Interaction of *Escherichia coli* Carbamyl Phosphate Synthetase with Glutamine†

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ABSTRACT: When glutamine-dependent carbamyl phosphate synthetase (from *Escherichia coli*) is incubated with L-[^{14}C]-glutamine in the absence of other substrates, a labeled enzyme complex is formed containing the equivalent of 1 mol of glutamine/mol (170,000 g) of enzyme. The bound label does not exchange at an appreciable rate with added unlabeled glutamine, and it reacts rapidly to form glutamate when bicarbonate, Mg^{2+}, and ATP are added. All of the label released after denaturation of the labeled enzyme complex by treatment with trichloroacetic acid can be accounted for as glutamine and glutamate. Carbamyl phosphate synthetase catalyzes the hydrolysis of L-glutamine in the absence of the other substrates at about 2% of the rate observed for glutamine-dependent carbamyl phosphate synthesis; the apparent K_m value for glutamine in the glutaminase reaction is very close to that for glutamine in the complete synthesis system. After treatment with the glutamine analog, L-2-amino-4-oxo-5-chloropentanoic acid, the enzyme did not bind glutamine nor was it active in catalyzing the hydrolysis of glutamine or the synthesis of carbamyl phosphate from glutamine, bicarbonate, and ATP. In the course of this work, the ability of the isolated enzyme to utilize a variety of glutamine analogs for carbamyl phosphate synthesis was examined; of the compounds tested, only α-methyl-L-glutamine was active. The latter compound, several other glutamine analogs and glutamate protected the enzyme against inactivation by L-2-amino-4-oxo-5-chloropentanoic acid, indicating that they can bind at the glutamine binding site.

Previous studies in this laboratory showed that *Escherichia coli* carbamyl phosphate synthetase can catalyze, in addition to glutamine-dependent and ammonia-dependent synthesis of carbamyl phosphate, the following "partial" reactions (Anderson and Meister, 1965, 1966): (1) bicarbonate-dependent hydrolysis of ATP to ADP and P_i, (2) synthesis of ATP and ammonia from carbamyl phosphate and ADP, and (3) ATP- and bicarbonate-dependent hydrolysis of γ-glutamylhydroxamate. It was also found that treatment of the enzyme with L-2-amino-4-oxo-5-chloropentanoic acid, a chloro ketone analog of glutamine (Khedouri *et al.*, 1966), destroyed the ability of the enzyme to catalyze the glutamine-dependent synthesis of carbamyl phosphate, but had no effect on the ammonia-dependent carbamyl phosphate synthesis reaction or the synthesis of ATP from carbamyl phosphate and ADP; the chloroketone-treated enzyme exhibited increased bicarbonate-dependent ATPase activity. The ability of the enzyme to hydrolyze γ-glutamylhydroxamate was also destroyed by treatment of the enzyme with the chloro ketone. Since these effects of the chloro ketone were prevented or greatly reduced by glutamine, it was concluded that the enzyme has a binding site for glutamine which can combine with the chloro ketone and that ammonia reacts on the enzyme at a different site.

In the present work we have carried out additional studies on the interaction of the enzyme with glutamine. Studies with a number of glutamine analogs indicate that the enzyme exhibits a high degree of specificity toward L-glutamine for carbamyl phosphate synthesis; the enzyme can also interact with several glutamine analogs. The experiments described here show that the enzyme can effectively bind close to 1 mol of L-glutamine/mol of enzyme in the absence of the other substrates, and that the bound glutamine does not exchange rapidly with free glutamine.[1] We have also found that the enzyme exhibits glutaminase activity. The findings are in accord with the view that a γ-glutamyl enzyme is formed during glutamine-dependent carbamyl phosphate synthesis.

Experimental Section

Materials. Carbamyl phosphate synthetase was isolated from *E. coli* as previously described (Anderson *et al.*, 1970). L-Glutamine, L-γ-glutamylhydroxamic acid, L- and D-asparagine, L-leucine, *S*-carbamyl-L-cysteine, and L-[G-^{14}C]glutamine were obtained from Schwarz-Mann. ATP was obtained from Sigma. Sodium [^{14}C]carbonate was obtained from the New England Nuclear Corp. L-2-Amino-4-oxo-5-chloropentanoic acid (chloro ketone) was obtained as described by Khedouri *et al.* (1966). α-Methyl-L-glutamine was obtained by the action of glutamine synthetase on α-methyl-DL-glutamic acid (Kagan *et al.*, 1965); L-[γ-^{14}C]glutamylhydroxamic acid was obtained in the same manner from L-[G-^{14}C]glutamic acid obtained from the New England Nuclear Corp. β-Methyl-DL-glutamine (mixture of four isomers) (Meister *et al.*, 1955), D-glutamine (Levintow and Meister, 1954), L- and D-homoglutamine, L-γ-glutamyldimethylamide (Meister, 1954), DL-β-glutamine, D-β-glutamine (Khedouri and Meister,

