain M region that appeared to be relatively buried in both CCT forms.

Comparison of the Arg-C Digestion Pattern of CCTsol and CCTmem—Because Arg-C digestion proceeded more slowly than chymotrypsin and because CCT has fewer sites for Arg-C, the fragmentation patterns were less complex than those generated by chymotrypsin. We identified by electrophoresis and immunoblotting a few large cleavage fragments (>34 kDa) generated by digestion for 1.5 min with Arg-C that did not react with anti-N (data not shown), indicating early cleavages that removed the N-terminal segment. Mass spectra obtained from a dried droplet preparation of the crude digestion mixture revealed three high mass fragments, Lys13–Arg309, Lys13–Arg353, and Lys13–Asp367, that account for the electrophoresis and immunoblotting observations (Fig. 4C). The peptides from a time course of Arg-C digestion were analyzed by CapLC-MALDI, and the peptide bond accessibility map derived from this data is shown in Fig. 6 (E and F, region III). There are only two Arg-C

![Figure 4](http://www.jbc.org/)

**Fig. 4.** Inaccessibility of the region between residues 274 and 303 in CCTsol. MALDI mass spectra of the peptides resulting from CCTsol digestion with chymotrypsin for 1 min (A) or 4 min (B) or Arg-C for 1.5 min (C) or 10 min (D). The samples were prepared by the dried droplet method. The peaks are annotated with the amino acid residue numbers comprising the detected peptide. The dashed boxes highlight the absence of high mass peptides spanning from the N terminus to potential chymotrypsin cleavage sites at 274, 278, 285, 289, 293, 299, or 303 or to the Arg-C site at 283.

![Figure 5](http://www.jbc.org/)

**Fig. 5.** Domain M- and P-derived high mass fragments reveal differences in the cleavage pattern of CCTsol and CCTmem. MALDI mass spectra of the peptides resulting from CCTsol and CCTmem digested with chymotrypsin for 2 min and prepared by the dried droplet method. The peaks are annotated with the amino acid residue numbers comprising the detected peptide. The inset shows an expanded view of the area in the dashed box, highlighting the heterogeneous phosphorylation of the domain P-derived peptides. Spectra of CCTmem sample is missing peptides resulting from cleavage at residues 234, 240, and 263 and is also missing Thr226–Asp367, indicated by broken arrows.
sites in domain M at Arg\textsuperscript{245} and Arg\textsuperscript{283}. Although no peptides ending at Arg\textsuperscript{283} were found in CCT\textsubscript{mem}, Gly\textsuperscript{224}–Arg\textsuperscript{245} as well as Lys\textsuperscript{13}–Arg\textsuperscript{145} were identified in the digest. This contrasts with the complete inaccessibility of Tyr\textsuperscript{246} after chymotrypsin digestion of CCT\textsubscript{mem}. This discrepancy is easy to rationalize. The membrane surface interaction of domain M would leave the polar side chain of Arg\textsuperscript{245} extending into the aqueous phase, whereas the phenol side chain of Tyr\textsuperscript{240} would insert into the membrane interface. This positioning is indicated from the NMR-derived structures of a peptide containing Arg\textsuperscript{245} and Tyr\textsuperscript{240} (10), and accordingly, Arg\textsuperscript{283} is inaccessible to Arg-C in CCT\textsubscript{mem} (Fig. 4).

Two other important differences between CCT\textsubscript{sol} and CCT\textsubscript{mem} revealed from chymotrypsin digestion, were also revealed by Arg-C digestion: (i) Sites within domain N at Arg\textsuperscript{36}, Arg\textsuperscript{42}, Arg\textsuperscript{88}, and Arg\textsuperscript{96} were cleaved to generate a variety of peptides in CCT\textsubscript{mem}, whereas none of these sites were cleaved in CCT\textsubscript{sol} for at least 10 min of digestion (Fig. 6C and Supplementary Fig. 1C). Thus the limited digestion pattern generated by two different proteases indicate that membrane binding increases the accessibility of the C-terminal side of domain N (Fig. 6, region II). (ii) Arg\textsuperscript{283} within the C-terminal side of domain M was cleaved in CCT\textsubscript{sol} (Fig. 6C and Supplementary Fig. 1C), but like Phe\textsuperscript{285}, this occurred only after cleavage of the hinge between domains C and M. As shown in Fig. 4 (C and D), large fragments corresponding to Met\textsuperscript{1}–Arg\textsuperscript{283} or Lys\textsuperscript{13}–Arg\textsuperscript{283} were not observed, although Lys\textsuperscript{13}–Arg\textsuperscript{221} and Lys\textsuperscript{13}–Arg\textsuperscript{292} were prominent. Thus Arg-C and chymotrypsin proteolysis revealed the relative inaccessibility of the C-terminal region of domain M in CCT\textsubscript{mem}.

