Substitution of Serine for Glycine-91 in the HXGH Motif of CTP:Phosphocholine Cytidylyltransferase Implicates This Motif in CTP Binding†

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ABSTRACT: The effect of mutations in the proposed catalytic domain of CTP:phosphocholine cytidylyltransferase was investigated by constructing the single mutants CT-S91 and CT-C114 from the double mutant CT-S91C114, previously shown to have 4-fold lower than wild-type activity [Walkey, C. R., Kalmar, G. B., & Cornell, R. B. (1994) *J. Biol. Chem. 269*, 5742−5749]. The constructs were overexpressed in COS cells. The mutation Gly-91 to Ser-91 was found to be responsible for the decreased activity, whereas Ser-114 to Cys-114 had no effect. An alanine substitution at position 91, CT-A91, had a lesser effect on cytidylyltransferase activity. CT-S91 and CT-WT were purified from COS cells, and their kinetic constants were determined. CT-S91 had a 4-fold lower V_{max}, and a K_m for CTP 25-fold higher than the wild-type enzyme, suggesting that substitution of Gly-91 with serine interferes with CTP binding. The K_m for phosphocholine was not affected in the CT-S91 mutant. There was no difference in the chymotrypsin sensitivities of CT-S91 and CT-WT, indicating that the mutation did not cause a global change in protein structure. However, the CT-S91 activity was more susceptible to inhibition by the denaturant urea than that of CT-WT, indicative of a perturbation of the active site folding. Gly-91 resides in the local sequence HSGH, which has been proposed to be a CTP-binding motif in the novel cytidylyltransferase superfamily [Bork, P., Holm, L., Koonin, E. V., & Sander, C. (1995) *Proteins: Struct., Funct., Genet. 22*, 259−266]. Our results represent the first experimental validation of this hypothesis.

Proteins have been proposed to belong to a limited set of superfamilies which include not only those proteins with sequence and functional similarity but also those with structural similarity (Orengo et al., 1994). Based on sequence homologies, it has been suggested that CTP: phosphocholine cytidylyltransferase (CT),[1] an important regulatory enzyme in phosphatidylcholine biosynthesis, is a member of a cytidylyltransferase superfamily which has a structural link to class I aminoacyl-tRNA synthetases (Bork et al., 1995). This superfamily contains a number of cytidylyltransferases as well as various other proteins that share the α−β phosphodiesterase function. A fundamental feature of this superfamily is the conserved motif His-X-Gly-His (HXGH), which is also characteristic of class I tRNA synthetases. The synthetase crystal structure with bound ATP indicates that the HXGH motif is a key participant in nucleotide binding, and the same motif has been proposed to mediate CTP binding in the cytidylyltransferase superfamily (Bork et al., 1995).

CT, which catalyzes the formation of CDP-choline from CTP and phosphocholine (Kent, 1990), undergoes an interconversion between an inactive, soluble form and an active, membrane-bound form (Vance, 1989; Tronchère et al., 1994). This interconversion is regulated by lipid binding and phosphorylation (Vance, 1989; Watkins & Kent, 1991). A tripartite structure was proposed for CT based on secondary structural predictions from the sequences of CT cDNA clones. This model consists of an N-terminal globular domain, an extended amphipathic helix, and a C-terminal region containing many turns (Craig et al., 1994; Cornell et al., 1995). Studies based on this model have identified and characterized the regulatory domains of CT. Phosphopeptide sequencing of CT overexpressed in insect cells has identified the C-terminal 53 amino acids as the phosphorylation domain. Residues 315−367 contain 16 serines, all of which were phosphorylated *in vivo* (MacDonald & Kent, 1994). The region from residues 238 to 299 (the amphipathic helix) corresponds to a discrete exon in the mouse CT gene (Rutherford et al., 1993), and there is strong evidence that this is the lipid-binding domain. After partial digestion with chymotrypsin, only those fragments of CT containing this region bound to activating lipid vesicles (Craig et al., 1994) and were photolabeled using a lipid soluble photoactivatable probe (J. Johnson, R. Aebersold, and R. Cornell, unpublished results). The C-terminal phosphorylation and lipid binding domains have been proposed to constitute the regulatory domain of CT (Cornell et al., 1995).

In contrast to the proposed regulatory domain, few studies have examined the N-terminal domain of CT, identified as a protease resistant 26 kDa fragment by chymotrypsin proteolysis (Craig et al., 1994). A polybasic sequence from residues 12−16 has been identified as the nuclear localization signal (Wang et al., 1995). The most highly conserved region of mammalian CTs is between residues 72 and 234 in which they share >99% identity. This conserved central

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[1] Abbreviations: CT, CTP:phosphocholine cytidylyltransferase; PC/OA, egg phosphatidylcholine/oleic acid (1:1); PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; PCR, polymerase chain reaction; CHO, chinese hamster ovary; CD, circular dichroism.

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domain also has 65% identity with CT from yeast (Tsukagoshi et al., 1987; Kalmar et al., 1994), 48% identity with CT from *Plasmodium falciparum* (Yeo et al., 1995), and 37% identity with residues 6−94 of *Bacillus subtilis* glycerol-3-phosphate cytidylyltransferase (Kalmar et al., 1994; Mauël et al., 1991). On the basis of this high degree of conservation, residues 72−235 have been proposed to be the catalytic domain of CT (Park et al., 1993; Kalmar et al., 1990). Recently, several mutational analyses have implicated the conserved domain in catalysis. Truncations at amino acids 228, 231, 236, or 239 resulted in catalytically active CT (Cornell et al., 1995; Wang & Kent, 1995; Yang & Jackowski, 1995; D. Veitch, unpublished results), although less than 10% of wild-type activity was found for truncations at 228, 231, or 239. A strain of CHO (CHO 58) contains a temperature-sensitive CT which has a 20-fold reduction in activity at the permissive temperature and 100-fold reduction in activity at the nonpermissive temperature (Esko et al., 1981; Sweitzer & Kent, 1994). This mutant CT contains a single mutation, Arg-140 → His-140, within the proposed catalytic domain (Sweitzer & Kent, 1994).

