Membrane-Binding Amphipathic α-Helical Peptide Derived from CTP:Phosphocholine Cytidylyltransferase†

Joanne E. Johnson and Rosemary B. Cornell*

Department of Chemistry and Institute of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

Received September 1, 1993; Revised Manuscript Received February 3, 1994®

ABSTRACT: A peptide corresponding to a portion of the amphipathic α-helical region of CTP:phosphocholine cytidylyltransferase was synthesized. This region of the enzyme was proposed to be the membrane-binding domain [Kalmar, G. B., Kay, R. J., Lachance, A., Aebersold, R., & Cornell, R. B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 6029]. We have shown that the peptide is physically associated with PG vesicles. CD of the peptide in buffer suggested a primarily random structure, while, in the presence of trifluoroethanol, the peptide was α-helical. Anionic lipid vesicles promoted an α-helical conformation, whereas neutral or cationic lipid vesicles did not alter the random structure of the peptide, suggesting a selective stabilization of the α-helix by anionic membranes. The fluorescence of the single tryptophan residue, which lies on the hydrophobic face of the amphipathic α-helix, was studied. Anionic lipid vesicles specifically induced a shift in the fluorescence to a lower wavelength. Fluorescence quenching by the aqueous-phase quencher, I^-, and the lipid-phase quencher 9,10-dibromo-PC was used to determine the accessibility of the tryptophan to each of these environments. The presence of anionic lipid vesicles, but not nonanionic lipid vesicles, decreased the quenching by I^- suggesting that, in the presence of anionic lipids, the tryptophan residue is poorly accessible to the aqueous I^-. Dibromo-PC significantly quenched the fluorescence only when present in anionic vesicles, confirming the membrane location of the tryptophan residue and the lipid specificity of this interaction. 9,10-Dibromo-PC quenched the fluorescence more efficiently than did 6,7-dibromo-PC or 11,12-dibromo-PC, localizing the tryptophan to the same depth in the bilayer as the ninth carbon on a fatty acid chain. These studies show that this peptide binds selectively to anionic membranes in an α-helical conformation, and the binding involves intercalation of the hydrophobic face of the helix into the membrane core. Thus, we propose that the mechanism of cytidylyltransferase activation by membranes involves lipid-specific stabilization of an amphipathic helical structure in the C-terminal region of the protein.

CTP:phosphocholine cytidylyltransferase (CT),[1] which catalyzes a key regulatory step in the biosynthesis of PC in mammalian systems, is an example of an amphitropic enzyme in that it is regulated by reversible translocation to membranes (Vance & Pelech, 1984; Vance 1989). The exact mechanisms for membrane binding and subsequent activation of the enzyme are yet to be resolved. Cell culture studies have demonstrated the involvement of a phosphorylation/dephosphorylation event in the regulation of CT (Watkins & Kent, 1991; Hatch et al., 1992; Wang et al., 1993). However, CT purified from rat liver can be activated by pure vesicles *in vitro* in the absence of kinases or phosphatases (Weinhold et al., 1986; Feldman & Weinhold, 1987; Cornell, 1991a,b). Activation of CT is dependent on the lipid composition of the membrane: membranes composed solely of PC are weakly activating, while those containing anionic phospholipids and fatty acids (Pelech et al., 1983; Feldman & Weinhold, 1987; Cornell, 1991a) or neutral lipids with small polar head groups such as diacylglycerol are strong activators (Cornell & Vance, 1987a,b; Slack et al., 1991; Cornell, 1991b). Cationic lipids such as sphingosine inhibit the effects of lipid activators (Sohal & Cornell, 1989). The membrane binding of CT involves both electrostatic and hydrophobic interactions (Cornell & Vance, 1987a,b; Cornell, 1991a,b).

The amino acid sequence of CT from rat liver has been determined (Kalmar et al., 1990; MacDonald & Kent, 1993), leading to a proposed model for its membrane interactions. The sequence contains no long hydrophobic regions for membrane-spanning nor any sites for covalent lipid attachment. There is, however, a region in the protein between residues 236 and 293 that is predicted to form a continuous, extended α-helix (Kalmar et al., 1990). If this domain is helical, it is strongly amphipathic throughout its length, with a mean helical hydrophobic moment of 0.52. Within this α-helix, an 11-mer motif is repeated three times in tandem. This type of structure has been proposed to mediate the membrane interactions of several proteins, including apolipoproteins (Segrest et al., 1990), mellitin (Dufourcq & Faucon, 1977), ion channel proteins and peptides modeled from these proteins (Agawa et al., 1991; Chung et al., 1992), influenza hemagglutinin (Lear & DeGrado, 1987), and other peptide hormones (Kaiser & Kezdy, 1984). Our model for the CT–membrane interaction involves this amphipathic α-helical region lying on the membrane surface with its hydrophobic face intercalated into the membrane core and its hydrophilic face interacting with the cytosol, with the lipid head groups, and with other regions of the protein.

† This work was funded by a grant from the Natural Science and Engineering Research Council of Canada. J.E.J. is supported by a Medical Research Council of Canada Studentship.

* To whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* March 15, 1994.

[1] Abbreviations: CT, CTP:phosphocholine cytidylyltransferase; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PA, phosphatidic acid; PS, phosphatidylserine; CL, cardiolipin; PI, phosphatidylinositol; HPLC, high-performance liquid chromatography; CD, circular dichroism; TFE, trifluoroethanol.

0006-2960/94/0433-4327$04.50/0

Evidence for an involvement of the putative amphipathic α-helix in the membrane interactions of CT has emerged recently from membrane binding studies of proteolytic fragments of CT (Craig et al., 1994). Chymotrypsin degradation of CT produces five discrete fragments which contain the N-terminus but are missing different lengths of the C-terminal region, as assessed using antibodies directed against these regions. Three of these fragments lack all or part of the putative amphipathic α-helical region and are unable to bind to activating lipid vesicles. The two fragments that contain this complete region bind to vesicles with similar lipid specificity as CT. These findings suggest that this region of the protein is necessary for membrane binding.

Few studies have examined the details of membrane binding of the enzyme at the molecular level. The protocol for purification (Weinhold et al., 1986) produces CT that is very dilute and bound to detergent micelles (Triton X-100) which interfere with analysis. This motivated us to design a model system involving the use of synthetic peptides to study the membrane-binding characteristics of CT. If the putative amphipathic helix operates as a discrete domain within the enzyme to mediate membrane binding, it is possible that a peptide corresponding to this region would behave in a similar manner. Synthetic peptides have previously been used to study the membrane interactions of the proposed N-terminal binding region of influenza hemagluttinin (Lear & Degrado, 1987; Claque et al., 1991), the putative anionic lipid-binding region of the erythrocyte Ca^{2+} pump (Filoteo et al., 1992), N-terminal signal sequences (Killian et al., 1990; McKnight et al., 1991), peptides derived from ion-channel proteins (Agawa et al., 1991; Chung et al., 1992), and other peptides which model the amphipathic helical structure (Kanellis et al., 1980; Ananthar-amaiah et al., 1985; Epand et al., 1987).

In order to investigate the structural and membrane-binding characteristics of the proposed amphipathic α-helix which is found in CT, we have analyzed a synthetic peptide that corresponds in sequence to a 33-residue portion of the helix containing the three 11-mer repeats. We have used circular dichroism to analyze the influence of membranes on the secondary structure of the peptide and have also studied the membrane interactions of the peptide by monitoring the fluorescence of a tryptophan residue on the hydrophobic face of the helix. Our results support our model involving this amphipathic helical region in the membrane interactions of CT.