Domain P Phosphopeptides Reveal the Degree of Phosphorylation of CCT\textsubscript{sol}—The PBAMs reveal that domain P in both CCT forms was highly accessible to both chymotrypsin and Arg-C. All Arg-C sites in domain P were cleaved immediately in CCT\textsubscript{mem}. However, only three of the four chymotrypsin sites in domain P were cleaved; Phe\textsuperscript{334} was not cleaved. The phosphorylation state of CCT purified by the method of MacDonald and Kent has been explored by proteolysis, high pressure liquid chromatography and sequencing (12). The phosphorylation state of CCT purified by the method of MacDonald and Kent (15), which we used, has not been explored. This information is important for studies on the membrane affinity of CCT purified by this method, because the phosphorylation state affects membrane affinity (33), and for consideration of CCT forms for crystallization. Cuts between Leu\textsuperscript{111} and the C terminus generated heterogeneously phosphorylated peptides with molecular masses <7000 Da. An average mass accuracy of <50 ppm in the 1000–7000 Da range (i.e. ±0.3 Da for a 6000-Da peptide) enabled facile identification of peptides sharing the same amino acid sequence but varying in the number of phosphoryl (80 Da) units (Table II). The average degree of phosphorylation for each peptide was calculated from the relative signal intensities within each set of phosphopeptides. For the peptide spanning the entire domain P, Gln\textsuperscript{312}–Asp\textsuperscript{367} enabled facile identification of peptides sharing the same amino acid sequence but varying in the number of phosphoryl (80 Da) units (Table II). The average degree of phosphorylation for each peptide was calculated from the relative signal intensities within each set of phosphopeptides. For the peptide spanning the entire domain P, Gln\textsuperscript{312}–Asp\textsuperscript{367}, the masses matched for four, five, six, and seven phosphates. Our analysis did not enable identification of each phosphorylated site; however, the results (Table II) indicate a phosphorylation pattern that is very similar to that obtained by MacDonald and Kent (12). The phosphorylated serines are spread over the entire 50-residue domain.

**DISCUSSION**

**Implications of the Peptide Bond Accessibility Data on CCT Structure**—The idea that domain M functions as an autoinhibitory domain in CCT\textsubscript{sol} is strongly supported by the constitutive activity of CCT\textsubscript{mem}. The present work has begun to unravel the nature of the inhibitory interactions of domain M by mapping its potential interdomain contact sites. The construction of the accessibility maps for CCT (Fig. 6) has generated a model that confirms the bipartite tertiary structure of CCT\textsubscript{sol} that of a compact head followed by a loosely folded and perhaps flexible tail. The maps show that the tertiary structure of domains N and C in CCT\textsubscript{mem} resembles that of CCT\textsubscript{sol} rather than CCT\textsubscript{mem}. The maps reveal that the central core of the catalytic domain remains inaccessible upon membrane binding and that
Protease Accessibility of the Catalytic Domain Remains Unaltered Upon Membrane Binding—Residues Phe\textsuperscript{165}–Phe\textsuperscript{185} of all three CCT forms are relatively inaccessible to chymotrypsin and residues Arg\textsuperscript{78}–Arg\textsuperscript{109} are inaccessible to Arg-C in both the soluble and membrane forms of CCT. Chymotrypsin-digested domain C progressively from the C terminus at Phe\textsuperscript{191}, Tyr\textsuperscript{182}, and Tyr\textsuperscript{173}, but only after cleavage in the very accessible hinge region (amino acids 216–225). After these primary cleavages secondary peptides were generated at Leu\textsuperscript{108}, Trp\textsuperscript{151}, Phe\textsuperscript{124}, Phe\textsuperscript{121}, Tyr\textsuperscript{107}, Leu\textsuperscript{76}, and Tyr\textsuperscript{60}. We did not observe any consistent differences in the chymotrypsin peptide bond accessibility in domain C between the three CCT forms with the exception of sites Tyr\textsuperscript{60} and Phe\textsuperscript{60} near the junction with domain N.

