Determination of Subunit Dissociation Constants in Native and Inactivated CTP Synthetase by Sedimentation Equilibrium

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ABSTRACT: Sedimentation equilibrium was used to correlate changes in aggregation state with active site modification of *Escherichia coli* CTP synthetase. The native enzyme equilibrated between monomers, dimers, and tetramers in the absence of substrates. At enzyme concentrations above 5 μM, tetramers represented 40% of the species in solution. Inactivation by 6-diazo-5-oxonorleucine (DON) or thiourea dioxide reduced the amount of tetramer to below detectable limits. However, inactivated enzyme still equilibrated between monomers and dimers. Simultaneous analysis of multispeed data at three protein concentrations yielded estimates of the dissociation constants for the monomer−dimer and dimer−tetramer equilibria. For multiple data sets of native enzyme, $K_{1,2}$ was between 1 and 2 μM, and $K_{2,4}$ was between 1 and 18 μM. For DON inactivated enzyme, $K_{1,2}$ was 3−4 μM, and for thiourea dioxide inactivated enzyme, $K_{1,2}$ was $\approx$1 μM. The values for $K_{1,2}$ are consistent with previously published studies by gel filtration, demonstrating that the enzyme dissociates to monomers in very dilute solution (Anderson, 1983). However, the sedimentation equilibrium experiments are the first to show that the enzyme forms tetramers in the absence of nucleotides. This result implies the presence of stable conformations in the native enzyme capable of dynamic equilibrium between monomers, dimers, and tetramers. The results presented here illustrate the sensitivity of sedimentation equilibrium for measuring the aggregation state of equilibrating enzyme species and demonstrate that active site modifications disrupt the quaternary structure of CTP synthetase.

Cytidine triphosphate synthetase catalyzes the conversion of UTP[1] to CTP in the presence of L-glutamine, Mg^{2+}, ATP, UTP, and GTP (Koshland & Levitzki, 1974). The enzyme contains a glutamine amide transfer domain fused to the C-terminus of a synthetase domain (Weng et al., 1986). The glutamine amide transfer domain catalyzes the conversion of glutamine to glutamate and NH_3 and requires GTP for allosteric activation. Ammonia produced by the amidotransferase activity then participates in the ATP-dependent conversion of UTP to CTP carried out by the synthetase domain. The enzyme carries out the terminal reaction in the *de novo* synthesis of pyrimidine nucleotides and provides precursor for RNA, DNA, phospholipids, and sialoglycoproteins. Rat and human tumor cells both exhibit increased CTP synthetase activity (Kizaki et al., 1980), and two inhibitors with anti-neoplastic activity, 3-deazauridine (McPartland et al., 1974) and cyclopentenylcytosine triphosphate (Kang et al., 1989), are known to inhibit CTP synthetase. Cyclopentenylcytosine is in phase I clinical trials (Politi et al., 1994).

The enzyme from *Escherichia coli* has been cloned and overexpressed as a single polypeptide chain of M_r 60 438 (Weng et al., 1986). The human (Yamauchi et al., 1990) and yeast (Ozier-Kalogeropoulos et al., 1994) enzymes also have been cloned. The yeast enzyme has been expressed and purified to homogeneity (Yang et al., 1994). Studies have shown that the *E. coli* enzyme displays positive cooperativity as a function of varied ATP and UTP (Long & Pardee, 1967; Levitzki & Koshland, 1972b) and negative cooperativity as a function of varied GTP (Levitzki & Koshland, 1972a). Additional studies have shown that it is a dimer under native conditions and that dimers associate to tetramers in the presence of ATP and UTP (Long et al., 1970; Anderson, 1983). In very dilute solutions, dimers dissociate to monomers (Anderson, 1983). The self-association properties of the enzyme are responsible for its cooperative behavior (Long & Pardee, 1967; Levitzki & Koshland, 1972b), and the general sequential model of cooperativity has been proposed to explain the various cooperative interactions in CTP synthetase (Koshland, 1970). Similar cooperativity has been observed in enzyme from rat liver (Thomas et al., 1988), Ehrlich ascites cells (Kizaki et al., 1981), and bovine liver in the presence of CTP (McPartland & Weinfeld, 1979), although early studies of bovine liver enzyme did not observe cooperative behavior in the absence of the end product CTP (Savage & Weinfeld, 1970).

The first study describing the quaternary structure of *E. coli* CTP synthetase used a combination of sedimentation equilibrium, sedimentation velocity, and gel filtration to characterize the native and denatured states of the enzyme (Long et al., 1970). Native CTP synthetase was reported to have a molecular weight of 105 000 by sedimentation equilibrium. By sedimentation velocity under denaturing conditions and by comparison to standards run under the same conditions, the monomer was suggested to have a molecular weight close to that of ovalbumin, or 45 000. Consequently, the enzyme was considered to be a dimer

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[1] Abbreviations: CTP, cytidine 5′-triphosphate; ATP, adenosine 5′-triphosphate; UTP, uridine 5′-triphosphate; DON, 6-diazo-5-oxonorleucine.

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under native conditions. Sedimentation velocity under native conditions gave an $s_{20,w}$ of 4.83. This was interpreted as evidence of an elongated shape in the dimer based on comparisons to $s_{20,w}$ values of other globular proteins of 100 000 molecular weight. Gel filtration on Sephadex G200 and G150 gave results consistent with a dimer in the native state. Additional gel filtration experiments in the presence of ATP and UTP showed that the enzyme is a tetramer in the presence of nucleotides (Long et al., 1970). All of these results were obtained before the *E. coli* enzyme was cloned and found to have a monomer weight of 60 438 (Weng et al., 1986).

Studies with bovine liver (McPartland & Weinfeld, 1976) and rat liver (Thomas et al., 1988) enzyme have shown that the mammalian enzyme also undergoes nucleotide-dependent polymerization. Sucrose gradient sedimentation has shown that bovine enzyme has sedimentation coefficients of 6.8 and 10.1 in the absence and presence of nucleotides, respectively. Gel filtration has shown that purified rat liver enzyme has molecular weights of 120 000 and 240 000, corresponding to dimers and tetramers, in the absence and presence of nucleotides, respectively. Gel filtration and detection by radiochemical assay have demonstrated the presence of dimers and small amounts of monomers and tetramers in rat liver cytosolic extracts, which have been presumed to contain endogenous concentrations of nucleotides (Thomas et al., 1988).