The first mammalian CT cDNA to be cloned (Kalmar et al., 1990) contained two mutations attributed to PCR error which resulted in two amino acid changes in the protein: Gly-91 to Ser-91 and Ser-114 to Cys-114 (MacDonald & Kent, 1993). Both mutations were in the proposed catalytic domain, and the double mutant had 4-fold lower activity than the wild-type enzyme when the cDNA was expressed in COS cells (Walkey et al., 1994). Several lines of evidence suggest that neither of the mutations at positions 91 nor 114 affect the membrane-binding properties. The double mutant was nearly identical to wild-type with respect to its distribution between soluble and membrane fractions in transfected COS cells. Translocation to membranes was inducible by oleic acid (Walkey et al., 1994). Moreover, the mutant enzyme was capable of binding a variety of lipid vesicles *in vitro*, and its activity was stimulated by these vesicles (Craig et al., 1994). In this paper we have constructed single mutants from the double mutant CT-S91C114 and have identified Gly-91 as being critical to CT activity. We have characterized the effect of mutations at this position by kinetic analysis, chymotrypsin proteolysis, and urea denaturation. The data suggest the involvement of Gly-91 in the binding of the substrate, CTP. Gly-91 resides within the HXGH motif proposed to participate in CTP binding in the cytidylyltransferase superfamily (Bork et al., 1995). Our results present the first positive test of that hypothesis.

MATERIALS AND METHODS

Materials. Restriction enzymes and Vent Polymerase were from New England Biolabs. Sequenase was from US Biochemicals. Dulbecco's Modified Eagle Medium and fetal calf serum were from Gibco BRL, and tissue culture supplies were from Corning. Hydroxyapatite was from Bio-Rad. Octyl glucoside, chymotrypsin (type II: from bovine pancreas), oleic acid, DTT, and CTP were from Sigma. [^{3}H]-Phosphocholine (10−15 nCi/nmol) was synthesized from [^{3}H]choline (Dupont-NEN) as described elsewhere (Cornell, 1989). Phosphatidylcholine (egg yolk) was from Avanti Polar Lipids. Electrophoresis reagents were molecular grade from Sigma or Gibco BRL. Urea and other reagents were analytical grade from British Drug House.

Construction of Mutants. CT-S91C114, the original rat liver CT clone, was constructed as described (Kalmar et al., 1990). It contains two mutations at nucleotides 271 and 340 of the coding region which result in two amino acid changes: Gly-91 to Ser-91 and Ser-114 to Cys-114. CT-WT is the clone of wild-type rat liver CT, constructed as described (Walkey et al., 1994). In this study, all mutants were inserted into the pAX142 vector, a derivative of pAX114 (Kay & Humphries, 1991) which uses the EF2 promoter in place of a CMV promoter. To generate the single mutants CT-S91 and CT-C114, CT-WT and CT-S91C114 were digested with *Sma*I at nucleotide 280 and *Cla*I in the pAX142 vector to yield 2919 and 1204 bp fragments. The 2919 bp fragment from CT-S91C114 was ligated with the 1204 bp fragment from CT-WT to form the CT-S91 construct, while the 1204 bp CT-S91C114 fragment was ligated with the 2919 bp WT-CT fragment to form the CT-C114 construct.

The CT-A91 construct containing the mutation Gly-91 to Ala-91 was generated by PCR amplification of a fragment between a vector-specific 5′ primer, 5′-CAAGCCTCA-GACAGTG-3′, and a CT-WT specific mutagenic primer, RC272G→C: 5′-CAGAGCCCGGGCATGA**G$_{272}$**CAGAGT-GAAAC-3′. The template was pAX142CT-WT. The PCR product was digested with *Mlu*I and *Sma*I, and the 324 bp fragment ligated with pAX142CT-WT in which the *Mlu*I/*Sma*I fragment had been removed. All mutant constructs were confirmed by DNA sequencing.

Transfection and Purification of CT from COS Cells. COS cells were transfected with wild-type and mutant cDNAs, and cytosols for initial characterization studies were prepared as previously described (Cornell et al., 1995). CT-WT and mutant enzymes were purified as described by MacDonald and Kent (1993) with some modifications. All buffers contained 0.2 mM PMSF but lacked the other protease inhibitors. Typically, COS cells from ten 15 cm plates were homogenized in 1 mL of homogenization buffer, and the soluble fraction was separated by centrifugation for 1 h at 100000*g*. CT was acid-precipitated from the cytosol after the addition of phosphatidylcholine/oleic acid (PC/OA; 1:2) vesicles as described by MacDonald and Kent (1993). The pellet was solubilized using 0.5 mL of octyl glucoside extraction buffer per mg of cytosolic protein. Chromatography over DEAE-Sepharose was bypassed by applying the octyl glucoside extract directly to a hydroxyapatite column (0.2 mL of hydroxyapatite per mg of cytosolic protein in a 1 cm diameter column) at a flow rate of 0.1 mL/min. The column was sequentially washed with five column volumes of buffers as described, at a flow-rate of 0.15 mL/min. Cytidylyltransferase activity was eluted in the final elution buffer containing 0.05% Triton X-100, and the active fractions were pooled, aliquoted, and stored at −85 °C. The purity of the enzymes was assessed by densitometry of SDS−polyacrylamide gels using a Microtek Scanmaker and NIH image software.