MATERIALS AND METHODS

Materials. Egg PC, dioleoyl-PG, 1-palmitoyl,2-oleoyl-PE, 1-palmitoyl,2-oleoyl-PA, beef heart CL, soy bean PI, 1-palmitoyl,2-stearoyl-6,7-dibromo-PC, 1-palmitoyl,2-stearoyl-9,10-dibromo-PC, and 1-palmitoyl,2-stearoyl-11,12-dibromo-PC were purchased from Avanti Polar Lipids (Alabaster, AL). Sphingosine, oleic acid, oleyl alcohol, and *o*-phthaldialdehyde were from Sigma. *sn*-1,2-Diacylglycerol was generated by digestion of egg PC using phospholipase C as described (Myher & Kuksis, 1979). Microcon-100 filtration units were purchased from Amicon (Beverly, MA). Lipids were stored in chloroform stocks under nitrogen at −20 °C. The concentration of the lipid stocks was determined periodically by measuring lipid phosphorus (Bartlett, 1959). The purity of the lipid stocks was checked by thin layer chromatography on silica plates (Merck). The solvent systems were chloroform/methanol/water, 65:35:4 (brominated lipids), chloroform/methanol/ammonia, 65:35:5 (other phospholipids), or hexane/diethyl ether/acetic acid, 60:40:1 (oleic acid, oleyl alcohol and diacylglycerol). Lipids were visualized using iodine vapor.

Peptide Synthesis. A 33-residue peptide was synthesized and HPLC purified by Dr. Ian Clark-Lewis and Philip Owen (University of British Columbia) as described (Clark-Lewis et al., 1991). The sequence, shown in Figure 1, corresponds to residues 256–288 of CT (Kalmar et al., 1990). The peptide was acetylated on the N-terminus and aminated on the C-terminus to eliminate the charged groups. The molecular weight of the peptide was 4038. The peptide was stored as a solid at −80 °C. For experiments, a stock solution of the peptide was made in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) with 3 or 4 equiv of NaOH added from a 1 M stock to maintain the pH. The peptide stocks were made by weighing 1.00–2.50 mg of solid peptide to an accuracy of ±0.01 mg. The peptide was dissolved in the appropriate volume of buffer (0.4–1.0 mL) with an accuracy of ±0.005 mL to give a final concentration of 0.6 mM, which was confirmed by measuring the absorbance at 280 nm. The solution was stored at −20 °C in aliquots which were thawed only once.

Preparation of Lipid Vesicles. Lipids from $CHCl_3$ stocks were mixed in a small round-bottom flask and dried on a rotary evaporator. The lipids were hydrated by vortexing in TE buffer. The resulting suspension was sonicated on ice (Branson sonicator with a fine probe operating at 80% power output) until the turbid solution was clear (>20 min). The sonicated solution was spun at 20000*g* for 5 min in a bench top microfuge to remove any multilamellar vesicles and any titanium debris from the sonicator probe. The resulting small unilamellar vesicles were used for spectroscopy measurements the same day as preparation.

Peptide–Vesicle Binding Assay. Peptide stocks and lipid vesicles used in this assay were made in 10 mM HEPES buffer, pH 7.4, due to interference by Tris with peptide quantitation (see below). Peptide (12 nmol) and varying amounts of lipid vesicles were incubated at room temperature for 10 min in a total volume of 200 μL. Samples were then transferred to a Microcon-100 filtration unit (molecular weight cutoff of 100 000) and centrifuged at 3000*g* for 4–7 min, until all but 5–10 μL had gone through the filter. The filter was rinsed with 100 μL of HEPES buffer and centrifuged again for 2–3 min. Lipid vesicles and bound peptide were recovered from the filter by rinsing with 100 μL of HEPES buffer which was then transferred to another vial. Unbound peptide was in the flow-through fraction under the filter. Unbound peptide was quantitated by reacting a sample with *o*-phthaldialdehyde in the presence of 2-mercaptoethanol and incubating at room temperature for 20 min, as described by Roth (1971). The fluorescence intensity (excitation at 340 nm, emission at 440 nm) was proportional to the peptide concentration over the range used. Lipids interfered with the quantification of peptide in the retentate. The percent of peptide unbound was (nmol of peptide in flow-through/nmol of peptide added) × 100%. Control proteins cytochrome *c* and apolipoprotein A-I were quantitated by the method of Bradford (1976).

Circular Dichroism Measurements. All measurements were taken on a Jasco 700 spectropolarimeter using a 1- mm quartz cell at 25 °C. The samples were in TE buffer with a peptide concentration of 30 μM and a lipid concentration of 4 mM, unless otherwise indicated. Each spectrum was measured twice at a 50 nm/min scan rate in steps of 0.5 nm, and the two were averaged and smoothed. Background spectra of the lipid vesicles in buffer were subtracted from the peptide spectra. Values are expressed as mean residue molar ellipticity. The percent helix was estimated using the equation $\theta_{222} = (f_h - i\kappa/N)[\theta_{h\,222\infty}]$ where θ_{222} is the mean residue molar ellipticity at 222 nm, f_h is the fraction in α-helical form, i is the number

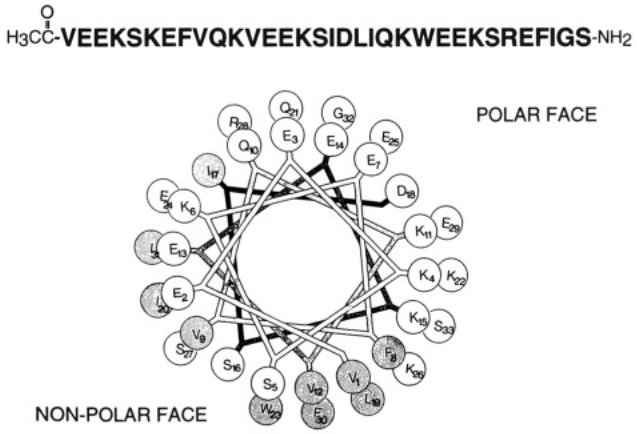

FIGURE 1: Amino acid sequence and helical wheel projection of the 33-residue peptide. Hydrophobic residues are shaded, demonstrating the amphipathic nature of the helix.

of helices (assumed to be one), κ is a wavelength-specific constant with a value of 2.6 at 222 nm, N is the number of residues in the peptide (33 residues), and $\theta_{h\,222\infty}$ is the molar ellipticity for a helix of infinite length at 222 nm and has a value of −39 500 deg·cm^2/dmol (Chen et al., 1974; Chang et al., 1978).

Fluorescence Studies. Spectra were recorded on a PTI-LS100 fluorescence spectrophotometer or a Perkin-Elmer MPF-44B fluorescence spectrophotometer, with emission and excitation slit widths of 3 and 8 nm, respectively. Samples were at room temperature in a 1-cm quartz fluorescence cuvette. The excitation wavelength was 280 nm, and the emission spectra were recorded from 300 to 440 nm. The sample in TE buffer contained 3.25 μM peptide and 0.5 mM lipid, unless otherwise indicated. At this concentration of well-sonicated lipid vesicles, there was no interference from sample turbidity. Lipid and peptide were preincubated at room temperature for >10 min prior to recording spectra. The fluorescence in the absence of peptide was subtracted from the fluorescence obtained in the presence of peptide. For iodide quenching experiments, samples in TE buffer contained peptide, lipid, 10 mM $Na_2S_2O_3$, and the indicated concentration of NaI added from a 4 M stock. NaCl, which does not quench fluorescence, was added to maintain a constant ionic strength of 0.2 M. In the case of quenching by brominated lipid, the specified dibromo-PC was incorporated at the indicated mol % into lipid vesicles from a $CHCl_3$ stock as described in vesicle preparation.