Generally, gel filtration has been used to assess the aggregation state of CTP synthetase under various conditions. For instance, the enzyme undergoes concentration-dependent hysteresis (Anderson, 1983), and it has been shown by gel filtration that this is related to enzyme polymerization (Anderson, 1983). At very dilute concentrations (7 μg/mL) and at 4 °C, gel filtration experiments demonstrate the presence of monomers. At 26 °C, increasing the protein concentration from 2.2 to 1050 μg/mL shifts the elution profile toward dimers. These studies are the basis for suggesting that the monomer−dimer equilibrium favors dimerization except at very low protein concentrations. However, quantitation of the monomer−dimer−tetramer equilibria has not been reported, and the primary equilibrium reaction has been thought to be equilibration between dimers and tetramers in the presence of nucleotides. No evidence has been presented that the enzyme forms tetramers in the absence of nucleotides.

In addition to its polymerizing and cooperative properties, CTP synthetase also displays half-of-sites reactivity (Levitzki et al., 1971). Active site labeling studies with the glutamine analog DON have shown that the analog covalently modifies the active site of the amidotransferase domain, but only modifies the enzyme to a final stoichiometry of 0.5 mol/mol of CTP synthetase monomer. This has been taken as evidence that the active site on one monomer must release glutamate before the active site on an adjacent monomer can bind glutamine and proceed with catalysis. However, DON-modified enzyme will still bind ATP and UTP, and DON-modified enzyme will catalyze the overall reaction with ammonia. The aggregation state of the DON-modified enzyme has been suggested to be a dimer by gel filtration studies (Long et al., 1970).

Other studies have shown that lysine modification by thiourea dioxide leads to complete inactivation of CTP synthetase (Robertson et al., 1992) and that CTP synthetase incorporates 3 mol of [^{14}C]thiourea dioxide/mol of CTP synthetase monomer. Substrate protection experiments have suggested that lysine modification occurs at the active site. Additional fluorescence experiments have suggested that the protein undergoes a structural change during modification. However, the aggregation state of the thiourea dioxide-modified enzyme also has not been determined.

Studies by sedimentation equilibrium provide a very well defined thermodynamic approach for understanding the properties of macromolecules in dilute solution (Yphantis, 1964). The technique does not rely on any external molecular weight calibrations and can provide quantitative measures of the binding interactions in associating systems. Previous studies have only presented qualitative measures of the binding interactions in CTP synthetase. The studies presented here were performed to obtain binding constants for the monomer−dimer and dimer−tetramer equilibria in CTP synthetase and to determine the aggregation state of the enzyme after modification by DON and thiourea dioxide.

MATERIALS AND METHODS

Reagents. Thiourea dioxide, DON, ATP, UTP, $MgCl_2$, dithiothreitol, BisTris propane, and EDTA were from Sigma. All other reagent grade chemicals were from Sigma.

Purification of CTP Synthetase. Enzyme was purified as described previously (Lewis & Villafranca, 1989). Enzyme used in these studies had a specific activity of 6−8 μmol of CTP min^{-1} mg^{-1}. Protein concentrations were determined by measuring the absorbance at 280 nm and using the previously determined extinction coefficient of $\epsilon^{0.1\%}_{280nm} = 0.89$ (Lewis & Villafranca, 1989; Levitzki & Koshland, 1972b). Purified enzyme was stored at −80 °C in 50 mM BisTris propane, pH 8.0, 0.5 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, 1 mM UTP, 20 mM $MgCl_2$, and 15% glycerol, at a final protein concentration of 0.5 mg/mL. Prior to use, aliquots of enzyme were dialyzed against 1 L of 50 mM BisTris propane, pH 8.0, and 1 mM dithiothreitol. A M_r of 60 438 was used for calculations of protein concentration. Enzyme was assayed under previously described conditions (Lewis & Villafranca, 1989), and the conversion of UTP to CTP was monitored at 291 nm in a Cary 3E spectrophotometer.

Inactivation of CTP Synthetase with Thiourea Dioxide and DON. Enzyme was incubated at 37 °C in 50 mM BisTris propane, pH 8.0, 100 mM NaCl, and 1 mM EDTA, at a final concentration of 0.72 mg/mL, for 60 min. Inactivation reactions contained either 2 mM DON or 10 mM thiourea dioxide. Aliquots of the inactivation reaction were removed at timed intervals, and CTP synthetase activity was measured. After 5 min, enzyme incubated with DON was >98% inactive. After 60 min, enzyme incubated with thiourea dioxide was >97% inactive. Inactivated enzyme samples were dialyzed exhaustively against three changes of 50 mM BisTris propane, pH 8.0, 100 mM NaCl, and 1 mM EDTA. The dialyzed samples then were used for analytical ultracentrifugation.

Analytical Ultracentrifugation. Enzyme was diluted with final dialysate to the concentrations shown in Tables 1−5. Analytical ultracentrifugation was performed in a Beckman XL-A using an An 60 Ti rotor and three sample cells with two channel centerpieces. Native CTP synthetase was permitted to come to equilibrium at three speeds and three

concentrations in a single run. Thiourea- and DON-modified samples were allowed to reach equilibrium at three speeds and three concentrations in separate runs. Scans were obtained at 280 nm with a step size of 0.001, and 99 scans were averaged. Samples were allowed to equilibrate for 48 h at 8000 rpm, and duplicate scans 2 h apart were overlaid to determine that there were no further changes in the sample cell. Data were collected at 8000 rpm, and then the sample was equilibrated at 12 000 rpm for the second data set and 18 000 rpm for the third data set. Data were fitted to the model for a single homogeneous species (1) or an nmer of a single species (2)

$$c_r = c_m \exp[M(1 - v\varrho)\omega^2(r^2 - r_m^2)/2RT] \quad (1)$$

$$c_r = c_{nmer} \exp[nM(1 - v\varrho)\omega^2(r^2 - r_m^2)/2RT] \quad (2)$$

where c_r is the concentration of the species at radial position r, c_m is the concentration of the species at the meniscus, n is the nmer value at fixed M, M is the weight-average molecular weight, v is the partial specific volume, ϱ is the solvent density, ω is the angular velocity, r is the radial distance from the center of rotation, r_m is the distance from the center of rotation to the meniscus, R is the gas constant, and T is the absolute temperature in kelvin. All runs were done at 4 °C. A value of 1.0017 was used for the solvent density. The partial specific volume of CTP synthetase was calculated by the method of Cohn and Edsall using partial specific volumes of amino acids as previously presented (Laue et al., 1992). The value for CTP synthetase was 0.7409. When M was fixed for fitting to eq 2, a value of 60 438 was used. Data analysis software running under Igor (Wavemetrics, Lake Oswego, OR) and incorporating a previously published nonlinear fitting algorithm (Johnson et al., 1981) was a generous gift from Dr. Preston Hensley at Smith Kline Beecham in King of Prussia, PA.

