Characterization of Metal Ion Activation and Inhibition of CTP Synthetase†

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Received June 15, 1992; Revised Manuscript Received January 11, 1993

ABSTRACT: A reinvestigation of the metal ion activation and specificity of CTP synthetase was begun in order to separate effects due to binding of free metal ions and binding of nucleotide-complexed ions. Apo-CTP synthetase was prepared by dialysis against 5 mM EDTA. Analysis of apo-enzyme by atomic absorption spectroscopy revealed that all bound metal ions could be removed. Thus, apo-enzyme contained no detectable amounts of Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, or Fe^{2+}. The half-saturation value for Mg^{2+}-dependent enzyme activation was approximately 2.6 mM at a total concentration of 2 mM nucleotides (ATP plus UTP). These data suggest that the enzyme requires more Mg^{2+} for full catalytic activity than required simply to complex the nucleotide substrates. Analysis of velocity versus $[Mg^{2+}]_{free}$ demonstrated that activity depends on $[Mg^{2+}]_{free}$. The half-saturation values for $[Mg^{2+}]_{free}$ were 660 and 280 μM in the NH_4^+- and glutamine-dependent assays, respectively. The half-saturation values for $[Mn^{2+}]_{free}$ and $[Co^{2+}]_{free}$ were approximately 2.6 and 3.8 μM in the NH_4^+-dependent assay and 2.8 and 4.7 μM in the glutamine-dependent assay. These results are consistent with the presence of a separate binding site for free metal ion on the enzyme. Over the range of 0.1–10 mM, neither Cu^{2+}, Zn^{2+}, Ni^{2+}, nor Ca^{2+} activated the enzyme. Also, both Cu^{2+} and Zn^{2+} were effective inhibitors of CTP synthetase in the absence of dithiothreitol at concentrations <50 μM. Inhibition by Zn^{2+} was reversed by EDTA, whereas inhibition by Cu^{2+} was not. In the presence of dithiothreitol, Zn^{2+}, Co^{2+}, and Ni^{2+} inhibited the enzyme at less than 200 μM metal ion. In steady-state inhibition experiments, Zn^{2+} and Cu^{2+} demonstrated noncompetitive inhibition with respect to $[Mg^{2+}]_{free}$, where K_{is} and K_{ii} were 0.3 and 0.8 μM for Zn^{2+} and 18 and 80 μM for Cu^{2+}, respectively. The suggestion that free metal ions bind to apo-CTP synthetase was confirmed by fluorescence and electron spin resonance measurements. Addition of Zn^{2+} to apo-enzyme resulted in an increase in the native tryptophan fluorescence, and addition of apo-enzyme to solutions of Mn^{2+}_{free} resulted in decreases in the EPR signal of Mn^{2+}_{free}. Both of these experiments provide the first physical measurements of free metal ion binding to CTP synthetase.

Cytidine triphosphate synthetase catalyzes the formation of cytidine 5′-triphosphate (CTP)[1] from UTP in two sequential steps (Koshland & Levitzki, 1974), as shown in eq 1 and 2.

$$\text{glutamine} \rightarrow \text{glutamate} + NH_3 \quad (1)$$

$$UTP + ATP + NH_3 \rightarrow CTP + ADP + P_i \quad (2)$$

In the first step (eq 1), an essential cysteine on the enzyme catalyzes the conversion of glutamine to NH_3 and glutamate. In step 2, NH_3 released by the glutaminase activity reacts with UTP in an ATP-dependent step to produce CTP, ADP, and inorganic phosphate.

The glutaminase activity (step 1) requires positive allosteric activation by GTP for maximal enzymatic rates (Levitski & Koshland, 1972a). However, GTP has no effect on the second step in the overall reaction. Formation of CTP may be measured in the presence of glutamine, GTP, ATP, and UTP, or by direct addition of exogenous NH_4^+ to the enzyme in the presence of ATP and UTP. The enzyme also catalyzes a UTP-dependent ATPase reaction (von der Saal et al., 1985).

Native CTP synthetase equilibrates between dimeric and tetrameric forms in the presence of nucleotides, and nucleotide-dependent tetramerization has been shown to be the basis of kinetic cooperativity in the enzyme (Koshland & Levitzki, 1974). For instance, saturating concentrations (1 mM) of ATP or UTP alone induce tetramerization, and the combination of ATP and UTP together synergistically lowers the effective nucleotide concentration required for tetramerization (Levitski & Koshland, 1972b). In the absence of UTP, ATP binding is cooperative, and in the absence of ATP, UTP binding is cooperative (Levitski & Koshland, 1972b). Similarly, allosteric GTP activation of the glutaminase reaction occurs in a cooperative manner for the tetrameric form (Levitski & Koshland, 1972a). Depending on temperature, GTP activation of the tetramer may occur with either positive or negative cooperativity (Levitski & Koshland, 1972a). The general sequential model (Koshland, 1970) has been proposed to explain the various cooperative interactions in CTP synthetase (Levitzki & Koshland, 1972b).

In general, nucleotide-dependent enzymes bind a metal–nucleotide complex as the substrate and are considered metal-activated enzymes (Morrison, 1979). In addition, some nucleotide-dependent enzymes may also bind a second metal ion, or may bind metal ions reversibly in the absence of nucleotides. Moreover, nucleotides themselves bind a variety of metals, and the specific metal–nucleotide complex bound to an enzyme will determine the activity of the enzyme.

In the case of CTP synthetase, five separate nucleotides bind to the enzyme as either substrate, product, or effector,

† This work was supported by National Research Service Award GM12340-02 (J.G.R.) and by NSF Grant DMB-8717646 (J.J.V.).

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[1] Abbreviations: CTP, cytidine 5′-triphosphate; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; UTP, uridine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid.

0006-2960/93/0432-3769$04.00/0

and there is no activity in the absence of Mg^{2+}. The original kinetic investigation of *Escherichia coli* CTP synthetase (Lieberman, 1956) demonstrated that 5–10 mM Mn^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+}, and Fe^{2+} would not support CTP synthetase activity and concluded that the enzyme is specific for Mg^{2+}. Similarly, CTP synthetase from Ehrlich ascites tumor cells is not active in the presence of 18 mM Mn^{2+}, Zn^{2+}, Co^{2+}, or Ca^{2+} (Kizaki et al., 1981). These results indicate that the enzyme must bind the Mg^{2+}–nucleotide complex for catalysis to occur and that high concentrations of other divalent cations will not support activity.

However, the metal ion specificity of CTP synthetase has not been investigated in any detail, and no studies have addressed the question of whether or not the enzyme will bind metal ions in the absence of nucleotides. In addition, the inhibitory effects of various metal ions have not been investigated. Thus, the previous studies may be interpreted in one of two ways: (1) that metal ions other than Mg^{2+} inhibit the enzyme; (2) that metal–nucleotide complexes other than MgNTP are not substrates for the enzyme. The studies presented here were conducted to determine whether or not other metal–nucleotide complexes at low concentrations will support activity and to determine whether or not other metal ions will bind to the enzyme in the absence of nucleotides.

MATERIALS AND METHODS

Materials. Ammonium chloride, $MgCl_2$, $MnCl_2$, glutamine, triethanolamine, EDTA, ATP, UTP, GTP, Taps, and atomic absorption standards were from Sigma. Hepes, Pipes, and Mes were from United States Biochemical Corp. Cobalt chloride was from Fisher, and $CuCl_2$ was from Aldrich. Zinc chloride, $NiCl_2$, and $CaCl_2$ were from J.T. Baker. PD-10 columns were from Pharmacia.