RESULTS

Properties of the Peptide. The sequence of the 33-residue synthetic peptide is shown in Figure 1. It corresponds to residues 256–288 in rat liver CT. The 33-residue stretch was chosen for peptide study for two reasons: (1) It consists of three 11-residue repeats, which implied a functional role in the protein. (2) This sequence contains a tryptophan residue, found on the nonpolar face of the proposed helix. The fluorescence of this tryptophan could be monitored to assess changes in the environment of the peptide. When plotted on an helical wheel projection (Figure 1), the peptide is observed to be highly amphipathic, with a highly-charged polar face and a nonpolar face. The peptide, with a net charge of −2, contains nine negatively charged and seven positively charged residues. These charged residues form streaks of alternating positive and negative charges running lengthwise along the polar face of the helix. There are three serine residues in register which interrupt the otherwise hydrophobic face of the helix. In dilute buffer, the peptide eluted from a Sephadex G-50 gel filtration column with an apparent molecular weight of approximately 6000 when compared to insulin α and β chains, aprotinin, and cytochrome c standards (data not shown), demonstrating that the peptide is not associated in a large aggregate.

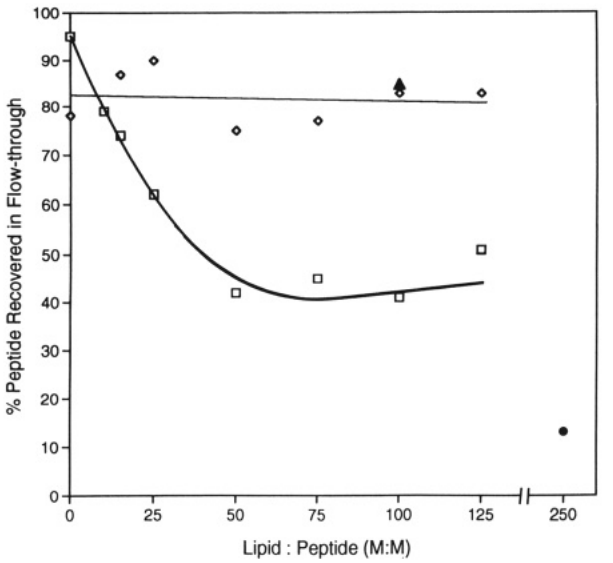

FIGURE 2: Binding of peptide to vesicles as a function of the lipid to peptide (molar) ratio. Peptide (12 nmol) was incubated with PG (□) or PC/DAG (3:1, ◇) vesicles of the indicated amount. The unbound peptide was separated from the vesicles by filtration and quantitated as described under Materials and Methods. Cytochrome c (▲) with PG vesicles or apolipoprotein A1 (●) with PC vesicles are shown as controls.

Physical Binding of the Peptide to PG Vesicles. The extent of peptide binding to PG vesicles was directly determined. Unbound peptide was separated from the bound peptide by filtration through a 100 000 molecular weight cut-off filter, which entrapped 90–95% of PG vesicles that were spiked with ^{3}H-labeled lipid (methyl ^{3}H-DPPC). Figure 2 shows the results of the binding assay. The amount of peptide recovered in the flow-through fraction decreased with increasing amounts of PG, reaching a minimum of 40–50 % recovered at lipid to peptide (mol/mol) ratios above 50:1. This would correspond to about 50–60% of the peptide bound by PG vesicles. In the presence of PC/DAG vesicles at various concentrations, approximately 80% of the peptide was recovered in the flow-through fraction. Thus, the binding of the peptide is lipid specific. 85% of cytochrome c, a nonmembrane protein, was recovered in the flow-through fraction when PG vesicles were present. Only 15% of apolipoprotein A-I , which is known to interact with PC vesicles (Wetterau & Jonas, 1982), was recovered in the flow-through fraction when PC vesicles were present. These analyses show that the binding assay is a valid monitor of peptide–lipid binding.

Circular Dichroism Indicates a Propensity of the Peptide for a Helical Conformation. The CD spectra were measured to determine the preferred secondary structure of the peptide, especially the propensity to form the predicted α-helix. The peptide in the simple TE buffer alone gave a CD spectrum that is indicative of a predominantly random structure with only a small amount of helix, ~14% (Figure 3). TFE is a solvent which promotes any favored secondary structure in peptides and proteins by promoting intramolecular hydrogen bonding (Sonnichsen et al., 1992). The presence of 50% TFE in TE buffer changed the spectrum of the peptide to one indicative of an α-helical structure, with the characteristic minima at 208 and 222 nm (Figure 3). The percent helix of the peptide in TFE was estimated to be approximately 54%.

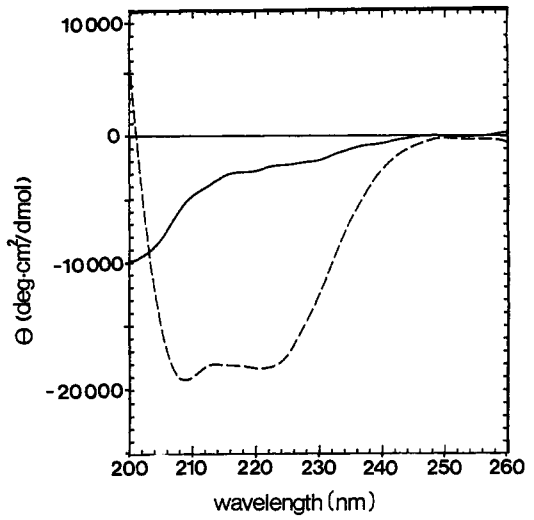

FIGURE 3: Circular dichroism spectra of the peptide in buffer (solid line) and in a 50% trifluoroethanol solution (dashed line). Peptide concentration was 30 mM in both samples.

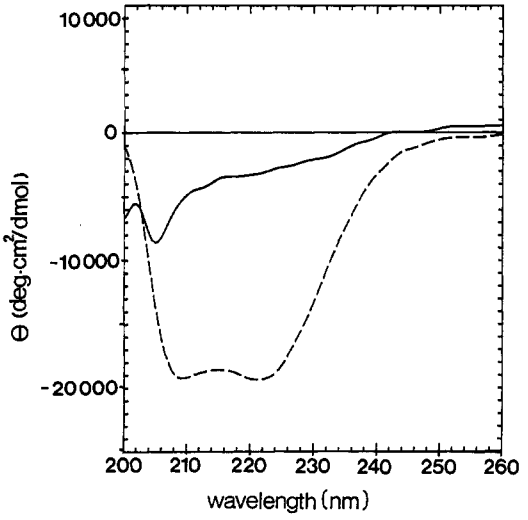

FIGURE 4: Circular dichroism spectra of the peptide (30 μM) in the presence of lipid vesicles (4 mM). Lipid vesicles were composed of egg PC (solid line) or DOPG (dashed line).

Anionic Lipid Vesicles Promote an α-Helical Structure. The preceding CD experiments show that the peptide does have the propensity to form an α-helical structure. Next, we sought to examine the effect of membranes, in the form of small unilamellar vesicles, on the secondary structure of the peptide in buffer. Figure 4 shows the CD spectrum of the peptide in the presence of lipid vesicles composed of PG, an anionic phospholipid. The presence of PG vesicles caused a change in the spectrum from that seen in buffer alone to one characteristic of an α-helical structure. A similar change in the CD spectrum was observed for the peptide in the presence of other anionic vesicles such as PC/oleic acid, PA, PI, PS, and PC/CL (Table 1). All of these lipids activate rat liver CT (Cornell, 1991a; Johnson et al., 1992). Addition of PC vesicles to the peptide caused no change in the CD spectrum from that in buffer alone (Figures 3 and 4). Other nonanionic lipid vesicles such as PC/PE, PC/diacylglycerol (3:1), and PC/oleyl alcohol (1:1) as well as the cationic vesicles PC/sphingosine (1:1) did not cause a change in the CD spectrum, indicating that the peptide remained in its predominantly random form. These results, summarized in Table 1, show that there is a lipid-induced change in the conformation of the peptide from a primarily random structure to an α-helix and

Table 1: Circular Dichroism Analysis of Peptide with Various Lipid Vesicles[a]

lipid	N	$[\theta]_{222}$ (deg cm²/dmol)[b]	estimated % helix[c]
none	12	2500 ± 300	14 ± 1
PC	4	3100 ± 700	16 ± 2
PC/PE (1:1)	2	3800 ± 400	18 ± 1
PC/diacylglycerol (3:1)	2	2300 ± 300	13 ± 1
PC/oleyl alcohol (1:1)	2	3000 ± 1000	15 ± 3
PC/sphingosine (1:1)	2	3500 ± 30	17 ± 0
PG	5	19000 ± 1500	57 ± 4
PC/oleic acid (1:1)	4	18000 ± 2000	55 ± 6
PA	3	21700 ± 900	63 ± 2
PI	2	25000 ± 3000	72 ± 8
PS	2	24000 ± 2000	69 ± 4
PC/CL (1:1)	3	22000 ± 2000	63 ± 4
in 50% TFE	7	18200 ± 700	54 ± 2

[a] Samples contained 30 μM peptide and 4 mM lipid, unless otherwise indicated. Spectra were measured as described under Materials and Methods. [b] $[\theta]_{222}$ is the mean residue molar ellipticity value calculated at 222 nm. [c] % Helix is estimated from $[\theta]_{222}$ by the method of Chen (1974) as described under Materials and Methods.