Additional data analysis was performed with the HID program from the Analytical Ultracentrifugation Facility at the University of Connecticut. Data for native and inactivated enzyme were analyzed with and without the second virial coefficient to test for nonideality. Fitted values of the second virial coefficient were <0.001, and inclusion of the second virial coefficient gave no significant improvements in the sum of the residuals squared or the square root of the variance. This is an indication that any nonideality in the system could not be detected. Therefore, the system was assumed to be ideal for all the analyses presented here.

For a monomer−dimer−tetramer equilibrium where species M is a monomer, species D is a dimer, and species T is a tetramer

$$M \overset{K_{1,2}}{\rightleftharpoons} D \overset{K_{2,4}}{\rightleftharpoons} T$$

data were fitted to the equation

$$c_r = c_{monomer} \exp[M\delta] + c_{dimer} \exp[2M\delta] + c_{tetramer} \exp[4M\delta] \quad (3)$$

where $\delta = (1 - v\varrho)\omega^2(r^2 - r_m^2)/2RT$ and $c_{monomer}$, c_{dimer}, and $c_{tetramer}$ are the concentrations of monomer, dimer, and tetramer, respectively. For model fitting to various equilibria, $c_{monomer}$, c_{dimer}, and $c_{tetramer}$ were either fixed or allowed to float, or fixed at values that eliminated any contribution from monomers or tetramers. Multispeed analysis of data at a given protein concentration was used to obtain the dissociation constants for the monomer−dimer and dimer−tetramer equilibria. From the general expression

$$K_d = (c_{mon})^n/(c_{nmer}) \quad (4)$$

and the various equilibria

$$M + M \overset{K_{1,2}}{\rightleftharpoons} D \qquad D + D \overset{K_{2,4}}{\rightleftharpoons} T$$

the expressions for monomer−dimer and dimer−tetramer dissociation constants can be written as

$$K_{1,2} = (c_{mon})^2/(c_{dimer}) \quad (5)$$

$$K_{2,4} = (c_{dimer})^2/(c_{tetramer}) \quad (6)$$

Rearranging (5) and (6) gives

$$c_{dimer} = (c_{mon})^2/K_{1,2} \quad (7)$$

$$c_{tetramer} = (c_{dimer})^2/K_{2,4} \quad (8)$$

and substitution of (7) in (8) gives

$$c_{tetramer} = [(c_{mon})^2/K_{1,2}]^2/K_{2,4} \quad (9)$$

Expressions 7 and 9 were substituted into eq 3 to yield an equation describing the model for a monomer−dimer−tetramer system

$$c_r = c_{mon} \exp[M\delta] + [(c_{mon})^2/(K_{1,2})] \exp[2M\delta] + \{[(c_{mon})^2/(K_{1,2})]^2/(K_{2,4})\} \exp[4M\delta] \quad (10)$$

where $\delta = (1 - v\varrho)\omega^2(r^2 - r_m^2)/2RT$. Merged data sets were fitted to eq 10 to obtain the dissociation constants $K_{1,2}$ and $K_{2,4}$.

RESULTS

Sedimentation Equilibrium of Native CTP Synthetase. Enzyme was sedimented to equilibrium at 1.5, 3, and 4.5 μM CTP synthetase and at three different speeds to measure the degree of self-association under noncatalytic conditions. Centrifuge speeds and protein concentrations were chosen to obtain equilibrium distributions over a range of absorbances from 0.005 to 0.975 at 280 nm, which corresponds to a range of protein concentrations from 92 nM to 18 μM. Assuming a single homogeneous species, data were fitted to eq 1 to determine the best fitting molecular weight as a function of speed and initial loading concentration. Each data set then was fitted to eq 2 with the molecular weight fixed at 60 438 to determine the best fitting nmer value. Table 1 presents the molecular weight averages and nmer values for each data set, and Figure 1 illustrates a representative data set obtained at the 3 μM loading concentration. Similar data were obtained at 1.5 and 4.5 μM loading concentrations.

For a single species, the molecular weight averages ranged from 120 000 to 151 000, and the nmer values ranged from 2.0 to 2.5. The data demonstrate that CTP synthetase self-associates to a higher order species approximating a dimer at 4 °C. Analysis as a single monomeric, trimeric, tetrameric, or pentameric species did not fit the data. This is generally

Table 1: Concentration and Speed Dependence of Apparent Molecular Weight Averages of Native CTP Synthetase

speed (rpm)	wave-length (nm)	temp (°C)	[protein][a]	M_r[b]	χ^2	nmer[c]	χ^2
8 000	280	4	1.5 μM	151 300 ± 9800	25	2.5 ± 7%	25
8 000	280	4	3.0 μM	141 800 ± 4400	27	2.3 ± 3%	27
8 000	280	4	4.5 μM	146 900 ± 5400	15	2.4 ± 4%	15
12 000	280	4	1.5 μM	137 900 ± 4400	41	2.3 ± 3%	41
12 000	280	4	3.0 μM	134 700 ± 2800	105	2.2 ± 2%	105
12 000	280	4	4.5 μM	129 600 ± 2200	157	2.1 ± 2%	157
16 000	280	4	1.5 μM	127 400 ± 3100	44	2.1 ± 2%	44
16 000	280	4	3.0 μM	129 100 ± 2900	264	2.1 ± 2%	264
16 000	280	4	4.5 μM	120 100 ± 1700	149	2.0 ± 1%	149
16 000	230	4	86 nM	93 500 ± 5500	6.4	1.5 ± 6%	6.5
16 000	230	4	129 nM	89 800 ± 5700	7.0	1.5 ± 6%	7.0
20 000	230	4	86 nM	73 100 ± 5100	5.0	1.2 ± 7%	5.0
20 000	230	4	129 nM	85 200 ± 900	6.2	1.5 ± 1%	6.2

[a] Concentration of protein loaded in centrifuge cells at the beginning of the experiment. [b] Molecular weights were obtained by fitting to eq 1 and are rounded to the nearest hundred. The error estimate represents the 65% confidence interval. [c] Values for the nmer at each protein concentration and speed were obtained by fitting to eq 2 with the molecular weight fixed at 60 438. The error estimate represents the 65% confidence interval.

consistent with previous data demonstrating that the enzyme is a dimer in the absence of substrates (Long et al., 1970). However, the fits to a single species all displayed some asymmetry in the residuals, as shown in Figure 1, and resulted in a distribution of molecular weights as a function of rotor speed. These variations are consistent with the idea that an equilibrium of more than one species exists under these conditions.