Enzyme Purification. Enzyme was purified as described previously (Lewis & Villafranca, 1989). Enzyme used in these studies had a specific activity of 5–8 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 $\epsilon^{0.1\%}_{280} = 0.89$ (Lewis & Villafranca, 1989; Levitzki & Koshland, 1972b). Purified enzyme was stored at −80 °C in 200 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 1 mM Na_4EDTA, 2 mM dithiothreitol, 2.5 mM ATP, 2.5 mM UTP, and 20% glycerol at a final protein concentration of approximately 15 mg/mL. A molecular weight of 60 300 was used for calculations of protein concentration.

Enzyme Assay. Enzyme activity was assayed spectrophotometrically at 37 °C in a Cary 2200 UV–visible spectrophotometer by following the increase in absorbance at 291 nm due to the conversion of UTP to CTP. Specific activity was calculated from the previously determined extinction coefficient $\Delta\epsilon = 1338$ M^{-1} cm^{-1} (Long & Pardee, 1967). The standard NH_4^+-dependent assay contained, in a final volume of 1 mL, 50 mM triethanolamine, pH 8.0, 1 mM ATP, 1 mM UTP, 50 mM NH_4Cl, 10 mM $MgCl_2$, and 10–30 μg of CTP synthetase, unless otherwise noted in the figure legends. The glutamine-dependent assay was the same as the NH_4^+-dependent assay except that the assay contained 0.2 mM GTP and NH_4^+ was replaced with either 2 or 10 mM glutamine. Either enzyme or NH_4^+ was added to start the reaction, and velocities were calculated from the linear portion of the progress curve within the first 2 min of the reaction. Nucleotide concentrations were determined by their absorbance and the following extinction coefficients: ATP, $\epsilon_{259} = 15\,400$ M^{-1}; UTP, $\epsilon_{262} = 10\,000$ M^{-1}; GTP, $\epsilon_{253} = 13\,700$ M^{-1} (Dawson et al., 1987). Concentrations of stock NH_4^+ solutions were determined by assay with glutamate dehydrogenase and reagents in a diagnostic kit from Sigma. Quartz cuvettes were rinsed in 1:1 HCl/HNO_3 to remove contaminating metal ions.

pH Optima. Enzyme activity was assayed as described above in a buffer system consisting of 50 mM Pipes, 50 mM Hepes, 50 Mes, and 50 Taps (PHMT buffer). Stock solutions of buffer were prepared by adjusting separate 20-mL volumes of PHMT buffer to the required pH with KOH. Substrates were dissolved in PHMT buffer, pH 7.5, and 50 μL of substrate solution was added to 880 μL of PHMT buffer in each cuvette. Enzyme then was added in a volume of 20 μL, and the reactions were started by addition of 50 μL of either glutamine or NH_4^+.

Determination of Metal–Nucleotide Concentrations. Metal–nucleotide concentrations were calculated as described previously (Morrison, 1979) by simultaneous solution of eq 3 and 4 where [M] is the free metal ion concentration, $[M]_t$

$$[M] = [M]_t - \{[MATP][1 + (K_1/K_2)([H]/K_H)]\} \quad (3)$$

$$[MATP] = [ATP]_t/\{1 + (K_1/[M]) + (K_1/[M])([H]/K_H) + (K_1/K_2)([H]/K_H)\} \quad (4)$$

is the total metal ion concentration, $[ATP]_t$ is the total ATP concentration, [MATP] is the metal–ATP concentration, [H] is the hydrogen ion concentration, K_1 is the dissociation constant for MATP, K_2 is the dissociation constant for MHATP, and K_H is the dissociation constant for HATP (Morrison, 1979). Values for K_1, K_2, and K_H were 1.37×10^{-5}, 2×10^{-3}, and 1.12×10^{-7} M, and were calculated as the reciprocal of the recommended stability constants for $MgATP^{2-}$, $MgHATP^-$, and $HATP^{3-}$ (O'Sullivan & Smithers, 1979). The stability constants for the various Mg^{2+} complexes of UTP and GTP were assumed to be the same as those for ATP (O'Sullivan & Smithers, 1979), and, therefore, in solving for the concentration of total Mg^{2+}–nucleotide, the total nucleotide concentration was substituted for the total ATP concentration. The stability constant used for MnATP was 100 000 M^{-1} (O'Sullivan & Smithers, 1979) and for CoATP was 162 000 M^{-1} (Sillen & Martell, 1971). Concentrations of stock solutions of Mg^{2+}, Mn^{2+}, and Zn^{2+} were determined by colorimetric titration against an EDTA standard in the presence of arsenazo I (Fritz et al., 1958). Concentrations of standard solutions of other metal ions were determined by atomic absorption spectroscopy.

Preparation of Apo-CTP Synthetase. Prior to use, 2-mL aliquots of enzyme were dialyzed against 1 L of 50 mM triethanolamine, pH 8.0, 5 mM Na_4EDTA, and 2 mM dithiothreitol to remove divalent cations and nucleotides. The enzyme then was dialyzed against six 1-L changes of 50 mM triethanolamine, pH 8.0, and 2 mM dithiothreitol over 36 h. Triethanolamine buffer was chosen to prevent adventitious metal ion chelation in subsequent experiments (Morrison, 1979). Apo-enzyme was analyzed for bound EDTA by laser-stimulated fluorescence emission in the presence of Eu^{3+} (Horrocks & Sudnick, 1981). Apo-enzyme contained <0.05 equiv of EDTA/monomer of CTP synthetase. Apo-enzyme was analyzed for the presence of Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, and Fe^{2+} by atomic absorption spectroscopy on a Perkin Elmer 1100 graphite furnace instrument. There were no detectable amounts of any of these seven metal ions in apo-CTP synthetase. The Mg^{2+}- and Mn^{2+}-bound forms of glutamine synthetase were used as positive controls for the presence of Mg^{2+} and Mn^{2+}. Phenoxazinone synthase and carboxypeptidase Y were used as positive controls for the presence of Cu^{2+} and Zn^{2+}.

Steady-State Intrinsic Fluorescence of Apo-CTP Synthetase. Fluorescence measurements were made on a Photon Technologies LS100 luminescence instrument. Enzyme solutions contained 50 mM triethanolamine, pH 8.0, 100 mM KCl, and 39 μg/mL CTP synthetase (0.64 μM monomer), with or without Zn^{2+} as indicated in Figure 9. When Zn^{2+} was added, the final concentration was 68 μM. Enzyme solutions were excited at 295 nm, and 15 scans were averaged.

Electron Spin Resonance on Mn^{2+}- and Mn^{2+}–CTP Synthetase. Electron spin resonance spectra were acquired on a Bruker ESP 300 E operating at a frequency of 9 GHz. Samples were placed in capillary tubes of 1-mm i.d., and the temperature was maintained at 25 °C with a Eurotherm temperature controller. All spectra were obtained with a modulation amplitude of 24.78 G and a power level of 2 mW. Apo-CTP synthetase was dialyzed against 50 mM triethanolamine, pH 8.0, to remove dithiothreitol. The spectrum of 100 μM Mn^{2+} was obtained in 50 mM triethanolamine, pH 8.0. The Mn^{2+}–CTP synthetase spectrum was obtained in 50 mM triethanolamine, pH 8.0, 115 μM CTP synthetase monomer, and 100 μM Mn^{2+}.

