Reversible Reaction of Cyanate with a Reactive Sulfhydryl Group at the Glutamine Binding Site of Carbamyl Phosphate Synthetase†

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ABSTRACT: Carbamyl phosphate synthetase from *Escherichia coli* reacts stoichiometrically (one to one) with [^{14}C]cyanate to give a ^{14}C-labeled complex which can be isolated by gel filtration. The formation of the complex is prevented if L-glutamine is present or if the enzyme is first reacted with 2-amino-4-oxo-5-chloropentanoic acid, a chloro ketone analog of glutamine which has been shown to react with a specific SH group in the glutamine binding site. The rate of complex formation is increased by ADP and decreased by ATP and HCO_3^-. The isolated complex is inactive with respect to glutamine-dependent synthetase activity. However, the reaction of cyanate with the enzyme is reversible. The rate of dissociation of the isolated complex is not affected by pH (over the pH range 6–10), is greatly increased by ATP and HCO_3^-, and is decreased by ADP. The allosteric effectors ornithine and UMP have no effect on either the rate of formation or the rate of dissociation of the complex; however, the apparent affinity of the enzyme for ATP is decreased by UMP and increased by ornithine. The site of reaction of cyanate with carbamyl phosphate synthetase, which is composed of a light and a heavy subunit, is with an SH group in the light subunit to give an *S*-carbamylcysteine residue. The binding of L-[^{14}C]glutamine to the enzyme and the inhibition of glutamine-dependent synthetase activity by the chloroketone analog are both prevented by the presence of cyanate. The reaction with cyanate is considered to be with the same essential SH group which is located in the glutamine binding site and is alkylated by 2-amino-4-oxo-5-chloropentanoic acid. The bicarbonate-dependent effects of ATP suggest that formation of the activated carbon dioxide intermediate is accompanied by changes in the heavy subunit which functionally alter the properties of the glutamine binding site on the light subunit. The allosteric effects of ornithine and UMP are probably not related to this intersubunit interaction.

We have previously reported that cyanate has the following effects on the catalytic activities exhibited by carbamyl phosphate synthetase from *Escherichia coli:* glutamine-dependent carbamyl phosphate synthesis and ATP- and bicarbonate-dependent L-γ-glutamyl hydroxamate hydrolysis activities are inhibited, bicarbonate-dependent hydrolysis of ATP (ATPase activity) is stimulated nearly threefold, stoichiometric synthesis of ATP from carbamyl phosphate and ADP (ATP synthesis activity) is not affected, and the apparent K_m for ammonia in the ammonia-dependent carbamyl phosphate synthesis activity is decreased (Anderson et al., 1973). A half-maximal effect of cyanate on these activities of carbamyl phosphate synthetase is observed when the concentration of cyanate in the assay mixtures is about 2 m*M*. These effects of cyanate on the different catalytic activities of carbamyl phosphate synthetase are similar to those of 2-amino-4-oxo-5-chloropentanoic acid, a chloro ketone analog of glutamine which apparently specifically alkylates an SH group at the glutamine binding site of the enzyme (Khedouri et al., 1966; Pinkus and Meister, 1972).

The studies on the nature of the interaction of cyanate with carbamyl phosphate synthetase reported in this paper indicate that, like the chloro ketone analog, cyanate reacts stoichiometrically and specifically with an SH group at the glutamine binding site, but, unlike the chloro ketone analog, the reaction is reversible.

Materials and Methods

Materials. Carbamyl phosphate synthetase was isolated from *Escherichia coli* B by the procedure described by Anderson et al. (1970) as modified by Matthews and Anderson (1972). ATP, ADP, L-glutamine, ornithine, UMP, and protease (from *Streptomyces griseus*) were obtained from Sigma Chemical Co. [^{14}C]Potassium cyanate, [^{14}C]Na_2CO_3, and L-[^{14}C]glutamine were obtained from New England Nuclear Corp. Reagent grade potassium cyanate was obtained from Mallinckrodt and was recrystallized before use.

Methods. The enzyme activity was determined by measuring the formation of ADP or [^{14}C]carbamyl phosphate as previously described (Anderson and Meister, 1965, 1966; Anderson et al., 1970). A unit of enzyme activity is defined as that amount of enzyme which will catalyze the synthesis of 1 μmol of carbamyl phosphate/hr at 37° under the assay conditions. Protein was determined from its absorbance at 280 nm (Anderson and Marvin, 1970).

The radiochemical purity of [^{14}C]cyanate was determined by measuring the percent of the total radioactivity which was converted to [^{14}C]urea after addition of 0.4 ml of 4 *M* NH_4Cl (pH 8.5) to a 1-ml aliquot of the [^{14}C]cyanate sample in water and boiling for 12 min. This procedure is the same as that used to measure [^{14}C]carbamyl phosphate in the presence of [^{14}C]bicarbonate. The [^{14}C]carbamyl phosphate is quantitatively converted to [^{14}C]cyanate and then to [^{14}C]urea under these conditions