On the basis of the data in Table 1 showing that CTP synthetase approximated a dimer at the 1.5, 3, and 4.5 μM loading concentrations, a second experiment was performed at very dilute loading concentrations to determine whether or not complete dissociation occurs at nanomolar concentrations of enzyme. Loading concentrations of 89 and 129 nM were sedimented to equilibrium at 16 000 and 20 000 rpm, and the protein distribution was determined by monitoring the peptide backbone at 230 nm. Table 1 lists the calculated molecular weight averages and nmer values obtained under these conditions.

In contrast to the higher loading concentrations, the calculated molecular weight averages were intermediate between monomeric and dimeric species. This is additional evidence for an equilibrating system and demonstrates that significant dissociation of dimers can be observed as a function of protein concentration under native conditions. The absorbance values ranged from 0 to 0.25 for the protein distributions at 230 nm, with standard errors of similar magnitude to the 280 nm data in Table 1. The lower χ^2 values in Table 1 are due to the same relative error applied to smaller absolute values.

Species Analysis of Native CTP Synthetase. As a consequence of the results in Table 1, the data were systematically analyzed for the presence of multiple equilibria. Table 2 presents the results of this analysis. Initially, as a reference point, data were fitted to a single monomeric or single dimeric species by fixing the molecular weight at 60 438 in eq 2 and by fixing the n value as either 1 or 2. The large χ^2 values for the fits to a monomeric species rule out the presence of a single monomeric species. In contrast, the fits

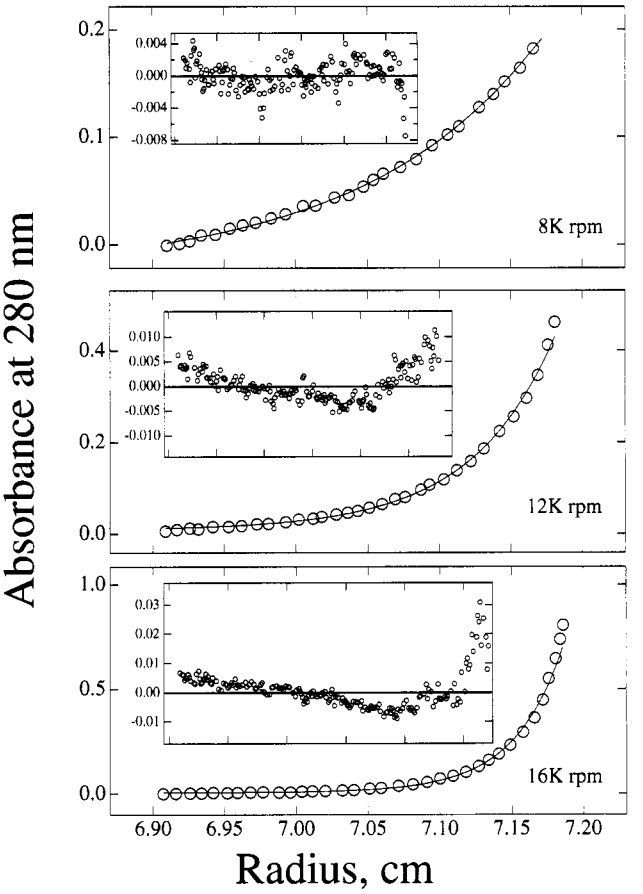

FIGURE 1: Sedimentation equilibrium of CTP synthetase at multiple rotor speeds. Native enzyme was allowed to reach equilibrium at three rotor speeds as described under Materials and Methods. The loading concentration was 3 μM for the data shown above. The figure only shows every fifth data point for clarity. The data at each speed were fitted to eq 1 with M as the fitted parameter, and the lines through the data represent the best fits with M = 141 800, M = 134 700, and M = 129 100 for the data at 8000, 12 000, and 16 000 rpm. Similar results were obtained with loading concentrations of 1.5 and 4.5 μM. Table 1 presents the best fits for all loading concentrations.

to a single dimeric species had much lower χ^2 values, although the residuals to these fits were all asymmetric. The asymmetric residuals indicated that the data cannot be described as a single species.

Consequently, the data then were fitted to a single-equilibrium two-species model. Data were fitted to eq 3 with the molecular weight fixed at 60 438 and either the tetramer or monomer term fixed at a vanishingly low value to reduce the equation to a single equilibrium of monomer−dimer or dimer−tetramer. For a single equilibrium at loading concentrations between 1.5 and 4.5 μM, the analysis would not converge on a fit to the monomer−dimer model. However, fitting to the dimer−tetramer model improved all the χ^2 values significantly, as compared to a single monomeric species or single dimeric species. This is consistent with the idea that, under native conditions at concentrations higher than 1 μM, the enzyme distributes between significant amounts of dimers and tetramers. In contrast, at the low nanomolar loading concentrations, the χ^2 values were more consistent with a monomer−dimer model than a dimer−tetramer model. This demonstrates a concentration-dependent shift in the equilibrium population of species.

Table 2: Species Analysis of Native CTP Synthetase

speed (rpm)	wavelength (nm)	temp (°C)	[protein][a]	χ^2 1[b]	2[b]	1 ↔ 2[c]	2 ↔ 4[c]	1 ↔ 2 ↔ 4[c]
8 000	280	4	1.5 μM	146	38	–	24	–
8 000	280	4	3.0 μM	571	61	–	23	–
8 000	280	4	4.5 μM	2126	320	–	128	–
12 000	280	4	1.5 μM	729	68	–	29	18
12 000	280	4	3.0 μM	3769	208	–	68	36
12 000	280	4	4.5 μM	7852	263	–	114	84
16 000	280	4	1.5 μM	1203	51	–	33	14
16 000	280	4	3.0 μM	6251	331	–	193	101
16 000	280	4	4.5 μM	8894	150	–	145	70
16 000	230	4	86 nM	17.3	11.5	5.6	32.1	4.7
16 000	230	4	129 nM	15.4	13.0	6.2	77.1	5.6
20 000	230	4	86 nM	6.2	14.4	4.4	15.1	114
20 000	230	4	129 nM	15.1	13.4	5.7	14.9	–

[a] Concentration of protein loaded in centrifuge cells at the beginning of the experiment. [b] Data were fitted to eq 2 with the molecular weight fixed at 60 438 and the nmer value fixed as shown at the head of each column. [c] Data were fitted to eq 3 with the molecular weight fixed at 60 438. For the 1 ↔ 2 equilibrium, the tetramer concentration was fixed at a vanishingly small value, and the monomer and dimer concentrations were allowed to float to obtain the best fit to a 1 ↔ 2 equilibrium. For the 2 ↔ 4 equilibrium, the monomer concentration was fixed at a vanishingly small value, and the dimer and tetramer concentrations were allowed to float to obtain the best fit to a 2 ↔ 4 equilibrium. For the 1 ↔ 2 ↔ 4 equilibrium, the concentrations of all the species were allowed to float to obtain the best fit. Dashes indicate that the data could not be fitted to the specified equilibrium.