To assess the positioning of the accessible and inaccessible sites on the catalytic domain, we took advantage of the solved structure for a homologous cytidylyltransferase, CCT (8). Residues Arg\textsuperscript{78}–Arg\textsuperscript{211} of CCTs share ~60% similarity and ~30% identity with residues 3–129 of GCT. Residues 78–211 of CCTs were modeled upon the atomic coordinates available for this enzyme (Protein Data Bank code 1COZ) using MODELLER. Fig. 7A shows the close match between peptide backbones (see “Experimental Procedures” for discussion of the modeling). Fig. 7B presents a surface rendering of the CCT domain C dimer, highlighting the chymotrypsin and Arg-C sites. The purpose of this representation is to localize the protease sites with respect to major domain C surfaces and secondary structural elements. In general the protease accessibility data correlate well with surface accessibility of sites on this model of the native domain C fold. The sites most accessible to chymotrypsin (Trp\textsuperscript{151}, Leu\textsuperscript{156}, Tyr\textsuperscript{173}, Tyr\textsuperscript{182}, and Phe\textsuperscript{191}, colored orange in Fig. 7B, bottom image) are on the periphery of the catalytic dimer, located in loops, the ends of structural elements, or within the peripheral 3–10 helix. Chymotrypsin sites that were cleaved more slowly, i.e. between 1 and 5 min (colored green in Fig. 7B), were at the start of β2 (Tyr\textsuperscript{107}), the end of α-B (Phe\textsuperscript{211}), or in loop L2 (Phe\textsuperscript{124}). Of the sites inaccessible to chymotrypsin, Phe\textsuperscript{35}, Phe\textsuperscript{60}, Leu\textsuperscript{66}, and Leu\textsuperscript{108} are completely buried on the model. The remaining uncleaved sites had only partially exposed side chains on the model (colored blue in Fig. 7B). Arg-C did not cleave any sites in the entire catalytic domain over a 10-min time-frame. Arg\textsuperscript{84} and Arg\textsuperscript{147} appear fairly exposed (Fig. 7B, top image), but they are positioned in the middle of α-helix A and β-3, respectively, impeding access of the protease to the peptide backbone. However, Arg\textsuperscript{84} and Arg\textsuperscript{147} are in turns and appear very exposed on the upper surface of the model in Fig. 7B. We suggest that this surface might be involved in interactions with domain N (Fig. 8). If domain M were bound tightly to the surface of domain C in CCT\textsubscript{mem}, one would anticipate that those domain C sites buried by domain M would become more accessible in CCT\textsubscript{sol} and CCT\textsubscript{236}. Because the Arg-C and chymotrypsin sites in domain C were relatively inaccessible in all three forms, we have not found data to support such a model. Our results suggest that the catalytic core is tightly folded and remains quite rigid upon membrane binding of domain M. Thus the changes at the active site upon membrane binding or truncation to generate CCT\textsubscript{236} do not involve major reorganization of structural elements in domain C that would be detected by protease accessibility.

Domain P Remains Highly Accessible Upon Membrane Binding—In contrast to domain C, domain P is the region of highest protease accessibility in both CCT\textsubscript{sol} and CCT\textsubscript{mem}. The rapid decrease in intensity of band 1 (Fig. 2), the decreased relative intensity of peak 1 to peak 2 in high mass MALDI-MS data (Fig. 3, B and C) and the absence of the peptide Tyr\textsuperscript{226}–Asp\textsuperscript{237} (Fig. 5) suggest that it may even be more accessible in CCT\textsubscript{mem}. This observation would appear to contradict a previous suggestion that domain P can serve as a second membrane-binding domain acting together with domain M. This was suggested by the lipid responsiveness of the activity of a CCT mutant missing residues 257–309, i.e.
most of domain M, but retaining domain P (34). This mutant was expressed in *Escherichia coli* (34), and thus was unphosphorylated, whereas the CCT we used had an average of ~6 phosphoryl groups/domain P. Dephosphorylation could change the membrane associations of domain P and hence its accessibility to the aqueous phase. The effect of the phosphorylation status of CCT on its tertiary structure is an important future research direction.

**The Region between Leu274 and Leu303 May Be a Contact Site for Interdomain Interactions in CCT**

Seven chymotrypsin sites in this region have >50% propensity for cleavage (23), but we did not detect any peptides with N or C termini at any of these positions except for Phe285. There is only one site for Arg-C cleavage in this region at Arg283. Both Phe285 and Arg283 are cut only after cleavage in the hinge region. (Chymotrypsin cleavage at Tyr225 precedes that at Phe285, Arg-C cleavage at Arg223 or Arg245 precedes cleavage at Arg283). We never observed high mass fragments terminating at these residues, even after the longest digestion time. This region of inaccessibility could be a contact site between domain M and another domain (e.g. domain N, as shown in Fig. 8), a potential contact site subject to modulation upon membrane binding.