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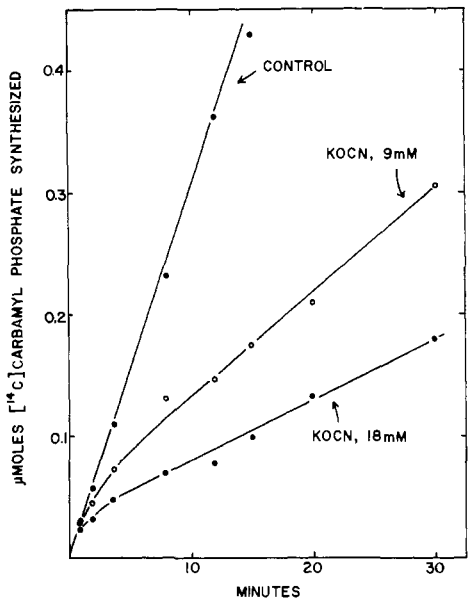

FIGURE 1: Inhibition of glutamine-dependent carbamyl phosphate synthetase activity by cyanate with time. The reactions were initiated by the addition of enzyme, and the μmoles of [^{14}C]carbamyl phosphate formed after incubation at 37° for the indicated times was determined as described in the text. The reaction mixtures contained potassium phosphate buffer (0.09 *M*, pH 7.8), ATP (20 m*M*), $MgCl_2$ (20 m*M*), [^{14}C]$NaHCO_3$ (20 m*M*, 8 × 10^5 cpm), L-glutamine (5 m*M*), enzyme (16 μg), and KOCN as indicated in a final volume of 1.0 ml.

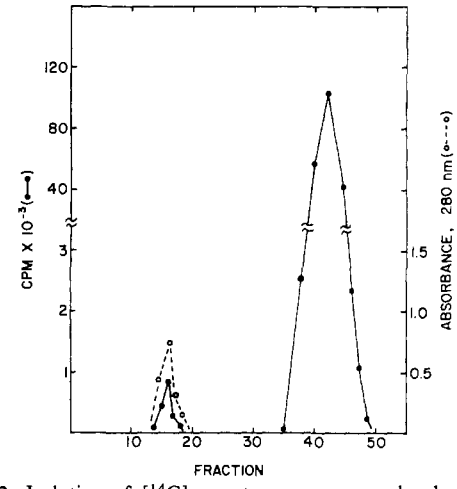

FIGURE 2: Isolation of [^{14}C]cyanate-enzyme complex by gel filtration. A mixture containing enzyme (1.5 mg), [^{14}C]potassium cyanate (2.1 m*M*, 2.3 × 10^6 cpm), potassium phosphate buffer (0.18 *M*, pH 7.5), and EDTA (5 mM) in a final volume of 0.11 ml was incubated at 22° for 1 min. The mixture was then subjected to gel filtration on a column (1 × 29 cm) of Sephadex G-50 previously equilibrated with 0.1 *M* potassium phosphate buffer (pH 7.8) at 4°. The column was eluted with the same buffer and 0.4-ml fractions were collected. The radioactivity was determined by counting an 0.05-ml aliquot of each fraction and the enzyme was measured by its absorbance at 280 nm.

(Anderson and Meister, 1965); the [^{14}C]urea is subsequently separated from [^{14}C]bicarbonate by passing the boiled solutions through small columns containing an anion exchange resin. It was found that no radioactivity passed through the anion exchange resin if the samples were not boiled and that 80–90% of the radioactivity present in the commercial preparations was actually [^{14}C]cyanate; since all of the radioactivity in the preparations was acid-labile and negatively charged, the radiochemical impurity was presumably [^{14}C]bicarbonate (Labbe, 1973). The actual concentration of [^{14}C]cyanate was determined chemically by the procedure described by Levine and Kretchmer (1971).

S-Carbamylcysteine was synthesized as described by McCord and Skinner (1963). The chloro ketone analog of glutamine, 2-amino-4-oxo-5-chloropentanoic acid, was synthesized as described by Pinkus and Meister (1972).

Measurements of radioactivity were made by liquid scintillation counting using a toluene-based cocktail with BIO SOLV (Beckman instruments, Inc.) as solubilizer.

Results

Effect of Cyanate on Carbamyl Phosphate Synthetase Activity as a Function of Time. The results in Figure 1 show that the rate of the glutamine-dependent carbamyl phosphate synthetase activity in the presence of cyanate decreases with time after mixing cyanate with the substrates and enzyme, reaching a reduced steady-state rate after 5 min which is proportional to the cyanate concentration. This reduced but constant rate is observed immediately, however, if cyanate is preincubated with the enzyme for 5 min at 37° in the presence of all components of the reaction mixture except glutamine; preincubation in the presence of glutamine and all other components except ATP and $MgCl_2$ has no effect. Double reciprocal plots of the final steady-state velocity at different concentrations of cyanate as a function of glutamine have revealed that cyanate behaves kinetically as a noncompetitive inhibitor of glutamine. The above results are interpreted to indicate that cyanate acts as an inhibitor by reacting with the enzyme at the glutamine binding site and that the rate of the reaction is relatively slow and is reversible. The reversibility of the reaction, for which direct evidence is given in a later section, suggests that the inhibition by cyanate is actually competitive rather than noncompetitive. This conclusion would not be inconsistent with the fact that noncompetitive inhibition is actually observed, since the rate of dissociation of the inhibitor from the enzyme is very low (Figures 3–5) and under these circumstances a competitive inhibitor will behave kinetically as a noncompetitive inhibitor (Westley, 1969).