However, though the χ^2 values for the dimer–tetramer model were improved as compared to the single monomeric or single dimeric species, the residuals still displayed nonrandom distributions at loading concentrations between 1.5 and 4.5 μM (data not shown). Consequently, all the data were fitted to the two-equilibrium, three-species, monomer–dimer–tetramer model. The data at all loading concentrations and at speeds of 12 000–16 000 rpm gave significantly improved fits to this model, as compared to the dimer–tetramer model, and all the fits gave more random residuals. This provides substantial evidence that CTP synthetase equilibrates between monomers, dimers, and tetramers under native conditions. At 8000 rpm, however, the data were more consistent with a dimer–tetramer equilibrium. This demonstrates that at the lower speed the enzyme did not distribute over a broad enough concentration range to sample very low concentrations, where the presence of monomer would be detected. This is additional evidence consistent with an equilibrating species model.

The data at low loading concentrations (86 and 129 nM) all fit better to the monomer–dimer model than to the dimer–tetramer model or the monomer–dimer–tetramer model. This is consistent with the idea that at the low loading concentrations the protein dissociated extensively and did not populate the tetrameric species.

Figure 2 illustrates a fit to the monomer–dimer–tetramer model for the data at 1.5 μm loading and 16 000 rpm. Similar results were obtained for all the data sets that best fit the monomer–dimer–tetramer model. The residuals were randomly distributed, in contrast to Figure 1, where the protein distribution was fitted to a single species model with the molecular weight as the floated parameter. The inset to Figure 2 illustrates the fraction of each species as a function of CTP synthetase concentration. Significant amounts of monomer and dimer predominate at protein concentrations

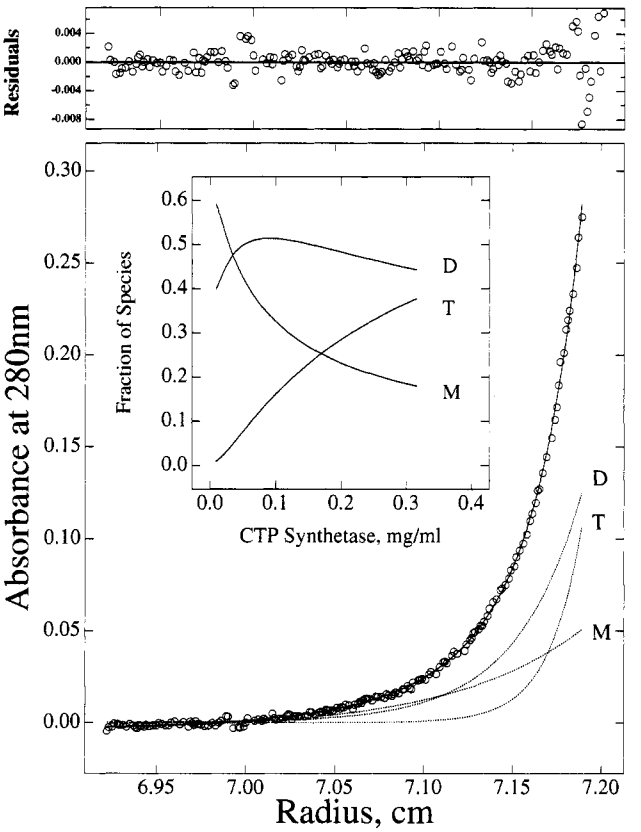

FIGURE 2: Best fit of CTP synthetase sedimentation equilibrium data to a monomer–dimer–tetramer model. Native enzyme was allowed to reach equilibrium as described under Materials and Methods. The loading concentration was 1.5 μM for the data shown above and the speed was 16 000 rpm. The data were fitted to eq 3 with the molecular weight fixed at 60 438, and the line through the data represents the best fit. Similar results were obtained with loading concentrations of 1.5 and 4.5 μM. Table 2 presents the best fits for all loading concentrations.

<0.05 mg/mL (0.8 μM), whereas at concentrations above 0.05 mg/mL the amount of monomer decreases to <20% of the total species present and the amount of tetramer increases to nearly 40% of the total species present. Most importantly, the data demonstrate that the enzyme does not exist solely as a dimer at any concentration in the experiment. The data also imply that at concentrations higher than 0.3 mg/mL (180 μM) the tetramer and dimer concentrations are almost equal. This is in significant contrast to gel filtration experiments that have not provided any evidence of the tetramer in the absence of nucleotides (Long et al., 1970; Anderson, 1983). The absence of evidence for a tetramer may simply mean that gel filtration experiments have not been done at high enough protein concentrations to observe this polymerization in the absence of substrates. Alternatively, the kinetics of tetramerization in the absence of nucleotides may be too slow to form separable species during a flowing gel filtration experiment.

Sedimentation Equilibrium of DON- and Thiourea Dioxide-Modified CTP Synthetase. Enzyme was inactivated by either DON or thiourea dioxide as described in Materials and Methods. The inactivated enzyme then was allowed to reach sedimentation equilibrium at three speeds and three concentrations, under conditions identical to those for native enzyme, to measure the degree of self-association of modified enzyme.

Table 3: Concentration and Speed Dependence of Apparent Molecular Weight Averages of Thiourea Dioxide- and DON-Modified CTP Synthetase

speed (rpm)	wavelength (nm)	temp (°C)	[protein][a] (μM)	M_r[b]	χ^2	nmer[c]	χ^2
Thiourea Dioxide-Modified CTP Synthetase							
8 000	280	4	2	82 500 ± 6900	10	1.4 ± 8%	10
8 000	280	4	4	107 800 ± 3600	11	1.8 ± 3%	11
8 000	280	4	6	117 000 ± 2300	15	1.9 ± 2%	15
12 000	280	4	2	89 700 ± 2400	13	1.5 ± 3%	13
12 000	280	4	4	102 300 ± 1700	26	1.7 ± 2%	26
12 000	280	4	6	106 600 ± 1100	31	1.8 ± 1%	31
16 000	280	4	2	88 200 ± 2100	35	1.5 ± 2%	35
16 000	280	4	4	93 900 ± 1600	42	1.5 ± 2%	42
16 000	280	4	6	100 500 ± 1000	81	1.7 ± 1%	81
DON-Modified CTP Synthetase							
8 000	280	4	2	117 700 ± 9900	11	2.0 ± 8%	11
8 000	280	4	4	112 000 ± 3800	8	1.9 ± 3%	8
8 000	280	4	6	117 400 ± 2900	10	1.9 ± 3%	10
12 000	280	4	2	109 500 ± 2500	6	1.8 ± 2%	6
12 000	280	4	4	110 800 ± 1800	11	1.8 ± 2%	11
12 000	280	4	6	110 300 ± 1200	12	1.8 ± 1%	12
16 000	280	4	2	108 300 ± 1900	7	1.8 ± 2%	7
16 000	280	4	4	110 600 ± 1500	22	1.8 ± 1%	22
16 000	280	4	6	107 600 ± 1400	34	1.8 ± 1%	34

