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Inactivation and covalent modification of CTP synthetase by thiourea dioxide

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Abstract

Thiourea dioxide was used in chemical modification studies to identify functionally important amino acids in *Escherichia coli* CTP synthetase. Incubations at pH 8.0 in the absence of substrates led to rapid, time dependent, and irreversible inactivation of the enzyme. The second- order rate constant for inactivation was 0.18 M^{-1} s^{-1}. Inactivation also occurred in the absence of oxygen and in the presence of catalase, thereby ruling out mixed-function oxidation/reduction as the mode of amino acid modification. Saturating concentrations of the substrates ATP and UTP, and the allosteric activator GTP prevented inactivation by thiourea dioxide, whereas saturating concentrations of glutamine (a substrate) did not. The concentration dependence of nucleotide protection revealed cooperative behavior with respect to individual nucleotides and with respect to various combinations of nucleotides. Mixtures of nucleotides afforded greater protection against inactivation than single nucleotides alone, and a combination of the substrates ATP and UTP provided the most protection. The Hill coefficient for nucleotide protection was approximately 2 for ATP, UTP, and GTP. In the presence of 1:1 ratios of ATP:UTP, ATP:GTP, and UTP:GTP, the Hill coefficient was approximately 4 in each case. Fluorescence and circular dichroism measurements indicated that modification by thiourea dioxide causes detectable changes in the structure of the protein. Modification with [^{14}C]thiourea dioxide demonstrated that complete inactivation correlates with incorporation of 3 mol of [^{14}C]thiourea dioxide per mole of CTP synthetase monomer. The specificity of thiourea dioxide for lysine residues indicates that one or more lysines are most likely involved in CTP synthetase activity. The data further indicate that nucleotide binding prevents access to these functionally important residues.

Keywords: chemical modification; cytidine triphosphate synthetase; kinetics; thiourea dioxide

Cytidine triphosphate synthetase from *Escherichia coli* catalyzes the formation of CTP from glutamine, ATP, and UTP (Koshland & Levitzki, 1974). The enzyme has a GTP-stimulated glutaminase activity, which catalyzes deamination of glutamine to provide nascent ammonia for attack on UTP. Positional isotope exchange experiments (von der Saal et al., 1985) and rapid quench kinetic data (Lewis & Villafranca, 1989) suggest that ammonia formed in the glutaminase reaction attacks a phosphorylated UTP intermediate that ultimately breaks down to CTP. The enzyme also catalyzes a UTP-dependent ATPase reaction (von der Saal et al., 1985) and displays both positive and negative cooperativity in the presence of GTP and substrates (Koshland & Levitzki, 1974). Native enzyme exists in equilibrium between dimeric and tetrameric forms, and saturating concentrations of nucleotides convert the enzyme to the more active tetrameric form (Koshland & Levitzki, 1974). The *E. coli* enzyme has been cloned and expressed (Weng et al., 1986).

The active site of CTP synthetase contains a reactive cysteine that participates in formation of a glutamyl-enzyme intermediate (Levitzki & Koshland, 1971). High sequence homology between CTP synthetase and several other amidotransferases suggests that Cys 379 in CTP synthetase functions as the reactive site of glutamyl-enzyme formation (Weng et al., 1986). The same comparisons also suggest that His 515 may have a role in proton transfer in the active site. However, no other active site amino acids have been identified, nor have any amino acids involved in nucleotide binding been identified. Therefore the specific interactions responsible for catal-

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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); Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

ysis and cooperativity in CTP synthetase remain to be elucidated.

Several anti-cancer drugs appear to target CTP synthetase for inactivation in the cell. Acivicin, an affinity analogue of glutamine, inactivates a number of glutamine amidotransferases and causes the depletion of CTP in mouse L1210 leukemia cells (Kemp et al., 1986). Cyclopentenylcytosine inhibits the growth of neoplastic cell lines, and its in vivo phosphorylation product, cyclopentenylcytosine triphosphate, inhibits partially purified CTP synthetase (Kang et al., 1989). Informed design of new drugs targeted at CTP synthetase would benefit from additional knowledge of active site amino acids and from knowledge of important structural features in molecules that bind to CTP synthetase. We have used thiourea dioxide as a site-directed reagent to probe the important structural features of the enzyme.

Previous studies have shown that thiourea dioxide (Fig. 1) effectively inactivates glutamine synthetase (Colanduoni & Villafranca, 1985; DiIanni et al., 1986; DiIanni, 1988). In these studies, inactivation kinetics demonstrated that a reversible complex forms between thiourea dioxide and glutamine synthetase. Moreover, structural comparisons suggested that the compound may mimic a γ-glutamyl phosphate intermediate in the glutamine synthetase reaction. Thiourea dioxide covalently modifies a lysine residue and the N-terminal serine in glutamine synthetase and also participates in the oxidation of His 269 (Colanduoni & Villafranca, 1985; DiIanni et al., 1986; DiIanni, 1988). Regarding the oxidation reaction, it has been postulated that thiourea dioxide reduces the active site metals of glutamine synthetase and that the reduced metals react with oxygen to generate hydrogen peroxide. In this mechanism, a reduced metal ion and hydrogen peroxide then react via Fenton chemistry to generate a hydroxyl radical that ultimately oxidizes His 269.

Both the glutamine synthetase and CTP synthetase reactions involve glutamine, ammonia, ATP, and ADP as either substrates or products, and therefore the potential similarity of the reactions suggested the possibility that thiourea dioxide might also be an inhibitor of CTP synthetase. Consequently, in order to explore the possibility that thiourea dioxide may be a general active site-directed reagent for glutamine-dependent enzymes and for CTP synthetase in particular, CTP synthetase was incubated with thiourea dioxide to test for specific inactivation. Thiourea dioxide caused specific, irreversible inhibition of CTP synthetase, and protection experiments with nucleotide substrates suggested that the reagent may interact with a site involved in cooperative effects in CTP synthetase.

