IDENTIFICATION OF THE DIMERIZATION DOMAIN OF
CTP: PHOSPHOCHOLINE CYTIDYLTRANSFERASE AND THE ROLE OF
DIMER FORMATION

by

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE

in the Department of Chemistry

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SIMON FRASER UNIVERSITY
August 1997

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ABSTRACT

CTP:Phosphocholine Cytidylyltransferase (CT) catalyses the key regulatory step in PC biosynthesis, the transfer of a cytidyl group from CTP to phosphocholine forming CDP-choline. Previous studies suggested that CT is dimeric and that the N-terminal two thirds mediates the interactions of the monomers in the homodimer (Cornell, R. (1989) *J. Biol. Chem.* **264**, 9077-9082; Craig, L., Johnson, J. E., and Cornell, R. B. (1994) *J. Biol. Chem.* **269**, 3311-3317). The goals of this research were to more definitively identify the domain(s) necessary and sufficient for dimerization and to determine the functional role of CT dimer formation. Purified rat liver CT was digested with chymotrypsin to produce a set of fragments that were mapped using anti-peptide antibodies. Chemical cross-linking of the CT fragments using glutaraldehyde, dithiobis(succinimidyl propionate) (DSP), or copper phenanthroline (Cu-PHT), followed by SDS-PAGE revealed that only those fragments that contained the N-terminal domain (28 kDa, ≈ 230 amino acids) could be covalently cross-linked. This finding was confirmed by two-dimensional SDS-PAGE, immunoblotting with an antibody against an N-terminal peptide, and by lack of phosphorylation (a marker for the C-terminus) associated with cross-linked fragments. CT that was cross-linked with DSP or glutaraldehyde appeared as a wide smear between 80-110 kDa. This was attributed to heterogeneity of cross-linked species. However cross-linking with Cu-PHT resulted in two clear bands corresponding to dimer (84 kDa) and trimer (~126 kDa). This suggested a monomer↔dimer↔trimer equilibrium. CT was treated with acetonitrile to disrupt dimer interaction. As the concentration of acetonitrile increased from 0 to 20%, the amount of CT dimer trapped by cross-linker decreased from 100 to 30%, but CT activity decreased only slightly, from 100% to 70% of full activity. This suggested that dimerization was not
necessary for activity. To examine the relationship between membrane binding and dimeric state, we added vesicles containing phosphatidylcholine (PC) and phosphatidic acid (PA). As the relative concentration of PA increased the amount of membrane-bound CT was expected to increase. The monomer/dimer ratio increased as the mol% PA increased, suggesting that membrane binding of CT is associated with dissociation of dimer to monomer. Moreover, vesicle-bound CT that was isolated following treatment with cross-linker was exclusively monomeric. Chemically cross-linked CT dimers could still bind to vesicles, indicating that dissociation to monomer must follow rather than precede the membrane binding step. Since membrane binding activates the enzyme, we propose that dissociation of dimer to monomer is required for enzyme activation. Cross-linked CT induced aggregation of vesicles containing phosphatidylglycerol (PG), suggesting that CT dimers can form cross bridges between two membrane bilayers. This finding suggests that CT monomers associate in an anti-parallel manner.
Dedication

For my parents, my lovely wife and daughter
Acknowledgements

I would like to thank my senior supervisor, Rosemary Cornell, for her guidance through this research project, for her patient help with any problem or question that arose over the past four years, for her thorough proof-reading of this thesis, and more……

To my labmates, Adrienne, Becky, Dallas, and Joanne, thank you for your friendly support and wonderful advice, which made me easily to get use to working and studying here.

Thank to Professor Bob Cushley, for his nicely to teach me using Nicomp Model 270 Submicron Particle Sizer.
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<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>CDP</td>
<td>Cytidine 5'-diphosphate</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>CT</td>
<td>CTP:phosphocholine cytidylyltransferase</td>
</tr>
<tr>
<td>CTP</td>
<td>Cytidine 5'-triphosphate</td>
</tr>
<tr>
<td>Cu-PHT</td>
<td>CuSO₄ + 1,10-phenanthroline</td>
</tr>
<tr>
<td>DG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DQPG</td>
<td>Dioleoyl-phosphatidylglycerol</td>
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<td>DSP</td>
<td>Dithio bis (Succinimidyl Propionate)</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>glu.</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>HGH</td>
<td>Human growth hormone</td>
</tr>
<tr>
<td>HIV-1 RT</td>
<td>Human immunodeficiency virus type 1 reverse transcriptase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>MLV</td>
<td>Multi-lamellar vesicle</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
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PC  Phosphatidylcholine
PE  Phosphatidylethanolamine
PG  Phosphatidylglycerol
PGM Phosphoglycerate Mutase
PI  Phosphatidylinositol
PMSF Phenylmethylsulfonyl fluoride
PS  Phosphatidylserine
PVDF Poly(vinylidene difluoride)
SDS Sodium dodecyl sulfate
SUV Sonicated unilamellar vesicle
TBS Tris-buffered saline
TFIIIA Transcription factor IIIA
TID 3-(trifluoromethyl)-3-(m-[125]liodophenyl)diazirine
TLC Thin layer chromatography
Tris Tris (hydroxymethyl) methylamine
TS buffer: 25 mM Tris, pH 7.5, 140 mM NaCl, 5 mM KCl,
0.5 mM Na₂HPO₄, 1 mM MgCl₂, 1 mM CaCl₂
CHAPTER ONE: INTRODUCTION

1.1 CTP:PHOSPHOCHOLINE CYTIDYLYTRANSFERASE (CT) IN PHOSPHATIDYLCHOLINE (PC) SYNTHESIS

1.1.1 Phosphatidylcholine

Phosphatidylcholine (PC) is the most abundant phospholipid in mammalian membranes. For instance, 40 to 60% of the phospholipids in the human red blood cell membrane are PCs and 60 to 70% of the phospholipids in sarcoplasmic reticulum (rabbit) are PCs (Gennis, 1989). PC is also a major phospholipid of mammalian lung surfactant and serum lipoproteins. A major function of PC is to generate a stable bilayer structure. Another key role of PC is as an important source of second messengers, such as diacylglycerol and arachidonic acid, in the signaling pathway of hormones and some growth factors (Exton, 1990, 1994; Pelech and Vance, 1989). Understanding the regulation of its metabolism will provide insights into how the PC content of cell membranes is controlled.

1.1.2 The role of CTP:Phosphocholine Cytidylyltransferase (CT)

In PC biosynthesis (Fig. 1.1), CTP:phosphocholine cytidylyltransferase catalyzes the key regulatory step, the transfer of a cytidyl group from CTP to phosphocholine, forming CDP-choline. CT exists in two different forms, an
Fig 1.1 PC metabolic pathway (Johnson thesis, 1996).
inactive soluble form and an active membrane-bound form (Vance and Pelech, 1984). The translocation of CT between the soluble and membrane compartments is influenced by two major types of regulation, the lipid composition of the membrane and phosphorylation / dephosphorylation of CT.

### 1.1.2.1 Regulation of CT Activity by Phospholipids

CT can be activated by two classes of activators. These two main classes of lipids are: Class I lipids, which are fatty acids and anionic phospholipids such as phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylylglycerol (PG) and phosphatidic acid (PA); and Class II lipids, which are neutral lipids with small polar head groups such as mono- and diacylglycerol. CT is not activated by zwitterionic phospholipids such as PC and phosphatidylethanolamine alone.

**Class I: Anionic lipids**

Fatty acids induce activation and translocation of CT *in vivo* (Feldman et al., 1981). A correlation was found between increased fatty acid content and increased CT activity in the membrane fraction of rat lung tissue immediately following birth (Weinhold, 1981). Treatment of cells with fatty acids stimulated PC synthesis and promoted translocation of CT from the soluble fraction to the membrane fraction (Pelech et al., 1983; 1984b; Whitlon et al., 1985; Aeberhard et al., 1986; Cornell & Vance, 1987a; Wang et al., 1993a; Houweling et al., 1994).

*In vitro*, fatty acids and anionic phospholipids were also effective at activating CT and causing translocation in cell extracts (Stern et al., 1976; Choy & Vance, 1978; Choy *et al.*, 1979; Feldman *et al.*, 1981; Feldman *et al.*, 1985).
While vesicles containing PC alone were ineffective at activating soluble CT, addition of oleic acid to the vesicles greatly stimulated activity. The greater the negative charge of the lipid the lower the mol % required for CT activation (Cornell, 1991a). CT does not recognize a specific lipid head group structure, but responds to the negative charge density of the lipidic surface (Cornell, 1991a; Arnold and Cornell, 1996). CT was activated by anionic lipids when presented in the form of lipid vesicles or as Triton X-100 mixed micelles.

**Class II: Neutral lipids with small polar head groups**

Elevation of the cellular diacylglycerol (DG) content, by incubation of cells with short chain soluble DGs, or by treatment with phospholipase C (PLC), stimulates translocation and activation of CT (Cornell and Vance, 1987a; Utal et al, 1991; Slack et al, 1991). The uncharged lipids, monoacylglycerol and oleyl alcohol, which like diacylglycerol, contain a small polar head group, promoted translocation of soluble CT *in vitro* (Cornell & Vance, 1987a; Johnson et al, 1992). CT was physically associated with sonicated PC vesicles containing monoacylglycerol, oleyl alcohol or diacylglycerol *in vitro* (Cornell and Vance, 1987b). Thus, there is not a strict specificity for diacylglycerol.

Purified CT was activated by PC/diacylglycerol mixtures only when in vesicle form (Cornell, 1991a). The requirement for a membrane or micelle structure for activation by lipid activators suggests CT is not activated by direct binding of a monomer of an activating lipid. The mole percent of lipid activator required for complete activation is 5-15%, depending on the lipid. Moreover, the lack of head-group specificity suggests CT does not possess a site designed to
recognize a specific chemistry of an activator lipid molecule(s). Perhaps the presence of lipid activators changes membrane properties which affect the CT-membrane interaction.

1.1.2.2 Regulation of CT Activity by Phosphorylation

Phosphorylation/dephosphorylation is a regulatory mechanism common in many cellular proteins. Protein kinases, which catalyze the phosphorylation, are a group of diverse and highly regulated enzymes that respond to various cellular signals by phosphorylating their specific target proteins. Cellular proteins are dephosphorylated by separate enzymes, the protein phosphatases.