[a] Concentration of protein loaded in centrifuge cells at the beginning of the experiment. [b] Molecular weights were obtained by fitting to eq 1 and are rounded to the nearest hundred. The error estimate represents the 65% confidence interval. [c] Values for the nmer at each protein concentration and speed were obtained by fitting to eq 2 with the molecular weight fixed at 60 438. The error estimate represents the 65% confidence interval.

Table 4: Species Analysis of Thiourea Dioxide- and DON-Modified CTP Synthetase

speed (rpm)	wavelength (nm)	temp (°C)	[protein][a] (μM)	χ^2 1[b]	χ^2 2[b]	χ^2 1 ↔ 2[c]	χ^2 2 ↔ 4[c]	χ^2 1 ↔ 2 ↔ 4[c]
Thiourea Dioxide-Modified CTP Synthetase								
8 000	280	4	2	16	28	10	–	–
8 000	280	4	4	122	19	11	–	41
8 000	280	4	6	50	17	15	–	–
12 000	280	4	2	119	110	13	–	13
12 000	280	4	4	935	172	24	–	25
12 000	280	4	6	3210	288	32	–	53
16 000	280	4	2	321	301	22	–	18
16 000	280	4	4	1059	493	27	–	27
16 000	280	4	6	6223	1280	55	–	55
DON-Modified CTP Synthetase								
8 000	280	4	2	35	11	11	–	–
8 000	280	4	4	99	11	8	–	7
8 000	280	4	6	237	11	10	–	–
12 000	280	4	2	135	12	6	–	9
12 000	280	4	4	545	28	10	–	9
12 000	280	4	6	1126	53	14	–	–
16 000	280	4	2	271	19	7	–	7
16 000	280	4	4	1181	55	15	–	11
16 000	280	4	6	2015	137	27	–	26

[a] Concentration of protein loaded in centrifuge cells at the beginning of the experiment. [b] Data were fitted to eq 2 with the molecular weight fixed at 60 438 and the nmer value fixed as shown at the head of each column. [c] Data were fitted to eq 3 with the molecular weight fixed at 60 438. For the 1 ↔ 2 equilibrium, the tetramer concentration was fixed at a vanishingly small value, and the monomer and dimer concentrations were allowed to float to obtain the best fit to a 1 ↔ 2 equilibrium. For the 2 ↔ 4 equilibrium, the monomer concentration was fixed at a vanishingly small value, and the dimer and tetramer concentrations were allowed to float to obtain the best fit to a 2 ↔ 4 equilibrium. For the 1 ↔ 2 ↔ 4 equilibrium, the concentrations of all the species were allowed to float to obtain the best fit. Dashes indicate that the data could not be fitted to the specified equilibrium.

For the first level of analysis, the enzyme was treated as a single species, and the molecular weight and nmer values were determined. Table 3 provides the results of this analysis. The weight-average molecular weights of thiourea dioxide-modified enzyme were in the range of 82 000−117 000 and gave nmer values of 1.4−1.9. For DON-modified enzyme, the weight-average molecular weights were in the range of 107 000−118 000 and gave nmer values of 1.8−2.0. In comparison to the results in Table 1, the molecular weights and nmer values were significantly lower than for native enzyme. As was the case for native enzyme, the single species analyses of modified enzyme all gave asymmetric residuals and suggested the presence of equilibrating species.

Consequently, as a second level of analysis, the species distributions were determined by fitting to eq 3 as described above for native enzyme. Table 4 contains the results of these analyses. None of the data were consistent with a single monomeric species. In general, the χ^2 values were much lower for fits to a single dimeric species. However, the χ^2 values were improved even more by fitting to a monomer−dimer equilibrium, especially at 12 000 and 16 000 rpm. None of the data fit a dimer−tetramer model. This provides strong evidence that inactivation by thiourea dioxide or DON decreases tetramer formation to below detectable levels. Finally, the data could be fitted to a monomer−dimer−tetramer model, but the χ^2 values did not improve over the monomer−dimer analyses. This is a further indication that tetramers do not contribute significantly to the overall equilibrium in thiourea dioxide- or DON-modified enzyme.

Multispeed Analysis of Native and Modified CTP Synthetase. Sedimentation equilibrium data for self-associating systems may be analyzed by either of two forms of the Lamm equation (Lamm, 1929). In one form, the concentration of a species at radial position c_r may be expressed as a unique term for each species. In a second form, the concentration of each species may be expressed in terms of equilibrium constants relating different species to each other. Equation 3 represents the Lamm equation in terms of species concentrations, whereas eq 10 represents the Lamm equation in terms of equilibrium constants.

Analysis by nonlinear least squares techniques in terms of species is generally more facile than analysis in terms of equilibrium constants because the equilibrium constants are highly correlated. That is, during the fitting process, a change in one equilibrium constant can be balanced by an offsetting change in a second equilibrium constant. The result is that the fit may not converge on a minimum because many pairs of equilibrium constants appear to give the same answer. One approach to overcoming this problem is to analyze multiple data sets simultaneously. This provides the inherent constraint that the fit must describe several equilibrium positions at once, and therefore it forces the fit to search a wider error space and increases the chance of finding a unique minimum.