In contrast to the time-dependent effect of cyanate on the glutamine-dependent carbamyl phosphate synthetase activity described above, we have observed that the increased steady-state rate of the bicarbonate-dependent ATPase activity (glutamine absent) which occurs in the presence of cyanate (18 m*M*) is attained virtually immediately after addition of all reactants.

Formation and Isolation of a Cyanate-Enzyme Complex. The isolation of a L-[^{14}C]glutamine-enzyme complex has been previously reported (Wellner et al., 1973); the complex is formed by simply incubating L-[^{14}C]glutamine with carbamyl phosphate synthetase for a short time and is isolated by removing the ^{14}C-labeled complex from unbound L-[^{14}C]glutamine by gel filtration chromatography at 4°. Although a portion of the bound ^{14}C-labeled moiety is apparently covalently attached to the enzyme, the complex is not stable and the chemical nature of the association has not been definitively established.

Experiments reported here demonstrate that an analogous, but relatively more stable [^{14}C]cyanate-enzyme complex can be isolated by gel filtration chromatography of carbamyl phosphate synthetase which has been incubated for several minutes with [^{14}C]cyanate. A typical result is shown in Figure 2. Binding of [^{14}C]cyanate is not observed

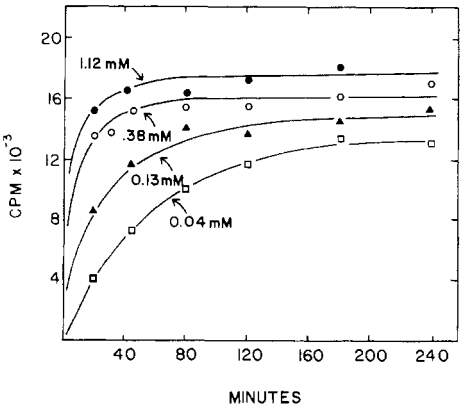

FIGURE 3: Formation of [^{14}C]cyanate-enzyme complex as a function of time and [^{14}C]cyanate concentration. Reaction mixtures containing potassium phosphate buffer (0.1 *M*, pH 7.6), EDTA (3 m*M*), enzyme (2.6 mg), and [^{14}C]potassium cyanate (1.5×10^7 cpm/μmol) as indicated in a final volume of 0.8 ml were incubated at 21°. At the indicated times an 0.1-ml aliquot was removed from each reaction mixture and subjected to gel filtration on small columns (0.3 × 7 cm) of Sephadex G-50 equilibrated with 0.1 *M* potassium phosphate buffer (pH 7.8) which also contained 0.01 *M* L-glutamine. The formation of [^{14}C]cyanate-enzyme complex was measured by determining the total radioactivity in the fraction (0.7 ml) containing enzyme.

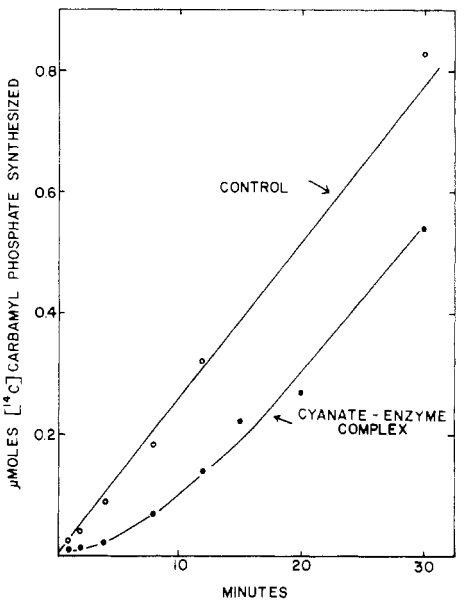

FIGURE 4: Reactivation of the glutamine-dependent carbamyl phosphate synthetase activity of the cyanate-enzyme complex with time of incubation. Cyanate-enzyme complex was prepared by incubating a mixture containing enzyme (3.0 mg), KOCN (4 m*M*), potassium phosphate buffer (0.18 *M*, pH 7.8), and EDTA (5 m*M*) in a final volume of 0.33 ml for 15 min at 22°; the complex was then isolated by gel filtration as described in Figure 2. The isolated cyanate-enzyme complex (0.16 mg) was then incubated with a mixture containing potassium phosphate buffer (0.1 *M*, pH 7.8), L-glutamine (5 m*M*), ATP (20 m*M*), $MgCl_2$ (20 m*M*), and [^{14}C]$NaHCO_3$ (20 m*M*, 8×10^5 cpm) in a final volume of 10 ml at 37°. Aliquots of 1 ml were removed at the indicated times and the μmoles of [^{14}C]carbamyl phosphate formed was determined as described in the text. Enzyme which had not been treated with KOCN was substituted at the same concentration for the cyanate-enzyme complex in the control.