Several studies found that addition of Mg-ATP to stimulate kinase function or NaF, a protein phosphatase inhibitor, to liver cytosol resulted in inhibition of CT activity and membrane binding (Niles & Makarski, 1979; Pelech et al., 1981; Pelech et al., 1982). Addition of okadaic acid, a specific inhibitor of protein phosphatases 1 and 2a, also reduced the amount of active membrane-bound CT (Hatch et al., 1990, 1991, 1992). These results suggested that phosphorylation / dephosphorylation also regulates CT. The soluble inactive form of CT appears to be stabilized by phosphorylation at specific sites, whereas the membrane-bound active form, is dephosphorylated (Watkins & Kent, 1990; Hatch et al., 1992). Recently Arnold et al. showed that dephosphorylation of purified CT enhances its affinity for anionic lipid vesicles (Arnold et al, 1997).
1.2 STRUCTURAL AND FUNCTIONAL DOMAINS OF MAMMALIAN CT

Rat liver CT was first purified in 1986 (Weinhold et al., 1986; Feldman & Weinhold, 1987). This achievement ushered in a new era of research on CT. In 1987 the first amino acid sequence was published for CT from yeast (Tsukagoshi et al., 1987). Later the primary structures of several mammalian CTs were determined (Kalmar et al., 1990, 1994; Rutherford et al., 1993; Macdonald & Kent, 1993; Sweitzer & Kent, 1994; Hogan et al., 1995) (Figure 1.2). The mammalian CTs are highly conserved with > 95% identity at the amino acid level. The protein contains 367 amino acid residues, and has a calculated molecular weight of 41,720 (rat liver CT). On the basis of structural predictions and sequence homologies, a model has been proposed for the domain structure of CT (Fig. 1.3) (Kalmar et al., 1990). The model contains three distinct domains: a globular N-terminal conserved catalytic domain (residues 1-235); a pair of amphipathic α-helices, the membrane binding domain (residues 236-315); and phosphorylation domain (residues 316-367) (Fig. 1.3).

1.2.1 Catalytic Domain and nuclear localization signal sequence

The N-terminus of CT (amino acid residues 74 to 235) has been proposed to be the catalytic domain. There are four pieces of experimental evidence for this domain identification:

1) The amino acid sequence of rat CT between residues 74-234 shares 63%
**Fig. 1.2 Amino acid sequence of rat-liver CT**—Sequence derived by Kalmar et al., (1990) and revised by MacDonald and Kent (1993). Putative amphipathic helix is in italics. Tandem 11-mer repeats are underlined.

```
MDAQSSAKVNSRKRKEVGPNGATEEDGIPSKVQRCAVGLRQPAFPSDE
IEVDFSKPYVRVTMEACRGTFCERPVRVYADGIFDFLSHMGARALMQK
NLFPNTLYLIVGCSDELTHNFLKFTVMNENERYDAVQHCRYVDEVVRNAP
WTLTPFLEAEHRIDFVAHDDIPYSSAGDDVYKHIKEAGMFAPTQRTEGI
STSDIITRIVRVDYVYARRNLQRTAKELNVSFINEKKYHLQERVDKV
KKVDVEEKSKEFVOKVEEKSIDLIQKWEESREFIGSFLEMFGPEGALK
HMLKEGKRMLQAISPKOSPSSSPHERSPSPSRWPSGKTSPPSSSPAS
LSRCKAVTCDSEED
```

**Fig. 1.3 Domain Structure for Mammalian CT.**
identity with residues 101-260 on the yeast CT sequence (Tsukagoshi et al., 1987).

II) This domain in rat liver CT is homologous (33% identity), with a related B. subtilis glycerolphosphate cytidylyltransferase (GCT) between residues 6-94 (Park et al., 1993).

III) A mutation at position 91 changing a single amino acid (Gly→Ser) in this domain resulted in defective CTP binding and CT activity (Veitch & Cornell, 1996). Mutation at His 89 and 92 also reduce CTP binding and Vmax (Veitch & Cornell, 1997 unpublished).

IV) A deletion mutant composed of this domain alone (amino acids 1-235) was catalytically active (Wang & Kent, 1995).

A 21 residue sequence at the N-terminus of CT (8-28) has been identified as the nuclear localization signal sequence (Wang et al., 1995). This domain is necessary for targeting CT to the nucleus (Wang et al., 1995). CT's intracellular distribution appears to be dynamic. Depending on cell type and stage of the cell cycle CT appears in nucleus or cytoplasmic compartments (Northwood and Cornell, unpublished). The function of the region between residue 29-73 is not known.
1.2.2 Membrane Binding Domain

In 1990, the amphipathic α-helix predicted between residue 236 - 299 was proposed as a potential membrane binding domain (Kalmar et al., 1990). The role for the amphipathic helix in membrane binding was demonstrated in membrane binding studies of proteolytic fragments of CT. Undigested CT and the 39 and 35 kDa fragments which contain the intact amphipathic helix did bind to anionic membranes, whereas fragments lacking this region did not (Craig et al., 1993). Recently, CT was photolabeled with the hydrophobic, photoreactive probe $^{125}$I-TID. Only fragments containing the amphipathic helix were covalently coupled to this membrane probe (Johnson et al., 1992). The results confirmed that residues 236-299 of CT is the membrane binding domain. The role of the second predicted amphipathic helix between 300-315 is not known.

1.2.3 Phosphorylation Domain

The C-terminal 55 residues (315 to 367) of CT contains many potential phosphorylation sites. The region is rich in serine-proline motifs that are potential substrates for cyclin dependent kinases, and contains potential sites for casein kinase II and glycogen synthase kinase II. This domain was highly phosphorylated in vivo (MacDonald & Kent, 1994; Cornell et al., 1995), yet deletion mutants lacking this domain, although not phosphorylated, were fully active and capable of membrane binding. Thus, this domain is not directly involved in catalysis or membrane binding, but may play a role in modulating these functions (Wang & Kent, 1995; Arnold et al., 1997).
Cornell et al. (1995) revealed that many kinases, such as casein kinase II, cdc2 kinase, GSK-3, and protein kinase-C, but not MAP kinase, catalyze the phosphorylation of CT in vitro. Casein kinase II phosphorylation was directed to Ser-362.

### 1.3 Dimerization Domain of CT

SDS-PAGE with or without β-mercaptoethanol revealed that purified CT is a 42 kDa protein. When the sulfhydryl reducing agent, dithiothreitol, was removed from the enzyme preparation by dialysis for 1-2 days, a new 84 kDa band appeared on the gel (Cornell, 1989; Craig et al., 1994). Reduction with dithiothreitol and electrophoresis in the second dimension showed that this 84 kDa protein was derived from the 42 kDa enzyme (Cornell, 1989). Three chemical cross-linking reagents, glutaraldehyde, Dithio bis (Succinimidyl Propionate) (DSP) and dimethyl-3,3'-dithiobispropionimidate (DSBP), were used to study dimerization of purified CT. The results suggested that CT forms a homodimer when it binds to membranes or Triton X-100 micelles (Cornell, 1989).

The subcellular forms of CT were separated by glycerol density centrifugation (Weinhold et al., 1986, 1989). Two forms of enzyme were present in cytosols, prepared from lung, Hep G2 cells, A549 cells or alveolar Type II cells: a high molecular weight H-Form and a low molecular weight L-Form. The H-form is a lipid-protein aggregate and is active. The L-Form resulted from the dissociation of H-Form and was dependent on added phospholipid vesicles for
activity. By SDS-PAGE the L-Form of the enzyme corresponds to the 42 kDa subunit. Weinhold, et al. (1989) found that the molecular weight of the L-Form was 97690 ± 10175, determined from sedimentation coefficients. This result suggested that the L-form of the enzyme is a dimer of the 42 kDa catalytic subunit.

Enzymatic proteolysis was used as a tool to study both the tertiary structure of CT and its membrane-binding domain (Craig et al., 1994). Limited chymotrypsin proteolysis of CT produced several distinct fragments: 39 kDa, 35 kDa, 28 kDa and 26 kDa (Fig. 1.4 a,b). All these fragments contain the N-terminus of CT as evidenced by their reactivity with an antipeptide antibody against CT's N-terminal 15 amino acids. Craig et al. observed a 56 kDa band that accompanied the major 28 kDa proteolytic fragment on SDS gels. It was suggested that the 56 kDa species was a dimer of the 28 kDa species. If this were true it would imply that the N-terminal domain of CT is involved in dimerization (Cornell, 1989; Craig et al., 1994).

1.4 Function of Protein Dimerization

Protein dimerization plays an important role in protein folding (Ekiel and Abrahamson, 1996), protein stabilization (Erhardt & Dirr., 1995; Tsigelny et al., 1995), and enzyme activity (Surette et al., 1996; Ekiel and Abrahamson, 1996; Bilwes et al., 1996; Alexander et al., 1995; Divita et al., 1993; Restle et al., 1992). In some cases dimer formation functions to inactivate a protein (enzyme);
Fig. 1.4 (a) Proposed sites for chymotrypsin digestion of CT. The molecular masses of the chymotryptic fragments were determined from a plot of log molecular mass of prestained standards versus the Rf on SDS-PAGE. The proposed sites of chymotrypsin cleavage are those sites C-terminal to F, W, Y, M, and L which generate theoretical fragments whose molecular mass is closest to the fragments determined by SDS-PAGE (Figure adapted from Craig et al., 1994).

Fig. 1.4 (b) Model of the membrane-bound CT dimer (Figure adapted from Craig et al., 1994)
while in other cases dimerization is required for the active state. Some examples of each mode are summarized.

1.4.1 Inactivation of Human Cystatin by dimerization

Human Cystatin C is an amyloidogenic protein, which functions as a small size inhibitor of cysteine proteinases. It is present in all human body fluids (Abrahamson et al., 1986). NMR spectroscopy, chromatographic, and electrophoretic methods revealed that the dimerization of cystatin involves properly folded molecules (Ekiel and Abrahamson, 1996). The dimerization interface of cystatin is at the site of reactivity with proteases. Dimerization leads to loss of inhibitor function towards the protease.

The pH in lysosomes is in the 4.6-5.0 region, which corresponds to the range where cystatin C dimerizes to some extent (Ekiel and Abrahamson, 1996). So cystatin C dimerization and inactivation may occur in acidified compartments in vivo, which could be relevant for the physiological regulation of cysteine proteinase activity.