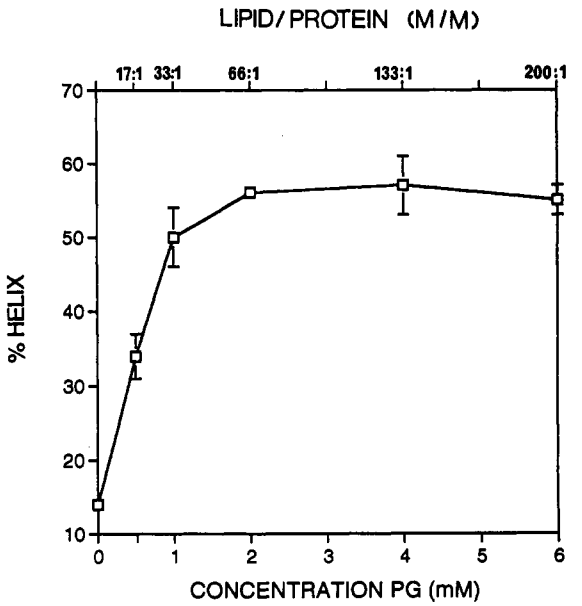

FIGURE 5: Dependence of percentage α-helical structure in peptide (30 μM) on the concentration of PG vesicles, as determined from CD. Spectra were measured and percent helix was calculated as described under Materials and Methods. Data are the average of two determinations.

that this conformational change is specific to anionic lipid vesicles.

The effect of anionic lipids on peptide conformation was concentration dependent, saturating at 1–2 mM PG (lipid to protein ratio of 33 to 66:1), which corresponds to ~60–120 peptide molecules for each vesicle, assuming 4000 PG molecules per vesicle (Figure 5). The peptide conformation showed sigmoidal dependence on the mol % of anionic lipid in the vesicles (Figure 6). The presence of PG (charge −1) at 30 mol % in PC vesicles caused little change in the peptide structure from that in the presence of PC, whereas 40 mol % PG induced the maximal degree of helicity. PA (charge of −2) promoted a conformational change between 20 and 30 mol %. This suggests that there is a minimum charge required on the membrane surface for the peptide to assume an α-helical conformation. A similar sigmoidal charge dependence has been observed for the activation of the native CT enzyme (Cornell, 1991a).

Anionic Lipid Vesicles Promote a Blue-Shift in the Fluorescence Emission. The results of the CD experiments

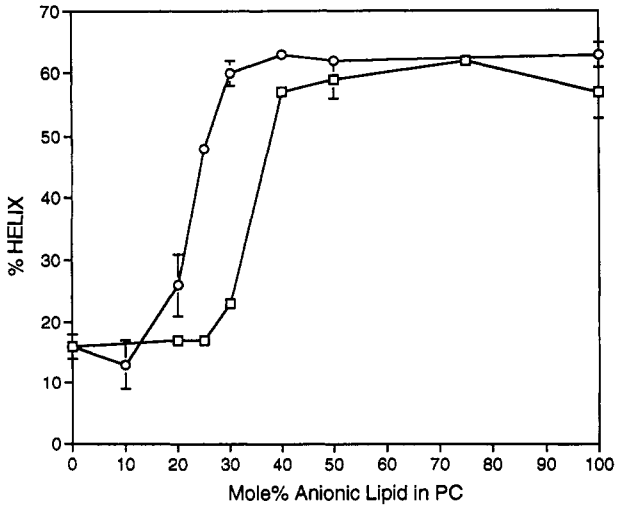

FIGURE 6: Dependence of percentage α-helical structure in the peptide (30 μM) on the mol % PG (□) or PA (○) in egg PC vesicles (4 mM total lipid), as determined from CD. Spectra were measured and percent helix was calculated as described under Materials and Methods. Data are the average of two determinations.

demonstrate that the peptide assumes an α-helical conformation in the presence of anionic lipid vesicles. Figure 1 shows that the helix formed by the peptide would be highly amphipathic, with a very highly charged polar face and a nonpolar face. This leads to a model of peptide–vesicle interaction involving the hydrophobic face of the helical peptide interacting with the hydrophobic membrane. To investigate this possibility, we monitored the fluorescence of a tryptophan residue which resides in the nonpolar face of the peptide helix. The tryptophan emission spectrum undergoes a characteristic shift in the emission maximum to a lower wavelength and an increase in quantum yield when the residue shifts from an aqueous to a hydrophobic environment (Lakowicz, 1983) This "blue-shift" in the tryptophan fluorescence would be evident if the aqueous peptide were to bind to lipid vesicles with its hydrophobic face intercalating into the membrane.

Figure 7 shows the fluorescence spectrum of the peptide in buffer, excited at 280 nm. The spectrum of the peptide is indistinguishable from that of free tryptophan in an aqueous environment, with a characteristic emission maximum of 350 nm. Upon addition of PG vesicles (Figure 7) the emission maximum shifted –20 nm, to 330 nm, and the intensity of the fluorescence increased, indicative of a shift of the tryptophan to a more hydrophobic environment. The spectra recorded on the Perkin-Elmer MPF-44B spectrofluorimeter showed a second peak, or shoulder, at 340–342 nm. However, this peak was not observed on spectra recorded on the PTI LS100 spectrofluorimeter, suggesting it was due to an artifact in the Perkin-Elmer instrument. Other anionic vesicles, such as PC/oleic acid, PI, PA, PS, and PC/CL caused a similar shift in the emission maximum of the fluorescence spectra. However, addition of PC vesicles to the peptide caused no change in the fluorescence spectrum (Figure 7). Similarly, other nonanionic lipid vesicles such as PC/PE, PC/sphingosine, PC/diacylglycerol, and PC/oleyl alcohol caused no change in the spectrum (data not shown).

The appearance of the peak at 330 nm was dependent on the concentration of anionic vesicles added. At 62.5 μM PG (lipid to protein ratio of 17:1) the 330-nm peak consisted of a shoulder on the 350-nm peak. At 125 μM (lipid to protein ratio of 33:1) the spectrum showed a peak at 330 nm, and further increases in the lipid concentration had no affect (data not shown). An all-or-nothing change in the spectrum was observed in these fluorescence experiments as in the CD

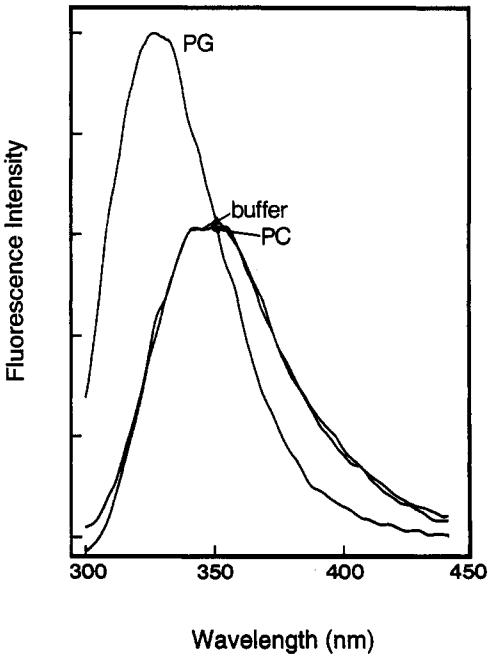

FIGURE 7: Tryptophan fluorescence of the peptide in buffer and in the presence of 0.5 mM DOPG or egg PC vesicles. The excitation wavelength was 280 nm. Peptide concentration was 3.25 μM.

experiments between 30 and 40 mol % PG in PC vesicles (data not shown). Thus, the induction of the helical conformation in the peptide, as observed in the CD experiments, correlated very well with a shift of the tryptophan to a more hydrophobic environment, as observed in the fluorescence experiments.