**Membrane Binding Buries Residues Phe234–Leu303**

Analysis of both high mass and low mass peptides were consistent in
showing that Tyr\textsuperscript{240}, Phe\textsuperscript{263}, and Phe\textsuperscript{285} as well as Arg\textsuperscript{283}, accessible in CCT\textsubscript{sol}, are uncleaved even at the longest digestion time in CCT\textsubscript{mem}. Residue Phe\textsuperscript{263} is also more protease-resistant in CCT\textsubscript{mem} than CCT\textsubscript{sol}. The residues flanking this region, Tyr\textsuperscript{225} and Arg\textsuperscript{229}, Leu\textsuperscript{311} and Arg\textsuperscript{298} are very rapidly cleaved in both forms. That Phe\textsuperscript{285} is relatively inaccessible to proteases in the presence of membranes may reflect its burial in lipid or a loss of flexibility of the peptide backbone in the near vicinity of the membrane embedded helix. Peptides spanning residues Asn\textsuperscript{216}–Leu\textsuperscript{298} fold into amphipathic \(\alpha\)-helices in the presence of anionic vesicles (35–37). The lipid interaction is accompanied by fluorescence blue shifts of Tyr\textsuperscript{240} and Tyr\textsuperscript{278}, indicative of transfer to a more nonpolar environment (35–37). The tryptophan fluorescence at positions 263 or 278 is sensitive to quenching by lipids with brominated acyl chains (35, 36). These data argue that the membrane-embedded region encompasses residues 234–285, at a minimum. The C-terminal flank of domain M between residues 286 and 303 is inaccessible in both forms; thus it could be argued that this region remains locked in interdomain contacts upon membrane binding rather than dissociating to bind lipids. However this scenario does not provide an explanation for the enhanced accessibility of domain N in CCT\textsubscript{mem} or CCT\textsubscript{236}. Thus the membrane binding region is likely to encompass residues 234–303.

Membrane Binding Increases the Accessibility of Sites in Domain N—Arg-C generated immediate cleavage at Arg\textsuperscript{13} and Arg\textsuperscript{14} in both CCT\textsubscript{sol} and CCT\textsubscript{mem}, showing for the first time that the N-terminal 15 residues of CCT are not a part of a compact fold (Fig. 8). Analysis of low mass peptides after chromatography and dislocation of domain M, the sites in domain N become accessible. The accessibility of this region of domain N in CCT\textsubscript{236}, which lacks domain M, also supports the notion of a domain N/domain M interaction in CCT\textsubscript{sol}. Because Arg-C and chymotrypsin cleavage sites do not mark the entire surface of domain C, it is quite possible that domain M contacts a portion of domain C as well as domain N in CCT\textsubscript{sol}. Upon membrane binding, domain N remains bound to domain C but repositions subtly (Fig. 8). We propose that the interaction of domain M with N contributes to autoinhibition of CCT in its soluble form. Our modified hypothesis for autoinhibition can now be tested using other approaches to specifically probe for interactions between domains M and N.

The domain N–domain M interaction could interfere with substrate docking or with the movements of helix B and loop L2 within the catalytic domain, which by analogy with GCT (9) may be important for catalysis (38). Comparison of the x-ray-derived structures of GCT in complex with substrate or with product has led to the proposal that during a catalytic cycle a \(\sim 10\)-residue segment encompassing the C terminus of helix B and the adjoining loop (L2) moves to engage the substrate, glycerol phosphate (3-Å shift at the point of maximal dislocation) and to stabilize the transition state at the \(\alpha\) phosphate of CTP (9). Charge stabilization of the transition state is accomplished by Lys\textsuperscript{122} and Lys\textsuperscript{460} in GCT (9) and Lys\textsuperscript{122} in CCT, which are situated in the L2 loop (38). Mutation of Lys\textsuperscript{122} to alanine in CCT decreases \(k_{\text{cat}}/K_m\) by a factor of \(\sim 200,000\) (38). A similar relocation of basic side chains is involved in catalysis of other structurally similar nucleotidylyltransferases (9). The contacts between domains N and M in CCT\textsubscript{sol} may constrain this movement to prevent ideal orientation of Lys\textsuperscript{122}.

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Cytidylyltransferase Interdomain and Membrane Interactions

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