Assays and Electrophoresis. Cytidylyltransferase activity was assayed as described (Cornell, 1989) and expressed as nmol of CDP-choline formed min^{-1} mg^{-1}. Under standard assay conditions, the substrate concentrations were 3 mM CTP and 1 mM phosphocholine. These concentrations were varied in assays for kinetic parameters. All assays contained 0.2 mM PC/OA (1:1) sonicated vesicles. Protein concentration was determined by the Bradford method using ovalbu-

Table 1: Activities of Wild-Type CT and Double and Single CT Mutants Overexpressed in COS Cells[a]

enzyme	residue 91	residue 114	specific activity (nmol of CDP-choline min^{-1} mg^{-1})
CT-WT	G	S	114 ± 5
CT-S91C114	S	C	25 ± 4
CT-S91	S	S	24 ± 4
CT-C114	G	C	121 ± 3
CT-A91	A	S	79 ± 5

[a] COS cells were transfected with the indicated CT cDNAs. Cytosols were prepared for the assay of CT activity using 3 mM CTP and 1 mM phosphocholine for 30 min. The data represent the average of CT activities determined from three separate experiments ± standard deviations.

min as a standard (Bradford, 1976). Proteins were separated using 12% or 13% SDS−polyacrylamide gels (Laemmli, 1970) and stained with either Coomassie blue or silver using a fast, sensitive microwave staining protocol (D. Harrison, University of Victoria, Canada, personal communication)

Determination of Kinetic Constants from Secondary Plots. Kinetic constants were determined from secondary plots of the slopes and intercepts of double reciprocal plots versus the reciprocal of the phosphocholine concentration. The K_m for CTP (K_a) was obtained from the ratio of the *y*-intercept of the slope replot (K_a/V) to the y-intercept of the intercept replot ($1/V$). The K_m for phosphocholine (K_b) and the V_{max} (V) were obtained from the secondary plot of the intercepts: $1/K_b = K_b/V \cdot (1/b) + 1/V$, where b is the concentration of phosphocholine.

Chymotrypsin Digestion. Purified enzymes were digested with chymotrypsin in the presence of 2 mM PC/OA (1:1) vesicles at varying mass ratios of cytidylyltransferase/chymotrypsin for 10 min at 37 °C. The reaction was stopped by the addition of PMSF to a final concentration of 2 mM, and the samples were incubated 1 min further at 37 °C. Aliquots were removed for cytidylyltransferase assays in the presence of 2 mM PMSF and 0.2 mM PC/OA (1:1) vesicles. Laemmli sample buffer was immediately added to the remaining samples, and the proteins were separated by SDS−PAGE.

Urea Denaturation Curves. Purified enzymes were incubated with varying concentrations of urea for 10 min at 37 °C in the presence of 0.2 mM PC/OA (1:1) vesicles and the conditions for assay of CT activity. The cytidylyltransferase assay was started by the addition of phosphocholine.

RESULTS

Generation and Initial Characterization of the Single Mutants CT-S91 and CT-C114. The double mutant CT-S91C114 has 4−5-fold lower activity than CT-WT using the standard assay conditions (Walkey et al., 1994; Table 1). In order to determine which mutation in the double mutant CT-S91C114 was responsible for its observed lower activity, we constructed the single mutants CT-S91 and CT-C114. CT-S91 contained the mutation Gly-91 to Ser-91, and CT-C114 contained the mutation Ser-114 to Cys-114. Both constructs contained the correct residue at the other position. CT-S91, CT-C114, CT-S91C114, and CT-WT were overexpressed in COS cells, and their activities in the cytosols were compared using the substrate concentrations of a standard CT assay. Approximately equal expression was obtained in all cases as determined by densitometry of

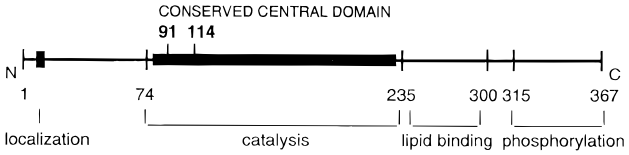

FIGURE 1: Schematic representation of the domain structure of mammalian CT. Indicated are the conserved central domain, which is proposed to function in catalysis, and the regulatory domain, consisting of the lipid binding and phosphorylation sites. The site of the nuclear localization signal and the positions of the Gly-91 to Ser-91 and Ser-114 to Cys-114 mutations are also shown.

Coomassie-stained SDS gels of cell homogenates (data not shown). CT-WT had a specific activity of 114 ± 5 nmol min^{-1} (mg of cytosolic protein)$^{-1}$, and CT-S91C114 activity was about 5-fold lower at 25 ± 4 nmol min^{-1} mg^{-1} (Table 1). There was no reduction in the specific activity of CT-C114 (121 ± 3 nmol min^{-1} mg^{-1}) from that of wild-type. However the specific activity of CT-S91, 24 ± 4 nmol min^{-1} mg^{-1}, corresponded to that of the double mutant. From these data, we conclude that the substitution of serine for glycine at position 91, and not the change from serine to cysteine at residue 114, is responsible for the reduced activity of the CT-S91C114.