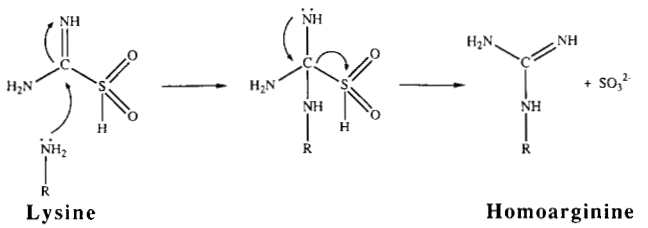

Fig. 1. Proposed mechanism for reaction of thiourea dioxide with lysine residues to form homoarginine.

Results

Inactivation with thiourea dioxide

Cytidine triphosphate synthetase was incubated with 20 mM thiourea dioxide at pH 8 and 37 °C in the absence of substrates to test for inactivation. Data in Figure 2 demonstrate the rapid, time-dependent inactivation of the enzyme by thiourea dioxide. Enzyme was inactivated as measured by either the glutamine-dependent or ammonia assay and was >99% inactive after 30 min of incubation at pH 8.0. Inactivation was specific for thiourea dioxide and did not occur with 20 mM urea, thiourea, or *O*-methylisourea. Inactivation was not reversed by extensive dialysis against 50 mM Hepes, pH 8.0, and the UV-visible spectrum of the inactivated enzyme was the same as the UV-visible spectrum of native enzyme (data not shown). Separate controls also showed that inactivation occurred in the absence of Mg^{2+}, and that a 13-fold molar excess of SO_3^{2-} over enzyme concentration had no inactivating effect for up to 60 min at 37 °C. These data indicate that there is no Mg^{2+}-dependent redox reaction involved in inactivation, and that the reaction product of thiourea dioxide and lysine does not contribute to inactivation.

Previous studies have shown that a mixed-function ox-

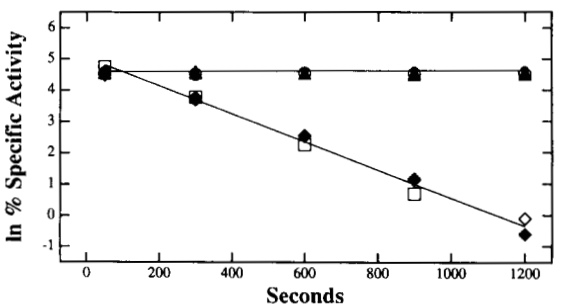

Fig. 2. Inactivation of CTP synthetase by thiourea dioxide. Enzyme was incubated as described under Materials and methods. In addition to buffer, $MgCl_2$, and enzyme, the reactions contained: no further addition (○), 20 mM thiourea dioxide (◆), 20 mM urea (△), 20 mM thiourea (▲), 20 mM *O*-methylisourea (×), 20 mM thiourea dioxide and 0.25 mg/mL catalase (◇), and 20 mM thiourea dioxide after degassing with argon (□). There was essentially no difference in the control incubations, and therefore all the symbols (○, △, ▲, ×) for these reactions overlap. Similarly, there was very little difference in the inactivation reactions, and therefore these symbols (◆,◇, □) also overlap. Symbols that are not distinctly visible at each time point may be assumed to be obscured by an overlying symbol.

idation system oxidizes a single histidine in glutamine synthetase (Levine, 1983), and that thiourea dioxide oxidizes His 269 in glutamine synthetase (DiIanni, 1988). As a result, it has been suggested that thiourea dioxide participates in the mixed-function oxidation system (DiIanni, 1988). This mechanism cannot occur in the absence of oxygen, and it has been shown that thiourea dioxide has no inactivating effect on glutamine synthetase in buffers purged with argon (DiIanni, 1988).

Consequently, CTP synthetase was reacted with thiourea dioxide under argon in order to test for an inactivation mechanism requiring oxygen. As shown in Figure 2, thiourea dioxide completely inactivated CTP synthetase even in the absence of oxygen. Similarly, the inactivation was done in the presence of catalase to remove hydrogen peroxide that might be involved in the reaction, and the presence of catalase also had no effect on the inactivation rate. These data suggest that an alternative mechanism must be responsible for thiourea dioxide inactivation of CTP synthetase.

Concentration dependence of thiourea dioxide inactivation.

Inactivation was measured as a function of thiourea dioxide concentration to determine whether or not there is evidence of equilibrium binding between the enzyme and thiourea dioxide. Data in Figure 3A show that plots of ln % specific activity against time were linear at all concentrations. This result indicates that there was no instability of thiourea dioxide during the experiment and that inactivation occurred as a first-order process. In addition, the replot of k_{inact} versus thiourea dioxide concentration in Figure 3B also was linear and showed no evidence of saturation at high thiourea dioxide concentrations. Thus, the linear replot indicates that the reagent does not form a specific, reversible complex with the enzyme prior to inactivation. The slope of the replot in Figure 3B yielded a second-order rate constant for inactivation of 0.18 M^{-1} s^{-1}.

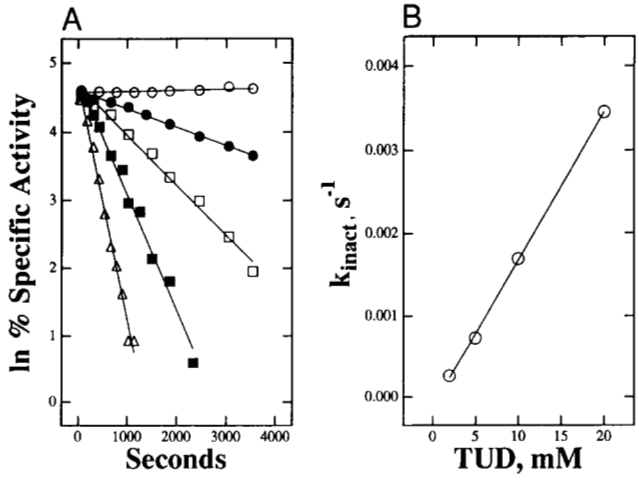

Fig. 3. Concentration dependence of thiourea dioxide inactivation. Enzyme was incubated as described under Materials and methods at four different concentrations of thiourea dioxide (TUD). In addition to buffer, $MgCl_2$, and enzyme, the reactions contained: no further addition (○), 2 mM thiourea dioxide (●), 5 mM thiourea dioxide (□), 10 mM thiourea dioxide (■), and 20 mM thiourea dioxide (△).