Consequently, data at three different speeds for each native and modified CTP synthetase sample were merged and fitted to eq 10 to obtain the equilibrium constants for the monomer−dimer and dimer−tetramer interactions. Figure 3 illustrates a representative multispeed analysis of native

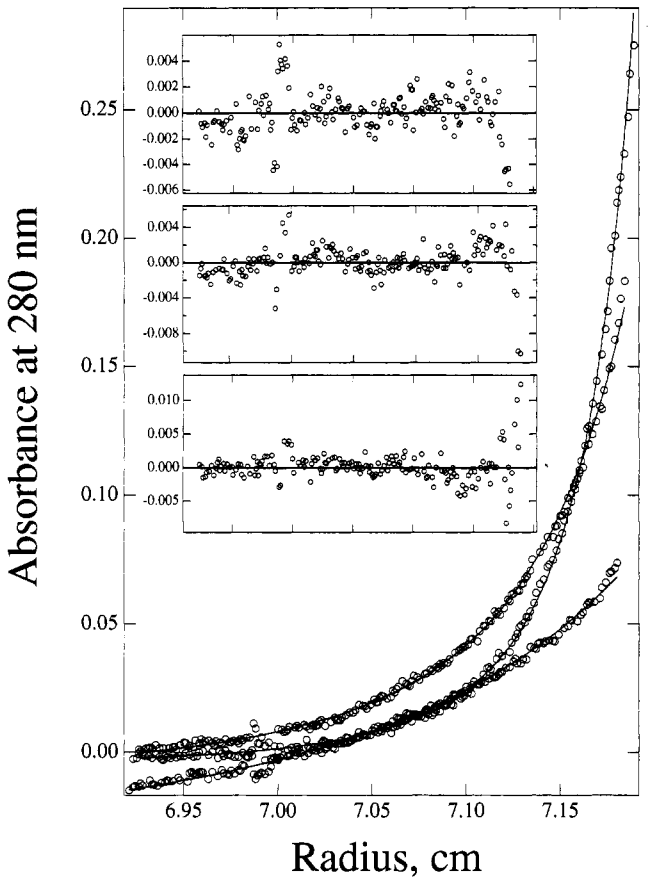

FIGURE 3: Best multispeed fit of CTP synthetase sedimentation equilibrium data to a monomer−dimer−tetramer model. Native enzyme was allowed to reach equilibrium at three speeds as described under Materials and Methods. The loading concentration was 1.5 μM for the data shown above, and the data from 8000, 12 000, and 16 000 rpm were pooled and fitted to eq 10 with the molecular weight fixed at 60 438. The lines through the data represent the best fit. Similar results were obtained with loading concentrations of 1.5 and 4.5 μM. Table 5 presents the best multispeed fits for all loading concentrations.

Table 5: Subunit Dissociation Constants from Multispeed Sedimentation Equilibrium Analysis

speed (rpm)	wavelength (nm)	temp (°C)	[protein][a] (μM)	$K_{1,2}$ (μM)	$K_{2,4}$ (μM)
Native CTP Synthetase[b]					
8 000, 12 000, 16 000	280	4	1.5	1.1 ± 1.6	1.2 ± 1.6
8 000, 12 000, 16 000	280	4	3.0	1.6 ± 1.8	4.0 ± 3.8
8 000, 12 000, 16 000	280	4	4.5	0.9 ± 1.2	18 ± 14
Thiourea Dioxide-Modified CTP Synthetase[c]					
8 000, 12 000, 16 000	280	4	2.0	4.3 ± 1.1	−
8 000, 12 000, 16 000	280	4	4.0	2.9 ± 0.7	−
8 000, 12 000, 16 000	280	4	6.0	2.9 ± 0.6	−
DON-Modified CTP Synthetase[c]					
8 000, 12 000, 16 000	280	4	2.0	0.2 ± 0.6	−
8 000, 12 000, 16 000	280	4	4.0	0.7 ± 0.4	−
8 000, 12 000, 16 000	280	4	6.0	1.3 ± 0.5	−

[a] Concentration of protein loaded in centrifuge cells at the beginning of the experiment. [b] Data were fitted to eq 10 with the molecular weight fixed at 60 438 and $K_{1,2}$ and $K_{2,4}$ as the floated parameters. [c] Data were fitted to eq 10 with the molecular weight fixed at 60 438 and $K_{1,2}$ as the floated parameter. Data could not be fitted to a 1 ↔ 2 ↔ 4 equilibrium, so $K_{2,4}$ was fixed at a value approaching infinity to eliminate any contribution from the $K_{2,4}$ equilibrium. Dashes indicate that the data could not be fitted when the $K_{2,4}$ equilibrium was included.

enzyme at the 1.5 μM loading concentration. Similar results were obtained for the other data sets. The monomer−dimer−tetramer model successfully described simultaneous data sets as judged by the random and small residuals, as well as by the low χ^2 values in comparison to monomer−dimer or dimer−tetramer fits. Additionally, the multispeed fit is strong evidence for the reversibility of the system.

Table 5 presents the fitted parameters from this analysis. The value of $K_{1,2}$ for native enzyme was in the range of 0.9−1.6 μM, whereas $K_{2,4}$ was in the range of 1.2−18 μM. Data obtained from modified enzyme could not be fitted to the 1↔2↔4 model, so the value of $K_{2,4}$ was fixed at a very high value to eliminate any contribution from the tetramer. The fits then converged to give a value for $K_{1,2}$ for modified enzyme. Relative to native enzyme, the $K_{1,2}$'s for modified enzyme were not changed significantly. For thiourea dioxide-modified enzyme, the $K_{1,2}$'s were in the range of 2.9−4.3 μM, whereas for DON-modified enzyme the $K_{1,2}$'s were in the range of 0.2−1.3 μM. The results of the multispeed analysis provide additional evidence that modified enzyme does not form detectable amounts of tetramers.

DISCUSSION

Bacterial CTP synthetase was first characterized as an oligomerizing enzyme with cooperative kinetic behavior over 25 years ago (Long & Pardee, 1967; Long et al., 1970). Since then the genes for the *E. coli*, human, and yeast enzymes have been cloned, and numerous studies have demonstrated various forms of cooperative behavior in the *E. coli*, yeast, and mammalian enzymes. All the studies to date have provided evidence for a dimer to tetramer transition in the presence of saturating concentrations of nucleotides and have interpreted the cooperative kinetic behavior in terms of this equilibrium.

However, most of these studies have relied on gel filtration as the sole technique for molecular weight determinations, and the few early studies that used analytical ultracentrifugation provided only qualitative analysis of the solution behavior of the enzyme. The widespread availability of computerized nonlinear data analysis and a new generation of analytical ultracentrifuge has made quantitative sedimentation equilibrium analysis more accessible to the average biochemist. The studies presented here were performed to take advantage of these recent advances and to quantitate subunit interactions in native CTP synthetase.