if L-glutamine (10 m*M*) is present during the incubation; the presence of L-glutamine has no effect, however, if it is added after the enzyme–cyanate complex has formed and a few minutes before the mixture is subjected to gel filtration chromatography. Binding of [^{14}C]cyanate is also not observed if the enzyme is first reacted with 2-amino-4-oxo-5-chloropentanoic acid as described by Pinkus and Meister (1972). The presence of L-asparagine (10 m*M*) during the incubation has no effect on the binding of [^{14}C]cyanate. Also, no radioactivity is eluted with the enzyme if [^{14}C]HCO_3^-, a probable contaminant of most [^{14}C]cyanate preparations, is substituted for [^{14}C]cyanate. The binding of L-[^{14}C]glutamine to carbamyl phosphate synthetase, carried out as previously described (Wellner et al., 1973), is prevented by the presence of potassium cyanate (5 m*M*) in the incubation mixture for 10 min at 22° prior to the addition of L-[^{14}C]glutamine. L-Glutamine and certain other glutamine analogs protect the enzyme against inhibition by 2-amino-4-oxo-5-chloropentanoic acid (Wellner et al., 1973). We have established that the presence of potassium cyanate (3 m*M*) in the incubation mixture for 15 min at 22° prior to addition of the chloro ketone also completely prevents inhibition of glutamine-dependent synthetase activity by the chloro ketone. This experiment was carried out essentially as described by Wellner et al. (1973), except that the assay for synthetase activity was carried out for 30 min and only the rate during the final 10 min was measured, thus eliminating the effect of cyanate itself on the synthetase activity (see Figure 4). These observations further suggest that cyanate interacts with carbamyl phosphate synthetase by reacting specifically at the glutamine binding site.

The formation of the complex as a function of time is shown in Figure 3 at several concentrations of cyanate. Although the rate of complex formation is dependent on cyanate concentration, the maximum amount of complex formed is apparently less at lower concentrations of cyanate regardless of the reaction time. This would indicate that a steady state is reached in which the rate of complex formation is the same as the rate of its decomposition. That decomposition is simply the result of reversibility of the reaction (dissociation of complex to yield cyanate and enzyme) is suggested by the fact that the maximum amount of complex formed in the presence of low concentrations of cyanate (about twice the enzyme concentration) does not decrease with time as would be expected if decomposition yielded another compound, such as [^{14}C]HCO_3^-, which could not react again with the enzyme. Direct evidence that the reaction is reversible is presented below. Experiments carried out as described in Figure 3 in which the concentration of [^{14}C]cyanate was 0.05 m*M* showed that the presence of ornithine (10 m*M*) or UMP (1 m*M*) had no effect on the rate of complex formation; however, the presence of ADP (20 m*M*) plus $MgCl_2$ (20 m*M*) resulted in an increase in the initial rate by about 35%, and the presence of ATP (20 m*M*) plus $MgCl_2$ (20 m*M*) and HCO_3^- (20 m*M*) resulted in a decrease in the initial rate by about 30%.

Dissociation of [^{14}*C*]*Cyanate–Enzyme Complex.* As shown in Figure 4, the isolated [^{14}C]cyanate–enzyme complex itself is apparently inactive with respect to glutamine-dependent carbamyl phosphate synthetase activity, but the effect is reversible and full activity is regained after a period of 15–20 min under the described conditions. This result also establishes that the isolated complex is not an artifact, but its formation is directly related to the observed effects of cyanate on the glutamine-dependent carbamyl phosphate synthetase activity, and, presumably, the other activities of carbamyl phosphate synthetase.

The amount of radioactivity which remains associated with the isolated [^{14}C]cyanate–enzyme complex decreases

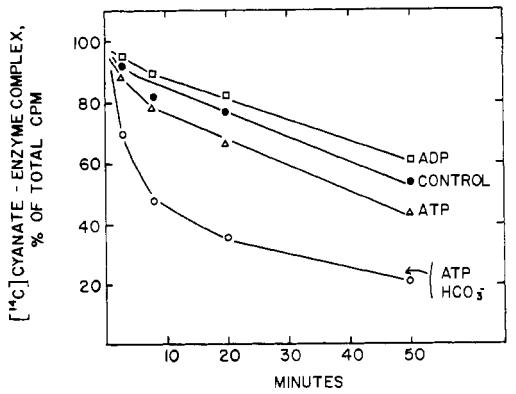

FIGURE 5: Effect of substrates on the rate of dissociation of the [^{14}C]cyanate-enzyme complex. Reaction mixtures containing potassium phosphate buffer (0.09 *M*, pH 7.8), [^{14}C]cyanate-enzyme complex (0.6 mg, 2.4 × 10^4 cpm), and ADP (10 m*M*), ATP (10 m*M*), or $NaHCO_3$ (10 m*M*) as indicated in a final volume of 0.5 ml were incubated at 37°. The mixtures containing ADP or ATP also contained $MgCl_2$ (10 m*M*). At the indicated times an 0.1-ml aliquot was removed from each reaction mixture and subjected to gel filtration at 4° on small columns (0.3 × 7 cm) of Sephadex G-50 equilibrated with 0.1 *M* potassium phosphate buffer (pH 7.8). Two fractions were collected, the first containing the [^{14}C]cyanate-enzyme complex (plus free enzyme) and the second containing the low molecular weight fraction, including [^{14}C]cyanate which had dissociated from the complex. The total radioactivity present in both fractions was determined. The results are expressed as the percent of the total cpm present as the [^{14}C]cyanate-enzyme complex. The ^{14}C-labeled material in the low molecular weight fraction was identified as [^{14}C]cyanate as described in the text.