1.4.2 Inhibition of Receptor Protein-Tyrosine Phosphatase-α by Dimerization

Receptor protein-tyrosine phosphatases (RPTPs) regulate the level of phosphotyrosine-containing proteins derived from the action of protein-tyrosine kinases (Hunter, 1995). RPTPs are type-I integral membrane proteins which contain one or two catalytic domains in their cytoplasmic region (Moureý, et al., 1994). The crystal structure of the membrane-proximal catalytic domain of a
typical RPTP, murine RPTPα, showed that the N-terminal segment of each monomer forms a helix-turn-helix structural wedge tucked into the active site of the opposing monomer in the RPTPα dimer. This arrangement renders the dimer form of RPTPα unable to bind phosphotyrosine-containing substrates. Therefore the dimer form of RPTPα is catalytically inactive (Bilwes et al., 1996).

Dimerization, which results in active-site blockage, may be a physiologically important mechanism for downregulating the catalytic activity of RPTPs.

1.4.3 Dimerization of Human Immunodeficiency Virus (HIV) Type 1 Reverse Transcriptase is Required for Activity

Human Immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) is one of the main targets in approaches to the chemotherapy of AIDS. This enzyme consists of one subunit of molecular weight of 66 kDa and another of molecular weight of 51 kDa, which represents a carboxy-terminally truncated form of the larger subunit (Divita et al., 1993). HIV-1 reverse transcriptase activity is confined exclusively to the dimeric forms (Divita et al., 1993; Restle et al., 1990; 1992 a; 1992 b). Using recombinant enzyme, Restle, et al. investigated the process of dimer formation, dimer dissociation and the properties of the mono- and dimeric forms of the enzyme. They used 20% acetonitrile to dissociate heterodimeric HIV-1 RT reversibly without denaturing the subunits (Restle et al., 1993). Dissociation resulted in transcriptase inactivation. On reducing the acetonitrile concentration to 4% by dilution, slow reassociation of monomers occurred, resulting in restoration of the original activity. They used
HPLC size exclusion chromatography to rapidly separate monomer from the dimer and determined their relative activities. Monomer was inactive or had low but measurable activity. Full enzyme activity of HIV-1 RT was confined to the dimeric forms.

From the reverse transcriptase crystal structure studies, Kohlstaedt et al. (1992) demonstrated that the 66 kDa subunit has a large cleft carrying the polymerase active site, whereas in the small subunit this site is not accessible due to an intramolecular rearrangement of the so-called connection domain. The formation of a heterodimer involves an interaction where by the smaller subunit has its connection domain in its active site cleft. The smaller subunit holds the bigger subunit in an active conformation. The heterodimer appears to have only one polymerase active site.

1.4.4 The Dimerization of the Protein Histidine Kinase CheA is required for activity

The CheA histidine kinase of *E. coli* is a member of the large family of histidine protein kinases involved in bacterial signal transduction. It plays an essential role in stimulus-response coupling during bacterial chemotaxis (Surette *et al.*, 1996).

This kinase is a homodimer that catalyzes the reversible transfer of a γ-phosphoryl group from ATP to the N-3 position of one of its own histidine residues. Using kinetic and chemical cross-linking approaches to study this protein, Surette *et al.* (1996) found that rates of autophosphorylation showed
second order dependence on CheA concentrations suggesting that this activity required two CheA molecules. Dimer formation would facilitate phosphorylation by an intermolecular process (Surette et al., 1996). The dissociation constant ($K_d = 0.2-0.4 \mu M$) was not affected by nucleotide binding, phosphorylation, or CheY (the response regulator) binding.

1.5 The Methodology of Protein Dimerization Studies

Several methods have been used to study protein dimerization, such as chemical cross-linking (Cornell, 1989; Shin & Sachs, 1996; Krehan et al., 1996), X-ray crystallography (Manna et al., 1996), site-directed mutagenesis (Manna et al., 1996; Alexander et al., 1996), and NMR spectroscopy (Ekiel & Abrahamson, 1996).

1.5.1 Chemical Cross-linking Approach

Chemical cross-linking reagents are well characterized as probes for analysis of oligomeric interactions of proteins (Habeeb and Hiramoto, 1968; Steck, 1972; Calpaldi et al., 1979; Middaugh et al., 1983; Cornell, 1989; Shin & Sachs, 1996; Krehan et al., 1996). For example, CT is a dimer; associated by noncovalent interaction. Dimer interaction can be monitored by chemically cross-linking (covalent bond formation) and separating the products of reaction on SDS-PAGE (Fig. 1.5 a). The cross-linker prevents the two subunits from dissociation in the denaturating conditions of SDS-PAGE.

The structure of the cross-linking reagents used in this thesis are shown in Fig. 1.5 (b). Glutaraldehyde reacts with lysine $\varepsilon$-amino groups, but also with
tyrosine, histidine, and cysteine side chains (Hab et al., 1968). These reactive groups must approach within ~7Å to be cross-linked by the aldehydes of glutaraldehyde.

DSP reacts specifically with primary amines (lysines), and has a maximum cross-linking length of 12Å. Cu-PHT catalyzes the oxidation of sulfhydryls (2RSH → R-S-S-R).

In this study, several approaches have been taken to identify the domain(s) necessary and sufficient for dimerization and to determine the functional role of CT dimer formation. At the start of this project, it was envisioned that CT was a dimer in both its membrane-bound, active form (Fig. 1.4 b) and its soluble inactive form. The results of this thesis suggest that dimer dissociation may be required for the activation of the membrane-bound enzyme.
Fig. 1.5 (a) Chemical Cross-linking Approach for CT Dimerization Study
Fig. 1.5 (b) Structure of chemical cross-linkers used in this thesis

1. Glutaraldehyde:

2. Dithio bis (Succinimidyl Propionate) (DSP)

3. Copper phenanthroline (Cu-PHT)
CHAPTER TWO: MATERIALS AND METHODS

2.1 MATERIALS

Phospholipids were from Avanti Polar Lipids (Alabaster, AL, USA). Oleic acid and choline kinase were from Sigma (St. Louis MO, USA). $[^3\text{H}]$choline chloride (80 Ci/mMol) was from New England Nuclear. Other chemicals were from Sigma or BDH (Vancouver, BC).

Coomassie Blue, DSP, and PHT were from Pierce. Glutaraldehyde was from British Drug House. Casein kinase II (the gift of Dr. J. Sanghera) was purified from sea star as described (Sanghera et al., 1990, 1992). $\gamma^{32}\text{P-ATP}$ (3000Ci/mmol) was from Amersham.

Chymotrypsin was from Sigma. $[^3\text{H}]$Phosphocholine was synthesized from $[^3\text{H}]$choline as described (Cornell, 1989).

Rabbit antiserum against the N-terminus of CT was as described (Cornell et al., 1995). Rabbit antiserum raised against a 33 residue peptide corresponding to a protein of the amphipathic helix domain of CT (residues 255-288) was generated by Dr. Adrienne Drobnies as described (Johnson et al., 1997).

Purified rat liver CT was provided by Joanne Johnson, Dallas Veitch, and Rebecca Arnold (Cornell et al., 1995). It was stored at -70°C in 50 mM Tris, pH 7.4, 0.15M NaCl, 0.2M K$_2$HPO$_4$, 2mM DTT, 1mM EDTA, 0.5mM-0.7 mM Triton X-100 and protease inhibitors (PMSF, 2.5 µg/ml leupeptin, 2.0 µg/ml chymostatin and pepstatin, 1.0 µg/ml antipain, 10 µg/ml each $\rho$-aminobenzamidine and benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride (MacDonald, 1993)).
2.2 BUFFERS, REAGENTS AND MEDIA

2.2.1. CT Assay

Carrier: CDP-choline, 10 mg / ml; phosphocholine, 60 mg / ml.

CT cocktail mix: 0.4M Tris-HCl, 0.24 M MgCl₂, 60mM CTP, 1.75M NaCl.

2.2.2 CT Dialysis

CT dialysis buffer (for DSP reaction): 0.1M K₂HPO₄, 2mM EDTA, 0.5mM Triton-100, pH 7.5.

CT dialysis buffer (to remove salt and unreacted cross-linkers): 10 mM Tris, pH 8.0, 0.01% SDS.

2.2.3 SDS-PAGE

Separating gel buffer (4 x conc.): 1.5 M Tris-HCl, 0.4% SDS, pH 8.7.

Stacking gel buffer (4 x conc.): 0.5 M Tris-HCl, 0.4% SDS, pH 6.8.

Sample buffer: 0.13 M Tris-HCl, 8% SDS, 2% b-mercaptoethanol, 20% glycerol, Bromophenol Blue, 0.1 mg / ml, pH 6.8.

Reservoir buffer (5 x conc.): 0.125 M Tris-HCl, 0.96 M glycine, 0.5% SDS.

Acrylamide stock: 30% acrylamide, 0.8% bis-acrylamide.

Coomassie Stain: 0.2% Coomassie blue, 45% methanol, 5% acetic acid.
2.2.4 Silver Staining

Fixer: 40% methanol, 10% acetic acid.

Developer: 0.28 M sodium carbonate, 0.5 ml of formalin (H₂O₂) per liter (Moreno, 1985)

2.2.5 Western Blots

Tris-buffered saline (TBS): 20 mM Tris-HCl, 150 mM NaCl, pH 7.4.

Transfer buffer: 39 mM Glycine, 48 mM Tris-HCl, 0.0375% SDS, pH 8.7.

Blotting buffer: 5% skim milk powder, TBS, 0.05% (w/v) Tween-20.

Diaminobenzadine (DAB) buffer: 50 mM Tris-HCl, pH 7.6, 0.15% NiCl₂.

Stripping buffer: 62.5 mM Tris-HCl, 100mM βME, 2% SDS, pH 6.7

2.2.6 Liposome

Liposome buffer: 10 mM Tris, 1 mM EDTA, pH 7.4.

2.2.7 Phosphorylation Reaction (Cornell, 1995)

Kinase assay buffer: 6 mM MgCl₂, 2 mM DTT, 25 mM Tris/HCl.

³²P-ATP Mix: 10 mM ATP, 2x10⁵ dpm / μl³²P-ATP, 25 mM Mops.
2.2.8 Chymotrypsin Stock

Chymotrypsin was stored as 10 mM stock at -20°C.

Homogenization buffer: 10 mM Tris-HCl, 1mM EDTA, 3mM MgCl₂,
2mM dithiothreitol, pH 7.4.