Anionic Lipid Vesicles Shield the Peptide from Aqueous Iodide Quenching. The tryptophan environment was also monitored by its accessibility to quenchers. An aqueous phase quencher, such as iodide, can quench the fluorescence of tryptophan upon collision with the molecule (Lakowicz, 1983; Eftink, 1991). If the tryptophan is not accessible to the aqueous environment, as is the case if the residue is buried in a membrane or in the interior of a protein, the aqueous quenchers will not affect the fluorescence.

Figure 8A shows the spectrum of the peptide in TE buffer + 0.2 M NaCl, which is identical to the spectra of peptide in TE. NaCl does not quench fluorescence and was present to maintain a constant ionic strength among all samples. When NaCl was replaced by 0.2 M NaI, there was a 48% reduction in the intensity of the fluorescence at 350 nm. Figure 8B shows the spectrum of the peptide in similar buffer containing PG vesicles, with its characteristic peak at 330 nm. Addition of iodide resulted in only a 14% quenching of the fluorescence peak in this case, suggesting that the presence of the vesicles shields the tryptophan from the aqueous iodide. A similar decrease in the iodide quenching was observed in the presence of other anionic lipid vesicles, whereas the quenching in the presence of nonanionic vesicles was similar to that obtained when no lipid is present (Table 2). Similar experiments were performed using the cation cesium as the aqueous quencher. Anionic vesicles protected the peptide from quenching by Cs^+, while nonanionic vesicles had no affect (data not shown), corroborating the results of iodide quenching.

A quantitative expression for this type of quenching is the Stern–Volmer plot, which relates the fluorescence quenching to the concentration of quencher by the following equation: $F_o/F = K_{sv}$[quencher] + 1, where F_o is the peak fluorescence intensity in the absence of quencher, F is the peak fluorescence intensity in the presence of quencher, and K_{sv} is the Stern–

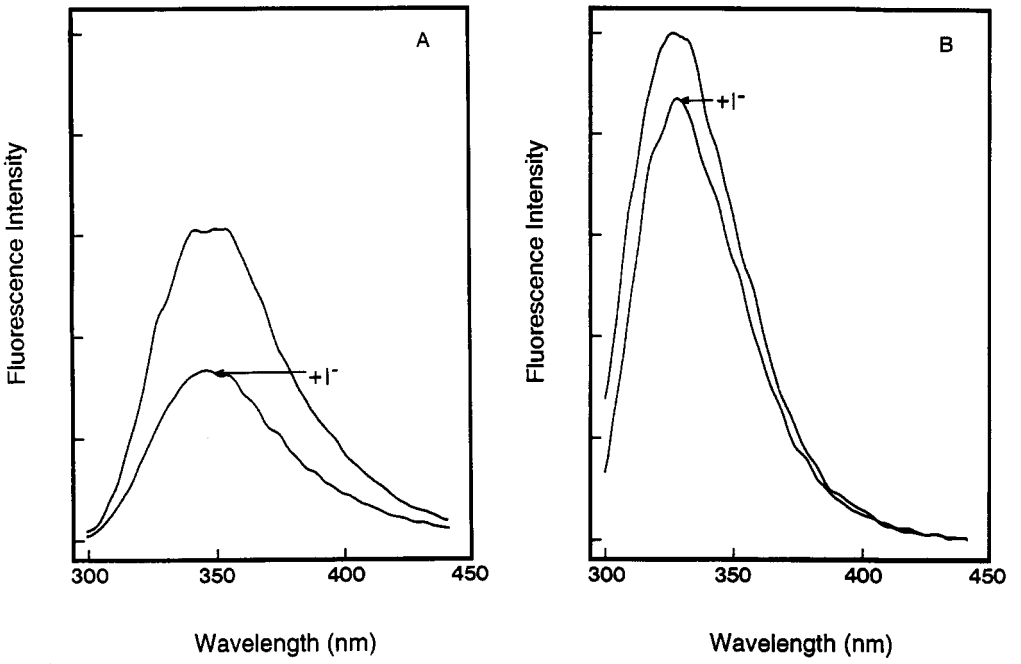

FIGURE 8: Quenching of tryptophan fluorescence of the peptide by aqueous iodide. The excitation wavelength was 280 nm. Peptide concentration was 3.25 μM. Unquenched peptide was in 0.2 M NaCl. Quenched peptide was in 0.2 M NaI. (A) Peptide in buffer. (B) Peptide in the presence of 0.5 mM DOPG vesicles.

Table 2: Quenching of Peptide Fluorescence by 0.2 M Aqueous Iodide[a]

lipid	% quench[b] 350 nm	% quench[b] 330 nm
none	48 ± 4	46 ± 4
PG	18 ± 7	15 ± 2
PC/oleic acid (1:1)	25 ± 1	24 ± 3
PC	45 ± 1	43 ± 3
PC/diacylglycerol (3:1)	47 ± 6	46 ± 5
PC/sphingosine (1:1)	51 ± 3	54 ± 2

[a] Lipid vesicles were present at 0.5 or 1 mM (for PC/oleic acid). Peptide concentration was 3.25 μM. Spectra were measured as described under Materials and Methods. Underlines indicate the peaks of fluorescence intensity of the spectra. Data are the average ± standard deviation of 2–4 separate experiments. [b] % Quench = $100(1 - F/F_0)$, where F is the fluorescence intensity of the quenched sample obtained in the presence of 0.2 M iodide, and F_0 is the fluorescence intensity of the unquenched sample with 0.2 M chloride in place of iodide.

Volmer quenching constant, which can be obtained as the slope of a plot of F_0/F vs concentration of quencher (Lakowicz, 1983; Eftink, 1991). If a fluorophore is inaccessible to the quencher, the lower quenching probability will be reflected in a lower K_{sv} value. Figure 9 shows the Stern–Volmer plot of iodide quenching for the peptide in buffer and in the presence of PC, PG, or PC/oleic acid. The similar slopes for peptide in buffer and in the presence of PC suggest that the peptide tryptophan remains in a similar aqueous environment upon addition of PC vesicles. However, the slope is much lower for the peptide in the presence of either PG or PC/oleic acid vesicles, suggesting that the tryptophan is much less accessible to the aqueous environment in the presence of these anionic lipid vesicles.