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[1] The binding of glutamine to carbamyl phosphate synthetase in the absence of the other substrates was first observed and reported briefly by Anderson and Meister (1966). Subsequent studies on formylglycinamide ribonucleotide amidotransferase (Mizobuchi and Buchanan, 1968) and CTP synthetase (Levitzki and Koshland, 1971) indicated that these enzymes can also bind glutamine under similar conditions. It seems therefore that such binding may be characteristic of a number of glutamine amidotransferases, and that it may reflect the presence in these enzymes of a site which can bind glutamine in a manner which is relatively independent of the binding of other substrates.

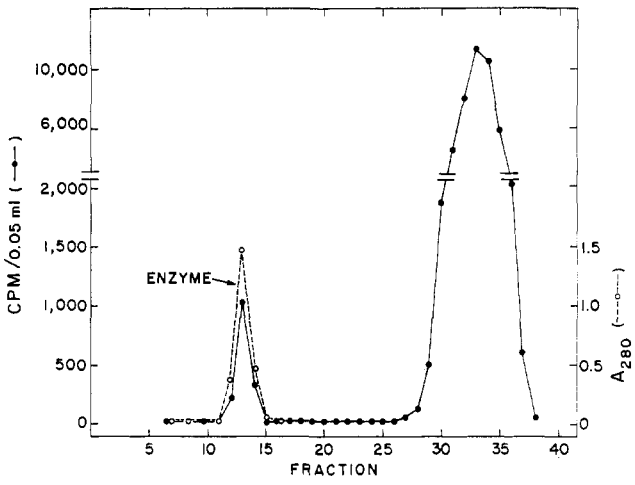

FIGURE 1: Isolation of enzyme-[^{14}C]glutamine complex by gel filtration. A mixture containing enzyme (2.15 mg), L-[^{14}C]glutamine (0.25 μmol; 625,000 cpm), potassium phosphate buffer (15 μmol; pH 6.8), and EDTA (0.5 μmol) in a final volume of 0.105 ml was incubated at 26° for 1 min. The mixture was then subjected to gel filtration on a column (1 × 36 cm) of Sephadex G-50 previously equilibrated with 0.1 M potassium phosphate buffer (pH 7.5) at 4°. The column was eluted with the same buffer and fractions (0.56 ml) were collected at about 3-min intervals. The absorbance at 280 nm was measured and the radioactivity was determined by scintillation counting.

1965), L-γ-glutamylmethylamide, L-γ-glutamylethylamide (Lichtenstein, 1942), L-γ-glutamylhydrazide (Le Quesne and Young, 1950), glutaramic acid (Jeffery and Vogel, 1934), and glutaramide (Fischer and Dilthey, 1902) were prepared as described.

Determination of Enzymatic Activities. Carbamyl phosphate synthesis activity was measured in an assay mixture (final volume, 0.3 ml) that contained 0.1 M Tris-HCl buffer (pH 7.8), 0.1 M KCl, 0.02 M ATP, 0.02 M $MgCl_2$, 0.02 M $NaHCO_3$, 0.01 M L-glutamine or 0.33 M NH_4Cl, and a sufficient amount of enzyme to form 0.02–0.4 μmol of carbamyl phosphate in 10 min at 37°. The reaction was stopped by addition of 0.1 ml of 1 N HCl; after standing at 0° for 5 min to inactivate the enzyme, 0.1 ml of 1 M Tris was added and the amount of ADP formed was determined from the decrease in absorbance at 340 nm after incubation of the samples with 2 ml of a solution containing 0.15 M Tris-HCl buffer (pH 7.6), 50 mM KCl, 2 mM $MgSO_4$, 5 mM phosphoenolpyruvate, 0.2 mM DPNH, pyruvate kinase (2 units/ml), and lactate dehydrogenase (4 units/ml). An aliquot of the reaction mixture in which carbamyl phosphate synthetase was omitted was employed as the blank. In some experiments, [^{14}C]bicarbonate was used and the formation of [^{14}C]carbamyl phosphate was determined as described by Anderson and Meister (1965). Bicarbonate-dependent ATPase was determined as described by Anderson and Meister (1966). Protein was determined from the absorbance at 280 nm. On the basis of dry weight determinations it was found that the absorbance of a solution of carbamyl phosphate synthetase containing 1 mg of protein/ml is 0.700 at 280 nm. A unit of carbamyl phosphate synthetase activity is defined as the amount of enzyme required for the synthesis of one micromole of carbamyl phosphate per hour under the conditions of assay described above.