Determination of Kinetic Parameters. Saturation plots of velocity versus total metal ion or GTP concentration were fit to a modified form of the Hill equation (eq 5), where A is the

$$v = A + B[M^n/(M^n + C^n)] \quad (5)$$

minimum value of v, B is the maximum value of v, M is the total concentration of added metal ion or GTP, n is the Hill coefficient, and C is the concentration of metal ion or GTP resulting in half-maximal velocity (Newton & Koshland, 1989). This equation was used rather than the Hill equation in order to account for any residual enzyme activity in the absence of metal ion or GTP. Saturation plots of velocity versus free metal ion or GTP concentrations were fit to eq 5 and 6, and complete data sets of velocity versus free metal ion concentration in the presence of several concentrations of inhibitory metal ion were fit to eq 7.

$$v = V_m S/(K_m + S) \quad (6)$$

$$v = V_m S/[K_m(1 + I/K_{is}) + S(1 + I/K_{ii})] \quad (7)$$

RESULTS

Saturation of Apo-CTP Synthetase Activity by Mg^{2+}. Preliminary kinetic experiments with holo-CTP synthetase indicated that maximal enzyme activity might require more $MgCl_2$ than required simply to complex the nucleotide substrates. Therefore, apo-CTP synthetase was prepared in order to characterize the full metal ion requirements of the enzyme. Apo-enzyme contained no detectable amounts of Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, or Fe^{2+}, as determined by atomic absorption spectroscopy. Apo-enzyme also contained less than 0.05 equiv of EDTA, as determined by laser-stimulated fluorescence emission (Horrocks & Sudnick, 1981).

Activity of apo-enzyme was determined as a function of added Mg^{2+} at saturating concentrations of UTP and ATP in the NH_4^+-dependent assay. Figure 1 shows the saturation curve for Mg^{2+} and the corresponding concentrations of Mg–nucleotide in the experiment. Very similar results were obtained in the glutamine-dependent reaction (results not shown). Both saturation curves displayed sigmoidal kinetics and were fit to eq 5.

The half-saturation values for Mg^{2+} were approximately 2.6 and 2.4 mM for the NH_4^+- and glutamine-dependent reactions, respectively. Referring to the data in Figure 1, at

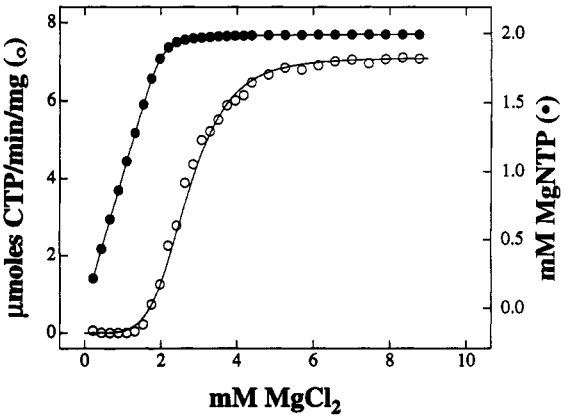

FIGURE 1: Saturation of apo-CTP synthetase activity by Mg^{2+}. Apo-CTP synthetase was prepared as described under Materials and Methods. Enzyme assays contained, in a final volume of 1 mL, 50 mM triethanolamine, pH 8.0, 1 mM ATP, 1 mM UTP, 25 mM NH_4Cl, and the indicated concentration of $MgCl_2$. Enzyme was added to start the reaction, and the final assay concentration of CTP synthetase was 36 μg/mL (0.6 μM subunit). The total Mg–nucleotide concentration was calculated from eq 3 and 4, as described under Materials and Methods. Open circles (○) represent enzyme velocity, and closed circles (●) represent the total Mg–nucleotide concentration. The line through the velocity data represents the best fit to eq 5. In a similar experiment, the Mg^{2+} saturation curve was determined in the glutamine-dependent assay (data not shown). For the glutamine-dependent experiment, the assay conditions were the same as those described above, except that the assay solutions contained 2 mM glutamine and 0.2 mM GTP, and did not contain NH_4Cl.

2 mM Mg^{2+} and pH 8.0, greater than 95% of the nucleotides would be present as the Mg^{2+}–nucleotide complex. Thus, at 2 mM Mg^{2+}, MgATP and MgUTP were both present at concentrations of greater than 950 μM. In the glutamine-dependent assay, the K_m values of ATP and UTP are 54 and 25 μM in the absence of GTP, and 116 and 71 μM in the presence of GTP (Lewis & Villafranca, 1989). Consequently, in Figure 1 at 2 mM Mg^{2+}, the Mg–nucleotide complexes were present at 8 to 35 times their K_m values.

However, the data in Figure 1 demonstrate that essentially complete Mg^{2+} complexation of nucleotide substrates only results in approximately 30% maximal velocity at 2 mM Mg^{2+}. This result suggests that additional Mg^{2+} must bind to the enzyme to attain maximal velocity and that a second metal ion binding site may be involved in enzyme activity. Additional experiments showed that excess Mg^{2+} up to 25 mM does not inhibit CTP synthetase (data not shown).

Similar results were obtained when Mg^{2+} activation was measured at less than saturating concentrations of ATP and UTP. Figure 2 shows the results of Mg^{2+} activation in the NH_4^+-dependent assay over the range of 0.5–18 mM Mg^{2+} at concentrations of 0.25 mM ATP and 0.25 mM UTP. At these nucleotide concentrations, the Mg^{2+}–nucleotide concentration was constant at 0.5 mM throughout the experiment. As Figure 2 shows, there is little or no activity at less than 1 mM Mg^{2+}, even though the nucleotides were fully complexed at all Mg^{2+} concentrations. The Mg^{2+} concentration at half-maximal activation was 5.6 mM, and the Hill coefficient was 3.4. These data demonstrate a clear requirement for excess Mg^{2+} in the CTP synthetase reaction. The data also demonstrate that at less than saturating concentrations the Mg–nucleotide complexes alone are insufficient for catalysis. The data in Figure 2 may be interpreted to mean that excess Mg^{2+}_{free} facilitates nucleotide binding at subsaturating Mg–nucleotide concentrations.

Saturation of Apo-CTP Synthetase Activity by Mg^{2+}, Mn^{2+}, and Co^{2+}. Several other divalent cations were tested to

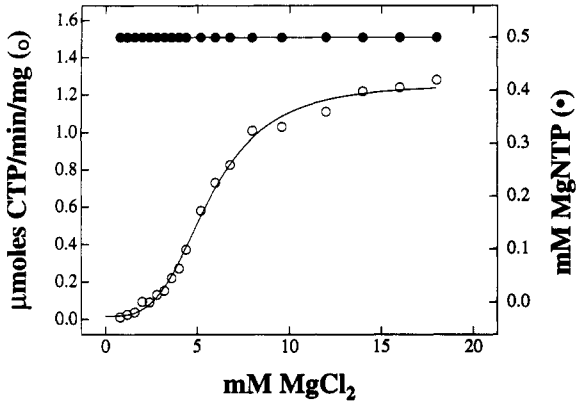

FIGURE 2: Saturation of apo-CTP synthetase activity by Mg^{2+} at subsaturating ATP and UTP. Apo-CTP synthetase was prepared as described under Materials and Methods. Enzyme assays contained, in a final volume of 1 mL, 50 mM triethanolamine, pH 8.0, 0.25 mM ATP, 0.25 mM UTP, 52 mM NH_4Cl, 26 µg/mL (0.4 µM subunit) CTP synthetase, and the indicated concentration of $MgCl_2$. Assays were started by the addition of NH_4Cl. The total Mg–nucleotide concentration was calculated from eq 3 and 4, as described under Materials and Methods. Open circles (O) represent enzyme velocity, and closed circles (●) represent the total Mg–nucleotide concentration. The line through the velocity data represents the best fit to eq 5.