Protection by glutamine and nucleotides

Enzyme was incubated with 20 mM thiourea dioxide in the presence of saturating (5 mM) concentrations of glutamine or nucleotides to test for protection against inactivation. Although the K_m for glutamine ranges between 0.17 and 0.5 mM, depending on the presence or absence of GTP (Lewis & Villafranca, 1989), a 5 mM concentration of glutamine had no effect on thiourea dioxide inactivation, as shown in Figure 4. The k_{inact} was 0.0034 s^{-1} in the absence of glutamine and 0.0032 s^{-1} in the presence of glutamine. Moreover, the inactivation rate in the presence of glutamine was linear, and this evidence indicates that the amino group of glutamine did not interfere substantially with the inactivation by reacting with thiourea dioxide itself.

In contrast, 5 mM concentrations of ATP, UTP, or GTP each provided substantial protection against thiourea dioxide inactivation. The observed rates of inactivation were 0.0002 s^{-1}, 0.0006 s^{-1}, and 0.0003 s^{-1} in the presence of ATP, UTP, and GTP, respectively, as compared to 0.0034 s^{-1} in the presence of thiourea dioxide alone. The concentrations of nucleotides in Figure 4 were sufficient to saturate nucleotide binding (Levitzki & Koshland, 1972a,b) and were high enough to induce tetramerization (Levitzki & Koshland, 1972a). Therefore, the data suggest that the individual nucleotides prevent thiourea

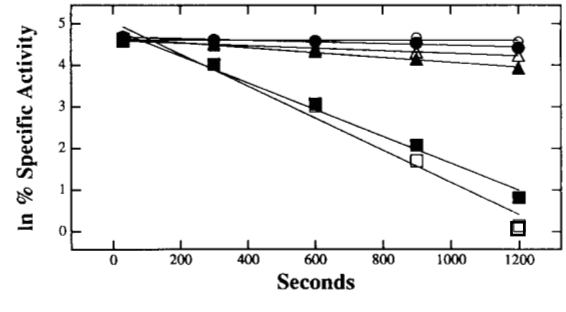

Fig. 4. Substrate protection of thiourea dioxide inactivation. Enzyme was incubated as described under Materials and methods. In addition to buffer, $MgCl_2$, and enzyme, the reactions contained: no further addition (○), 20 mM thiourea dioxide and 5 mM ATP (●), 20 mM thiourea dioxide and 5 mM UTP (△), 20 mM thiourea dioxide and 5 mM GTP (▲), 20 mM thiourea dioxide and 5 mM glutamine (■), and 20 mM thiourea dioxide (□). Symbols that are not distinctly visible at each time point may be assumed to be obscured by an overlying symbol.

dioxide from reacting with amino acid residues in the CTP synthetase tetramer.

Concentration dependence of nucleotide protection

The concentration of individual nucleotides was varied in separate inactivations to determine the concentrations of nucleotides required for half-maximal protection against thiourea dioxide inactivation. In addition, pairs of nucleotides were used in a 1:1 ratio to determine whether or not combinations of nucleotides would be more effective in protection against thiourea dioxide inactivation. The profiles in Figures 5 and 6 demonstrate the sigmoidal character of nucleotide protection against thiourea dioxide inactivation. Consequently, data were fit to a modified form of the Hill equation that describes a sigmoidal curve with a positive intercept on the Y axis (Newton & Koshland, 1989). Table 1 lists the best fitting parameters for the data in Figures 5 and 6.

Data in Figure 5 show that all three nucleotides provided similar protection. The concentrations required for half-maximal protection by ATP, GTP, and UTP were approximately 5, 6, and 7 mM, respectively. The values of $1/k_{inact}$ at maximum protection by ATP and GTP were the same at 6.9×10^3 s, and both of these values were higher than the value for UTP protection at 5.4×10^3 s. However, the Hill coefficient was approximately 2 in each case. The values of the Hill coefficients indicate that there are, at minimum, two nucleotide binding sites under these conditions.

In contrast, pairs of nucleotides at a 1:1 ratio provided more effective protection against thiourea dioxide inactivation than did single nucleotides alone. The data in Figure 6 and the fits to the data in Table 1 show that a 1:1 ratio of ATP:UTP gave the most effective protection. For example, the concentration required for half-maximal protection was only 0.88 mM for the ATP:UTP combination, compared to 3.11 mM and 3.13 mM for the ATP:GTP and UTP:GTP combinations, respectively. In the presence of two nucleotides, the Hill coefficients were all approximately 4. In this case, the Hill coefficients indicate that there are, at minimum, four nucleotide binding sites under these conditions.

Table 1. *Kinetic constants for nucleotide protection against thiourea dioxide inactivation*

	Constants[a]			
Nucleotide	$1/k_{inact}$ (s)	$1/k^*_{inact}$ (s)	C (mM)	n
ATP	233 ± 79	6,920 ± 640	4.98 ± 0.5	2.05 ± 0.22
UTP	182 ± 160	5,410 ± 1890	7.24 ± 2.40	2.03 ± 0.63
GTP	197 ± 190	6,990 ± 1700	5.93 ± 1.50	1.77 ± 0.45
ATP:GTP[b]	493 ± 180	6,030 ± 310	3.11 ± 0.13	3.98 ± 0.69
UTP:GTP	331 ± 110	4,340 ± 250	3.13 ± 0.19	3.75 ± 0.71
ATP:UTP	685 ± 190	8,890 ± 300	0.88 ± 0.05	4.43 ± 0.75

[a] Data in Figures 5 and 6 were fit to Equation 3 (see Materials and methods) and Table 1 depicts the best fitting constants from those fits. In Table 1, $1/k_{inact}$ represents A in Equation 3, or the value $1/k_{inact}$ in the absence of substrates; $1/k^*_{inact}$ represents B in Equation 3, or the value $1/k_{inact}$ at maximal protection by substrates; C represents the nucleotide concentration required for half-maximal protection; and n is the Hill coefficient.

[b] Data for protection by two nucleotides were obtained at a 1:1 ratio of the indicated nucleotides.