Generation and Characterization of CT-A91. The above results suggest that a serine substitution at that position is detrimental to CT function and raised the possibility that Gly-91 is essential for catalytic function. To further probe the importance of residue 91 to CT catalysis, we substituted the glycine with an alanine at this position. We expressed the CT-A91 construct in COS cells, and found the specific activity of the mutant in the cytosol to be 79 ± 5 nmol min^{-1} mg^{-1}, a value intermediate between the activities of CT-WT with glycine and CT-S91 with a serine at residue 91 (Table 1). This suggests that the lower activity of CT-S91 is at least partially due to the specific substitution of the glycine with serine and that alanine is less disruptive than serine at this position.

Purification of Wild-Type and Mutant CTs from COS Cells. We purified the wild-type and mutant CTs for further study from overexpressed COS cells by using a purification scheme based on the method of MacDonald and Kent (1993). The scheme, which differs from that of MacDonald and Kent primarily in that it bypasses the DEAE column and applies the octyl glucoside extract directly on the hydroxyapatite column, resulted in a 19−28-fold purification with a 52−72% recovery of total activity from the COS cell cytosols. The enzymes appeared greater than 90% pure (as determined by densitometry) when analyzed by SDS−PAGE (Figure 2). This method compares favorably with the purification of CT from a baculovirus overexpression system in which a 35-fold purification with a 20% recovery of activity from the cytosol was reported (MacDonald & Kent, 1993). No difference was seen in the behavior of CT-WT and the mutants during purification, implying that their ionic and hydrophobic characteristics do not vary substantially. The purification of wild-type and mutant enzymes allowed us to investigate the effect of these mutations at position 91 on their kinetic properties.

Kinetic Analysis of Purified Enzymes. Kinetic constants were initially determined for purified CT-WT, CT-S91, and CT-A91 using double-reciprocal plots of initial velocity versus varying concentrations of one substrate at saturating

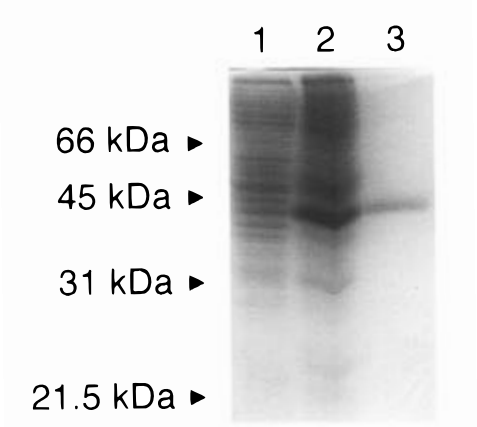

FIGURE 2: Purification of CT-S91 from COS cells transfected with CT-S91 cDNA. Protein samples from each purification step were run on a 12% SDS−polyacrylamide gel and stained with Coomassie Brilliant Blue. CT-WT and CT-A91 gave similar results. Lanes: (1) COS cell cytosol; (2) extract following octyl glucoside solubilization of PC/OA precipitate; (3) hydroxyapatite pool. The position of molecular weight standards is shown.

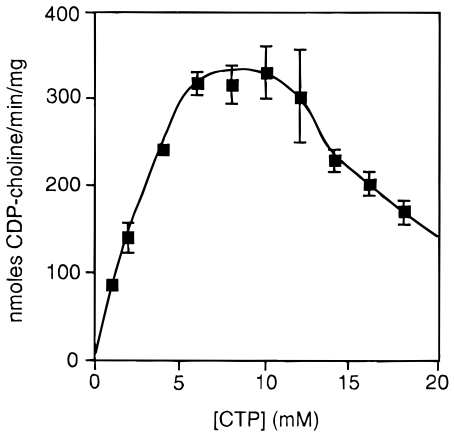

FIGURE 3: Substrate inhibition of CT-S91 by CTP. Purified CT-S91 was assayed (0.6 μg per assay) for CT activity for 10 min at 37 °C in the presence of increasing concentrations of CTP. The phosphocholine concentration in the assay was 1 mM. The data represent the average of a triplicate experiment ± standard deviation. Absence of visible error bars indicates an error within the limits of the symbol.

concentrations of the other. CT-WT and CT-A91 showed Michaelis-type behavior toward both CTP and phosphocholine over the concentration ranges used for kinetic analysis (0.2−3 mM CTP; 0.14−3 mM phosphocholine). Both CT-WT (data not shown) and CT-S91 (Figure 3) were inhibited by CTP at concentrations above 10 mM. In the case of CT-S91, the substrate inhibition occurred at concentrations near the K_m. CTP concentrations were chosen from the hyperbolic portion of the curve and used to determine an apparent K_m for CTP of 13.3 ± 0.3 mM, 20-fold higher than for CT-WT (0.63 ± 0.09 mM). The CT-A91 mutant had an apparent K_m for CTP 2.5-fold higher than wild-type at 1.6 ± 0.03 mM. An optimal concentration of CTP of 12 mM was used in the determination of the apparent phosphocholine K_m for the CT-S91 mutant. Both mutants showed an increase in the apparent K_ms for phosphocholine: 2.02 ± 0.15 mM for CT-S91 and 1.55 ± 0.19 mM for CT-A91 compared to 0.48 ± 0.06 mM for CT-WT.