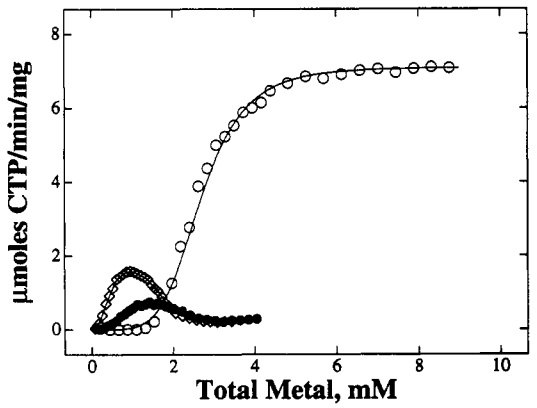

FIGURE 3: Saturation of apo-CTP synthetase activity by Mg^{2+}, Mn^{2+}, and Co^{2+}. Apo-CTP synthetase was prepared as described under Materials and Methods. Enzyme assays contained, in a final volume of 1 mL, 50 mM triethanolamine, pH 8.0, 1 mM ATP, 1 mM UTP, 75 mM NH_4Cl, 22 µg/mL apo-CTP synthetase (0.4 µM subunit), and the indicated concentration of either $MgCl_2$, $MnCl_2$, or $CoCl_2$. Enzyme was equilibrated in the assay mixture for 1 min at 37 °C, and the assay was started by addition of NH_4Cl. Open circles (O) represent Mg^{2+}-dependent activity, open diamonds (◇) represent Mn^{2+}-dependent activity, and closed circles (●) represent Co^{2+}-dependent activity. In a similar experiment, the metal ion saturation curves were determined in the glutamine-dependent assay (data not shown). For the glutamine-dependent experiment, the assay conditions were the same as those described above, except that the assays contained 10 mM glutamine and 0.2 mM GTP, and did not contain NH_4Cl.

determine whether or not CTP synthetase is specific for Mg^{2+}. Figure 3 demonstrates that both Mn^{2+} and Co^{2+} will substitute for Mg^{2+} in the CTP synthetase reaction at saturating concentrations of nucleotides. However, both Mn^{2+} and Co^{2+} yield lower maximal activities than Mg^{2+}, and both of these metal ions become inhibitory at concentrations greater than 2 mM. In the glutamine-dependent reaction, Co^{2+} activation was minimal and in some enzyme preparations was not measurable at all (data not shown). However, Co^{2+} activation was always measurable in the NH_4^+-dependent assay. Additional experiments indicated that Ca^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+} produced no measurable CTP synthetase activity over the range of 0.1–10 mM.

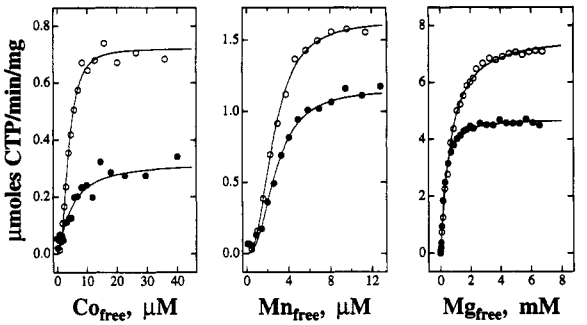

FIGURE 4: Saturation of apo-CTP synthetase activity as a function of $[Mg^{2+}]_{free}$, $[Mn^{2+}]_{free}$, and $[Co^{2+}]_{free}$. Data from Figure 3 were used to calculate free metal ion concentrations and to plot velocity versus free metal ion concentration. Data for Mn^{2+} and Co^{2+} were analyzed over their activation ranges. Open circles (O) represent enzyme activity in the NH_4^+-dependent assay, and closed circles (●) represent activity in the glutamine-dependent assay. Lines through the data represent the best fits to eq 5. Table I lists the best-fitting parameters from the data fits.

Table I: Kinetic Parameters for Apo-CTP Synthetase Activation by Mg^{2+}_{free}, Mn^{2+}_{free}, and Co^{2+}_{free}[a]

	ammonia-dependent assay	glutamine-dependent assay
Mg^{2+} activation		
A[b]	0	0
B	7.7 ± 0.1	4.7 ± 0.04
C	660 ± 21	280 ± 9
n	1.2 ± 0.04	1.3 ± 0.05
Mn^{2+} activation		
A	0	0
B	1.6 ± 0.03	1.2 ± 0.03
C	2.6 ± 0.1	2.8 ± 0.1
n	2.4 ± 0.1	2.4 ± 0.2
Co^{2+} activation		
A	0	0
B	0.72 ± 0.01	0.32 ± 0.02
C	3.8 ± 0.1	4.7 ± 0.9
n	2.4 ± 0.2	1.3 ± 0.22

[a] Data from Figure 4 were fit to eq 5. Parameter *A* was fixed at 0 during fitting. [b] Units of *A* and *B* are micromoles of CTP per minute per milligram, units of *C* are micromolar, and *n* represents the Hill coefficient.

All three activating metal ions were analyzed over the range of concentrations resulting in activation of both the NH_4^+- and glutamine-dependent reactions. The data were solved for the free metal ion concentration by simultaneous solution of eq 3 and 4, and the velocity was plotted as a function of free metal ion concentration, as shown in Figure 4. The data in Figure 4 were fit to both eq 5 and eq 6. In all cases, the data fit better to eq 5, as judged by the sum of squares and randomness of residuals. Table I shows the kinetic parameters for the fits to eq 5. The half-saturation values for Mn^{2+}_{free} and Co^{2+}_{free} were approximately 2.6 and 3.8 µM, respectively, in the NH_4^+ assay and 2.8 and 4.7 µM, respectively, in the glutamine assay. These values were significantly lower than the half-saturation values for Mg^{2+}_{free} in the NH_4^+- and glutamine-dependent reactions, which were 660 and 280 µM, respectively.

The Mg^{2+}, Mn^{2+}, and Co^{2+} binding constants for ATP are all similar at 13.7, 10, and 6.2 µM, respectively (Sillen & Martel, 1971), and, consequently, the binding constants for the corresponding metal–UTP complexes would be expected to be the same as for the metal–ATP complexes. Therefore, concentration-dependent changes in the formation of the metal–nucleotide complexes cannot account for the shift to lower half-saturation values in the presence of Mn^{2+} and Co^{2+}. The data suggest that MnNTP and CoNTP complexes bind

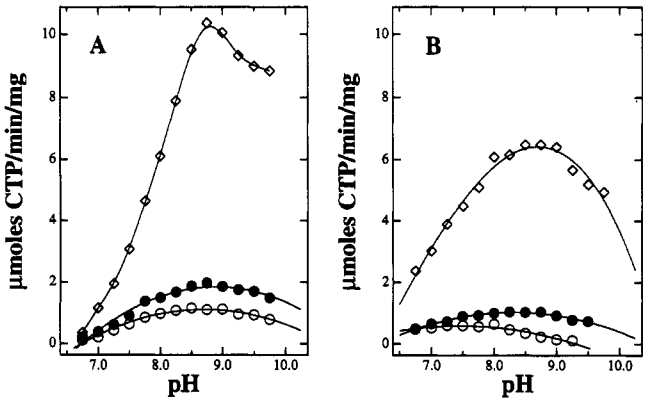

FIGURE 5: pH–activity profiles at saturating Mg^{2+}, Mn^{2+}, and Co^{2+}. Apo-CTP synthetase was prepared as described under Materials and Methods and was dialyzed to remove dithiothreitol. Enzyme assays were done as described under Materials and Methods and contained, in a final volume of 1 mL, 1 mM ATP, 1 mM UTP, 23 μg/mL apo-CTP synthetase (0.4 μM subunit), saturating metal ion, and PHMT buffer at the indicated pH. Saturating metal ion concentrations were 10 mM Mg^{2+}, 0.5 mM Mn^{2+}, and 1 mM Co^{2+}. Panel A gives the activities in the NH_4^+-dependent assay, which contained 50 mM NH_4Cl, and panel B gives the activities in the glutamine-dependent assay, which contained 0.2 mM GTP and 10 mM glutamine. Open diamonds (◇) represent Mg^{2+}-dependent activity, closed circles (●) represent Mn^{2+}-dependent activity, and open circles (○) represent Co^{2+}-dependent activity.

to the enzyme tighter than the MgNTP complexes. Inhibition by Mn^{2+} and Co^{2+} at concentrations higher than 2 mM is consistent with the possibility of more than one free metal ion binding site on the enzyme.