Fluorescence Quenching by Bromine-Labeled PC Is Selective for Anionic Vesicles. There are two possible explanations for the lipid specific changes in the tryptophan environment: (1) The peptide binds to the lipid vesicles, and the tryptophan residue, along with the nonpolar face of the helix, intercalates into the hydrophobic interior of the membrane bilayer. (2) The tryptophan becomes shielded from the aqueous environment by other residues in the peptide upon

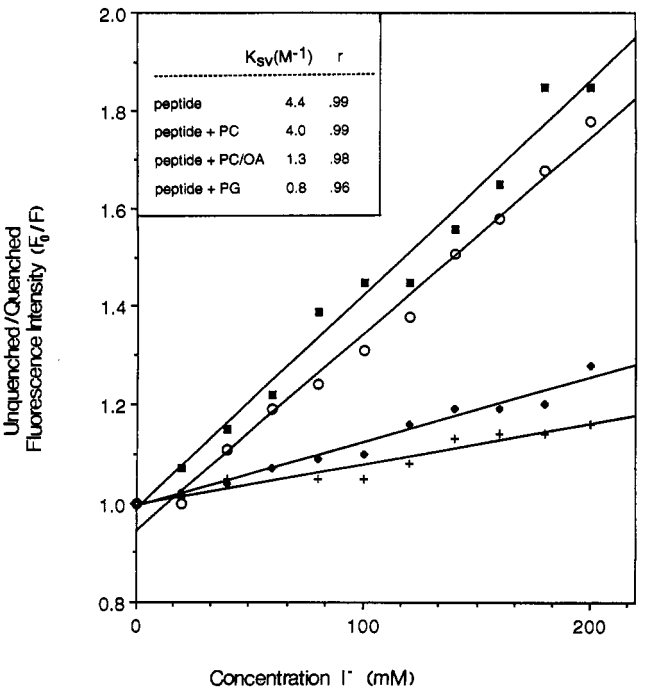

FIGURE 9: Stern–Volmer plot for iodide quenching of tryptophan fluorescence. Samples contained 3.25 μM peptide in buffer (■), or in the presence of 1 mM vesicles composed of egg PC (○), egg PC/oleic acid (1:1, ◆), or DOPG (+). Iodide concentration was as indicated. The ionic strength was kept constant at 0.2 M by the addition of NaCl.

the transition to a helical conformation. To discriminate between these two possibilities, we used a quencher that is located in the membrane bilayer: 9,10-dibromo-PC. Quenching of the tryptophan fluorescence would occur only if the residue were intercalated into the hydrophobic core of the membrane bilayer, where the bromide is located. Figure 10 shows the spectra of the peptide with PC/PG vesicles containing either 50% egg PC (unquenched) or 50% 9,10-dibromo-PC (quenched). The introduction of bromide into the bilayer quenched the fluorescence of the tryptophan by 58 ± 4% ($N = 3$), demonstrating the location of the tryptophan

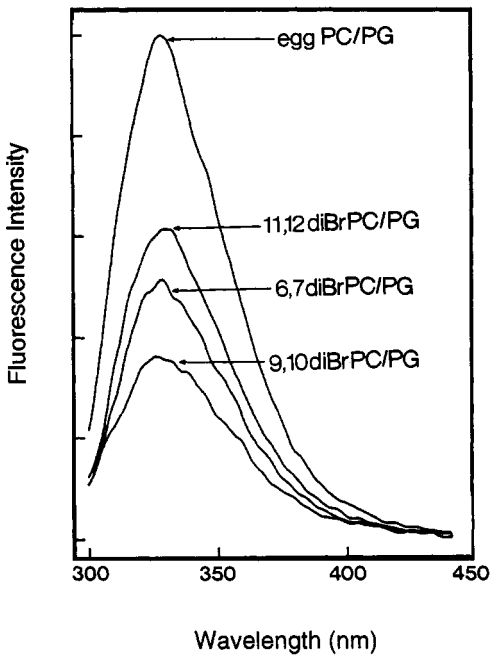

FIGURE 10: Quenching of tryptophan fluorescence of the peptide by 9,10-dibromo-PC. The excitation wavelength was 280 nm. Peptide concentration was 3.25 μM. Unquenched sample contained 0.5 mM egg PC/DOPG (1:1) vesicles. Quenched samples contained 0.5 mM vesicles composed of either 9,10-dibromo-PC/DOPG (1:1), 6,7-dibromo-PC/DOPG (1:1), or 11,12-dibromo-PC/DOPG (1:1).

Table 3: Quenching of Peptide Fluorescence by 50 mol % 9,10-dibromo-PC[a]

lipid[b]	% quench[c] 350 nm	% quench[c] 330 nm
PC/PG (1:1)	55 ± 4	57 ± 2
PC/oleic acid (1:1)	56 ± 7	67 ± 3
PC	1 ± 1	0
PC/diacylglycerol (3:1)	8 ± 2	13 ± 9
PC/sphingosine (1:1)	16 ± 2	18 ± 2

[a] Lipid vesicles were present at 0.5 mM. Peptide concentration was 3.25 mM. Spectra were measured as described under Materials and Methods. Underlines indicate the peaks of fluorescence intensity of the spectra in the presence of each lipid. Data are the average ± standard deviation of 2–4 separate experiments. [b] For unquenched samples, PC indicates egg PC. For quenched samples, 50% of all vesicles was composed of 9,10-dibromo-PC, the remainder of the PC being egg PC. [c] % Quench = $100(1 - F/F_0)$, where F is the fluorescence intensity of the quenched sample obtained with vesicles containing 9,10-dibromo-PC, and F_0 is the fluorescence intensity of the unquenched sample with egg PC in place of 9,10-dibromo-PC.

in the bilayer. Similar quenching results were seen when 9,10-dibromo-PC was incorporated into PC/oleic acid vesicles, whereas 9,10-dibromo-PC incorporated into nonanionic lipid vesicles caused minimal or no quenching, suggesting no peptide intercalation into these vesicles (Table 3). Thus, the tryptophan residue on the nonpolar face of the peptide helix does intercalate into the hydrophobic bilayer of anionic lipid vesicles.

To examine the extent of intercalation of the tryptophan residue into the bilayer, the amount of quenching by bromide at different positions on the fatty acyl chain of PC was analyzed (Figure 10). PC with bromide at the 6,7-, 9,10-, or 11,12-positions of the fatty acyl chain were incorporated into PC/PG vesicles. The 6,7- and 11,12-dibromo-PCs quenched the fluorescence of the peptide tryptophan by 42 ± 5% (N = 3) and 38 ± 2% (N = 3), respectively, suggesting they are approximately equidistant from the fluorophore; whereas, the 9,10-dibromo-PC quenching was more efficient (58%), suggesting it is closer to the fluorophore. A simple conclusion would be that the tryptophan intercalates to between the 6,7- and 11,12-positions, near the 9,10-positions of the fatty acyl chain in the outer leaflet of the membrane bilayer.

DISCUSSION

We have used circular dichroism to study the secondary structure and fluorescence to study the membrane interactions of a peptide derived from the proposed amphipathic α-helical membrane-binding domain of CT. The results of these spectroscopic experiments have led to three major conclusions about the membrane-binding characteristics of this peptide. (1) The peptide interacts with membranes in an α-helical conformation. (2) A portion of the peptide intercalates into the hydrophobic core of the membrane. (3) The peptide interacts specifically with anionic membranes. These results implicate a membrane-binding role for the corresponding region of CT.

The Peptide Interacts with Membranes in an α-Helical Conformation. The results of the CD studies demonstrate that the peptide, while random in buffer solution, forms an α-helical structure in the presence of anionic membranes. In all cases examined, the lipid vesicles that caused a change in the fluorescence properties of the peptide also promoted the helical conformation. There was a close correlation between the lipid to protein ratio required, or mol % anionic lipid required, for maximum induction of helix and for complete disappearance of the 350-nm tryptophan emission peak. These observations argue that the peptide binds to these vesicles as a helical structure.

The percentage α-helix in the membrane-bound structure was estimated to be 55–70%, while in buffer solution the peptide was only ~14% helical. For both forms of the peptide, the remainder of the structure seems to be largely random with <10% β-sheet structure, by comparison to the reference polylysine spectra of Greenfield and Fasman (1969). Similar estimations for percentage helicity were obtained for other membrane-binding amphipathic peptides: a peptide derived from the N-terminal region of influenza hemagglutinin was 45% helical (Lear & DeGrado, 1987); model ion-channel peptides were 60–70% helical (Agawa et al., 1991; Chung et al., 1992); and peptides modeled from apolipoproteins were up to ~50% helical (Kanellis et al., 1980; Anantharamaiah et al., 1985; Epand et al., 1987; Yoshimura et al., 1992).