Intrinsic fluorescence and circular dichroism

The intrinsic fluorescence of the 3 tryptophans and 16 tyrosines in CTP synthetase was used as a means of detecting structural changes in the protein induced by inactivation with thiourea dioxide. Excitation at 280 nm was used to excite both tyrosines and tryptophans, and excitation at 295 nm was used to excite tryptophans selectively. Data in Figure 7 demonstrate a 24% decrease in the combined fluorescence of tyrosine and tryptophan due to modification with thiourea dioxide and a 14% decrease in the tryptophan fluorescence due to modification. The fluorescence decreases suggest changes in protein conformation that affect the environment of both tyrosines and tryptophans.

Similarly, the circular dichroism (CD) spectrum of

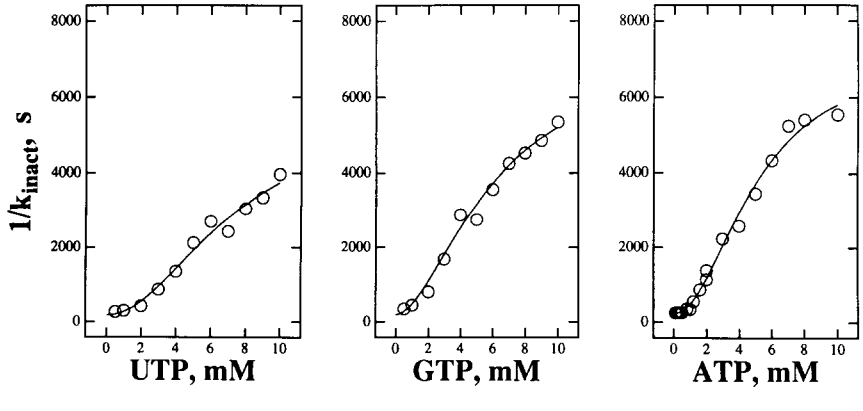

Fig. 5. Concentration dependence of nucleotide protection against thiourea dioxide inactivation. Enzyme was incubated as described under Materials and methods. In addition to buffer, $MgCl_2$, and enzyme, the reactions contained the indicated concentration of either ATP, UTP, or GTP. Data in each panel were fit to Equation 3 (see Materials and methods). Table 1 lists the constants from each fit.

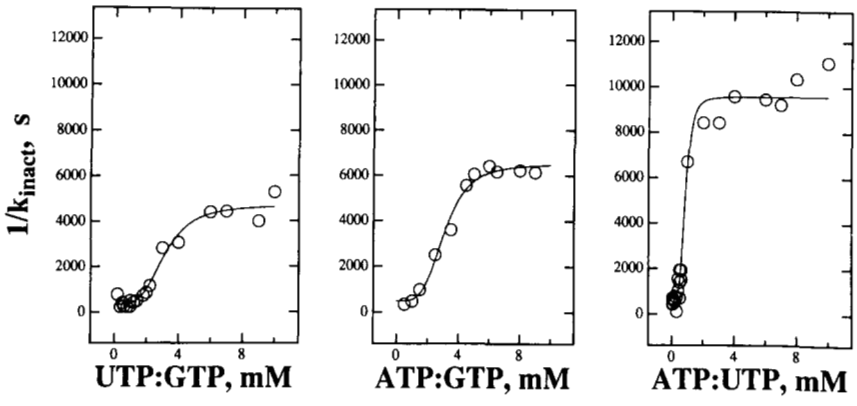

Fig. 6. Concentration dependence for pairs of nucleotides in protection against thiourea dioxide inactivation. Enzyme was incubated as described under Materials and methods. In addition to buffer, $MgCl_2$, and enzyme, the reactions contained the indicated concentration of total nucleotide, which was present as a 1:1 ratio of the indicated pair. Data in each panel were fit to Equation 3 (see Materials and methods). Table 1 lists the constants from each fit.

CTP synthetase also was used as a means of detecting structural changes in the protein due to modification by thiourea dioxide. Data in Figure 8 demonstrate a small but reproducible change in the mean ellipticity of the protein over the region of 205–220 nm. The maximum decrease over this region was approximately 5%, and the decrease indicates that thiourea dioxide modification alters the α-helical content of the protein.

Native gel electrophoresis

Native and thiourea dioxide-modified CTP synthetase were electrophoresed through nondenaturing gels in the presence of 1 mM ATP and 0.5 mM UTP to determine whether or not the modified protein forms tetramers. The result in Figure 9, lane 7, shows that native CTP synthetase migrated in this gel system with an apparent molecular weight of ≈300,000, based on extrapolation from the plot of log molecular weight versus relative mobility of the standards. The native enzyme migrated with a larger than expected molecular mass in this gel system but always was present as a single band.

In contrast, CTP synthetase modified to <1% activity migrated as a continuous band over a range of molecular weights from 60,000 to 260,000. This same continuous band was observed in each of two gels where the proteins were analyzed in the presence of nucleotides and in a separate gel in the absence of nucleotides. These data indicate that thiourea dioxide caused variable modification of individual subunits. It is possible that modified subunits formed tetramers in the presence of nucleotides but that subunits modified at different sites, or at more than one site, or less than the maximum number of sites, did not form tetramers and migrated at some intermediate molecular weight. Alternatively, this result may indicate the presence of unmodified subunits that combined with modified subunits to varying degrees. However, this result clearly demonstrates a distribution of subunit forms due to modification by thiourea dioxide.

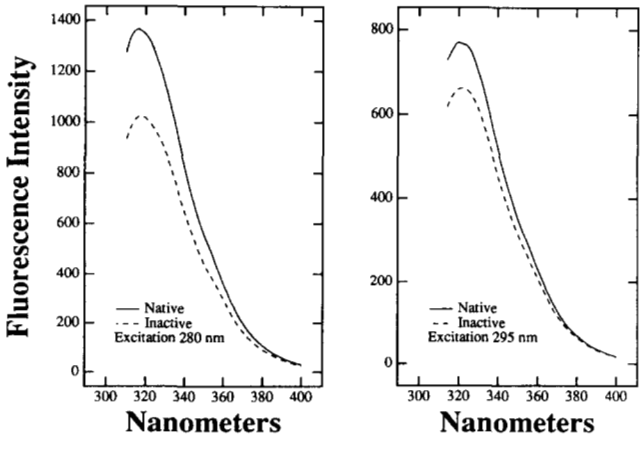

Fig. 7. Fluorescence spectra of native and inactivated CTP synthetase. Inactivated and control samples of CTP synthetase were prepared as described under Materials and methods. Individual spectra represent the average of 10 scans.