Determination of pH Optima for Metal Ion Activation. Enzyme was assayed in both the NH_4^+-dependent and glutamine-dependent assays at optimal Mg^{2+}, Mn^{2+}, and Co^{2+} concentrations as a function of varied pH. Figure 5 presents the pH–activity profiles for each assay. The pH optimum for all metal ions was approximately 8.7 in the NH_4^+-dependent assay. In the glutamine-dependent assay, the pH optimum for both Mg^{2+} and Mn^{2+} also was approximately 8.7, whereas for Co^{2+} the optimum was approximately 8.0. All activities in the NH_4^+-dependent assay were higher than in the glutamine-dependent assay. The results for Mg^{2+} activation in both the NH_4^+- and glutamine-dependent assays were very similar to those reported previously (Levitzki & Koshland, 1971), except that the optimum in Figure 5 was approximately pH 8.7 for the NH_4^+-dependent assay as compared to pH 10 in previous studies (Levitzki & Koshland, 1971).

Allosteric Activation by GTP in the Presence of Mg^{2+} and Mn^{2+}. Enzyme was assayed in the glutamine-dependent assay at optimal Mg^{2+} and Mn^{2+} concentrations as a function of varied [GTP]. Excess Mg^{2+} in the Mg^{2+}-dependent activation experiment permitted the use of saturating concentrations of ATP and UTP. However, due to progressive inhibition by excess Mn^{2+}, as shown in Figure 3, the Mn^{2+} concentration in this experiment was limited to 1.2 mM. Consequently, the total permissible nucleotide concentration also was limited, and subsaturating concentrations of ATP and UTP were used. Under these conditions, the maximal activity in the presence of Mn^{2+} was reduced. Under similar conditions, the Co^{2+}-dependent activity could not be measured.

Figure 6 shows that both Mg^{2+} and Mn^{2+} support activation by GTP. The K_m for GTP in the presence of Mg^{2+} was 59 μM, and the $K_{m,app}$ for Mn^{2+} was 8 μM. Neither of the activation curves demonstrated kinetic cooperativity. The lower $K_{m,app}$ for Mn^{2+}-dependent GTP activation suggests that the MnGTP complex binds to the enzyme tighter than

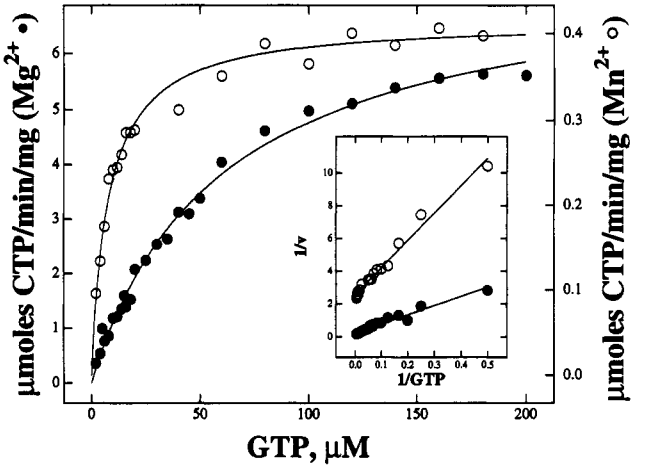

FIGURE 6: GTP activation in the presence of Mg^{2+} and Mn^{2+}. Apo-CTP synthetase was prepared as described under Materials and Methods. Enzyme assays were done as described under Materials and Methods. For the Mg^{2+}-dependent activation curve, assays contained, in a final volume of 1 mL, 100 mM triethanolamine, 1 mM ATP, 1 mM UTP, 10 mM Mg^{2+}, 10 mM glutamine, 7 μg/mL apo-CTP synthetase (0.1 μM subunit), and the indicated concentration of GTP. For the Mn^{2+}-dependent activation curve, assays contained, in a final folume of 1 mL, 100 mM triethanolamine, 0.5 mM ATP, 0.5 mM UTP, 1.2 mM Mn^{2+}, 10 mM glutamine, 26 μg/mL apo-CTP synthetase (0.4 μM subunit), and the indicated concentration of GTP. Assays were started by addition of glutamine. Open circles (○) represent Mn^{2+}-dependent activity, and closed circles (●) represent Mg^{2+}-dependent activity. Lines through the data represent the best fits to eq 6.

the MgGTP complex, and this is consistent with the data in Figure 3 suggesting that MnNTP complexes bind to the enzyme tighter than the MgNTP complexes.

Stimulation by Mn^{2+} and Co^{2+} in a Mixed Metal Ion System. The results in Figure 2 suggested that it might be possible to test for Mn^{2+} and Co^{2+} stimulation under conditions where free Mg^{2+} is present and there is little or no CTP synthetase activity. Consequently, enzyme assays were set up to contain 0.25 mM ATP, 0.25 mM UTP, and 1 mM Mg^{2+}, conditions which produce fully complexed MgNTP, 0.5 mM excess Mg^{2+}, and undetectable CTP synthetase activity. Using these initial conditions, additional Mg^{2+}, Mn^{2+}, or Co^{2+} was added over a range of 0.02–1 mM, thus producing a total metal ion concentration of 1–2 mM. In separate controls, Mn^{2+} or Co^{2+} was added to assay solutions containing no Mg^{2+}.

Figure 7, panels A and B, shows that there was no detectable activity in the presence of 1 mM Mg^{2+}, even after an additional 0.02–1 mM Mg^{2+} was added. In contrast, in the presence of 1 mM Mg^{2+}, addition of 0.02–1 mM Mn^{2+} or Co^{2+} stimulated enzyme activity very effectively. Comparison of the Mn^{2+} and Co^{2+} activation curves in the presence and absence of Mg^{2+} demonstrates that the presence of Mg^{2+} shifts the activation curves to lower Mn^{2+} and Co^{2+} concentrations and abolishes the cooperative behavior observed in the absence of Mg^{2+}. Also, the presence of Mg^{2+} doubled the total activity due to Co^{2+} addition.