The solution properties of native enzyme were analyzed at several levels, as illustrated in Tables 1 and 2 and in Figures 1 and 2. The results in Tables 1 and 2 illustrate the need to study the enzyme at multiple speeds and concentrations in order to observe the full range of subunit interactions. All the data were consistent with an oligomerizing system. Furthermore, the data were best described as a monomer−dimer−tetramer system, except at low nanomolar loading concentrations, where the primary equilibrium is between monomers and dimers, and at low speeds and micromolar concentrations, where the primary equilibrium is between dimers and tetramers.

These results confirm previous work demonstrating that the enzyme dissociates to monomers in very dilute solutions (Anderson, 1983). Most importantly, however, they demonstrate that the native enzyme, in the absence of nucleotides, forms significant amounts of tetramers. Previously, the

enzyme has always been thought to be a dimer in the absence of nucleotides (Anderson, 1983; Levitzki & Koshland, 1972b). This result demonstrates that gel filtration does not adequately measure the aggregation state of CTP synthetase.

In general, the enzyme elutes from gel filtration columns in a single peak, regardless of the loading concentration or the presence or absence of nucleotides (Anderson, 1983). Based on the broadness of the peak, it has not been possible to rule out the presence of tetramers, but no tetramers have been resolved cleanly. This is most likely due to the equilibrating nature of the enzyme but also may reflect the running conditions of the experiment and possible protein interactions with the gel. For instance, in previous studies, loading concentrations of 37 nM to 17 μM CTP synthetase also included 2 mg/mL bovine serum albumin (Anderson, 1983). The carrier protein in the previous experiments may have had a stabilizing effect on the species distribution in the column. All the present experiments were performed on CTP synthetase alone.

Temperature also may be important to the aggregation state of CTP synthetase. In this regard, the protein displays cold lability in dilute solutions (17 μg/mL, 300 nM) (Anderson, 1983). That is, after incubation of the enzyme in dilute solutions at 4 °C without nucleotides, the progress curve in activity assays shows hysteresis and does not reach maximal activity. Gel filtration of enzyme under these conditions results in monomeric enzyme. Addition of nucleotides or reincubation at 26 °C reverses the hysteresis (Anderson, 1983).

The sedimentation equilibrium experiments here were all done at 4 °C, but the loading concentrations were between 1.5 and 6 μM, except for the one experiment at nanomolar loading concentrations. At these concentrations, the experiments began well above the point of cold lability but came to equilibrium at concentrations across the cell from 92 nM to 18 μM, thus spanning the range where cold lability is present. However, even under these conditions, the enzyme demonstrated the presence of monomers, dimers, and tetramers. Thus the presence of cold lability did not appear to dissociate the enzyme irreversibly at equilibrium at 4 °C. All the data were consistent with equilibrating species, even at 4 °C.

Covalent modification of native enzyme by DON or thiourea dioxide greatly weakens tetramerization. Several levels of analysis demonstrated that the weight-average molecular weights at various speeds and concentrations of modified enzyme were all lower than for native enzyme and that the species distribution did not include tetramers. For instance, modified enzyme could be described by a monomer−dimer−tetramer system, but inclusion of the tetramer term did not improve the χ^2 value, and none of the data on the modified enzyme could be fitted to a dimer−tetramer model. This is substantial evidence that any formation of tetramers in modified enzyme could not be detected.

Thus, modification of the active site by DON affects the interactions at the dimer−dimer interface. Similarly, thiourea dioxide modification must affect the dimer−dimer interface. While DON inactivation must occur at the active site, substrate protection experiments have only suggested that thiourea dioxide modification occurs at the active site (Robertson et al., 1992). It remains possible that these modifications (3 mol of thiourea dioxide/mol of enzyme) also may occur at other positions on the protein. Thiourea dioxide modifies lysine residues, and thus one or more of these modification sites might be expected to be on the surface. This would be consistent with direct effects at a dimer−dimer interface. In contrast, DON modification at the active site would be expected to exhibit its effects through conformational changes, unless the active site is between dimers.

Quantitation of the subunit dissociation constants by model fitting indicates that $K_{1,2}$ is in the range of 1−2 μM and does not change significantly after enzyme inactivation. Thus, modification of the active site by DON does not transmit an effect to the interactions involved in dimerization. Similarly, the sites of thiourea dioxide do not affect these interactions.

Native enzyme gave $K_{2,4}$'s of 1.2, 4.0, and 18 μM. Except for the value of 18 μM, the $K_{2,4}$'s were in the range of the $K_{1,2}$'s for native and inactivated enzyme. The high value of 18 μM and the large standard errors relative to the fitted dissociation constants in Table 5 may be related to parameter correlation during minimization. The correlation coefficients were in the range of 0.90−0.95 where values below 0.95 are acceptable and values above 0.95 indicate unresolved parameters (Hensley et al., 1986). As discussed above, the form of the fitted function contributes to the difficulty in resolving parameters, and the K_d's are interrelated in eq 10. Global analysis of multiple data sets provides stringency to overcome this problem but does not guarantee resolved parameters. The high correlation coefficients indicate the difficulty of resolving the dissociation constants in Table 5 and are consistent with the large standard errors.

The available crystal structures of cooperative enzymes show that cooperative effects are transmitted by rotation of subunits rather than translation and that there are generally no changes in secondary structure involved in alternating between the R and T states of two-state cooperative enzymes (Lipscomb, 1991). In the absence of a crystal structure of CTP synthetase, these results, if generally true, might suggest that CTP synthetase undergoes rotation of subunits in a dimer. The evidence in Figure 2 that the native enzyme forms significant amounts of tetramers might suggest that the dimer is in equilibrium between two angles of rotation and that the preferred angle leads to further tetramerization. Ligand binding might be envisioned to lock the rotation of dimers to the preferred angle and thus stabilize tetramerization.

While the molecular mechanism of tetramerization must remain speculative until a crystal structure is available, measurement of the extent of tetramerization might be relevant to drug discovery efforts (Kang et al., 1989). For instance, evaluation of inhibitors usually involves determination of a K_i by some steady-state technique, and for systems where the enzyme equilibrates between more than one species, it may be necessary to account for multiple equilibria in order to obtain informative inhibition constants (Pargellis et al., 1994). The studies presented here directly reveal changes in the aggregation state of CTP synthetase as a result of active site modification. Drug candidates might also affect the aggregation state, and sedimentation equilibrium provides a sensitive tool for monitoring these changes. Increasingly, the visibility of important drug targets such as HIV-1 reverse transcriptase (Lebowtiz et al., 1994) and HIV-1 protease (Pargellis et al., 1994) illustrates the benefits of considering the macromolecular association in drug design,

and drug design efforts on other targets such as CTP synthetase might benefit from similar considerations.

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