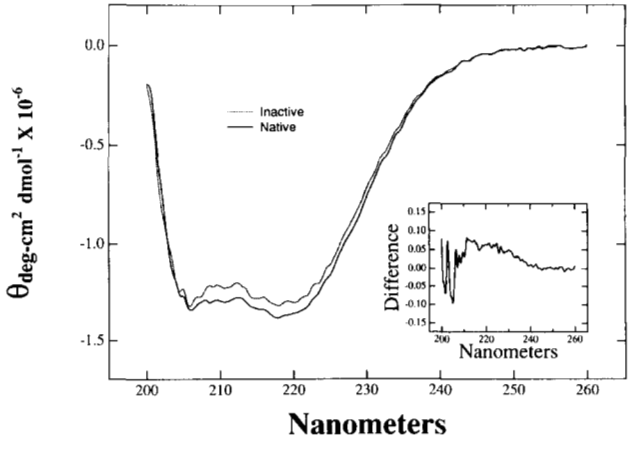

Fig. 8. CD spectra of native and inactivated CTP synthetase. Inactivated and control samples of CTP synthetase were prepared as described under Materials and methods. Individual spectra represent the average of five scans.

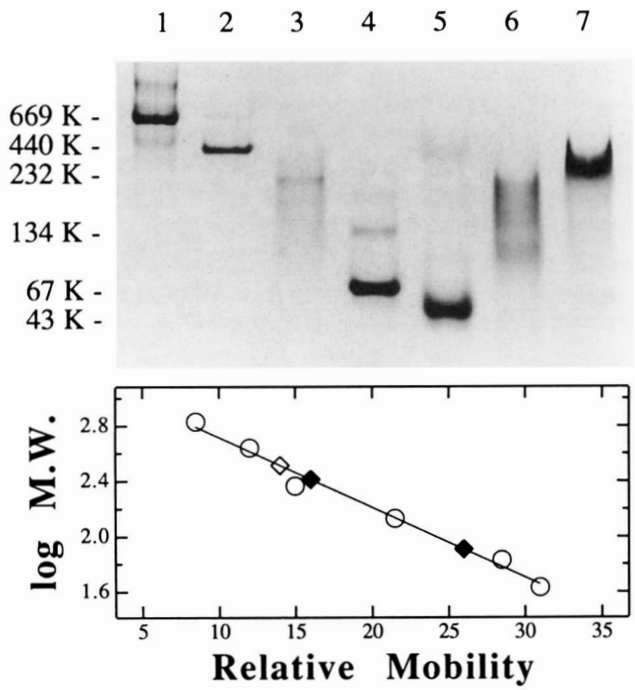

Fig. 9. Native gel electrophoresis of active and thiourea dioxide inactivated CTP synthetase. Inactivated and active samples of CTP synthetase were prepared and analyzed on nondenaturing gels as described under Materials and methods. Individual lanes on the gel contained: lane 1, thyroglobulin; lane 2, ferritin; lane 3, catalase; lane 4, bovine serum albumin; lane 5, ovalbumin; lane 6, inactivated CTP synthetase; and lane 7, active CTP synthetase. The relative mobilities of the standards were used to construct the plot shown beneath the gel. The relative mobilities of both monomers and dimers of bovine serum albumin were calculated from lane 4. Open circles correspond to the standards. The open diamond corresponds to the mobility of active CTP synthetase, and the closed diamonds correspond to the minimum and maximum mobilities of inactivated CTP synthetase.

Radiolabeling

The stoichiometry of thiourea dioxide modification was determined by measuring the incorporation of [^{14}C]thiourea dioxide into CTP synthetase. Data in Figure 10 demonstrate that complete inactivation correlated with incorporation of approximately 3 mol of [^{14}C]thiourea dioxide per mole of CTP synthetase monomer.

pH dependence of k_{inact}

The pH dependence of inactivation was measured at pH values between 6 and 9. The titration curve in Figure 11 shows that there was very little inactivation below pH 7, but that k_{inact} increased at all pH values above 7.0. The best fit of the data in Figure 11 to Equation 4 (see Materials and methods) indicated that the titratable group involved in thiourea dioxide inactivation has a K_a of $4.88 \times 10^{-8} \pm 5.8 \times 10^{-9}$, which corresponds to a pK_a of 7.3. This pK_a falls in the range of values for imidazole (pK_a = 7.0) and α-NH_2 (pK_a = 7.8) groups of free

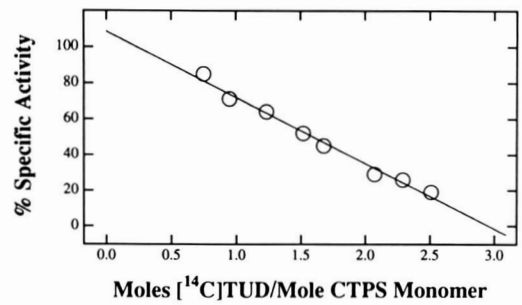

Fig. 10. Stoichiometry of [^{14}C]thiourea dioxide incorporation into CTP synthetase. Incorporation of radioactivity into CTP synthetase was measured as described under Materials and methods.

amino acids (Bell & Bell, 1988). In contrast, the ε-NH_2 group of free lysine in solution has a pK_a of 10.2 (Bell & Bell, 1988). However, as discussed below, the pK_a's of protein-bound lysines may be lowered several pH units, and therefore the data in Figure 11 are still consistent with thiourea dioxide modification of lysine residues.