Glutaminase activity was determined in reaction mixtures (final volume, 0.1 ml) containing 0.01 M L-[^{14}C]glutamine (30,000 cpm), 0.1 M potassium phosphate buffer (pH 7.6), and sufficient enzyme to catalyze the formation of 0.04–0.2 μmol of glutamate in 15–30 min at 37°. The reaction was stopped by adding 0.01 ml of 1 N HCl. The [^{14}C]glutamate formed was separated from the [^{14}C]glutamine by paper electrophoresis in 0.05 M sodium acetate buffer (pH 5.5). Electrophoresis was carried out in Beckman Model R apparatus using strips (1 × 12 cm) of Whatman No. 3MM paper, and 30 V/cm for 30 min at 24–26°. The areas of the paper strips containing the labeled compounds were identified by spraying the dried papers with a 0.25% solution of ninhydrin in 95% acetone; the strips were cut into sections and the radioactivity present was determined with a gas-flow counter.

Pyrrolidonecarboxylate was determined after separation from glutamine and glutamate (Krishnaswamy *et al.*, 1962).

TABLE I: Protection of the Enzyme by L-Glutamine and Certain Glutamine Analogs against Inhibition by L-2-Amino-4-oxo-5-chloropentanoic Acid.[a]

Compound Present during Preincubation	Carbamyl Phosphate Formed (μmol)	Protection %
None	0.34	[0]
L-Glutamine	0.85	[100]
D-Glutamine	0.33	0
α-Methyl-L-glutamine	0.73	77
β-Methyl-DL-glutamine	0.80	90
L-Homoglutamine	0.42	16
D-Homoglutamine	0.35	2
DL-β-Glutamine	0.42	16
D-β-Glutamine	0.34	0
L-Asparagine	0.40	12
D-Asparagine	0.34	0
L-γ-Glutamylhydrazide	0.85	100
L-γ-Glutamylhydroxamic acid	0.83	96
L-γ-Glutamylmethylamide	0.45	22
L-γ-Glutamyldimethylamide	0.34	0
L-γ-Glutamylethylamide	0.35	2
L-Glutamate	0.60	51
Glutaramide	0.34	0
Glutaramic acid	0.31	0
S-Carbamyl-L-cysteine	0.27	0
L-Leucine	0.34	0

[a] The enzyme (2.9 μg) was preincubated in a solution containing L-2-amino-4-oxo-5-chloropentanoic acid (0.015 μmol), potassium phosphate buffer (10 μmol; pH 7.8), EDTA (0.025 μmol), KCl (10 μmol), and L-glutamine or analog as indicated (2 μmol) in a final volume of 0.1 ml. After preincubation at 37° for 10 min, 0.9 ml of a solution containing L-glutamine (20 μmol), ATP (20 μmol), $MgCl_2$ (20 μmol), $NaH^{14}CO_3$ (20 μmol; 850,000 cpm), and potassium phosphate buffer (100 μmol; pH 7.8) was added. After further incubation for 10 min at 37°, the amount of [^{14}C]carbamyl phosphate formed was determined as described by Anderson and Meister (1965).

Results

Specificity of the Enzyme toward L-Glutamine. The ability of several structurally related compounds to replace L-glutamine in carbamyl phosphate synthesis was examined in the

standard assay system previously described (Anderson and Meister, 1965) except that 20 mM glutamine (or analog) was used; in these experiments [^{14}C]bicarbonate was used and the formation of [^{14}C]carbamyl phosphate was determined. Under these conditions, α-methyl-L-glutamine was about 10% as active as L-glutamine while β-methyl-DL-glutamine (mixture of four isomers), D-glutamine, L-homoglutamine, D-homoglutamine, DL-β-glutamine, D-β-glutamine, L-asparagine, and D-asparagine were less than 0.5% as active as L-glutamine. These and several other compounds were also examined to see whether they would protect the enzyme against inhibition by 1.5×10^{-4} M L-2-amino-4-oxo-5-chloropentanoic acid (Table I). In these studies the enzyme was preincubated for 10 min in a mixture containing the chloro ketone and glutamine analog, after which activity was determined in a reaction mixture containing L-glutamine, ATP, Mg^{2+}, [^{14}C]HCO_3^-, and potassium phosphate buffer. Significant protection of the enzyme was achieved with α-methyl-L-glutamine, β-methyl-DL-glutamine, L-γ-glutamylhydrazide, L-γ-glutamylhydroxamic acid, and a number of other compounds as indicated in Table I; it is of interest that L-glutamate gave about 50% of the protection observed with L-glutamine.