The preliminary kinetic analysis suggested that both CT-S91 and CT-A91 have higher K_m values than wild-type for both substrates. The largest effect by far on the kinetic constants was that of CT-S91 on the K_m for CTP, and this change was further studied. Initial velocity measurements were made for CT-WT and CT-S91 at seven concentrations of CTP and six concentrations of phosphocholine, and the data were analyzed using double-reciprocal plots (Figure 4A,C). Kinetic constants were determined by secondary plots of the slopes and intercepts of the plots in 4A and 4C versus the reciprocal of the phosphocholine concentration (Figure 4B,D). Sets of nonparallel lines intersecting to the left of the *y* axis were obtained for both CT-WT and CT-S91, consistent with a random order sequential mechanism (Feldman & Weinhold, 1987; Park et al., 1993). All kinetic values reported are the average of two separate experiments ± the half range (Table 2). The CT-S91 mutant had a K_m for CTP of 8.6 ± 3.4 mM, 25-fold higher than the value for CT-WT of 0.34 ± 0.04 mM. The V_{max} for CT-S91 was 4-fold lower than for CT-WT at 840 ± 15 nmol min^{-1} mg^{-1} compared to 3180 ± 150 nmol min^{-1} mg^{-1}. However, the mutation at position 91 did not affect the K_m for phosphocholine. Values of 0.56 ± 0.07 mM for CT-WT and 0.52 ± 0.26 for CT-S91 were obtained from the intercept replot. Due to the low activity of CT-S91, it was difficult to obtain activity values at the low substrate concentrations. Although the error between the two sets of analysis for the serine mutant is large, there is a clear difference between the K_m values for CTP of CT-WT and CT-S91 (Table 2 and Figure 4). These kinetic studies suggest the involvement of Gly-91 in the binding of CTP and confirm that the catalytic domain of CT resides in the conserved central domain.

Chymotrypsin Proteolysis of CT-WT and CT-S91. To determine whether the defective CTP-binding of the CT-S91 mutant was caused by large structural changes in the protein, we used chymotrypsin proteolysis. Chymotrypsin proteolysis is a useful tool for examining the tertiary structure of proteins and has been used to identify tertiary structural features of enzymes such as GPI-specific phospholipase D (Li et al., 1995) and the α-subunit of RNA polymerase (Negishi et al., 1995). Limited chymotrypsin proteolysis of wild-type CT generates a characterized series of fragments, digesting sequentially from the C-terminus of the whole enzyme (42 kDa), via 39, 35, 30, and 28 kDa fragments, to a protease-resistant 26 kDa fragment corresponding to the N-terminal conserved domain (Craig et al., 1994; Cornell et al., 1995). The digestion also produces C-terminal low molecular mass fragments of 11 to 19 kDa[2] (Cornell et al., 1995; J. Johnson, R. Aebersold, and R. Cornell, unpublished results). A change in the chymotrypsin sensitivity or in the size of the digestion fragments might indicate a large change in protein structure. As different preparations of purified enzyme contained variable amounts of endogenous lipid, and chymotrypsin digestion of CT is sensitive to lipid concentrations (Craig et al., 1994), the chymotrypsin digestion of CT-WT and CT-S91 was compared in the presence of saturating lipid vesicles using different CT/chymotrypsin mass ratios. The addition of 2 mM PC/OA (1:1) lipid vesicles ensured that CT was bound to the vesicles and not to the Triton micelles in the buffer used to elute and stabilize purified CT (J. Johnson, R. Aebersold, and R. Cornell, unpublished results). No further proteolysis occurred after digestion was stopped with 2 mM PMSF (data not shown). Thus we were able to correlate activity with the fragments generated.

[2] The C-terminal fragment runs anomalously on SDS−PAGE, having a calculated molecular mass of 16.2 kDa.