2.2.9 Glutaraldehyde Stock

Glutaraldehyde 10mM stock stored as water solution at -20°C.

2.2.10 Dithio bis (Succinimidyl Propionate) (DSP) Stock

DSP was dissolved in dimethyl sulfoxide and stored as 10 mM stock at -20°C.

2.2.11 Phospholipids Stocks

Phospholipids were dissolved in CHCl₃ and stored as 15-100 mM stock at -20°C.
2.3 METHODS

2.3.1 Chymotrypsin Digestion

Chymotrypsin, diluted in homogenization buffer (10 mM Tris-HCl, pH 7.4, 1mM EDTA, 3mM MgCl₂, 2mM dithiothreitol, 0.2M K₂HPO₄) was added to purified CT at a mass ratio of 15:1 (CT : Chymotrypsin) unless otherwise specified. The samples (30 - 50 µl) were incubated for 10 min at 37°C. The reaction was stopped by adding PMSF from a 1.6 mM stock in homogenization buffer, 0.2 M K₂HPO₄ or H₂O to a final concentration of 1 mM PMSF. The proteins were concentrated in a Savant Speed-Vac evaporator and then were separated by SDS-PAGE.

2.3.2 Cross-linking Reactions

The buffer used in the purification of CT contains 2mM DTT and 50mM Tris. Prior to cross-linking with DSP, these interfering compounds were removed by dialysis against 3x500 ml of the buffer (0.2M K₂HPO₄, pH 8, 2mM EDTA, 0.5mM Triton X-100), the recovery of CT activity after dialysis was 76±12%(N=6) (Cornell, 1989). Removal of Tris and DTT was not necessary for cross-linking with glutaraldehyde or Cu-PHT.

Cross-linking was initiated by adding DSP (1.0 mM), glutaraldehyde (1.0-2.0 mM), or Cu-PHT (0.2 mM CuSO₄ and 0.6 mM 1,10-phenanthroline) in a sample volume of 0.03-0.10ml. DSP was dissolved in dimethyl sulfoxide and
then diluted in water. The concentration of dimethyl sulfoxide in the reaction was 1%. Dimethyl sulfoxide itself had no effect on the gel patterns (Cornell, 1989). For DSP reactions, samples were incubated at 37°C for 30 min. and the reaction was quenched with ammonium acetate, pH 8.5, added to a final concentration of 0.1M. Glutaraldehyde reactions were for 15 min at 37°C, and were stopped with 0.1M ethanolamine. Cu-PHT reactions were for 15 min and were stopped with Laemmli electrophoresis sample buffer without β-ME. For DSP and Cu-PHT reactions, the samples were subsequently electrophoresed in the absence of the reducing agent, β-mercaptoethanol.

2.3.3 Gel Electrophoresis

Three SDS-PAGE systems were used in these studies:

1) 12% polyacrylamide gel (Laemmli, 1970):

Salts and unreacted cross-linkers were removed from some samples by dialysis against 400 volumes of 10 mM Tris, pH 8, 0.01% SDS. Samples were concentrated in a Savant Speed-Vac. Dried samples were dissolved in Laemmli sample buffer (with or without mercaptoethanol) and boiled for 3 min. In cross-linking experiments with DSP, all samples were heated for 30 min at 37°C instead of boiling.
2) Two dimensional analysis (Cornell, 1989):

Slab gel (mini apparatus) were run for both first and second dimensions. The outside lane of the first dimension 10% gel was sliced off and soaked in 20ml of 40 mM DTT, 100 mM Tris, pH 6.8, 0.1% SDS for 30 min at 30°C. This gel strip was overlayed horizontally onto a second 12% gel using a 5% acrylamide stacking gel to seal it in place. Molecular mass standards were run on every gel. The standards were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and hen egg white lysozyme (14 kDa). Gels were stained with Coomassie Blue or by the combined Coomassie-silver method (Moreno et al., 1985).

3) Electrophoresis of small molecular weight peptides (Schagger, et al., 1987):

This gel system was employed to analyze chemical cross-linked synthetic peptides. In this system, the stacking gel contained 0.74M Tris, pH 8.25, 0.07% SDS, 3.8% acrylamide, 0.12% Bis-acrylamide, 0.048% APS and 0.096% TEMED. There was ~ 1cm spacer gel between separating gel and stacking gel. This space gel contained 1M Tris, pH 8.25, 0.1% SDS, 10% acrylamide, 0.3% Bis-acrylamide, 0.05% APS and 0.1% TEMED. The separating gel contained 1M Tris, pH 8.25, 0.1% SDS, 15.5% acrylamide, 1% Bis-acrylamide, and 13.3% glycerol. Proteins were loaded on the gel after boiling in sample buffer and the
gel was electrophoresed with an anode buffer of 0.2 M Tris, pH 8.9, and a cathode buffer of 0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.25, at 75 V at 4°C for ~15 hr.

2.3.4 Western Blots

Gels were transferred to a PVDF membrane using an LKB 2117 multiphor II electrophoresis unit at 1.33 mA/cm² for 2 h. The transfer buffer consisted of 39 mM glycine, 48 mM Tris, and 0.0375% SDS. The PVDF filters were blocked in "blotting buffer" (TBS: 20 mM Tris, pH 7.4, 0.15 M NaCl; 0.05% (w/v) Tween-20; 5% (w/v) skim milk powder) for 2 hr at room temperature with gentle agitation. The filters were incubated with the primary antibody at 4°C overnight. The filters were washed with blotting buffer and then incubated at room temperature in blotting buffer with a 1/2000 dilution of goat anti-rabbit horseradish peroxidase for 1.5 h. After a final wash the filters were developed with 1.6 mM diaminobenzidine in 50 mM Tris, pH 7.6, 0.03% (w/v) NiCl₂ with 0.03% (v/v) hydrogen peroxide. The reaction was stopped with a large excess of distilled water.

2.3.5 Phosphorylation Reactions

Phosphorylation reactions contained 12.0 μg CT in 30 μl kinase assay buffer (6 mM MgCl₂, 2 mM DTT, 25 mM Tris/HCl), and 10 μl (25 ng) casein kinase II. γ³²P ATP was added to start the reaction from a 0.1 mM stock in 25
mM Mops, pH 7.4 to a final concentration of 0.02 mM ATP and 20 μCi/ml. Another 10μl kinase was added at 30 min. The samples were incubated for 60 min at 30°C and analysed by SDS-PAGE. For autoradiography, Coomassie-stained gels were dried and exposed to reflections film (Dupont) with or without the intensifying screen at -80°C for the indicated time.

2.3.6 CT Assay

The enzymatic activity of CT was analyzed by monitoring the incorporation of the radioactive substrate [³H]phosphocholine into [³H]CDP-choline. The solution, with a final volume of 50 μl, contained 20 mM Tris, pH 7.4, 87.5 mM NaCl, 12 mM MgCl₂, 3 mM CTP, 10 mM DTT, and variable amounts of lipid and enzyme. [³H]phosphocholine (final concentration 1-2 mM; S.A=7-15 Ci/mol) was added to start the reaction, which was incubated in a shaking 37 °C H₂O bath for 10 to 45 min, then quenched by addition of 30 μl MeOH/NH₃ (9:1). Samples were centrifuged at 15000 rpm for a few seconds, and a portion of the supernatant was spotted on a silica plastic-backed TLC plate (Merck # 5748) and developed in 0.6 % (w/v) NaCl /MeOH /NH₃ (5:5:1 volume ratio). The CDP-choline band was visualized with 0.02% (w/v in methanol) dichlorofluorescein under UV light, and the silica was scraped from the plate. [³H]CDP-choline was quantified by liquid scintillation counting. Units of CT activity are nmol CDP-choline formed per minute.
2.3.7 Preparation of Sonicated Unilamellar Vesicles (SUVs)

Lipids in CHCl₃ stocks were dried in small round bottom flasks under vacuum with a rotary evaporator for >10 min. The dried lipids were redissolved in TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA) and sonicated on ice (Bronson sonicator with a fine probe operating at 80% power output) until the turbid solution was clear (10 to 20 min for lipid dispersion containing anionic lipids, 20 to 45 min for vesicles containing PC). The sonicated solution was centrifuged at 15000 rpm for 5 min to remove any titanium debris from the sonicator probe. Lipids vesicles were used the same day as preparation except for the PC/oleic acid vesicles used for the CT activity assay. These were sometimes stored and used within a few days of preparation.

2.3.8 Preparation of Multi-lamellar Vesicles (MLVs)

Lipids from chloroform stocks were dried in a culture tube under N₂, and were then vacuum dried for 4-10 hr at room temperature, and then hydrated in Liposome buffer (10 mM Tris, 1 mM EDTA, pH 7.4). The suspension was incubated for 10 min at 37°C, followed by vigorous vortexing for 1 min. MLVs were used the same day as preparation.

2.3.9 Preparation of [³H]phosphocholine

[³H]Phosphocholine was prepared from [³H]choline by reaction with choline kinase in the presence of ATP. 1 to 2 mCi [³H]choline in ethanol was
dried under nitrogen and reacted with 0.3 units choline kinase for 1 h at 37 °C in the presence of 0.1 M Tris pH 8.0, 10 mM ATP, 10 mM MgCl₂.

[^3]H]Phosphocholine was separated from unreacted[^3]H]choline on a Dowex ion exchange column: choline eluted with H₂O, phosphocholine eluted with 50 mM NaCl. Ca^{2+}-Free phosphocholine (unlabelled) was added to give a final phosphocholine concentration of 10 to 15 mM and a specific activity of 10 to 15 Ci/mol (Cornell, 1989).

2.3.10 Light Scattering

Purified CT was treated with Cu-PHT (or glutaraldehyde) for 15 min at 37°C or was untreated. SUVs of DOPG (0.2 mM) were added and the sample was incubated for up to 10 min before estimation of the particle size by Nicomp Model 270 Submicron Particle Sizer (relative volume, distribution analysis mode).