Discussion

The present data show that thiourea dioxide irreversibly inactivates CTP synthetase. In contrast to the case for glutamine synthetase, the inactivation by thiourea dioxide does not show saturation at high thiourea dioxide concentrations. This evidence indicates that the molecule does not participate in an equilibrium binding step with the enzyme prior to inactivation, or, alternatively, that the equilibrium step is so fast in the forward direction that the model reduces to two consecutive first-order reactions. However, the second alternative implies a very small K_d for thiourea dioxide, and this is not consistent with the millimolar concentrations needed for CTP synthetase inactivation. Consequently, thiourea dioxide may be considered as a specific chemical modification reagent for CTP synthetase but not as a mimic of a reaction intermediate. Nevertheless, because substrates protect against

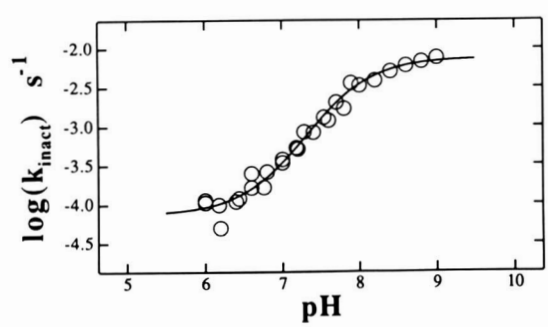

Fig. 11. Dependence of k_{inact} on pH. Enzyme was incubated with 20 mM thiourea dioxide at different pH values as described under Materials and methods. The line through the data represents the best fit to Equation 4 (see Materials and methods) and contains an inflection point with a pK_a of 7.3.

enzyme inactivation, we have used the reagent to begin to identify functionally important amino acids in CTP synthetase.

Previous studies have shown that thiourea dioxide modifies both histidine and lysine residues in glutamine synthetase (Colanduoni & Villafranca, 1985; DiIanni et al., 1986; DiIanni, 1988). Histidine modification in glutamine synthetase requires the presence of a mixed-function oxidation system to generate hydrogen peroxide, whereas lysine modification does not, and thiourea dioxide will act as a reductant in this system. Consequently, several experiments were done to determine whether or not thiourea dioxide inactivation of CTP synthetase also requires a mixed-function oxidation system.

The data in Figure 2 show that CTP synthetase inactivation by thiourea dioxide does not require oxygen. This tends to rule out the requirement for a mixed-function oxidation system. Alternatively, it may be possible that thiourea dioxide inactivation only requires a very small amount of oxygen, which may have been present even after dilution into an argon-purged solution of thiourea dioxide. Nevertheless, a reaction requiring oxygen should have shown some concentration dependence, and the oxygen concentration in a solution of enzyme diluted into an argon-purged reaction mixture would have been substantially lower than in controls where the reaction mixture was not purged. Therefore, purged inactivation solutions should have had slower inactivation rates, but the data in Figure 2 show that they did not. In addition, catalase might also have been expected to have an effect on the inactivation rate if the inactivation required oxygen and the generation of hydrogen peroxide, but catalase also had no observable effect on the inactivation rate. These data suggest that histidine oxidation is not the mechanism of inactivation.

More importantly, CTP synthetase inactivation by thiourea dioxide correlated with the incorporation of 3 mol of [^{14}C]thiourea dioxide per mole of monomer. This is direct evidence that the inactivation mechanism involves covalent modification of the enzyme. Previous studies have shown that thiourea dioxide and lysine react to produce homoarginine, as shown in Figure 1 (Colanduoni & Villafranca, 1985). The chemical reactivity of thiourea dioxide and the stable incorporation of [^{14}C]thiourea dioxide strongly indicates that lysine modification is the mechanism of CTP synthetase inactivation in these experiments. Preliminary analysis of peptides derived from labeled CTP synthetase also supports the idea that covalent modification of lysine residues is the mechanism of inactivation. Initial sequence data indicate that radioactivity is associated with at least one lysine residue in a mixture of four peptides that eluted in a single peak in the peptide map. However, work is still in progress to isolate the single radioactive peptide in this mixture and to identify the specific modification site. Several other radioactive peaks in the peptide map are also under investigation.

The pH profile of inactivation demonstrates a pK_a of 7.3 for the titratable group, which appears to be inconsistent with the pK_a of lysine residues, which is 10.2 for free lysine. However, previous studies have shown that the environment within a protein may lower the pK_a of lysine residues by as much as four pH units. For instance, both acetoacetate decarboxylase (Schmidt & Westheimer, 1971) and mandelate racemase (Landro et al., 1991) have been shown to contain catalytic lysine residues with pK_a's of 5.9 and 6.4, respectively. It has been speculated that electrostatic destabilization of the positive charges on these lysine residues by other active site residues may account for their lower pK_a's. These precedents may account for the observed pK_a in Figure 11 and the chemical evidence for modification of a lysine group or groups involved in CTP synthetase inactivation.

However, the distribution of molecular weights in the inactivated protein, as demonstrated by native gel electrophoresis, may suggest that inactivation does not uniformly lead to labeling the same lysines on each subunit. In this experiment, in the ideal case, complete inactivation would produce an homogeneous set of subunits containing three extra guanidino moieties from thiourea dioxide, which would be expected to be positively charged at pH 8.8. These subunits might migrate as tetramers if modification has no effect on protein structure in the presence of nucleotides, or as dimers or monomers if modification disrupts the native structure required for tetramerization. The lack of discrete bands in the native gel indicates heterogeneity of charge on the subunits, and the range of molecular weights observed, from 60,000 to 260,000, suggests multiple combinations of subunits with varying charge.

It is significant that the presence of nucleotides resulted in almost complete protection against inactivation. Three basic mechanisms may be envisioned for protection. First, nucleotide binding may have changed the electronic environment at an adjacent site and thereby prevented the chemistry of modification, or, second, nucleotide binding may have physically blocked the modification site, or, third, nucleotide binding may have induced conformational changes that buried the modification site.

Both ATP and UTP must bind at the active site for catalysis, and GTP, as an allosteric activator, presumably must bind at a separate, spatially distant site. Therefore, the nucleotides define at least three separate sites. Data in Figure 5 show that nucleotide binding to any one of these three separate sites provides protection against inactivation. This might be explained if thiourea dioxide modifies an amino acid where all three nucleotide binding sites overlap, in which case physical protection would be the mechanism of protection. Similarly, if all three nucleotides merely contact a common amino acid, this might be sufficient to prevent inactivation.