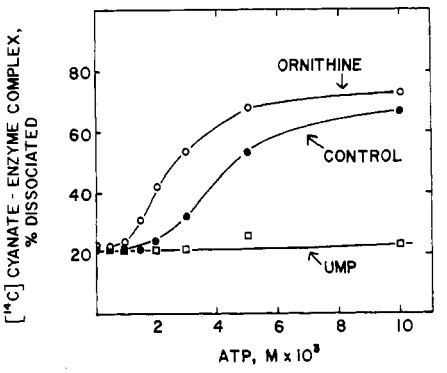

FIGURE 6: Effect of ornithine and UMP on the rate of dissociation of the [^{14}C]cyanate-enzyme complex as a function of ATP concentration. The reaction mixtures contained potassium phosphate buffer (0.09 *M*, pH 7.8), sodium bicarbonate (9 m*M*), [^{14}C]cyanate-enzyme complex (0.6 mg, 3 × 10^3 cpm), $MgCl_2$ in concentrations that were equimolar with ATP, ATP as shown, and where indicated ornithine (10 m*M*), or UMP (5 m*M*) in a final volume of 0.115 ml. After incubation for 20 min at 37° 0.01 ml of 0.1 *M* UMP was added and the entire reaction mixture was subjected to gel filtration at 4° as described in Figure 5. The results are expressed as the percent of the complex which had dissociated (percent of the total radioactivity present in the small molecular weight fraction).

slowly with time when the complex is incubated at 37°. The data in Figure 5 show that the rate of this decomposition is greatly increased by the presence of ATP and that this effect of ATP is dependent on the additional presence of bicarbonate. The presence of ornithine, UMP, or glutamine had no effect on the rate of decomposition, while the presence of ADP had a small inhibitory effect on the rate of decomposition. The effect of ATP concentration in the presence and absence of ornithine or UMP on the rate of decomposition is shown in Figure 6. These results are very similar to previously reported studies where it was shown that the availability of a specific SH group located in the light subunit of carbamyl phosphate synthetase for reaction with various reagents is dependent on the presence of ATP plus bicarbonate and that this effect is enhanced by ornithine and can be eliminated by UMP (Foley et al., 1971; Matthews and Anderson, 1972). Thus, the data in Figure 6 further substantiate the conclusion that ornithine and UMP act as positive and negative allosteric effectors, respectively, by effecting a change in the affinity of the enzyme for the substrate ATP.

The radioactive product resulting from the decomposition (dissociation) of the [^{14}C]cyanate-enzyme complex is [^{14}C]cyanate. This was established by boiling the ^{14}C-labeled small molecular weight fractions which separated from the [^{14}C]cyanate-enzyme complex for each experimental point described in Figure 5 in the presence of NH_4Cl and passing this mixture through an anion exchange resin as described under Methods. All of the radioactivity which was no longer associated with the complex could be accounted for as [^{14}C]urea (derived from [^{14}C]cyanate as a result of boiling with NH_4Cl); as controls it was shown that no radioactivity passed through the anion exchange resin if the [^{14}C]cyanate-NH_4Cl mixtures were not boiled.

Stoichiometry of Reaction of Cyanate with Carbamyl Phosphate Synthetase. The data given in Table I show that 1 mol of carbamyl phosphate synthetase will apparently bind up to 1 mol of [^{14}C]cyanate. The results are similar to those reported for the stoichiometry of the binding of glutamine, i.e., up to 1 mol of L-[^{14}C]glutamine will bind to 1 mol of the enzyme, but the decrease in specific activity of the enzyme with time which is observed on storage is accompanied by a decreased availability of glutamine (or cyanate) binding sites (Wellner et al., 1973).

Table I: Stoichiometry of Binding of [^{14}C]Cyanate to Carbamyl Phosphate Synthetase.[a]

Expt No.	Carbamyl Phosphate Synthetase Activity (units/mg)	Mol of [^{14}C]-Cyanate/ 170,000 g of Enzyme	Mol of [^{14}C]-Cyanate/ 170,000 g of Active Enzyme
1	273	0.82	0.89
2	199	0.67	0.98
3	167	0.55	0.97

[a] Reaction mixtures containing potassium phosphate buffer (0.13 *M*, pH 7.6), EDTA (0.25 m*M*), enzyme (0.86, 0.81, and 1.47 mg in experiments 1, 2, and 3, respectively), and [^{14}C]cyanate (1.6 m*M*, 84,000 cpm) were incubated at 20° for 20 min. The reaction mixtures were then subjected to gel filtration as described in Figure 5 and the quantity of [^{14}C]cyanate associated with enzyme (mol of [^{14}C]cyanate bound/mol of carbamyl phosphate synthetase) was determined by measuring the percent of the total radioactivity present in the fraction containing enzyme. The highest specific activity which we have observed for the purified enzyme is 295 units/mg. The data for moles of [^{14}C]cyanate/mole of active enzyme were calculated assuming that cyanate binds only to active enzyme and the fraction of the total enzyme which is active is simply the observed specific activity divided by 295.