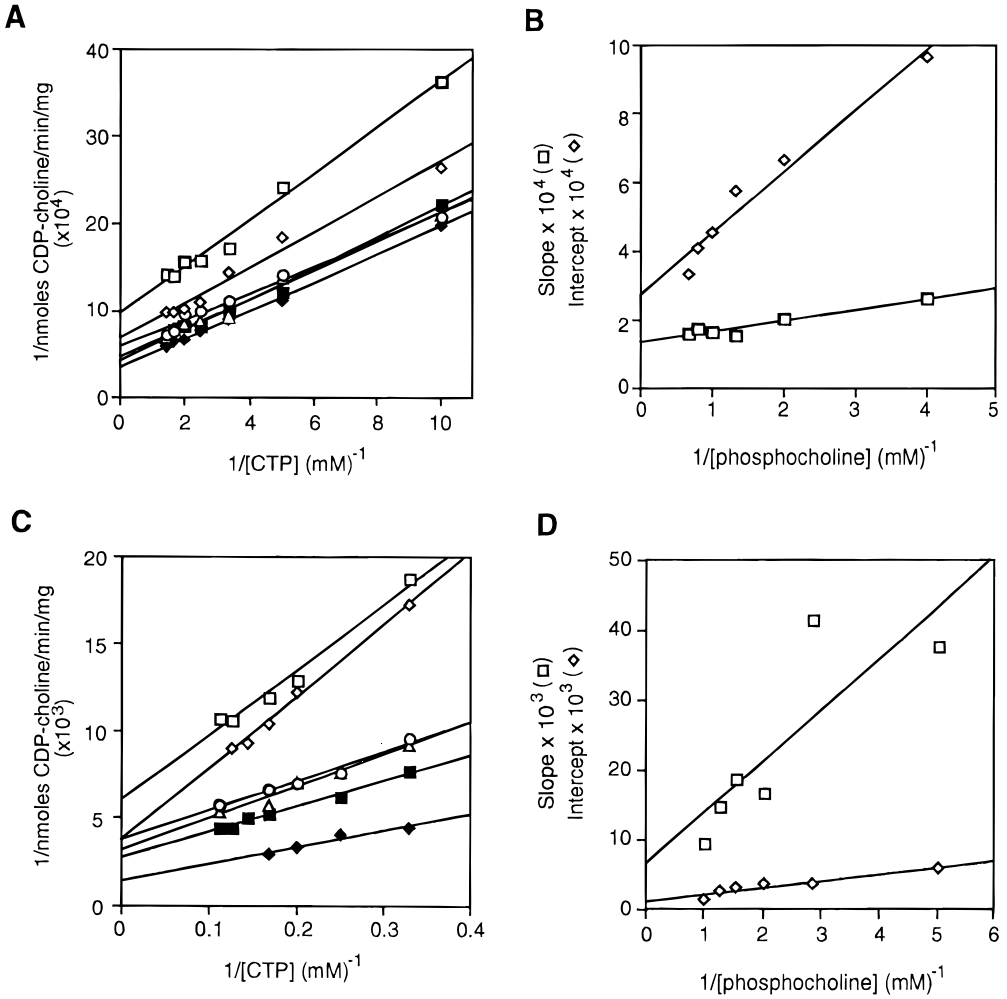

FIGURE 4: Initial rate analysis of CT-WT and CT-S91. CT activity was assayed for 10 min at 37 °C. (A) Double-reciprocal plot of CT-WT activity at variable CTP concentrations and a fixed phosphocholine concentration of 0.2 mM (□), 0.4 mM (◇), 0.6 mM (○), 0.8 mM (△), 1.0 mM (■), and 1.2 mM (◆). (B) Secondary plots of slopes (□) and intercepts (◇) from (A) versus the reciprocal phosphocholine concentrations. (C) Double-reciprocal plot of CT-S91 activity at variable CTP concentrations and a fixed phosphocholine concentration of 0.2 mM (□), 0.35 mM (◇), 0.5 mM (○), 0.65 mM (△), 0.8 mM (■), and 1.0 mM (◆). (D) Secondary plots of slopes (□) and intercepts (◇) from (C) versus the reciprocal phosphocholine concentrations. The line fit to the data was done by linear regression. The experiments were repeated with similar results.

Table 2: Kinetic Constants for CT-WT and CT-S91[a]

enzyme	expt.	K_m phosphocholine (mM)	K_m CTP (mM)	V_{max} (nmol of CDP-choline min^{-1} mg^{-1})
CT-WT	I	0.63	0.30	3330
	II	0.49	0.37	3030
	average	0.56	0.34	3180
CT-S91	III	0.26	12	850
	IV	0.78	5.2	825
	average	0.52	8.6	840

[a] V_{max}s and substrate K_ms for CT-WT and CT-S91 were determined from the secondary plots of double-reciprocal plots as shown in Figure 4B,D. Figure 4 plots the data from experiments I and IV.

There was no discernible difference in the chymotrypsin sensitivity or fragmentation pattern of CT-WT and CT-S91 (Figure 5). In either case, the 42 kDa parent molecule was digested to produce 39, 28, 26, and 19 kDa and one or more of the low molecular weight bands running near the dye front. Very little of the 35 and 30 kDa fragments were seen as these sites are protected from the protease by activating lipids (Craig et al., 1995). This suggests that the Gly-91 to Ser-91 substitution does not generate any large perturbation in the tertiary structure of CT. Activity appeared to correspond primarily to the 42 and 39 kDa bands which were completely digested at a CT/chymotrypsin mass ratio of 20:1 (Figure 5, lane 4). The protease-resistant 26 kDa fragment persisted at higher amounts of protease, and no activity was associated with this or the 28 kDa fragment in either enzyme. The complementary 19 kDa C-terminal fragment has been sequenced, and its N-terminus is at Thr-226 (J. Johnson, R. Aebersold, and R. Cornell, unpublished results) implying that the 26 kDa fragment terminates at Tyr-225 and thus lacks 10 residues of the conserved central domain. A CT truncation mutant at Lys-228 has less than 8% of wild-type activity (Cornell et al., 1995; Veitch & Cornell, 1994); thus it is not surprising that the 26 kDa fragment has no detectable activity.

Urea Denaturation of CT-WT and CT-S91. Differences in urea sensitivity can reflect the effect of mutations on the stability of a protein (Matouschek & Fersht, 1991). Urea decreases the ΔG for unfolding and has been proposed to denature proteins by perturbing protein−water interactions and/or by binding to the protein directly (Johnson & Fersht, 1995). We were unable to directly monitor the folding process of the mutant and wild-type CTs, because both must be purified in the presence of Triton X-100 which interferes