The Parameters of the light scattering measurements:

Channel Width = 3.0E1 USEC
Prescale Factor = 1
Temperature = 23 °C
Viscosity = 0.9325
Index of Refrac = 1.333
Autoprint at CH1 = 100 K Counts
Fit Error < 5.0
Run Time: ~ 28 min
Scanning Range: 10 - 540 nm

2.3.11. Densitometry Measurements

Gels were scanned with OFOTO program and densitometry was performed using Image 1.44 program.
CHAPTER THREE: IDENTIFICATION OF CT DIMERIZATION

DOMAIN

3.1 RESULTS

3.1.1 Chemical Cross-linking Prevents Dissociation of Proteolysis

Fragments

To test the proposal that the N-terminal domain of CT is involved in dimerization, we developed protocols using proteolysis, chemical cross-linkers and SDS PAGE. After reaction with glutaraldehyde, the 42 kDa CT monomer disappeared, and a new diffuse band between 84 kDa and 120 kDa emerged (Fig. 3.1, lane 2), as has been previously reported (Cornell, 1989). The chymotrypsin fragments at 40, 39, 28, and 26 kDa have been previously identified as fragments containing the N-terminus (but not the C-terminus) of CT (Craig et al., 1994) (Fig. 1.4). When chymotrypsin was added to digest the cross-linked product, the diffuse glutaraldehyde cross-linked band was not separated by SDS-PAGE (Fig. 3.1, lane 4). Upon varying the chymotrypsin concentration, it was found that even at a mass ratio of 5:1 (CT:chymotrypsin), the diffuse band was still undissociated by SDS PAGE (data not shown). The likely reason is that CT has multiple sites for chemical cross-linking, and the glutaraldehyde can react intra-molecularly to produce linkages between the C-terminal and the N-terminal domain of CT. Even if the protease could digest the protein, the
Fig. 3.1 Chemical Cross-linking affects Apparent Sensitivity to Chymotrypsin—Purified CT was reacted with glutaraldehyde and quenched with ethanolamine, prior to treatment with Chymotrypsin. Lane 1, original untreated CT (12 μg); Lane 2, CT (12 μg) was treated with 1 mM glutaraldehyde; Lane 3, CT (12 μg) was treated with chymotrypsin (CT/protease = 1:20, W/W); Lane 4, 12 μg of CT was reacted with glutaraldehyde, after 15 min. chymotrypsin was added (CT/protease = 1:20) for another 15 min. Gel was stained with Coomassie Blue.
fragments would still be covalently linked with the N-terminal region of CT through the cross-linker, glutaraldehyde. This would prevent dissociation of the 84-110 kDa band by SDS PAGE. Another possible reason for lack of resolution of the cross-linked bands into component fragments is that the chemical cross-linker may have inhibited the activity of the protease.

In order to test these possibilities, the cleavable reagent DSP was used. First we cross-linked CT with DSP (Fig. 3.2, lane 4). After quenching the cross-linker, chymotrypsin was added to digest the cross-linked products (Fig. 3.2, lane 3). Finally fresh DTT was added to reduce disulphide bonds (Fig. 3.2, lane 6). DTT reduction of the DSP cross-linked CT generated chymotrypsin fragments at 39, 30, 28 and 26 kDa (Fig. 3.2, lane 6). This result shows that the chemical cross-linker DSP does not inhibit the digestion by chymotrypsin. Rather, this result supports the hypothesis that CT may have tertiary interactions between the C-terminal and the N-terminal domains. These domains would approach each other to within 12 Å during the incubation with cross-linker. In all subsequent experiments to analyze the domain responsible for dimerization the enzyme was digested prior to chemical cross-linking.

3.1.2 Evidence That the CT dimerization Domain Resides in The N-terminal Domain

The approach taken to identify the dimerization domain was to digest CT
Fig. 3.2 Chemical Cross-linking with DSP does not interfere with chymotrypsin digestion—Lane 1, molecular weight standards; Lane 2, original untreated CT (12 µg); Lane 3, 12 µg CT was reacted with 1 mM DSP as described in Methods; after 15 min. chymotrypsin was added (CT/protease = 1:20) for 15 min.; Lane 4, CT was treated with 1 mM DSP; Lane 5, CT (12 µg) was treated with chymotrypsin (CT/protease = 1:20) for 15 min.; Lane 6, CT was treated as lane 3, after which the sample was incubated with fresh DTT at 30°C for 30 min.. Gel was stained with Coomassie blue.
into characterized fragments followed by cross-linking and identification of cross-linked species by SDS-PAGE and immunoreactivity.

The results of an experiment using glutaraldehyde cross-linker are shown in Fig. 3.3. CT was first treated with chymotrypsin to produce fragments at 40, 39, 28, and 26 kDa (lane 2). Three new bands appeared following the cross-linking step (lanes 3,4): 84 kDa, 65 kDa and 56 kDa. The later two bands were present only in samples treated with both chymotrypsin and glutaraldehyde. In order to confirm that the 84 kDa species was a dimer of the 42 kDa CT monomer and to identify the 56 kDa and 65 kDa species, we used the cleavable cross-linker DSP and two dimensional gel analysis.

CT was digested with chymotrypsin at a mass ratio of 15:1 (CT : chymotrypsin). The cross-linker DSP was added to react with the chymotrypsin digestion fragments. After cross-linking with DSP the same pattern of bands was observed as with glutaraldehyde (Fig. 3.4, lane 5). DSP is a cleavable cross-linker, so the sample was then incubated with fresh DTT to resolve cross-linked species into their monomeric components, and electrophoresed in the second dimension. The 84 kDa band migrated into the second gel to a position corresponding to 39-42 kDa. The protein 65 kDa band was separated into two bands: 39-42 kDa and 28 kDa. The 56 kDa band migrated to a position corresponding to 28 kDa. The 42 kDa and other lower molecular weight bands
Fig. 3.3 Glutaraldehyde cross-linking of chymotrypsin digested CT—Lane 1, original untreated CT (12 μg); Lane 2, CT was treated with chymotrypsin (chymotrypsin/CT = 1:20); Lane 3, 4 and 5, CT was treated with chymotrypsin, following reacted with glutaraldehyde (lane 3, 0.5 mM glu., lane 4, 1.0 mM glu. and lane 5, 1.5 mM glu.); Lane 6, CT was reacted with glutaraldehyde (1.5 mM), without prior chymotrypsin digestion. Gel was stained with Coomassie blue.
Fig. 3.4 Resolution of DSP Cross-linked CT Fragments by 2-D PAGE—A. first dimension 10% polyacrylamide gel. Samples were run in the absence of β-mercaptoethanol or DTT. Lane 1, molecular weight standards; lane 2, untreated CT (12 μg); lane 3, CT was treated with chymotrypsin (CT/protease = 1:20); lane 4, CT was treated with 1 mM DSP for 15 min; lane 5, 12 μg CT was treated with chymotrypsin followed by reaction with DSP. B, second dimension. A lane identical with lane 5 was reduced with DTT and electrophoresed into a second 12% gel. Gels were stained with Coomasie blue and silver.
migrated to the same position in both the first and second dimensional gels. These results suggest that only protein species of 42 kDa, 39 kDa and 28 kDa can form cross-linked dimers, and imply that the N-terminal 28 kDa domain is involved in CT dimerization.

3.1.3 Antibody Mapping of the Fragments

The results of the two dimensional gel shown in Fig. 3.4, suggested that the proteins 84kDa, 65kDa and 56kDa contain the N-terminus. To test this we transfered the proteins from SDS gel to a PVDF filter and blotted the filter with antibody raised against the N-terminal peptide of CT (amino acids 1-15). A pattern identical to that from the Coomassie-stained gel emerged (Fig. 3.5) indicating that each of the proteins (84 kDa, 65 kDa and 56 kDa) contained the N-terminal region of CT. The weak bands at 84, 65 kDa and 56 kDa are also visible in the sample that was treated with only chymotrypsin (Lane B-2). These species are present because the SDS (100°C) treatment is not always sufficient to dissociate the non-covalent interactions of the dimer. The amount of glutaraldehyde used in this experiment caused excessive smearing of cross-linked bands due to greater than optimal intramolecular cross-linking. These findings provided evidence that the dimerization domain of CT is located in the N-terminus.
Fig. 3.5 Antibody mapping of the dimer fragments-- (A) Coomassie stain 10% gel: Lane 1, untreated CT (12 μg); lane 2, 12 μg of CT was treated with chymotrypsin (CT/protease = 1:20, W/W); Lane 3, CT was treated with 1.0 mM glutaraldehyde for 15 min; Lane 4, Chymotrypsin-treated CT was cross-linked with 1.0 mM glutaraldehyde for 15 min.; (B) Western blot: The gel in A was blotted with anti-N antibody.
Fig. 3.6 Identification of fragments containing the C-terminal phosphorylation domain—12 μg of CT was $^{32}$P labeled using $^{32}$P-ATP and casein kinase II. (A) Lane 1, $^{32}$P labeled CT (12 μg); Lane 2, $^{32}$P labeled CT was incubated with chymotrypsin at 37°C for 15 min. (CT/protease = 1:20), after which it was treated with 1.0 mM glutaraldehyde; Lane 3, $^{32}$P labeled CT was treated with chymotrypsin (CT/protease = 1:20); Lane 4, $^{32}$P labeled CT was treated with 1 mM glutaraldehyde. Gels were stained with Coomasie Blue and silver. (B) The autodiographic film of the same gel shown in A. Arrows indicate the 84, 65, and 56 kDa species.
3.1.4 Analysis for the presence of the C-terminal phosphorylation domain in CT cross-linked fragments

The antibody mapping and two-dimensional SDS-PAGE experiments suggested that the 56 kDa species was a complex of the N-terminal 28 kDa fragment; thus it should not contain C-terminus. The 65 kDa species appeared to be a complex of the 28 kDa fragment plus 42 kDa or 39 kDa fragments. If so, the 65 kDa species would contain all or a portion of the C-terminal phosphorylation domain, but the 56 kDa fragment should not. To test this, we phosphorylated CT using $^{32}$P-ATP and casein kinase II. The casein kinase II site has been mapped to serine 362 at the C terminus (Cornell, et al., 1995). The labeled CT was then treated with chymotrypsin at 37°C for 15 min., and finally glutaraldehyde was added to the solution. The products were analyzed by SDS PAGE and autoradiography (Fig. 3.6). The result showed that the 84 kDa and 65 kDa fragments were labeled, and the 56 kDa band was not. The $^{32}$P labeling intensity of the 65 kDa band was very weak compared to its relative intensity by Coomassie staining.