It seems more likely, however, that nucleotide protection involves a functional relationship with the inacti-

vation site(s). In other words, the fact that nucleotide protection displays sigmoidal behavior in all cases suggests an effect transmitted through conformational changes in the protein. In this mechanism, the modification site may not contact the nucleotide binding sites, but its accessibility to thiourea dioxide may be controlled functionally by changes induced upon nucleotide binding. Protection by conformational changes predicts that combinations of nucleotides would be more effective in protection because of their known synergistic effects. Data in Figures 5 and 6 confirm this synergism. For instance, it is known that ATP or UTP alone will induce tetramerization at concentrations above 2 mM (Levitzki & Koshland, 1972b). It also is known that ATP and UTP in combination shift the equilibrium toward tetramer formation more effectively than either nucleotide alone (Levitzki & Koshland, 1972b), and Figure 6 demonstrates that a combination of ATP and UTP provided the lowest concentration for half-maximal protection and the highest value of $1/k_{inact}$.

All the data for nucleotide protection demonstrate positive cooperativity, including the data for GTP protection against inactivation. This agrees with previous results showing positive cooperativity in nucleotide binding (Levitzki & Koshland, 1972a,b). Also, the ranges of nucleotide concentrations in Figures 5 and 6 were wide enough to imply polymerization from the dimer to the tetramer in the course of the experiment (Levitzki & Koshland, 1972b), except in the case of GTP, where there appears to be no evidence that GTP alone induces tetramerization. However, it is known that GTP causes conformational changes in the enzyme (Levitzki & Koshland, 1972a), and therefore all the data in Figures 5 and 6 support the argument that protection occurs by induced conformational changes.

In addition, modification with thiourea dioxide caused detectable changes in the fluorescence and CD properties of the enzyme. This is consistent with the idea that maximum enzyme activity requires the structural flexibility to transmit cooperative effects to neighboring subunits, and that disruption of this cooperativity would prevent maximal activity. Modification by thiourea dioxide clearly does change the native structure, and, correspondingly, we observed that inactivation occurs. Thiourea dioxide modification blocks both glutamine-dependent and ammonia-dependent activity, and therefore structural changes are not localized simply to the glutamine amide transfer domain.

In summary, the results here demonstrate the usefulness of thiourea dioxide as a probe of important sites in CTP synthetase. The data firmly support modification of at least three residues on the enzyme, which are most likely lysine residues. The implication is that the amino acid residues (lysines?) are functionally involved in CTP synthetase activity. Future experiments will focus on identifying the specific modification sites so that site-directed mutagenesis may be used to study the role of these amino acid residues in enzyme function.

Materials and methods

Materials

Thiourea dioxide, *O*-methylisourea, thiourea, urea, ammonium chloride, magnesium chloride, glutamine, bis-tris-propane, triethanolamine, EDTA, ATP, UTP, and GTP were from Sigma. Catalase was from Boehringer Mannheim. Hepes, Mes, and Pipes were from United States Biochemical Corp. New England Nuclear was the source of [^{14}C]thiourea and Amersham was the source of the [^{14}C]hexadecane reference standard. Gradient polyacrylamide gels (4–15%) were purchased from Bio-Rad. Ecolume was from ICN, and PD-10 gel filtration 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 7.8 μmol CTP min^{-1} mg^{-1}. Protein concentrations were determined by measuring the absorbance at 280 nm and using the previously determined extinction coefficient of $\varepsilon^{280}_{0.1\%} = 0.89$ (Levitzki & Koshland, 1972b; Lewis & Villafranca, 1989). 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. Prior to use, aliquots of enzyme were dialyzed against three 1-L changes of 50 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 1 mM Na_4EDTA, and 2 mM dithiothreitol to remove nucleotides. A molecular weight of 60,300 was used for calculations of protein concentration.

Enzyme assay

Enzyme activity was assayed spectrophotometrically 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 $\Delta\varepsilon = 1{,}338\ M^{-1}\ cm^{-1}$ (Long & Pardee, 1967). The NH_4^+-dependent assay contained, in a final volume of 1 mL, 50 mM Hepes, pH 8.0, 1 mM ATP, 1 mM UTP, 20 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM Na_4EDTA, and 10–20 μg CTP synthetase. The glutamine-dependent assay was the same as the NH_4^+-dependent assay except that the assay contained 0.2 mM GTP, and NH_4Cl was replaced with 10 mM glutamine. Enzyme 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.

Inactivation with thiourea dioxide

Inactivation reactions contained, in a final volume of 200 μL, 200 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 20 mM thiourea dioxide, 0.9 mg/mL CTP synthetase, and additions as noted in the figures. Enzyme was equilibrated with buffer at 37 °C for 3 min, and thiourea dioxide was added to start the reaction. Aliquots of the reaction were removed at various times and added immediately to assay mixture to measure enzyme activity. Glutamine, nucleotides, and thiourea dioxide were dissolved in 200 mM Hepes, pH 8.0, in order to maintain the ionic strength and pH of the incubations. For the inactivation under argon, thiourea dioxide, buffer, and $MgCl_2$ were mixed and bubbled with argon for 20 min. The inactivation was started by addition of 15 μL of enzyme to 185 μL of degassed reaction mixture. Nucleotide concentrations were determined by their absorbance and the following extinction coefficients: ATP $\varepsilon_{259} = 15{,}400\ M^{-1}$, UTP $\varepsilon_{262} = 10{,}000\ M^{-1}$, GTP $\varepsilon_{253} = 13{,}700\ M^{-1}$ (Dawson et al., 1969).