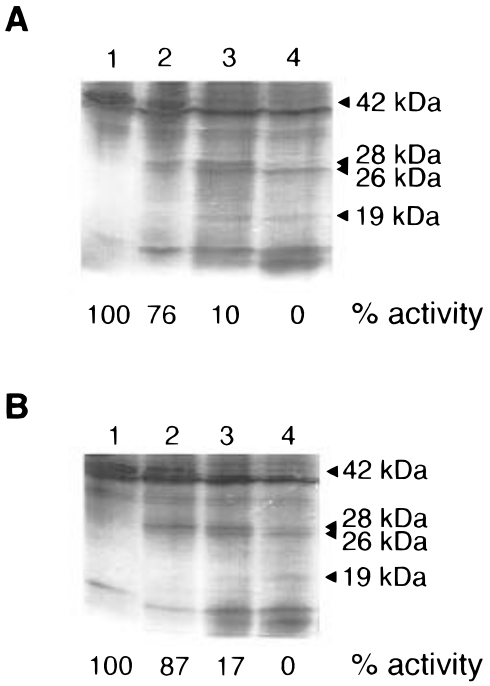

FIGURE 5: Chymotrypsin proteolysis of CT-WT and CT-S91. Purified CT-WT and CT-S91 were digested (3.75 μg/digestion) at 37 °C in the presence of 2 mM PC/OA vesicles at varying mass ratios of CT:chymotrypsin. The reaction was stopped with 2 mM PMSF at 10 min. A portion (0.375 μg) of each digested sample was removed and assayed for CT activity for 30 min in the presence of 2 mM PMSF and 0.2 mM PC/OA vesicles. Substrate concentrations for the CT assay were 3 mM CTP, 1 mM phosphocholine for CT-WT, and 12 mM CTP, 2 mM phosphocholine for CT-S91. The activity of each sample is expressed as percent of undigested activity. The remaining sample was electrophoresed on a 13% gel and silver stained. (A) CT-WT. (B) CT-S91. CT/chymotrypsin mass ratios: Lanes (1) no chymotrypsin; (2) 100:1; (3) 50:1; (4) 20:1. Bands at 40, 34 (doublet), and 17 kDa are artifacts of silver staining.

with most spectroscopy (fluorescence, CD). Therefore, we investigated the stability of the active sites of CT-WT and CT-S91 by comparing the effect of increasing urea concentration on their activities. If the Gly-91 to Ser-91 mutation had no effect on the folding of the active site, then the sensitivity of enzyme activity to urea denaturation would not be expected to change. The purified enzymes were subjected to urea denaturation in the presence of saturating PC/OA vesicle concentrations to circumvent the variations in endogenous lipid in the purified enzymes as described above. The CTP concentration was at the determined K_m values for either CT-WT or CT-S91. CT-S91 was more sensitive to urea than CT-WT (Figure 6): 50% of CT-S91 activity was lost at approximately 0.65 M urea compared with the approximately 1.3 M urea required for a 50% loss of CT-WT activity.

DISCUSSION

Both Gly-91 and Ser-114 are highly conserved in mammalian, *C. elegans*, yeast, and *Plasmodium* CTs, with Gly-91 also conserved in the *B. subtilis* cytidylyltransferase, implying that these residues might play an important role in CT function. Analysis of single mutants generated from the double mutant, CT-S91C114, indicated that Gly-91 is important for CT activity whereas the function of Ser-114 was not apparent. While substitution of serine for cysteine is conservative, there is no amino acid that can substitute

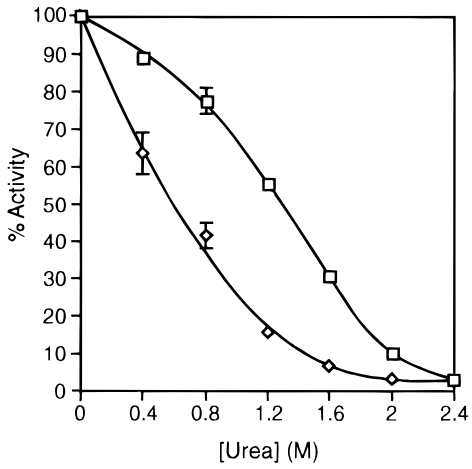

FIGURE 6: Urea denaturation of CT-WT and CT-S91. Purified CT-WT and CT-S91 (0.35 μg per assay) were incubated in CT assay mix in the presence of 0.2 mM PC/OA vesicles and increasing concentrations of urea for 10 min. CT activity was subsequently assayed for 30 min at 37 °C. Substrate concentrations for the CT assay were 0.34 mM CTP, 1 mM phosphocholine for CT-WT and 8.6 mM CTP, 1 mM phosphocholine for CT-S91. Activity for each enzyme is expressed as percent of activity with no urea. The data represent the average of two experiments (each in triplicate) ± half range. (□) CT-WT; (◇) CT-S91. Absence of visible error bars indicates an error within the limits of the symbol.

for glycine. Preliminary kinetic analysis suggested that substitution of either serine or alanine at position 91 affected CTP binding. Further analysis confirmed that Ser-91 increased the K_m for CTP 25-fold, reduced the V_{max} four-fold, but had no significant effect on the K_m for phosphocholine.

Mutations at Position 91 Perturb the Structure of the Active Site. The effect of a serine substitution at position 91 on the stability of the active site was examined by measuring the loss of catalytic activity during urea denaturation. The increased susceptibility of CT-S91 to urea denaturation compared to that of CT-WT suggests that this mutation perturbs the folded structure and probably reduces the number of stabilizing interactions at the active site. This perturbation did not lead to a global structural change since the chymotrypsin proteolysis sensitivity and fragmentation pattern were unaltered. Secondary structural predictions support the idea that a small change in structure may occur with this mutation. Gly-91, unlike Ser-91, is predicted by the method of Garnier to disrupt an α-helix, and the Chou−Fasman algorithm predicts Gly-91 to have a higher propensity for forming a turn than Ser-91. A small change in structure caused by the mutations at position 91 may be sufficient to explain the changes in kinetic constants. This is supported by the localization of Gly-91 in a conserved motif that participates in ATP binding in class I amino acyl tRNA synthetases.