Site of Reaction of [^{14}C]Cyanate with Carbamyl Phosphate Synthetase. Carbamyl phosphate synthetase is composed of two nonidentical subunits (Trotta et al., 1971; Matthews and Anderson, 1972). The SH group which is specifically alkylated by the chloro ketone 2-amino-4-oxo-5-chloropentanoic acid is located in the smaller (light) subunit. The radioactivity associated with the [^{14}C]cyanate-

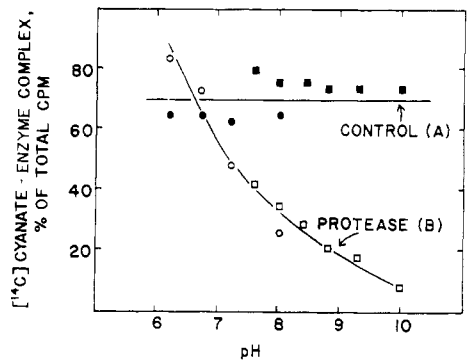

FIGURE 7: Effect of pH and proteolysis on the stability of the [^{14}C]cyanate-enzyme complex. Two reaction mixtures (A and B) containing potassium phosphate buffer (1 m*M*, pH 7.8), $CaCl_2$ (2 mm), [^{14}C]cyanate-enzyme complex (1.6 mg, 1.4×10^4 cpm), and protease (4 mg, reaction mixture B only) in a final volume of 3.4 ml were incubated at 22° for 35 min. Aliquots (0.2 ml) were then removed and added to 0.05 ml of either 0.2 *M* glycylglycine buffer at the indicated pH values (squares) or 0.2 *M* potassium phosphate buffer at the indicated pH values (circles) and incubated at 37° for 25 min. The solutions were then acidified by the addition of 0.05 ml of 1 *N* HCl. The radioactivity in the acidified samples was measured after [^{14}C]CO_2, derived from decomposition of the [^{14}C]cyanate-enzyme complex to [^{14}C]cyanate and the subsequent treatment with acid, was removed by flushing the acidified solution with excess unlabeled CO_2 ($NaHCO_3$ and Dry Ice). The quantity of [^{14}C]cyanate-enzyme complex remaining (acid stable radioactivity) is expressed as the percent of the total initial radioactivity remaining in the acidified solutions after treatment as described above.

enzyme complex is also covalently attached to the light subunit of the enzyme. This was established by isolation of the [^{14}C]cyanate-enzyme complex as described above and then subjecting the enzyme complex to sodium dodecyl sulfate gel electrophoresis as previously described (Matthews and Anderson, 1972). The two bands corresponding to the heavy and light subunits were located and the radioactivity associated with each was determined by counting in a liquid scintillation counter. No radioactivity was associated with the heavy subunit, whereas radioactivity corresponding to about 60% of that expected was associated with the light subunit.

Preliminary studies showed that the radioactivity associated with the [^{14}C]cyanate-enzyme complex remains associated with the protein when the complex is precipitated and washed several times with 5% trichloroacetic acid, but that the complex is labile to base. As shown above, the intact complex does dissociate slowly at 37°. The rate of dissociation is not affected by pH over the pH range 6–10 (Figure 7). However, if the conformational integrity of the complex is first destroyed by extensive hydrolysis with a protease, the rate of breakdown to [^{14}C]cyanate increases with increasing pH and is quite rapid at pH 10 (Figure 7). These results suggest that the complex is the result of the reaction of cyanate with an SH group in the glutamine binding site to give an *S*-carbamylcysteine residue which by itself would be quite labile to base (Stark, 1972; Twu and Wold, 1973), but that the *S*-carbamyl group is protected from rapid decomposition when it exists within the confines of the active glutamine binding site. *S*-Carbamylcysteine, like the complex, is stable under acid conditions and dissociates at pH values above 7 to give cyanate (Stark, 1972).

Direct evidence that the reaction of cyanate with carbamyl phosphate synthetase is with a cysteine residue was obtained by isolation of *S*-[^{14}C]carbamylcysteine from a mixture resulting from the treatment of the [^{14}C]cyanate-

Table II: Rate of Dissociation of *S*-Carbamylcysteine and ^{14}C-Labeled Compound Isolated from the [^{14}C] Cyanate–Enzyme Complex.[a]

Time of Analysis (min)	% of Total *S*-Carbamylcysteine Dissociated	% of Total Radioactivity Dissociated
0.2	4	4
1.6	15	19
3.2	25	24
6.5	43	31
11.2	58	56
37.0	78	71
70.0	68	62

[a] The dissociation of *S*-carbamylcysteine to cysteine and cyanate was determined by including the SH reagent 5,5′-dithiobis(2-nitrobenzoic acid) in the reaction mixture and recording with time the increase in absorbance at 412 nm due to the reaction of this reagent with cysteine (Ellman, 1959). The reaction was carried out at 22° in a 10-mm spectrophotometer cell located in the sample compartment of a Beckman Model 25 spectrophotometer. The reaction mixture contained 5,5′-dithiobis(2-nitrobenzoic acid) (5 m*M*), *S*-carbamylcysteine (0.1 m*M*), a small amount of the ^{14}C-labeled unknown (4×10^3 cpm, less than 2×10^{-4} m*M*), and potassium phosphate buffer (0.1 *M*, pH 8.0); the reference cell was identical except no *S*-carbamylcysteine was present. At the indicated times an aliquot (0.1 ml) was removed from the reaction mixture and added to 0.1 ml of 0.1 *N* HCl in a liquid scintillation vial. The total radioactivity remaining in each vial after incubation overnight at 20° was then determined (undissociated ^{14}C-labeled *S*-carbamylcysteine would be stable under these conditions, whereas [^{14}C]cyanate derived from dissociation would decompose to [^{14}C]CO_2 and be lost). The percent dissociation by both measurements reaches a maximum and then decreases after 37 min. This probably reflects a slow reaction of the liberated acid-labile cyanate with the product which absorbs at 412 nm, 2-nitro-5-mercaptobenzoic acid; the product would be acid-stable and would no longer absorb at 412 nm.