3.1.5 The Interface of CT Dimer Contains a pair of Cysteines

Cu-PHT (CuSO$_4$ + 1,10-phenanthroline) catalyses disulfide bond formation between two cysteines that contact each other in a folded protein. A
Fig. 3.7 Cu-PHT cross-linking of untreated and chymotrypsin treated CT--12 μg of CT was reacted with Cu-PHT, prior to treatment with chymotrypsin, or treated with chymotrypsin, prior to addition of Cu-PHT (see Methods). lane 1, untreated CT (12 μg); lane 2, CT reacted with Cu-PHT only; lane 3, CT was treated with chymotrypsin (CT/protease = 1:20) only; lane 4, 12 μg of CT was incubated with Cu-PHT at 37°C for 15min., followed by treated with chymotrypsin; lane 5, CT was treated by chymotrypsin first, followed by Cu-PHT.
previous report suggested that oxidizing conditions promoted covalent cross-linking of CT monomers to form the 84 kDa dimer (Cornell, 1989). There are seven cysteine residues in CT. Five of them are in the N-terminus at position 37, 68, 73, 113, 139 and the other two residues are near the C-terminus at position 354 and 359. Purified CT was incubated with Cu-PHT at 37°C for 15 min, followed by the treatment with chymotrypsin. The products were analyzed by SDS PAGE (Fig. 3.7). The same pattern of cross-linked fragments emerged as generated using glutaraldehyde or DSP (Fig. 3.3, 3.4, 3.5, and 3.6). New species at 56, 65, and 84 kDa were observed in the presence of Cu-PHT (Fig. 3.7, lane 5). When the reaction order was changed (protease digestion first, followed by Cu-PHT), the same cross-linked pattern resulted (Fig. 3.7, lane 5). A clear band at ~120 kDa was also clearly resolved from the 84 kDa band in the samples cross-linked with Cu-PHT (Fig. 3.7, lane 4,5). These data indicated that the cysteines at position 354, 359 do not form disulfides with the cysteines in the N-terminal domain. Otherwise the cleavage patterns would have been different when cross-linking succeeded digestion vs succeeded digestion. Since the N-terminal domain was cross-linked by Cu-PHT, there must be at least two cysteine residues present in the N-terminus of CT close to each other in the interface of CT dimer which covalently link the 2 monomers upon oxidation.
**Fig. 3.8 Glutaraldehyde cross-linking of Pep-62** — lane 1, 4 µg Pep-62 untreated; lane 2, 4 µg Pep-62 reacted with 1 mM glutaraldehyde for 15 min at 37°C in the absence of PC/oleic acid; lane 3, 4 µg Pep-62 reacted with 1.5 mM glutaraldehyde in the presence of PC/oleic acid.
3.1.6 Pep62 Cross-linking Reaction

The region of CT between amino acids 240-300 contains the membrane-binding segment (domain M). In the presence of activating lipid vesicles this region assumes an amphipathic α helical structure and intercalates into the lipid bilayer where it obtains some protection from proteases (Craig et al., 1994; Johnson and Cornell, 1994). Since amphipathic helices are common dimerization motifs, we asked whether this domain could also contribute to the dimerization of CT. First we probed the potential of a 62mer peptide (Pep-62) corresponding to domain M to form dimers or oligomers that could be covalently trapped with chemical cross-linkers. The cross-linking was performed in the presence or absence of PC/oleic acid vesicles, which have been shown to bind Pep62 (Johnson et al., 1997). This would test the role of the peptide-lipid bilayer interaction in the oligomerization.

Pep-62 migrated to a position of ~ 8 kDa (Fig. 3.8, lane 2). In the presence or absence of PC/oleic acid, reaction with glutaraldehyde produced bands at 8, 16, and 22 kDa, representative of peptide monomer, dimer, and trimer. The ratio of oligomers to monomer was very low (under cross-linking conditions that result in near complete dimerization of whole CT enzyme). These results suggest that in isolation domain M does have a weak capacity to oligomerize, and that the PC/oleic acid vesicles do not appear to modulate this oligomerization.
3.2 DISCUSSION

3.2.1 The Dimerization Domain of CT Resides in N-terminal Region

Previous studies using chemical cross-linking revealed that CT forms a homodimer in the Triton X-100 complexed form (Cornell, 1989) and suggested that the dimerization domain is in the N-terminus of CT (Craig et al, 1994). Here we supply some strong evidence to support this suggestion.

CT was treated with chymotrypsin, prior to cross-linking with glutaraldehyde or Cu-PHT. Three new bands appeared in the cross-linked samples: 84 kDa, 65 kDa, and 56 kDa (Fig. 3.3; 3.7). We expected that the 84 kDa species was a dimer of the 42 kDa CT monomer, the 56 kDa species was a dimer of the 28 kDa CT fragment. We hypothesized that the 65 kDa species would contain the N-terminal domain of CT. These expectations were confirmed using the cleavable cross-linker DSP and two dimensional gel analysis (see 3.1.2): The 56 kDa species resolved into two 28 kDa CT fragments which contain the N-terminus (by antibody reactivity), but not the C-terminus (lack of $^{32}$P labeling). The 65 kDa species is a complex of the 42 kDa CT monomer and the 28 kDa fragment. Antibody mapping showed that the 65 kDa band contained the N-terminus (3.1.3). The kinase-catalyzed $^{32}$P incorporation showed that the 65 kDa fragments were labeled, but only weakly compared to the 84 kDa band (3.1.4). The composition of the 65 kDa species is described in Fig. 3.9.
Fig. 3.9 Methionine-tRNA synthetase Structure (Traut, 1994).
A series of chymotrypsin fragments at apparent molecular weights of 19, 17, 15 and 11 kDa, can be visualised in the appropriate gel system (Johnson et al., 1997). These have been mapped as C-terminal fragments with an anti-C-terminal peptide antibody (Johnson et al., 1997) (Fig. 1.4). The 42 kDa polypeptide is digested readily into 26 + 16 kDa fragments; The 16 kDa band migrates anomalously as a 19 kDa band. If the C-terminal domain was participating in dimerization we should have observed a cross-linked band at ~38 kDa. This is not apparent in the gel shown in Fig. 3.6-B, in which the C-terminal domain was tagged with $^{32}$P. This would suggest a lack of participation of the C-terminal domain in dimerization. Since in isolation domain M does have capacity to oligomerize (3.1.6), lack of cross-linking suggested that not only is the C-terminal domain of CT unnecessary for dimerization, but that the two C-termini of the dimer do not interface each other in the CT dimer.

Recently, the Kent group has obtained crystals of B. subtilis glycerolphosphate cytidylyltransferase (GCT) (unpublished) and the crystal structure has been solved. This enzyme is homologous to the catalytic domain of cholinephosphate CT (Park et al., 1993), and it is a dimer in the crystal. There are 4 $\beta$-sheets and 3 $\alpha$-helices in GCT. The structure of GCT is similar to methionine-tRNA synthetase (Fig. 3.9). There appear to be four contacts between the monomers of GCT - two helix-helix and two loop-loop (Kent, personal communication). The dimer interaction of cholinephosphate CT may be very similar to that of GCT.
These findings provide evidence that the dimerization domain of CT is located solely in the N-terminus. The original model for CT dimer interactions (Fig. 1.4 b) is incorrect.

3.2.2 The Tertiary Interaction Between C-terminal and N-terminal Domain of CT

When CT was cross-linked with glutaraldehyde or DSP, digestion with chymotrypsin did not yield fragments that could be separated by SDS PAGE. The likely reason is that CT has multiple sites for chemical cross-linking, and the cross-linkers can react intra-molecularly to produce covalent linkages between the C-terminus and the N-terminus of CT, thus trapping a tightly globular form. Even if the cleavage reagent could digest CT, the fragments would still be covalently linked with the N-terminal region of CT through the cross-linker, and this would cause the 84-110 kDa band to be undissociated by SDS-PAGE. A trivial explanation, that cross-linker may inhibit the protease, was ruled out by the finding that DSP-cross-linked fragments could be resolved after reduction of the disulfide by DTT. The N- and C-terminal domains may become covalently cross-linked by glutaraldehyde or DSP during random collisions of the two domains (to within 8 - 12 Å). Alternatively the two domains may have stable, long lived associations.

Another indication of interactions between N- and C-terminal domains came from the resolution of the 84 kDa and 65 kDa DSP-cross-linked species in
A. 65 kDa fragment

\[
\begin{align*}
\text{84 kDa} & \xrightarrow{\text{Chymotrypsin}} 65 \text{ kDa} + 65 \text{ kDa} \\
\text{C} & \text{DSP} \\
\text{N} & \text{SDS-PAGE} \rightarrow \text{N} + \text{N} + 2 \text{□□□}
\end{align*}
\]

B. 84 kDa fragment

\[
\begin{align*}
\text{84 kDa} & \xrightarrow{\text{Chymotrypsin}} 84 \text{ kDa} \\
\text{C} & \text{DSP} \\
\text{N} & \text{SDS-PAGE} \rightarrow \text{N} + \text{N} + 2 \text{□□□}
\end{align*}
\]

Fig. 3.10 Tertiary Interactions Between C-terminus and N-terminus of CT Explain the Composition of Digested, Cross-linked Species.
the second dimension. In the second dimension gel, the 84 kDa band was separated into 42 kDa, but the 28 kDa fragment also appeared. The ratio of 42 kDa/28 kDa is \( \sim 1:1 \) (Fig. 3.4). An explanation for this finding is that cleavage of the polypeptide at the hinge between the N-terminal and C-terminal domains or within the C-terminal domain, does not cause complete dissociation of the two domains due to covalent cross-links. Thus the 84 kDa band consists of a \( \sim 1:1 \) mixture of intact 42 kDa band plus a 42 kDa species that is cleaved at the polypeptide backbone at one or more sites in the C-terminal domain, but these C-terminal fragments remain in complex with the N-terminal 28 kDa domain due to the covalent cross-links. The 65 kDa band was separated on the second dimension gel into two bands: 39-42 kDa and 28 kDa. Interestingly, the ratio of 42 kDa/28 kDa is 1:5, not 1:1. A scheme that can explain this stoichiometry is shown in Fig. 3.10. The 65 kDa band is composed of one 42 (or 39) kDa polypeptide plus one 28 kDa, but the 42 kDa species is cleaved at the peptide backbone in two thirds of the complexes. Despite the cleaved peptide backbone the small fragments remain associated with the large 26 kDa domain (see Fig. 3.10).