Incubation with Zn^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+} and Co^{2+}. Apo-enzyme was incubated with several different divalent cations to determine whether or not activating metal ions and other metal ions might also inhibit the enzyme. For the experiment in Figure 8, apo-enzyme was equilibrated for 1 min at 25 °C with either Zn^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+}, or Co^{2+} to permit metal ion binding to unoccupied binding sites on the protein. An aliquot of the equilibrated mixture then was assayed in the presence of 10 mM Mg^{2+} and saturating concentrations of

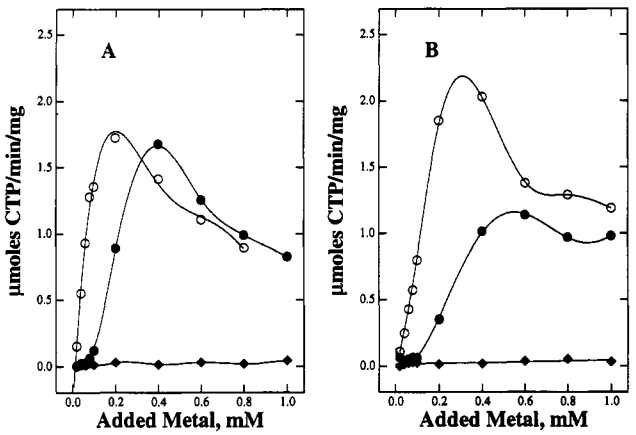

FIGURE 7: Mixed metal ion activation. Apo-CTP synthetase was prepared as described under Materials and Methods. Enzyme assays contained, in a final volume of 1 mL, 100 mM triethanolamine, pH 8.0, 0.25 mM ATP, 0.25 mM UTP, 50 mM NH_4Cl, 26 μg/mL apo-CTP synthetase (0.4 μM subunit), and metal ions as described below. Assays were started by addition of NH_4Cl. (Panel A) Closed diamonds (♦), each assay contained a starting concentration of 1 mM Mg^{2+}, and then additional Mg^{2+} was added as shown; open circles (○), each assay contained a starting concentration of 1 mM Mg^{2+}, and then additional Mn^{2+} was added as shown; closed circles (●), each assay contained no metal ions to begin with, and then additional Mn^{2+} was added as shown. (Panel B) Closed diamonds (♦), each assay contained a starting concentration of 1 mM Mg^{2+}, and then additional Mg^{2+} was added as shown; open circles (○), each assay contained a starting concentration of 1 mM Mg^{2+}, and then additional Co^{2+} was added as shown; closed circles (●), each assay contained no metal ions to begin with, and then additional Co^{2+} was added as shown.

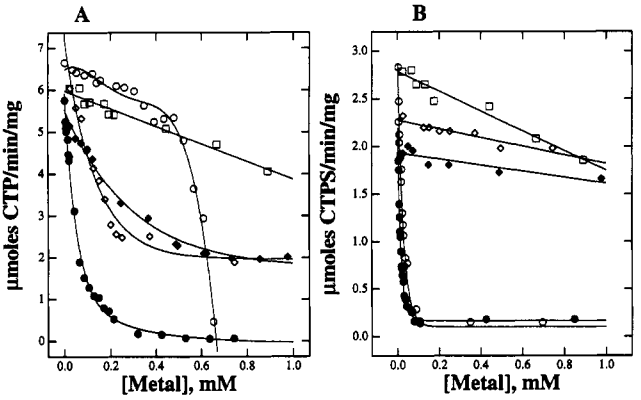

FIGURE 8: Inhibition of apo-CTP synthetase activity by Cu^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+}, and Co^{2+}. In panel A, enzyme incubations contained, in a final volume of 100 μL, 100 mM triethanolamine, pH 8.0, 270 μg/mL apo-CTP synthetase (4.5 μM subunit), 0.5 mM dithiothreitol, and the indicated concentration of either $CuCl_2$, $ZnCl_2$, $NiCl_2$, $MnCl_2$, or $CoCl_2$. Enzyme was equilibrated with the divalent cation for 1 min at 25 °C, and then 75 μL of the incubation was transferred to a cuvette containing the standard NH_4^+ assay mixture as described under Materials and Methods. Enzyme activity was measured immediately after addition of enzyme to assay mixtures. In panel B, apo-CTP synthetase was desalted rapidly by passage through a PD-10 column to remove dithiothreitol. Therefore, the incubations and assays in panel B were the same as those in panel A, except that they contained no dithiothreitol. Closed circles (●) represent Zn^{2+}, open circles (○) represent Cu^{2+}, open squares (□) represent Mn^{2+}, open diamonds (◇) represent Co^{2+}, and closed diamonds (♦) represent Ni^{2+}.

nucleotides to test for any inactivating effect of either Zn^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+}, or Co^{2+}. In Figure 8 panel A, the incubation solutions contained a final concentration of 0.5 mM dithiothreitol due to the dithiothreitol routinely included in all CTP synthetase solutions to maintain maximal activity. In Figure 8 panel B, dithiothreitol was removed from the enzyme solution by rapid gel filtration on a PD-10 column. In the absence of reductant, the enzyme retained only 50% of its activity, but 100% activity could be regenerated by adding back dithiothreitol.

Figure 8 demonstrates that enzyme equilibration with Zn^{2+}, Ni^{2+}, and Co^{2+} in the presence of dithiothreitol causes 50–95% inhibition of enzyme activity in the range of 10–400 μM metal ion. In this concentration range, Cu^{2+} and Mn^{2+} only had a slight inhibitory effect. However, concentrations of Cu^{2+} above 500 μM resulted in almost complete inhibition of enzyme activity. The sharp downturn of the Cu^{2+} inhibition curve occurs at the concentration where dithiothreitol and Cu^{2+} are equimolar. This result suggests that dithiothreitol protects the enzyme from Cu^{2+} inhibition by complexing the Cu^{2+} ions.

In the absence of dithiothreitol, both Zn^{2+} and Cu^{2+} produced almost complete inhibition at concentrations less than 100 μM. The potent Cu^{2+} inhibition in the absence of dithiothreitol confirms the protective effect of dithiothreitol. In contrast, both Ni^{2+} and Co^{2+} had little inhibitory effect in the absence of dithiothreitol. This is consistent with the fact that both Ni^{2+} and Co^{2+} appeared to undergo reduction or complexation in buffers containing dithiothreitol, as evidenced by a color change from clear to red. The same color change occurred in enzyme incubations in the presence of dithiothreitol, but did not occur in the absence of dithiothreitol. Thus, the inhibition by Ni^{2+} and Co^{2+} in Figure 8 panel A is due to the reduced or complexed forms of Ni^{2+} and Co^{2+}.

Steady-State Inhibition by Cu^{2+} and Zn^{2+}. The results in Figure 8 do not differentiate between inactivation by metal ions and reversible inhibition by metal ions carried into the assay from the incubations. In a separate experiment, 10.3 μM CTP synthetase was incubated at 25 °C with 177 μM Zn^{2+} for 10 min in 50 mM triethanolamine buffer, pH 8.0, at which point the enzyme was only 15% active. Addition of 5 mM EDTA to the incubation solution resulted in recovery of 100% enzyme activity in 15 min, which indicates that Zn^{2+} inhibition is reversible.

When 19 μM CTP synthetase was incubated with 145 μM Cu^{2+} at 25 °C in 50 mM triethanolamine, pH 8.0, the enzyme activity was reduced to 1% in 10 min. Enzyme activity continued to decrease to <1% over an additional 30 min in the presence of 5 mM EDTA. Thus, in contrast to Zn^{2+} inhibition, Cu^{2+} inhibition was irreversible.

Additionally, steady-state inhibition experiments were carried out in the presence of Cu^{2+} and Zn^{2+} to determine whether or not these ions compete with Mg^{2+} for binding to the enzyme. Apo-enzyme was assayed at varied concentrations of Mg^{2+} at two fixed concentrations of Zn^{2+} or Cu^{2+}. The assays all contained saturating concentrations of nucleotides (1 mM ATP and 1 mM UTP). Under these conditions, in the presence of Cu^{2+}, the NH_4^+-dependent assay was linear for only about 30 s, at which point the enzyme activity rapidly decreased. In this experiment, the assays were started by ammonia addition immediately after enzyme addition to assay mixtures containing Cu^{2+} and substrates.

In these experiments, the velocity versus Mg^{2+} saturation curves were all sigmoidal, and there was very little discernible inhibition below 2 mM Mg^{2+}, the region of concentrations where the nucleotides would be less than fully complexed with Mg^{2+}. These results suggest that Cu^{2+} and Zn^{2+} do not inhibit the enzyme by altering nucleotide binding but that inhibition may result from interactions at a second metal ion site required for full activity in the presence of Mg^{2+}.