Binding of L-*Glutamine to the Enzyme.* When the enzyme was incubated with L-[^{14}C]glutamine and the mixture then subjected to gel filtration, a significant amount of radioactivity (equivalent to about 1 mol of [^{14}C]glutamine/170,000 g of enzyme) eluted from the column with the enzyme; as indicated in Figure 1, a good separation of the enzyme from low molecular weight compounds was achieved. Most (88%) of the label in the low molecular weight peak was present as glutamine as determined by paper electrophoresis. A result similar to that shown in Figure 1 was obtained when sodium phosphate was used in place of potassium phosphate buffer. The experiment described in Figure 2 was carried out to determine whether the enzyme-bound glutamine exchanges at a significant rate with free glutamine, and whether the bound glutamine readily reacts in the presence of other substrates. In this experiment the Sephadex column was equilibrated with a phosphate buffer containing L-glutamine and bicarbonate; Mg^{2+} and ATP were then added in a small volume to the top of the column and the column was eluted with the same buffer until 15 fractions (8.4 ml) were collected. Then, a mixture containing the enzyme and L-[^{14}C]glutamine was added in a small volume to the top of the column and elution with buffer was carried out. In this procedure the enzyme–L-[^{14}C]glutamine complex was separated from the unbound L-[^{14}C]glutamine and in the process of elution passed through the band of ATP and Mg^{2+} previously applied to the column. Under these conditions, very little radioactivity remained associated with the enzyme and an intermediate peak of radioactivity, all of which was present as glutamate (as shown by paper electrophoresis at pH 5.5 and 2.0), appeared in the same fractions that contained nucleotide. Quantitative analysis by paper chromatography showed that more than 97% of the ninhydrin-positive material in this intermediate peak was present as glutamine. This indicates that the specific activity of the [^{14}C]glutamate was very close to that of the [^{14}C]glutamine used and thus that the labeled and unlabeled glutamine had not equilibrated.

Experiments analogous to those described in Figures 1 and 2 were carried out with L-[γ-^{14}C]glutamylhydroxamate. The results obtained were very similar to those shown in Figures 1 and 2; thus, radioactivity equivalent to about 0.9 mol of γ-glutamylhydroxamate/170,000 g of enzyme eluted from the column with the enzyme. An experiment identical with that

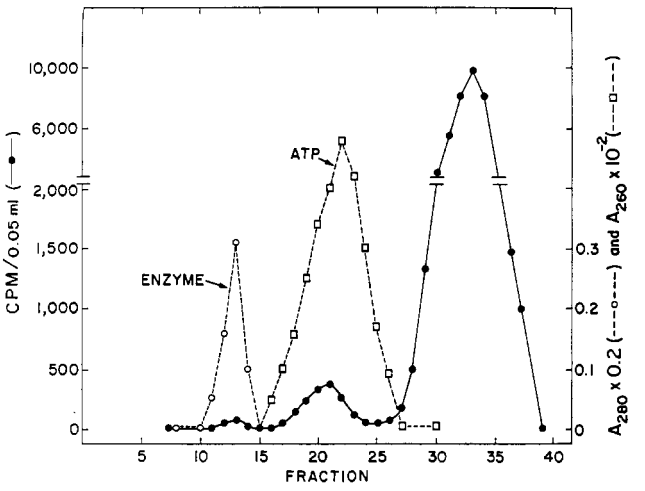

FIGURE 2: Evidence for the reactivity of the enzyme–glutamine complex. The column of Sephadex G-50 described in Figure 1 was equilibrated with a solution containing 0.1 M potassium phosphate buffer (pH 7.5), 0.01 M L-glutamine, and 0.01 M sodium bicarbonate at 4°; 0.1 ml of a mixture containing ATP (10 μmol) and $MgCl_2$ (10 μmol) was then added to the top of the column and the column was eluted until 15 fractions (8.4 ml) were collected. A mixture containing enzyme, L-[^{14}C]glutamine, potassium phosphate buffer, and EDTA (composition given in Figure 1) was then applied to the top of the column and elution was carried out as in Figure 1, except that the buffer contained glutamine and bicarbonate. The absorbances at 260 and 280 nm were measured and the radioactivity was determined by scintillation counting.

shown in Figure 1 except that L-[^{14}C]glutamate was substituted for L-[^{14}C]glutamine was also carried out; radioactivity equivalent to 0.3 mol of glutamate/170,000 g of enzyme eluted with the enzyme. When the enzyme was inactivated with respect to glutamine-dependent carbamyl phosphate synthetase activity by incubation with 5×10^{-4} M L-2-amino-4-oxo-5-chloropentanoic acid as described previously (Khedouri *et al.*, 1966), and then mixed with L-[^{14}C]glutamine and subjected to gel filtration as described in Figure 1, no radioactivity remained associated with the enzyme.