enzyme complex with a protease. [^{14}C]Cyanate-enzyme complex (8.5 mg, 5×10^5 cpm), isolated as described above, was precipitated and washed with 5% trichloroacetic acid. The excess trichloroacetic acid was removed by extraction with ether. The precipitate was suspended in 2 ml of a solution containing potassium phosphate buffer (2 m*M*, pH 7.0) and $CaCl_2$ (1 m*M*). Protease was added and hydrolysis was allowed to proceed for 60 min at pH 7.0 at 37°; protease was added in 0.2-ml aliquots containing 4 mg of protease in the phosphate-$CaCl_2$ buffer at 1, 10, and 20 min. The pH was maintained at 7.0 by addition of 0.1 *M* NH_4OH as needed with a pH Stat. The reaction was terminated by adding 0.12 ml of 1 *N* acetic acid which adjusted the pH to 4.0. The radioactivity recovered at this point totaled 3.2×10^5 cpm. The entire solution was subjected to paper chromatography (Whatman 3MM) with butanol–acetic acid–water (12:3:5, v/v). The majority of the radioactivity (2.1×10^5 cpm) migrated with an R_f of 0.17. Radioactivity at the origin (4×10^4 cpm) and two other areas (R_f values of 0.55 and 0.62, respectively, totalling 2×10^4 cpm) were shown to also be labile to base. The radioactive component which migrated with an R_f of 0.17 was eluted and shown to be *S*-[^{14}C]carbamylcysteine. The radioactivity had the same R_f (0.24) as *S*-carbamylcysteine after paper chromatography on Whatman 1 paper with butanol–acetic acid–water (2:1:1, v/v). In addition, the rate of dissociation to give [^{14}C]cyanate at pH 8.0 was the same as that of *S*-carbamylcysteine (Table II).

Discussion

Previous studies on carbamyl phosphate synthetase from

E. coli have shown that this enzyme is composed of two nonidentical subunits of different molecular weight (Matthews and Anderson, 1972; Trotta et al., 1971, 1974). The isolated light subunit contains the glutamine binding site and has glutaminase activity only, whereas all of the binding sites for the allosteric effectors and the other substrates are located on the heavy subunit and all of the activities which do not involve glutamine or a glutamine analog can be catalyzed by the heavy subunit alone (Trotta et al., 1971, 1974). Both subunits are required for glutamine-dependent carbamyl phosphate synthetase activity. As has been proposed for other amidotransferases (Hartman, 1973), the reaction with glutamine probably involves the formation of a γ-glutamyl intermediate with an SH group at the glutamine binding site (Wellner et al., 1973; Pinkus and Meister, 1972). In the case of carbamyl phosphate synthetase the NH_3 made available by this reaction on the light subunit subsequently must react stoichiometrically with an intermediate formed on the heavy subunit (Trotta et al., 1971).

Significant interaction between the two different subunits of carbamyl phosphate synthetase and their binding sites has been demonstrated. The reaction of 2-amino-4-oxo-5-chloropentanoic acid with a specific SH group in the glutamine binding site on the light subunit results in an increased ATPase activity and a lowered K_m for NH_3, both of which are functions of the heavy subunit (Khedouri et al., 1966; Pinkus and Meister, 1972). Another SH group which is also located in the light subunit reacts with *N*-ethylmaleimide only when ATP and HCO_3^-, both of which presumably interact on the heavy subunit to give an activated form of carbon dioxide, are present (Matthews and Anderson, 1972). Additional evidence for interaction between these subunits is the observation that the presence of ATP and HCO_3^- stimulates the rate of catalytic hydrolysis of γ-glutamyl hydroxamate by the light subunit (Anderson and Meister, 1965; Wellner et al., 1973), and the binding of γ-glutamyl hydroxamate to the light subunit has the same effect as the chloro ketone on ATPase activity (Anderson et al., 1973).

The results presented in this paper indicate that cyanate reacts with a specific SH group in the glutamine binding site on the light subunit of carbamyl phosphate synthetase and provide additional evidence for interaction between this site and the remainder of the active site located on the other subunit. The properties of the reaction of cyanate with carbamyl phosphate synthetase are very similar to the reaction of the above chloro ketone with this enzyme. Like the chloro ketone, cyanate reacts specifically with a single SH group located in the light subunit, the reaction with cyanate prevents the binding of L-[^{14}C]glutamine, the reaction with cyanate is prevented by the presence of L-glutamine, the presence of the allosteric effectors ornithine and UMP have no effect on the rate of the reaction with cyanate, and the rate of the reaction with cyanate is significantly decreased by the presence of ATP and HCO_3^-. As noted above, these two compounds also have similar effects on the different activities catalyzed by carbamyl phosphate synthetase (Anderson et al., 1973). Reaction of the enzyme with the chloro ketone prevents reaction of [^{14}C]cyanate with the enzyme to form a [^{14}C]cyanate–enzyme complex, and formation of the cyanate–enzyme complex prevents reaction of the chloro ketone with the enzyme. The reaction with cyanate is therefore considered to be with the same essential SH group which is located in the glutamine binding site and is alkylated by the chloro ketone analog of glutamine. It is perhaps significant, as discussed below, that the presence of ADP increases the rate of reaction of cyanate with carbamyl phosphate synthetase, an effect which is opposite to that observed with the addition of ATP and HCO_3^-. The effect of ADP on the rate of the reaction of the enzyme with the chloro ketone has not been reported.