That the peptide is only ~60% helical in the presence of vesicles can be explained in two ways. The first possibility is that, when bound to lipids, each peptide is 60% helical and 40% random conformation. Although in the native CT protein this whole region is predicted by the algorithms of Garnier et al. (1978) or Chou and Fasman (1978) to be in an α-helical conformation, some loss of structure at the ends of the peptide could occur, perhaps accounting for up to 40% of the total structure. The other explanation is that the peptide is bound to membranes in an all helical conformation, but only 60% of the peptide is bound. The other 40% of the peptide is unbound in random conformation. A direct quantification of peptide binding to PG vesicles revealed that, even at the highest ratio of lipid to peptide, ~40% of the peptide was unbound (Figure 2), which supports the latter explanation. In buffer solution, the random structure is the most probable, and the peptide is predominantly in this conformation. Anionic lipid vesicles stabilize the peptide in the helical structure, and this becomes the predominant conformation. Thus, there would be two populations of peptide, bound and unbound, which would be exchanging. This was also evident in the case of a synthetic model amphipathic peptide where not all of the peptide was tightly associated with the lipid vesicles in a density gradient ultracentifugation (Kanellis et al., 1980).

A Portion of the Peptide Intercalates into the Hydrophobic Core of the Membrane. Once the helical structure of the peptide was confirmed, the tryptophan located in the nonpolar face proved to be a useful handle in assessing the location of the nonpolar helix face with respect to membranes. The maximum fluorescence emission wavelengths and quenching abilities of iodide demonstrated that the tryptophan was less accessible to the aqueous environment in the presence of anionic lipids, while the quenching abilities of the hydrophobic dibromo PCs in anionic lipid vesicles confirmed the location of the tryptophan in the hydrophobic interior of the membrane. The use of aqueous iodide as a quencher showed that the tryptophan was not as accessible to the buffer when anionic lipids were present. However, the tryptophan was not completely inaccessible to the iodide as it was still about 20% quenched even though the quenching experiments with dibromo-PC show that the tryptophan is located well within the bilayer. Thus, this residual quenching is likely due to the presence of unbound peptide in the aqueous environment, which exists in dynamic equilibrium with the membrane-bound peptide, as discussed above.

The use of the three dibromo-PCs, with the bromine quencher at different positions in the membrane, allowed us to localize the depth of intercalation of the tryptophan. The bromines in 9,10-dibromo-PC, which was the most efficient quencher of the tryptophan in our peptide, have been localized by X-ray diffraction to 8 Å from the bilayer center, or 7 Å from the head group/hydrocarbon boundary (Wiener & White, 1991). The bromines in 6,7-dibromo-PC and 11,12-dibromo-PC have been localized to 11 and 6.5 Å, respectively, from the center of the bilayer, or 4 and 8 Å, respectively, from the head group/hydrocarbon boundary (McIntosh & Holloway, 1987). Quenching by these two lipids was approximately the same but less than that of the 9,10-dibromo-PC, suggesting that the tryptophan was located at a position approximately equidistant from the two probes. This would place the tryptophan at the same depth in the bilayer as the 9 position on the fatty acyl chain, 9 Å from the bilayer center or ~6 Å from the head group/hydrocarbon boundary. In studies of a model ion-channel peptide that interacts with the membrane via an amphipathic helical structure intercalating on the surface of the bilayer, a tryptophan on the hydrophobic face of the helix was found to intercalate to 7–9 Å from the bilayer center (Chung et al., 1992). These results are similar to ours, which lends support for our model of the peptide helix interacting parallel to the membrane surface, with its hydrophobic face intercalated.

The Peptide Interacts Specifically with Anionic Membranes. We have found, in both CD and fluorescence studies, that our peptide responds specifically to anionic lipid vesicles. Vesicles composed of zwitterionic lipids or cationic lipids caused no change in the fluorescence characteristics or secondary structure of the peptide relative to that of the peptide in buffer solution, demonstrating an inability to induce an α-helical structure which predicates membrane intercalation. The peptide interacted with a variety of anionic lipids, showing no preference for any specific head group, which demonstrates that the peptide is indeed responding to the negative charge on the lipids. There was a minimum surface charge required on the membrane for peptide binding, as illustrated by the sigmoidal dependence of the percentage α-helical structure on the mol % PG or PA in the zwitterionic PC membrane. Furthermore, the maximal helicity (60%) was achieved at a lower mol % of PA (charge of −2) than PG (charge of −1). These responses to the negative surface charge were also reflected in the shift of the fluorescence emission spectra, which occurred at the same mol % as the helical conformational change.

The stringent lipid selectivity that the CT peptide shows is quite uncommon among membrane-binding amphipathic α-helical peptides. Apolipoprotein-derived model amphipathic helical peptides (Kanellis et al., 1980; Anantharamaiah et al., 1985; Epand et al., 1987), the N-terminal peptide from influenza hemagglutinin, (Lear & DeGrado, 1987), and model ion channel peptides (Agawa et al., 1991; Chung et al., 1992) all bind to the nonanionic PC vesicles in an amphipathic α-helical conformation. The PhoE model signal peptide (Killian et al., 1990), mellitin (Dufourq & Faucon, 1977), and a model fusogenic peptide (Yoshimura et al., 1992) are examples of amphipathic helical peptides that preferentially interact with anionic lipid vesicles, but each peptide also interacts with PC vesicles to a lesser extent.

In contrast, the CT peptide interacts *only* with anionic vesicles. This lipid selectivity is not just due to a "nonspecific" electrostatic attraction because the peptide has a net charge of −2 and thus should be attracted to positively charged vesicles. There must be some specific feature in its structure which confers this lipid selectivity. In the theoretical amphipathic α-helical structure (Figure 1), there exists a cluster of cationic lysine residues at the interface of the nonpolar and polar face, which could function to attract the peptide to a negatively charged membrane surface. These residues could stabilize the membrane-bound structure by interacting with the phospholipid head groups when the nonpolar face is intercalated into the membrane. However, these lysine clusters are a key feature of the apolipoprotein amphipathic helices which do not show anionic lipid specificity (Segrest et al., 1990). The lysine clusters, found at both interfaces of the nonpolar and polar helical faces, have been proposed to extend the hydrophobic face of the helix because they have long hydrocarbon chains that could be located in the membrane, with the positively charged groups interacting at the membrane surface with the phosphate group on the phospholipid head groups. Unlike the apolipoprotein amphipathic helices which have cationic clusters at each interface of the nonpolar and polar helical faces, the CT peptide contains a cationic cluster at one interface and an anionic cluster at the other interface. Possibly the negative surface charge requirement on the membrane involves a localized decrease in the pH near the membrane surface, leading to protonation of some of the glutamate residues on the peptide. This would simultaneously increase the positive charge on the peptide, leading to stronger electrostatic binding to the negatively charged membrane. The CT derived peptide is also unique in that it contains three serine residues on its nonpolar face. Whether these features confer lipid specificity is a question yet to be answered.

Relation to the Native Cytidylyltransferase. This paper presents evidence for a CT domain that may be responsible for the binding and activation of the enzyme by anionic lipid vesicles. We propose that the mechanism whereby anionic phospholipid vesicles activate CT involves stabilization of the α-helical conformation of residues 256–288 and perhaps the contiguous region N-terminal to this 33-mer. Stabilization of an α-helix transforms this region into a highly amphipathic structure, the nonpolar face of which can readily intercalate into the hydrophobic core of a membrane.