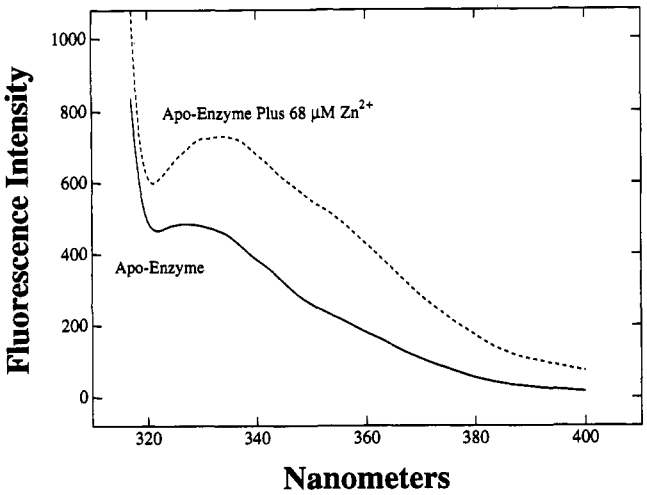

FIGURE 9: Intrinsic fluorescence of CTP synthetase with and without Zn^{2+}. Fluorescence measurements were made as described under Materials and Methods.

When the same data were plotted as velocity versus $[Mg^{2+}]_{free}$, all the saturation curves displayed hyperbolic kinetics. Therefore, the velocity versus $[Mg^{2+}]_{free}$ data were fit to eq 7, the equation for noncompetitive inhibition, and were plotted in double-reciprocal form. The K_{is} and K_{ii} from these fits were 0.3 ± 0.1 and 0.8 ± 0.2 μM, respectively, for Zn^{2+} inhibition, and were 18 ± 4 and 80 ± 13 μM, respectively, for Cu^{2+} inhibition.

Intrinsic Tryptophan Fluorescence in the Presence of Metal Ions. After equilibration with various metal ions, the intrinsic tryptophan fluorescence of apo-enzyme was measured in an effort to detect structural changes due to metal ion binding. Saturating concentrations of Mg^{2+} (10 mM) and Mn^{2+} (7 mM) produced no changes in the fluorescence spectrum of apo-enzyme. In contrast, 0.7 mM Co^{2+} decreased the intrinsic fluorescence by 16%, and 58 μM Cu^{2+} decreased the fluorescence by 46%. These decreases are probably due to dynamic quenching, especially in the case of Cu^{2+} (Lakowicz, 1983). However, 68 μM Zn^{2+} produced a 33% increase in the tryptophan fluorescence and caused a 7-nm red-shift in the absorption peak, as shown in Figure 9. The increase in fluorescence can only be due to Zn^{2+} binding, and therefore this experiment provides direct evidence for free metal ion binding to the enzyme. The red-shift in the absorption peak is an indication that Zn^{2+} binding causes greater tryptophan exposure to solvent, but the enzyme contains three tryptophan residues, and therefore the data do not identify a specific site for the conformational change.

Detection of Mn^{2+} Binding by Electron Spin Resonance. Room temperature solutions of Mn^{2+}_{free} provide a well-characterized EPR signal due to the five unpaired electrons in $[Mn(H_2O)_6]^{2+}$. In contrast, protein-bound Mn^{2+} has a diminished signal intensity due to changes in zero-field splitting and induced asymmetry by enzyme ligands (Swartz et al., 1972). Thus, the disappearance of Mn^{2+}_{free} may be used to measure Mn^{2+} binding to proteins (Cohn & Townsend, 1954). Figure 10 shows the decrease in the Mn^{2+}_{free} signal due to addition of apo-CTP synthetase to a solution of 100 μM Mn^{2+}. This experiment and the fluorescence experiment described above provide direct physical evidence of free metal ion binding to CTP synthetase, and thereby support all the kinetic evidence suggesting that free metal ions may bind to the enzyme at a site separate from the nucleotide binding site.

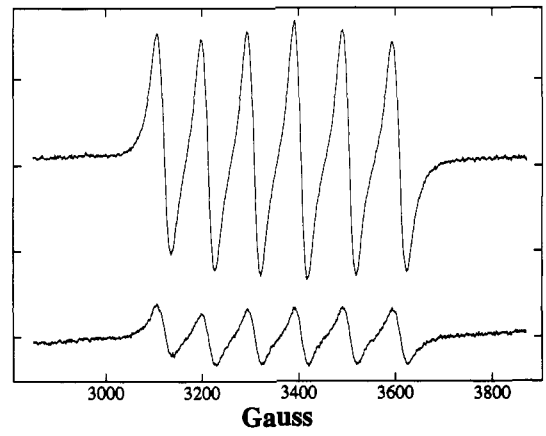

FIGURE 10: Electron spin resonance measurement of Mn^{2+} binding. Electron spin resonance measurements were made as described under Materials and Methods. The top spectrum represents 100 μM Mn^{2+}_{free}, and the bottom spectrum represents 115 μM CTP synthetase subunit and 100 μM Mn^{2+}.

DISCUSSION

The results presented here show that CTP synthetase may be activated by metal ions other than Mg^{2+}. In particular, Mn^{2+} and Co^{2+} activate the enzyme at concentrations <1 mM in the absence of Mg^{2+}, whereas the enzyme is inactive at higher concentrations of either metal ion. In addition, the results show that Cu^{2+} and Zn^{2+} are both potent inhibitors of the enzyme. The kinetic results indicate that the enzyme contains a metal ion binding site(s) apart from the sites involved in binding the MgNTP complexes. In addition, both fluorescence and EPR measurements provided direct physical evidence that the enzyme binds a free metal ion.

The kinetics of metal ion dependencies exhibited by CTP synthetase are similar to those demonstrated by other enzymes. For instance, glutamine synthetase (Hunt et al., 1975) and carbamoyl-phosphate synthetase (Raushel et al., 1979) may be activated by either Mg^{2+}, Mn^{2+}, or Co^{2+}, and have a metal ion site in addition to a metal–nucleotide site, while $NADP^+$–malic enzyme (Brown & Cook, 1981), NAD^+–malic enzyme (Milne & Cook, 1979), NAD^+-specific isocitrate dehydrogenase (Barratt & Cook, 1978), and galactosyltransferase (O'Keeffe et al., 1980) may be activated by either Mg^{2+} or Mn^{2+}. In addition, the biphasic response of CTP synthetase to Mn^{2+} and Co^{2+} is similar to the response of carbamoyl-phosphate synthetase, where these metal ions activate the enzyme and then inhibit the enzyme as their concentrations increase.

When plotted as velocity versus total metal ion concentration, the activation kinetics of CTP synthetase yielded sigmoidal saturation curves for all divalent cations. The sigmoidal regions of these curves can be attributed both to nucleotides binding the metal ions and to cooperative binding of metal–nucleotide complexes to the protein. Plots of velocity versus free metal ion concentration demonstrated variable cooperativity, but in all cases, the data fit better to a cooperative model than to a hyperbolic model, even in cases where the n value was only 1.2–1.3. This may indicate that free metal ion binding is cooperative or that free metal ion binding influences metal–nucleotide binding, which in turn produces an allosteric effect in the saturation curve.