Unlike the reaction with the chloro ketone which results in irreversible alkylation of the SH group, the reaction with cyanate to give the *S*-carbamyl derivative is reversible. In contrast to that of an *S*-carbamyl group fully exposed to solvent (e.g., *S*-carbamylglutathione or *S*-carbamylcysteine) the rate of dissociation of the cyanate–enzyme complex is relatively slow and not dependent on pH (Stark, 1964, 1972), indicating that the *S*-carbamyl group, like the SH group itself (Pinkus and Meister, 1972), is "buried" and that the rate of dissociation is controlled by the nature of the glutamine binding site. As with the rate of complex formation the presence of the allosteric effectors ornithine and UMP have no effect on the rate of dissociation. However, the presence of ATP and HCO_3^- greatly increases the rate of dissociation whereas the presence of ADP decreases the rate to a small extent, effects which are just opposite to those observed on the rate of the reaction of cyanate with the enzyme to form the complex.

The fact that the effect of ATP on the rates of formation and dissociation of the cyanate–enzyme complex requires the presence of HCO_3^- suggests that the formation of the activated carbon dioxide intermediate on the heavy subunit is at least in part responsible for the changes in the heavy subunit which significantly affect the glutamine binding site. This bicarbonate-dependent interaction is probably functionally related to the transfer of NH_3 and/or the hydrolysis of the postulated γ-glutamyl intermediate. The binding of the allosteric effectors ornithine or UMP to site(s) on the heavy subunit appears to have no direct effect on the properties of the glutamine binding site, indicating that the allosteric effects of these two compounds are probably not related to the interaction between the two subunits. Instead, the evidence presented here and elsewhere supports the view that ornithine and UMP act by increasing and decreasing, respectively, the affinity of the enzyme for ATP and ADP. The two different conformational states of the enzyme which have been shown to exist in the presence of ornithine or UMP, respectively, are therefore probably related to changes in the heavy subunit only (Anderson and Marvin, 1970; Foley et al., 1971; Matthews and Anderson, 1972).

As indicated above, the reaction of glutamine and glutamine analogs (γ-glutamyl hydroxamate and the chloro ketone) with the SH group in the glutamine binding site affects the properties of the heavy subunit. It is interesting that cyanate, which is not a structural analog of glutamine, also reacts specifically with this SH group and that this reaction affects the properties of the heavy subunit in the same way as the glutamine analogs. The conformational change(s) which probably occur, therefore, appear to be related to the reaction with the SH group per se rather than to unique binding properties of glutamine or its analogs.

Assuming that the reaction with glutamine does involve a γ-glutamyl thiol ester intermediate and that the reaction with cyanate involves carbamylation of this same SH group, it might be anticipated that the *S*-carbamyl group formed as a result of the reaction with cyanate would be subject to hydrolysis by the same mechanisms involved in hydrolysis of the postulated γ-glutamyl intermediate. We have not

been able to demonstrate the catalytic hydrolysis of cyanate, however, under a number of conditions involving procedures which would detect a very small amount of turnover.

Schroeder et al. (1969) have shown that the enzyme formylglycinamide ribonucleotide amidotransferase is also inactivated by low concentrations of cyanate. As with carbamyl phosphate synthetase, the glutamine-dependent activity is completely inhibited, but the ammonia-dependent activity is not affected, and the reaction occurs with the SH group at the glutamine binding site which reacts with other analogs of glutamine such as albizziine. Unlike the reaction with carbamyl phosphate synthetase the reaction with cyanate is irreversible. Several other enzymes have been reported to be inhibited by cyanate (Haines and Zamecnik, 1967; Shaw et al., 1964; Glader and Conrad, 1972; Veronese et al., 1972; Huang and Madsen, 1966; Chabas and Grisolia, 1972), but most of these studies have involved rather high cyanate concentrations (0.05–1.0 *M*) and extended reaction times. An exception is the reaction of cyanate with an SH group of papain which has properties similar to those reported in this paper (Sluyterman, 1967); however, this reaction apparently does not involve an essential SH group at the active site, since considerable enzyme activity remains after reaction with cyanate is complete. The inhibition of formylglycinamide ribonucleotide amidotransferase and carbamyl phosphate synthetase, both amidotransferases, by cyanate is unique, therefore, since the reaction involves rapid and stoichiometric carbamylation by low concentrations of cyanate of a specific and essential SH group in the glutamine binding site of both enzymes. The reaction is active-site-directed in the sense that reaction occurs at a rapid rate under conditions where other exposed and reactive SH groups are not affected, at least in the case of carbamyl phosphate synthetase. Since the presence of a glutamine binding site and the transfer of the amide nitrogen of glutamine from the glutamine binding site to an acceptor molecule, presumably located at another contiguous site, are common features of the above two amidotransferases and of amidotransferases in general, the possibility that cyanate may interact specifically with the glutamine binding site(s) of other amidotransferases is currently being investigated.

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