These results add further evidence that CT may have stable tertiary interactions between the C-terminus and the N-terminus, because cleavage of the polypeptide at the hinge between the two domains or within the C-terminal domain did not cause complete dissociation of fragments (Fig. 3.10).
3.2.3 CT Trimer

Chemical cross-linked CT (using glutaraldehyde, DSP, and DTBP) generated a diffuse 84-120 kDa band in SDS gels (Cornell, 1989; 3.1.1). This diffuse band may be caused by excessive chemical cross-linking. Since glutaraldehyde reacts with several amino acids, such as lysine, tyrosine, histidine and cysteine, multiple cross-links within and between monomers would generate heterogeneous products with varying SDS binding properties. Highly cross-linked polypeptides may not unfold as much as the same uncross-linked polypeptides and would bind less SDS.

Cu-PHT (CuSO₄ + 1,10-phenanthroline), on the other hand, catalyses disulfide bond formation between two cysteines that contact each other in a folded protein. When this reagent was employed to study CT dimerization, the maximum number of covalent cross-links between two monomers was 7 (7 cysteines in CT monomer). Cu-PHT cross-linking generated distinct, well resolved bands at 84 and ~120 kDa (Fig. 3.5, lane 2). The ~120 kDa band also appeared in the DSP cross-linking experiment (Fig. 3.2, lane 3). The molecular weight of this protein is about 3 x 42 kDa (CT monomer). This band was labelled in the phosphorylation experiment (Fig. 3.6 B, lane 2). Thus it contains the C-terminus. It also was labelled by N-terminal antibody (Fig. 3.5 B). Thus we propose that it represents CT homotrimer. The ~120 kDa CT band was not
present in the membrane fractions (Fig. 4.3; 4.4). From these results, we suggest that CT may form a trimer in the soluble form. Why CT forms a trimer is still a puzzle. A proposal for trimer formation is shown in Fig. 5.1.
CHAPTER FOUR: THE RELATIONSHIP BETWEEN THE DIMERIZATION
AND ACTIVITY OF CT

The results of previous studies were interpreted to mean that mammalian
CT exists as a homodimer in soluble form and also forms a homodimer when it
binds to membranes or Triton X-100 micelles (Cornell, 1989; Weinhold, 1989;
Craig, et al., 1994). What is the function of CT dimerization? Does it play an
important role for its activity? The role of CT dimerization has not been
addressed previously.

4.1 RESULTS

4.1.1 Effect of Acetonitrile on CT Dimerization and Activity

The interactions of the CT dimer are relatively strong since some dimer
persistence on SDS gels under reducing conditions even without chemical cross-
linking. We sought a means of disrupting the interactions between monomers
without denaturing them. Acetonitrile is a protein denaturant which has been
used to study the dimerization of HIV type 1 reverse transcriptase (Restle 1990).
Acetonitrile 20% dissociated the reverse transcriptase from dimer to monomer
without destruction of the transcriptase tertiary and secondary structure. We
used this characteristic of acetonitrile to disrupt quaternary structure prior to
tertiary or secondary structure in our analysis of the function of CT dimerization.
Purified CT was incubated with 0%, 5%, 10%, 15% and 20% of acetonitrile at room temperature for 5 min. Two sets of samples were prepared in parallel to measure dimer interactions or enzyme activity. Both sets contained the CT assay mix and PC/oleic acid vesicles. Set one contained glutaraldehyde for analysing the ratio of monomer/dimer and set two contained 3H-phosphocholine for the CT activity assay. Both sets contained the same ratio of CT/acetonitrile. The results of the SDS PAGE analysis are shown in Fig. 4.1. As the acetonitrile concentration increased more of the CT migrated as the monomer, and less as the dimer and trimer species.

Comparison of Fig 4.2B with Fig. 4.2A shows that oligomerization is more sensitive to acetonitrile than is CT activity; 15% acetonitrile led to a 70% decrease in the percent of CT associated as a dimer or trimer, but only a 30% reduction in enzyme activity compared with untreated CT. This result suggested that CT dimerization may not be necessary for its activity.

4.1.2 Effect of Phospholipid Vesicles on CT Dimerization

The purified CT used in these studies contained 0.5-0.7 mM Triton X-100 as a stabilizer. The Triton concentration was diluted only to 0.1-0.5 mM. Thus the "soluble CT" was in fact complexed to a Triton micelle. CT is not active under these conditions.

CT binding to anionic phospholipids results in enzyme activation. Phosphatidic acid (PA) and phosphatidylycerol (PG) are potent phospholipid
Fig. 4.1. Acetonitrile Inhibits CT Dimerization—Lane 1, untreated CT; Lane 2-5, 12 µg CT was incubated with 0 to 15% acetonitrile at room temperature for 5 min, followed by glutaraldehyde (1.5 mM) addition for 15 min at 37°C. Lane 2, 0%; Lane 3, 5%; Lane 4, 10%, Lane 5, 15%. Gel was stained with Coomassie Blue. This experiment was repeated two times with similar results.
Fig. 4.2 (A) Effect of Acetonitrile on CT activity

12 μg CT was incubated with 0 - 20% acetonitrile at room temperature for 5 min as in Fig 4.1. The CT activity assay was initiated with ³H-phosphocholine. This experiment was repeated two times with similar results.

Fig. 4.2 (B) Effect of Acetonitrile on dimerization

The gel shown in Fig. 4.1 was analysed by densitometry, the density of the dimer plus trimer relative to the combined density of all bands is plotted.
activators of CT. The effect of acetonitrile on CT dimerization and activity suggested that CT may not require dimerization for enzyme activation. We then asked, does activation require dissociation of the dimer to the monomeric state, and does binding to phospholipids dissociate the dimer? To test this hypothesis, we examined the effects of increasing PA (or PG) concentration on the dimerization state, assessed by chemical cross-linking. CT was cross-linked with glutaraldehyde in the presence of pure PC vesicles (Fig. 4.3, lane 3). This resulted in complete crosslinking of the CT dimer. The PA (or PG) concentration was varied from 0%-100 mol %, with PC as the other phospholipid component. As the concentration of PA (or PG) increased from 0-100% the ratio of CT monomer/dimer increased (Fig. 4.3; Fig. 4.4) (The ratio of monomer / dimer plus trimer was determined by densitometry of the scanned gels. Fig. 4.3: 0% PA, 0; 40% PA, 0; 60% PA, 0.28; 80% PA, 0.32; 100% PA, 0.36. Fig 4.4: 20 % PG, 0.29; 50% PG, 0.70; 100% PG, 0.71). It was reasoned that the higher the PA (or PG) content of the vesicles the longer the lifetime of CT in the membrane-bound state (Arnold et al, 1997). The experiment was repeated using Cu-PHT as cross-linker, with similar results (not shown). Because two very different types of cross-linking reagents had reduced effectiveness in the presence of 100% PA (or PG), it is unlikely that the effect was due to an inhibition of the cross-linking reaction by PA (or PG).
Fig. 4.3 Effect of PA vesicles on CT dimerization-- Lane 1, untreated CT; Lane 2, 12 μg CT was treated with 1.5 mM glutaraldehyde; Lane 3-8: 12μg CT was incubated with sonicated PC/PA vesicles (0.5 mM) at 37°C for 10 min., followed by glutaraldehyde addition. Lane 3, 0% PA; Lane 4, 20% PA; Lane 5, 40% PA; Lane 6, 60% PA; Lane 7, 80% PA; Lane 8, 100% PA.
Fig. 4.4 Effect of PG vesicles on CT dimerization—Lane 1, untreated CT; Lane 2, 12 μg CT was treated with 1.5 mM glutaraldehyde; Lane 3-6: 12 μg CT was incubated with sonicated PC/PG vesicles (0.5 mM) at 37°C for 10 min., followed by glutaraldehyde addition. Lane 3, 0% PG; Lane 4, 20% PG; Lane 5, 50% PG; Lane 6, 100% PG.
4.1.3 Phosphatidic Acid (PA) Does Not Effect Phosphoglycerate Mutase (PGM) Cross-linking Reaction

As the concentration of PA (or PG) increased in the previous experiments, up to 25% (PA) or 40% (PG) of CT could not be cross-linked to form a dimer. One could argue that phospholipid vesicles may affect the reactivity of cross-linkers. To answer this question a dimeric protein, phosphoglycerate mutase (PGM), which is not a membrane binding protein, was used to test the effect of PA. In the presence or absence of PA, the same cross-linked products of PGM were obtained by SDS PAGE (Fig. 4.5). This result showed that effects of PA on glutaraldehyde cross-linking of CT are not due to an inhibition of the glutaraldehyde reaction.

4.1.4 Effect of MLV PA on CT Dimerization

The results of the experiments in section 4.1.2 suggested that PA (or PG) sonicated vesicles reduced the efficiency of the cross-linking reaction. In that experiment, we did not determine how much of the CT was membrane bound nor did we determine the ratio of CT dimer/monomer in the supernatant vs the membrane fraction. To solve this problem, MLVs were used instead of the sonicated vesicles. This would enable separation of the phospholipids from solution by a simple centrifugation. Prior to addition of the cross-linker, Cu-PHT, CT was incubated with MLVs composed of various concentrations of PA or
Fig. 4.5 Phosphatidic acid (PA) Does not Effect Glutaraldehyde Cross-linking of PGM-- PGM is a dimeric protein and is not a membrane binding protein. Lane 1, untreated PGM; lane 2, 12 μg PGM was treated with glutaraldehyde (1.5 mM) at 37°C for 15 min. in the absence of PA; lane 3, same as lane 2, but in the presence of 100% PA vesicles (0.5 mM).

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Fig. 4.6 Membrane-bound CT is monomeric--Lane 1, untreated CT; lane 2, CT was treated with Cu-PHT in the absence of phospholipids; lanes 3-5, CT was incubated with 0.5 mM MLVs (PA/PC), after which it was treated with Cu-PHT; lane 3, 100% PC; lane 4, 50% PA and 50% PC; lane 5, 100% PA. (S-supernatant; P-pellet; CT: 12μg; MLVs: 0.5mM; Cu-PHT: 0.3mM Cu²⁺, 0.5mM PHT).
**Fig. 4.7. Cross-linked CT dimers can bind to lipid vesicles**—Lane 1, untreated CT; lane 2, CT was treated with Cu-PHT in the absence of PA MLVs; lane 3, CT was incubated with PA MLVs only; lane 4, CT was treated with Cu-PHT, after which it was incubated with PA MLVs (S-supernatant; P-pellet; CT: 12μg; PA MLVs: 0.5mM; Cu-PHT: 0.3mM Cu²⁺, 0.5mM PHT).
without MLVs for 10 min at 37°C. The mixture was centrifuged at 15000 rpm for 30 min. The supernatant and pellet fractions were analysed by SDS-PAGE (Fig. 4.6). In the absence of phospholipids, cross-linked CT was in the supernatant (lanes 2A, 2B). When 100% MLV PA was added, we found that the ratio of CT dimer/monomer in the supernatant was 1:1, and there was no CT dimer in the pellet, only CT monomer. This result suggested that CT dimers dissociate to monomers when they bind to the membrane. This experiment has been repeated 2 times with the same results.