In contrast to the metal ion activation kinetics of CTP synthetase, activation by the allosteric effector GTP in the presence of Mg^{2+} and Mn^{2+} was hyperbolic (Figure 6). Previous GTP binding studies have shown that GTP binding to the CTP synthetase dimer is hyperbolic and that GTP

binding to the tetramer is negatively cooperative at 25 °C (Levitski & Koshland, 1972a). For the experiments in Figure 6, the MgATP and MgUTP concentrations were saturating, providing conditions where the enzyme would be in the tetrameric form. This predicts that GTP binding would be negatively cooperative, assuming that our results at 37 °C would be the same as previous results at 25 °C (Levitski & Koshland, 1972a) and that the kinetic GTP activation curve would be the same as the GTP binding curve. However, for the experiment in Figure 6, the MnATP and MnUTP concentrations were less than saturating, and these would be conditions favoring the dimer, which would be more consistent with hyperbolic GTP activation.

There is some indication in the double-reciprocal plots (Figure 6) of downward curvature that might be indicative of negative cooperativity, but fits of the actual data to eq 5 were equivocal. For MnGTP activation, the Hill coefficient was 0.84 ± 0.03, and for MgGTP activation, it was 1.4 ± 0.12, even though both replots might have predicted Hill coefficients of ≤ 1. In addition, the saturating nucleotide concentrations for the MgGTP activation curve would have favored tetramerization and negative cooperativity, when in fact the fitted Hill coefficient was 1.4 and suggested positive cooperativity. Previous studies with MgGTP and CTP synthetase have reported a Hill coefficient of 0.6 (Levitski & Koshland, 1972a).

In fitting to eq 5 to test for negative cooperativity, we have progressed from a two-parameter equation (eq 6) to a four-parameter equation, and would expect a significantly lower variance if eq 5 represents the true underlying model. This was not the case. The variances of the fits to the Michaelis–Menten equation and to the modified Hill equation were 0.0039 and 0.0033 for the MnGTP data, and were 0.66 and 0.45 for the MgGTP data. Adding extra parameters to an equation usually gives a slightly better fit, but these small changes are not enough to conclude that GTP activation is negatively cooperative, especially in view of the fact that the Hill coefficient was 1.4 for the MgGTP data. Therefore, we conclude that the GTP activation data in this study are more consistent with hyperbolic activation and probably do not involve cooperative interactions within the protein.

The pH–activity profiles indicate that enzyme activity increases between pH 7 and 8 and that the enzyme is maximally active between pH 8 and pH 9. Several pK_a's for ATP, ADP, GTP, UTP, and CTP are in the region of pH 6.5–7.0 (Morrison, 1979), and, therefore, all the nucleotides would be fully ionized as the pH approaches 8.0 and would bind divalent ions to produce MNTP complexes. The fact that the pH optima are all above pH 8.0 is consistent with the idea that the MNTP complexes are the true substrates for the enzyme.

The mixed metal ion experiment described in Figure 7 presents one of the most intriguing results in this report. This experiment appears to separate binding and catalytic events, and thereby may suggest that a free metal ion site is involved in catalysis. Under the conditions in Figure 7, there is no catalysis due to the presence of MgNTP alone. Under these same conditions, low micromolar concentrations of Mn^{2+} or Co^{2+} activate the enzyme. Most importantly, this activation is not cooperative, as it is in the presence of Mn^{2+} or Co^{2+} alone. This loss of cooperativity and the shift to lower activation concentrations of Mn^{2+} or Co^{2+} in the presence of Mg^{2+} may mean that the MgNTP's are bound to the enzyme and that Mn^{2+} or Co^{2+} simply initiate catalysis by binding at a separate site that may be part of the catalytic site.

In order to explain Zn^{2+} and Cu^{2+} inhibition, we first examined the sequence of CTP synthetase for the presence of possible metal ion binding sequences. For instance, the sequence of CTP synthetase was examined for the presence of a Cys_2, His_2 zinc finger motif (Klevit, 1991), but there was no evidence for such a motif. Although the enzyme contains five His and eight Cys, there are no His-X_3-His or Cys-X_2-Cys sequences in the protein. It remains possible, however, that subunit interactions in the dimer or tetramer might form a zinc-specific binding site by aligning His and Cys residues in the proper orientation between subunits.

Also, the sequence of CTP synthetase was examined for the presence of contiguous or closely spaced His residues that might provide nitrogenous ligands to a Cu^{2+} atom. There is one His-Arg-His sequence in the protein, and additional His residues might fold into the proper orientation to create a tight binding, square-planar, nitrogen-ligated Cu^{2+} site, such as is found in various copper-containing enzymes.

However, it may be more plausible to consider cysteine ligation as the mechanism of Zn^{2+} and Cu^{2+} inhibition. It is known that the glutaminase reaction in CTP synthetase produces a glutamyl-enzyme intermediate at a critical cysteine residue (Levitzki & Koshland, 1971). Metal ion binding at this cysteine could certainly block the active site. It also is known from the available enzyme crystal structures involving a Zn^{2+} structural binding site that each of these sites provides four cysteine ligands to a Zn^{2+} atom (Vallee & Auld, 1990). The Zn^{2+} thiolate clusters in metallothioneins provide another example of Zn^{2+} ligation to cysteine (Vallee & Auld, 1990).

Both Zn^{2+} and Cu^{2+} produced noncompetitive inhibition in relation to Mg^{2+}_{free}. This indicates that Zn^{2+} and Cu^{2+} can bind to a form of the enzyme containing bound Mg^{2+} and rules out direct competition with Mg^{2+}_{free} as the exclusive mode of inhibition. In turn, this implies that Zn^{2+} and Cu^{2+} can bind to a site separate from the Mg^{2+} site. These results are consistent with the idea that Zn^{2+} and Cu^{2+} may bind at the catalytic cysteine residue while Mg^{2+} binds at a separate metal ion binding site also accessible to Mn^{2+} and Co^{2+}. If Zn^{2+} and Cu^{2+} do bind at the catalytic cysteine residue, these results would also mean that NH_4^+ cannot enter the active site in the presence of Zn^{2+} or Cu^{2+}, because all the inhibition studies were done in the NH_4^+-dependent assay.

The kinetic studies presented here cannot resolve these different possibilities. However, the changes in the fluorescence and EPR signals shown in Figures 9 and 10 should provide a way to determine the number of metal ion sites and the binding constants of each of the various metal ions. Moreover, binding of various metal ions and metal–nucleotide species to the enzyme should also provide access to other physical techniques such as NMR that can be used to measure distances between different sites on the enzyme. In conjunction with kinetic studies, these physical methods can provide a detailed picture of the structure–function relationships in CTP synthetase. Studies are now in progress to exploit these physical techniques to elucidate further the role of metal ions in CTP synthetase.

Lastly, it should be noted that the activity of mammalian CTP synthetase is 2–3 orders of magnitude lower than the activity of *E. coli* CTP synthetase (Kizaki et al., 1981; Weinfeld et al., 1978). The results presented here may indicate that the mammalian enzyme has a different metal ion specificity than the *E. coli* enzyme or that metal ion inhibition may contribute to the lower activities of mammalian enzyme. For instance, both the bovine liver and the Ehrlich ascites enzymes have been purified in the absence of EDTA, except for a

calcium phosphate absorption step, wherein both procedures bind the enzyme to calcium phosphate and wash the bound gel with 50 mM EDTA. However, if there is any irreversible Cu^{2+}-dependent inhibition prior to this step, the purified enzyme might only reflect the residual activity of inhibited enzyme. Therefore, the metal ion specificity of these enzymes should be examined to determine whether or not this is a cause of the large differences in enzyme activities from different sources.

ACKNOWLEDGMENT

We thank Dr. John Freeman for samples of phenoxazinone synthase, Cynthia Millis for samples of carboxypeptidase Y, and Luis Reynaldo for samples of glutamine synthetase. We also thank Dr. William Horrocks and JoAnne Bruno for the analysis of enzyme-bound EDTA by laser-stimulated fluorescence emission.

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