Gly-91 Resides in HXGH, the Proposed CTP-Binding Motif. Gly-91 resides in the local sequence His_{89}-Ser-Gly-His_{92}, of which the glycine and both histidines are conserved in the mammalian, *C. elegans*, yeast, *Plasmodium*, and *B. subtilis* cytidylyltransferases. Serine is replaced with leucine in yeast and *Plasmodium* CTs and histidine in *C. elegans* CT. In addition, this HXGH motif lies in the most conserved region of the cytidylyltransferase superfamily which includes enzymes that cleave the α−β phosphodiester bonds in either CTP or ATP and have NMP transferase activities (Bork et

al., 1995). A similar motif, His-Ile-Gly-His (HIGH), has been shown to be involved in ATP binding in class I amino acyl tRNA synthetases (Moras, 1992). In this motif, both histidines and the glycine are invariant, while the isoleucine can be replaced by leucine, valine, or methionine (Moras, 1992). Mutations which replaced the first histidine with asparagine, glutamine, or glycine, or the last histidine with asparagine or glycine in the HIGH motif of tyrosyl tRNA synthetase, all destabilized ATP binding (Jones et al., 1986; Leatherbarrow & Fersht, 1987; Wells et al., 1991). Mutation of the HIGH motif glycine to alanine in this enzyme resulted in a ~5-fold decrease in activity. Preliminary analysis of crystals of this mutant suggested that the alanine methyl group blocked access of the adenine ring to the peptide backbone at this site (Brown et al., 1986).

The crystal structure of the *Escherichia coli* glutaminyl tRNA synthetase (Perona et al., 1993) demonstrated that this motif ($H_{40}IGH_{43}$) is involved in binding the ATP phosphates in the catalytic cleft of the enzyme. Specificity for binding the adenine ring is conferred through contacts elsewhere in the protein. In the glutaminyl enzyme, both histidines are oriented such that the protonated N of the imidazole ring forms contacts with the ATP phosphates: His-40 hydrogen bonds via a water molecule to the β-phosphate oxygen. The distance between the His-43 imide hydrogen and the α-phosphate oxygen is too great to permit a hydrogen bond in its ground state tetrahedral geometry (Perona et al., 1993). However, Perona et al. (1993) propose that, in the trigonal-bipyramidal geometry of the transition state, the distance is shortened providing hydrogen bonding stabilization by the His-43 imidazole hydrogen. Gly-42 is vital for two reasons: (1) to form a turn that allows the histidines to orient correctly, and (2) to allow the adenine ring to rest on the polypeptide backbone. Substitution of any other residue in this position could perturb the structure, disrupting the hydrogen bonding, and sterically interfering with the positioning of the adenine ring (Perona et al., 1993).

The HXGH motif has been proposed to fulfill a similar function in the cytidylyltransferase superfamily. A three-dimensional model of the *Bacillus* glycerol phosphate CT has been developed using type I tRNA synthetases as a template for aligning predicted secondary structures. This model positions the HXGH motif in a loop between a β-strand and an α-helix, in a $\beta-\alpha-\beta$ structure which represents a variation of the classic nucleotide binding fold (Bork et al., 1995). As protein superfamilies often show a general similarity of folding, especially in conserved regions (Orengo et al., 1994), it is plausible that rat liver CT has a similar nucleotide binding structure. Our results suggest that this may be the case and can be explained in this context.

The wild-type residue, glycine, with no side chain and a symmetrical C_α, is able to assume a broad range of conformations and is especially important in turns and hinge regions (Richardson & Richardson, 1989). As discussed, glycine may be instrumental in forming a turn in the HXGH sequence of CT and allowing contacts to be made with the nucleotide phosphates. Both serine and alanine are more conformationally constrained due to their side chains. The hydroxyl side chain of serine may interfere more so than alanine with the cytidine ring resting on the peptide backbone at this position. The CT-S91 mutant had a 4-fold decrease in V_{max}. This reduction in catalysis may be caused by a small displacement of His-43, which would no longer be able to participate via hydrogen bonding in the stabilization of the transition state geometry of the α-phosphate. Mutations of His-89 and His-92 in CT and analysis of their functional consequences will provide further tests of the role of the HXGH sequence in CTP binding and the stabilization of the transition state.

The Conserved Central Domain Contains the Active Site of CT. There is now strong evidence that CT consists of structurally distinct regulatory and catalytic domains. Our data support the model of CT structure in which the active site resides within the conserved central domain, and this conclusion is supported by studies of C-terminal truncation mutants. The removal of parts or all of the proposed regulatory domain result in an enzyme with full or partial activity, depending on the position of the truncation. Truncation mutants that terminate between residues 311 and 314, and that lack the entire phosphorylation domain but contain the lipid binding domain, have been reported to have wild-type activity or greater (Cornell et al., 1995; Wang & Kent, 1995; Yang & Jackowski, 1995). Moreover, a truncation mutant at residue 236, lacking the entire putative regulatory domain and containing the entire conserved domain, also had wild-type activity and was able to support PC synthesis upon expression in CT-deficient CHO 58 cells (Wang & Kent, 1995). In contrast, truncations at residues 228, 231, 239, and 257 result in partially active enzymes with only 3% to 17% of wild-type activity (Cornell et al., 1995; Veitch & Cornell, 1994; Yang et al., 1995; Veitch and Cornell, unpublished results). The chymotrypsin generated 26 kDa fragment ending at residue 225 had no detectable activity. Differences in the activities of similar truncation mutants have yet to be reconciled. These may reflect in part the differences in preparations from the various expression systems employed, as none of the truncation mutants have yet been purified. However, these results provide evidence that the conserved central domain contains the active site of CT and suggest that the C-terminal end of the conserved central domain is necessary for full activity although it is not yet clear where the exact C-terminal boundary of the catalytic domain lies. An N-terminal deletion ($\Delta 2-32$) was as active as the wild-type enzyme (Wang et al., 1995). Additional N-terminal deletions will be useful to determine the N-terminal boundary of the catalytic domain.

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