The membrane interactions of the isolated 33-residue domain of CT mimic in several respects the interactions of the native enzyme. The interactions of both seem to involve electrostatic and hydrophobic components. Like the peptide, CT responds poorly to PC vesicles, while anionic lipid vesicles promote enzyme binding and are potent activators. For both

the peptide and the enzyme, the negative surface charge on the membrane rather than the chemical structure of the head groups is a determining factor in the binding (Cornell, 1991a). The CT–membrane interaction also involves a hydrophobic component. Detergents inhibit the membrane interactions of the enzyme, while high salt washes were not effective (Cornell & Vance, 1987a). Features which disturb the packing of a lipid bilayer, such as high vesicle curvature or the gel to liquid crystalline phase transition, increase the enzyme activity (Cornell, 1991b). These observations suggest an intercalation of a portion of the enzyme into the bilayer, an event that is observed for the peptide. Thus, it is reasonable to propose that the region of CT that is represented by this peptide is involved in membrane binding.

One major difference in the membrane-binding characteristics of CT and the peptide is that the enzyme is activated by a second class of lipids, those with small polar head groups, such as diacylglycerol and oleyl alcohol, when present in a PC membrane (Cornell & Vance, 1987a; Cornell, 1991b). These lipids had no effect on the peptide, either in the CD structural analysis or in the fluorescence characteristics. Thus, it seems that this 33-residue sequence does not contain all of the elements necessary to impart the lipid specificity of the native CT. Possibly, a separate domain exists in the enzyme which is responsible for interactions with this class of activators. Removal of the amphipathic α-helical region by chymotrypsin degradation produces fragments unable to bind to either class of activating lipid (Craig et al., 1994), suggesting that this is the main domain which mediates lipid binding. There may be another distinct region of the protein which is involved in imparting the actual specificity for these neutral, polar lipids. Another possibility is that the membrane-binding domain for the two classes of lipids is overlapping, and our peptide corresponds to only a portion of this membrane-binding domain. The likely candidate for this membrane-binding domain is the entire amphipathic α-helical domain (residues 236–293).

ACKNOWLEDGMENT

We thank Dr. Ian Clark-Lewis and Philip Owen for the peptide synthesis, Dr. Steven Holdcroft for the use of the PTI spectrofluorimeter, and Carl Johansson for advice regarding the fluorescence measurements.

REFERENCES

Agawa, Y., Sannamu, L., Ono, S., Aoyagi, H., Ohno, M., Taniguchi, T., Anzai, K., & Kirino, Y. (1991) *J. Biol. Chem. 266*, 20218–20222.

Anantharamaiah, G. M., Jones, J. L., Brouillette, C. G., Schmidt, C. F., Chung, B. H., Hughes, T. A., Bhown, A. S., & Segrest, J. P. (1985) *J. Biol. Chem. 260*, 10248–10255.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–253.

Chang, C. T., Wu, C.-S. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13–31.

Chen, Y.-H., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350–3359.

Chou, P. Y., & Fasman, G. D. (1978) *Annu. Rev. Biochem. 47*, 251–276.

Chung, L. A., Lear, J. D., & DeGrado, W. F. (1992) *Biochemistry 31*, 6608–6616.

Clague, M. J., Knutson, J. R., Blumenthal, R., & Herrmann, A. (1991) *Biochemistry 30*, 5491–5497.

Clark-Lewis, I., Moser, B., Walz, A., Baggiolini, M., Scott, G. J., & Aebersold, R. (1991) *Biochemistry 30*, 3128–3135.

Cornell, R. B. (1991a) *Biochemistry 30*, 5873–5880.

Cornell, R. B. (1991b) *Biochemistry 30*, 5881–5888.

Cornell, R. B., & Vance, D. E. (1987a) *Biochim. Biophys. Acta 919*, 26–36.

Cornell, R. B., & Vance, D. E. (1987b) *Biochim. Biophys. Acta 919*, 37–48.

Craig, L., Johnson, J. E., & Cornell, R. B. (1994) *J. Biol. Chem. 269*, 3311–3317.

Dufourcq, J., & Faucon, J.-F. (1977) *Biochim. Biophys. Acta. 467*, 1–11.

Eftink, M. R. (1991) in *Biophysical and Biochemical Aspects of Fluorescence Spectroscopy* (Dewey, T. G., Ed.) Plenum Press, New York.

Epand, R. M., Gawish, A., Iqbal, M., Gupta, K. B., Chen, G. H., Segrest, J. P., & Anantharamaiah, G. M. (1987) *J. Biol. Chem. 262*, 9389–9396.

Feldman, D. A., & Weinhold, P. A. (1987) *J. Biol. Chem. 262*, 9075–9081.

Filoteo, A. G., Enyedi, A., & Penniston, J. T. (1992) *J. Biol. Chem. 267*, 11800–11805.

Garnier, J., Osguthorpe, D. J., & Robson, B. (1978) *J. Mol. Biol. 120*, 97–120.

Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.

Hatch, G. M., Jamil, H., Utal, A. K., & Vance, D. E. (1992) *J. Biol. Chem. 267*, 15751–15758.

Johnson, J. E., Kalmar, G. B., Sohal., P. S., Walkey, C. J., Yamashita, S., & Cornell, R. B. (1992) *Biochem. J. 285*, 815–820.

Kaiser, E. T., & Kezdy, F. J. (1984) *Science 223*, 249–255.

Kalmar, G. B., Kay, R. J., Lachance, A., Aebersold, R., & Cornell, R. B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6029–6033.

Kanellis, P., Romans, A. Y., Johnson, B. J., Kercret, H., Chiovetti, R., Jr., Allen, T. M., & Segrest, J. P.(1980) *J. Biol. Chem. 255*, 11464–11472.

Killian, J. A., Keller, R. C. A., Struyve, M., de Kroon, A. I. P. M., Tommassen, J., & de Kruijff B. (1990) *Biochemistry 29*, 8131–8137.

MacDonald, J. I. S., & Kent, C. (1993) *Protein Expression Purif. 4*, 1–7.

McIntosh, T. J., & Holloway, P. W. (1987) *Biochemistry 26*, 1783–1788.

McKnight, C. J., Rafalski, M., & Gierasch, L. M. (1991) *Biochemistry 30*, 6241–6246.

Myher, J., & Kuksis, A. (1979) *Can. J. Biochem. 57*, 117–124.

Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.

Lear, J. D., & DeGrado, W. F. (1987) *J. Biol. Chem. 262*, 6500–6505.

Pelech, S. L., Pritchard, P. H., Brindley, D. N., & Vance, D. E. (1983) *J. Biol. Chem. 258*, 6782–6788.

Roth, M. (1971) *Anal. Chem. 43*, 880–882.

Segrest, J. P., deLoof, H., Dahlman, J. G., Brouillette, C. G., & Anantharamaiah, G. M. (1990) *Proteins: Struct., Funct., Genet. 8*, 103–117.

Slack, B. E., Breu, J., & Wurtman, R. J. (1991) *J. Biol. Chem. 266*, 24503–24508.

Sohal, P. S., & Cornell, R. B. (1990) *J. Biol. Chem. 265*, 11746–11750.

Sonnichsen, F. D., Van Eyk, J. E., Hodges, R. S., & Sykes, B. D. (1992) *Biochemistry 31*, 8790–8798.

Vance, D. E. (1989) in *Phosphatidylcholine Metabolism* (Vance, D. E., Ed.) pp 33–45, CRC Press, Boca Raton, FL.

Vance, D. E., & Pelech, S. L. (1984) *Trends Biochem. Sci. 9*, 17–20.

Wang, Y., MacDonald, J. I. S., & Kent, C. (1993) *J. Biol. Chem. 268*, 5512–5518.

Watkins, J. D., & Kent, C. (1991) *J. Biol. Chem. 266*, 21113–21117.

Weinhold, P. A., Rounsifer, M. E., & Feldman, D. A. (1986) *J. Biol. Chem. 261*, 5104–5110.

Wetterau, J. R., & Jonas, A. (1982) *J. Biol. Chem. 257*, 10961–10966.

Wiener, M. C., & White, S. H. (1991) *Biochemistry 30*, 6997–7008.

Yoshimura, T., Goto, Y., & Aimoto, S. (1992) *Biochemistry 31*, 6119–6126.