4.1.5 Cross-linked CT Dimers Can Bind to Lipid Vesicles

There are two possible sequences for the dissociation of CT dimer to monomer promoted by lipid vesicles: (1) Dissociation of dimer in the aqueous phase, followed by membrane binding of monomers; (2) Dimer binding to membrane, followed by dissociation to monomers. To distinguish between these possibilities we cross-linked CT with Cu-PHT prior to addition of membranes. Cross-linked CT should bind to membranes only if model (2) is correct. CT was treated with Cu-PHT at 37°C for 15 min, followed by incubation with MLVs composed of 100% PA for another 10 min. The mixture was centrifuged at 15000 rpm for 30 min. The supernatant and pellet fractions were analysed by SDS-PAGE (Fig. 4.7). In the absence of phospholipids, untreated CT and cross-linked CT were in the supernatant (lanes 1A, 1B, 2A, 2B). At least 50% of the total CT bound to the MLVs (lanes 3A, 3B, 4A, 4B), and covalent cross-linking
did not inhibit the binding (lane 4B). These results suggest that the cross-linked CT dimers can bind to the membranes, supporting model (2).

4.1.6 Cross-linked CT Induces Phospholipid Vesicle Aggregation: Study Using Light Scattering

The previous experiments suggested that CT may dissociate when it binds to membranes and that the C-terminal domain of CT was not necessary for dimerization (see 3.1.4). There are at least two models for association of CT dimer via the N-terminal domain, a “parallel” arrangement (original model) or an “anti parallel” arrangement (Fig. 4.8). If the antiparallel model were true, cross-linked CT might induce cross-links between phospholipid vesicles with CT as the cross-bridge. To test which model is correct, light scattering was used to measure the vesicle diameter with or without cross-linked CT. Fresh sonicated PG vesicles have an average diameter around 35 nm (Fig. 4.11). This size distribution did not change upon addition of glutaraldehyde (or Cu-PHT) or CT (25 CT monomers per 35 nm vesicle) (Fig. 4.11 Panel B and C). However, incubation with previously glutaraldehyde cross-linked CT produced a second peak between 65 and 130 nm (Fig. 4.11 Panel D). This peak was not observed when the sample contained only cross-linked CT (data not shown). The integrated ratio of the two peaks in panel D was 0.22 (35 nm peak : 65-130 nm peak).
1. Original model, parallel arrangement

2. "Anti parallel" arrangement

Fig. 4.8 Two models for CT dimer—In an antiparallel arrangement, one could envision CT acting as a bridge between two membrane bilayers as shown in 2. This would not be possible in the original model.
Fig. 4.9 Cross-linked CT Caused PG Aggregation—78 µg CT was treated with glutaraldehyde (glu) for 15 min at 37°C or was untreated. Sonicated DOPG vesicles (0.2mM) were added and the particle size was determined by Nicomp Model 270 Submicron Particle Sizer. This experiment was repeated three times with similar results.
These results provide evidence that CT may be dissociated after it binds to the membranes. Otherwise, one would expect that CT would form cross-linkages even in the absence of cross-linker. The vesicle cross-linking of CT confirmed that the tails of the CT dimer are not close to each other but far apart.

4.2 DISCUSSION

The data from chapter 3 suggested that CT forms a dimer or a trimer when in the soluble form. The function of the CT dimer or trimer and the effect of activating membranes on CT's oligomeric state have been addressed in this section.

At the onset of the project we believed that the dimeric form of CT was active. The experiments shown in this section of the thesis suggest that CT may be inactive as a dimer. Firstly, the effects of acetonitrile on CT activity and chemical cross-linking potential suggested that CT dimers are not required for the active state of CT, since the activity was much more resistant to this reagent than the cross-linking reaction. The CT activity only declined above 10% acetonitrile probably due to denaturation of the enzyme tertiary and/or secondary structure. One possible alternative explanation for the effects of acetonitrile on CT cross-linking is that glutaraldehyde reactivity may have been inhibited by the acetonitrile. Acetonitrile could potentially react with glutaraldehyde in strongly basic conditions, but would not react in neutral or acidic conditions. In these experiments the system pH was ~7, so acetonitrile...
would likely not inhibit the reactivity of glutaraldehyde. Moreover 20% acetonitrile was sufficient to completely dissociate HIV RT dimers (Restle et al., 1993), hence would be expected to disrupt CT dimers as well. Secondly, the effects of PL vesicles suggested the possibility that CT may dissociate to monomer after it binds to the membranes and that the role of CT dimerization may be to inactivate the enzyme. The monomer form of CT appeared to be increased by raising the anionic phospholipid concentrations, and there was no CT dimer in the membrane fraction (only CT monomer) when 100% MLV PA was added (Fig. 4.6). Our interpretation of this result is that the CT dimers dissociate into monomers when they bind to the membrane. Although we showed that PA vesicles do not inhibit the cross-linking by glutaraldehyde of a soluble enzyme (PGM), we have not yet conducted appropriate controls to exclude the possibility that the efficiency of glutaraldehyde cross-linking is reduced in the milieu of an acidic membrane.

All cross-linkers, including Cu-PHT (data not shown), inhibit CT activity (Cornell, 1989). However, we were unable to distinguish whether the inhibition was due to covalent trapping of the dimers or due to inactivation of critical residues in the active site.

Cornell previously proposed that CT is a homodimer when bound to a detergent micelle or membrane vesicle (Cornell, 1989). This hypothesis was based on the results of chemical cross-linking much like the present studies. In the presence of a Triton X-100 micelle or a membrane vesicle containing
PC/oleic acid, CT was treated with glutaraldehyde (or DSP, DTBP) and cross-linked CT dimer (84-110 kDa) appeared in SDS gels as the major or sole product. However, because CT binding is reversible, during the incubation with the cross-linker, the monomeric enzyme will exchange off the vesicles and during the lifetime of its unbound state, it would likely reform dimers and react with the cross-linker, becoming a covalently trapped dimer (Fig. 4.3, 4.4).

The light scattering experiments revealed that cross-linked CT, but not untreated CT, can induce aggregation of small vesicles. In these experiments particles with diameters ~ 2-4 times the diameter of the pure SUVs were generated by cross-linked CT. The ratio of CT monomers to 35nm vesicles was estimated roughly to be ~ 25 using an average PG molecular area of 70Å² to determine the number of PG molecules/vesicle. In the future titrations of CT/vesicle should be performed to show that the proportion of the vesicle aggregate is a function of the CT/vesicle ratio, that is, the number of potential cross-bridges. Recently mitochondrial creatine kinase octomers were found to cross-link cardiolipin-containing vesicles (Stachowiak et al., 1996). In cells this enzyme is found at contact sites between inner and outer mitochondrial membrane. Perhaps CT also makes contacts between interfacing membranes within the cell.

The ability of covalently cross-linked CT dimers to cross-bridge vesicles has implications for the manner of association of the dimers. This finding strongly supports the antiparallel model (Fig. 4.8). It is difficult to envision how cross-
linked CT dimers would induce vesicle cross-linking if they are arranged "in parallel" (Fig. 4.8). The finding that only cross-linked CT induces vesicle aggregation supports our hypothesis that CT dimers (non-covalently associated) may dissociate to monomer after it binds to the membranes, and that only the monomer form of CT may be active.

In the GCT crystal structure, there are two loop-loop contacts. One of the loop-loop interactions is the HXGH loop. This motif was suggested as the CTP binding site (Veitch & Cornell, 1996; Veitch & Cornell 1997 unpublished). If the CTP binding site (HXGH) were involved in dimerization of CT, this site would not be available for CTP binding in the dimer form of CT. In the other words, dimerization of CT would block the CTP binding site leading to an inactive enzyme. Only when the dimer dissociates to the monomer would this site be available for CTP binding. Together all of these findings support the hypothesis that the dimeric form of CT may be an inactive enzyme, and that the activation of CT may be induced by dimer dissociation to monomer upon binding to the membrane.
CHAPTER FIVE: THE MODEL OF CT ACTIVITY

Our current data lead us to propose a new model for the regulation of CT. In this model, the soluble inactive form of CT is a dimer and / or trimer and the active, membrane-bound form is a monomer. The increased anionic phospholipid concentrations in a membrane promote binding of the dimer(Fig. 5.1; I, II). The CT dimer dissociates into monomers when both of the opposite M domains bind to the membranes (Fig. 5.1, III). The driving force for dimer dissociation is the stronger interaction of domain M with the membrane. Only the monomeric, membrane bound form is catalytically active. There are two ways that CT could be released from membrane: (1) By a decrease in the anionic lipid content of the membrane which destabilizes the helical conformation of domain M with loss of lipid affinity; (2) In addition, some dimeric forms may contact the membrane binding CT monomer and drawing it back into the aqueous phase (Fig. 5.1, IV, V). The second process would create CT trimer. Since the trimer form of CT was unstable comparing with the dimer form (In the experiment 3.1.5, we can see that there is more CT dimer than trimer.), two trimers may rearrange to form three dimers (Fig. 5.1, VI)). In this model, all steps are reversible.
Fig. 5.1 A proposed Model for the Activation of CT.
CHAPTER SIX: CONCLUSIONS

The results of our research have led us to six conclusions:

(1). The dimerization range located in the N-terminal domain of CT;

(2). CT may have stable tertiary interactions between the C-terminus and the N-terminus, because cleavage of the polypeptide at the hinge between the two domains did not cause complete dissociation;

(3). CT forms some trimer in the soluble (Triton-complexed) form;

(4). Since the N-terminal domain was cross-linked by Cu-PHT, there must be at least two cysteine residues present in the N-terminus of CT close to each other in the interface of CT dimer which covalently link the 2 monomers upon oxidation;

(5). Dimerization is not required for activity.

(6). CT is an antiparallel arranged "head to head" dimer, which, upon binding to apposing membranes, will dissociate into monomers. The dissociation of dimer to monomer may be required to generate the active form of the enzyme.
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