Fluorescence and CD

Enzyme was inactivated as described above to <1% activity and then was dialyzed against four successive changes of 10 mM bis-tris-propane, pH 8.0. The control enzyme sample was incubated with buffer and then dialyzed separately against 10 mM bis-tris-propane, pH 8.0. Fluorescence spectra were obtained with a Photon Technologies LS-100 Luminescence System. Samples contained 3 mL of 100 mM triethanolamine, pH 7.5, and 30 μL of either 2.9 mg/mL native CTP synthetase or 30 μL of 2.9 mg/mL inactive CTP synthetase. Spectra were obtained at 25 °C, and 10 scans were averaged to produce a final spectrum. CD spectra were obtained with an Aziz 62 DS spectropolarimeter. Samples contained 450 μL 10 mM bis-tris-propane, pH 8.0, and 50 μL of either 2.9 mg/mL native CTP synthetase or 2.9 mg/mL inactive CTP synthetase. Spectra were obtained at 25 °C, and five scans were averaged to produce a final spectrum.

Native gel electrophoresis

Bio-Rad Mini-PROTEAN II 4–15% gradient polyacrylamide gels containing 0.375 M Tris-HCl, pH 8.8, were used for native gel electrophoresis. Running buffer contained 24.8 mM Tris base, 192 mM glycine, 1 mM ATP, 0.5 mM UTP, and 5 mM $MgCl_2$ without pH adjustment. Sample buffer contained 75 mM Tris-HCl, pH 6.8, 50% glycerol, and 0.1% bromophenol blue. Gels were equilibrated with running buffer by electrophoresis for 30 min at 200 V constant voltage. The buffer tanks then were drained and fresh running buffer was added. Molecular weight markers and native and inactive CTP synthetase were dissolved 1:1 in sample buffer, and approximately 30 μg of each protein was loaded on the gel. Electrophoresis was performed for 30 min at 200 V constant voltage, and the gels were stained with Coomassie G-250.

Synthesis of [^{14}C]thiourea dioxide

Radioactive thiourea dioxide was prepared as described previously (Colanduoni & Villafranca, 1985), except that the reaction was done on a smaller scale. Solid [^{14}C]thiourea (57 mCi/mmol) was dissolved in 100 μL of water. In the synthetic reaction, 6.3 mg of thiourea and 80 μL of [^{14}C]thiourea were dissolved in 90 μL of water and were chilled on ice. The reaction was started by addition of 25 μL of 30% H_2O_2, and was left to react for 35 min on ice. The precipitate was collected by centrifugation and was washed with 1 mL of hot methanol. The precipitate was dissolved in 200 μL of 50 mM triethanolamine, pH 8.0, 10 mM $MgCl_2$, and 1 mM EDTA. The final concentration was determined using an extinction coefficient of $\varepsilon_{270} = 400$. The final specific radioactivity was 3,200 dpm/nmol.

Stoichiometry of [^{14}C]thiourea dioxide incorporation

Incorporation of [^{14}C]thiourea dioxide into CTP synthetase was determined by a filter paper assay. The inactivation reaction contained 50 mM triethanolamine, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM EDTA, 5 mg/mL CTP synthetase, and 20 mM [^{14}C]thiourea dioxide (3,200 dpm/nmol) in a final volume of 600 μL. The reaction was started by addition of enzyme, and aliquots were removed at timed intervals for the measurement of activity and protein bound [^{14}C]thiourea dioxide. Protein-bound radioactivity was measured by transferring 60 μL of reaction mixture to squares of Whatman 3 MM filter paper and quenching the filter papers in 500 mL of cold 10% trichloroacetic acid. Quenched filter papers then were washed twice with 200 mL of cold ethanol and once with 50 mL of ethyl ether. The washed papers were dried in air, and radioactivity on the filter papers was quantitated by liquid scintillation counting in 10 mL of Ecolume. The control reaction contained no enzyme. The amount of radioactivity on control filter papers was subtracted from the corresponding experimental filters. Separate aliquots of reaction mixture were removed and assayed for enzyme activity immediately after each quenched sample.

Inactivation with thiourea dioxide as a function of pH

Inactivations with 20 mM thiourea dioxide were done as described above, except that the buffer was 200 mM Hepes-Mes-Pipes. Equal volumes of 200 mM Hepes, 200 mM Mes, and 200 mM Pipes were mixed to form the buffer system, and separate 10-mL volumes of the buffer

system were adjusted to the required pH with either 1 M HCl or 2 M NaOH. The final pH of each incubation was measured at the end of the inactivation.

Data analysis

All inactivation reactions contained a large excess of thiourea dioxide (2–20 mM) over CTP synthetase (14.9 μM monomer). Therefore, the inactivation reactions may be regarded as pseudo-first order, and the rate of inactivation, V_{inact}, may be written as in

$$V_{inact} = \frac{-d[E]}{dt} = k_{inact}[E] \quad (1)$$

(Bell & Bell, 1988). This may be rearranged and integrated to give

$$\ln \frac{E_t}{E_0} = -k_{inact}t, \quad (2)$$

where E_t is the specific activity at time t and E_0 is the initial specific activity (Bell & Bell, 1988). Consequently, $\ln(E_t/E_0)$ represents the natural log of the percent specific activity remaining at any time t, and a plot of ln % specific activity versus time yields a value for the pseudo-first-order rate constant of inactivation k_{inact}. Data in Figures 2, 3A, and 4 were fit to the equation of a straight line, and the slope of each line was taken as k_{inact}. Values for k_{inact} in Figures 5, 6, and 11 were calculated from plots of ln % specific activity versus time as a function of added nucleotide or pH.

Data for the concentration dependence of nucleotide protection were fit to a modified form of the Hill equation (Newton & Koshland, 1989),

$$Y = A + B\left(\frac{S^n}{C^n + S^n}\right), \quad (3)$$

where A is the value of k_{inact} in the absence of nucleotides, B is the maximal value of k_{inact} due to nucleotide protection, S is the concentration of nucleotide, C is the concentration of nucleotide resulting in half-maximal protection, and n is the Hill coefficient. Data for the inactivation of CTP synthetase as a function of pH were fit to

$$\log Y = \log\left[\frac{Y_L + Y_H * (K/H)}{1 + (K/H)}\right], \quad (4)$$

where Y_L is the constant value at low activity, Y_H is the constant value at high activity, K is the dissociation constant of the ionizing group, and H is the hydrogen ion concentration. A nonlinear least-squares program described previously was used to fit the data (Duggleby, 1984).

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