The most highly purified preparations of carbamyl phosphate synthetase thus far obtained exhibit a specific activity close to 280 units/mg. For reasons which are not yet fully understood, storage of such enzyme preparations at 0–5° is frequently accompanied by a gradual decrease in specific activity which usually levels off in the range of 90–130 units/mg. The variation in the glutamine binding capacity of different enzyme preparations was examined in relation to their specific activities determined at the time of the experiments. As indicated in Table II, the binding of glutamine varied from 0.32 to 0.88 mol per 170,000 g of protein; when binding is expressed in terms of active enzyme (*i.e.*, assuming a specific activity of 280 units/mg for active enzyme), values ranging from 0.96 to 1.2 were obtained.[2]

Several studies were carried out in which the binding of L-[^{14}C]glutamine to the enzyme was determined by gel filtration carried out under equilibrium conditions; a representative study is described in Figure 3. The enzyme used in this experi-

[2] It is possible that the light subunit of the enzyme (which contains the glutamine binding site as shown by Trotta *et al.*, 1971, and Pinkus and Meister, 1972) becomes inactivated or dissociates on storage; specific study of this phenomenon has not yet been carried out.

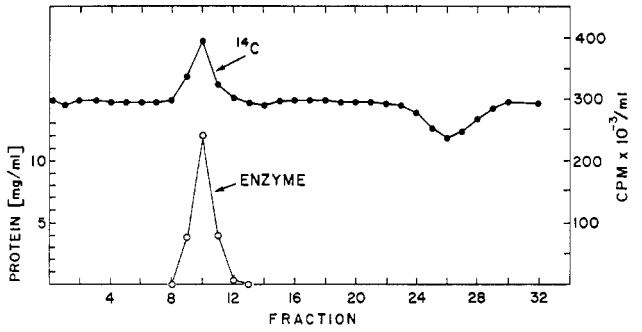

FIGURE 3: Binding of glutamine to the enzyme as determined by gel filtration under equilibrium conditions. A column of Sephadex G-50 (1 × 23 cm) was equilibrated with potassium phosphate buffer (0.2 M; pH 7.6), EDTA (0.5 mM), and L-[^{14}C]glutamine (0.14 mM). The enzyme (17 mg; specific activity 195 units/mg; 1 ml) was added to the top of the column and elution was carried out with the same buffer. Fractions of 1 ml were collected using a flow rate of 0.2 ml/min. Enzyme was determined from the absorbance at 280 nm and radioactivity was determined by scintillation counting.

ment exhibited a specific activity of 195 units/mg and the binding of radioactivity observed was equivalent to 0.86 mol/mol of enzyme. These experiments indicate that the enzyme has only a very high-affinity binding site for glutamine; thus, the binding studies carried out under equilibrium conditions do not provide evidence for binding greater than that found in the gel filtration separation studies (Figure 1).

Studies on the Nature of the Bound Material. Several experiments were carried out in an effort to determine the nature of the bound ^{14}C-labeled compound. When fractions containing the labeled protein (obtained by gel filtration as described in Figure 1) were treated with trichloroacetic acid, about half of the radioactivity precipitated with the protein. Thus, in a typical experiment, 0.2 ml of the eluate from the column containing 0.5 mg of enzyme and 12,000 cpm of ^{14}C was mixed with 0.05 ml of 25% trichloroacetic acid and then centrifuged. The precipitate, which contained 5670 cpm, was suspended in 0.1 ml of potassium phosphate buffer (0.5 M, pH 6.8). An aliquot of this suspension was spotted on a paper strip, which was then subjected to electrophoresis at pH 5.5 as described under Methods. No radioactivity was found at the point of application of the sample (which contained the protein), and 50.6% of the radioactivity moved with authentic glutamine; the remainder of the radioactivity moved with glutamate. Similar study of the supernatant solution showed that 93.2% of the radioactivity was present as glutamate and that the remainder was glutamine. No pyrrolidonecarboxylate was found. These studies indicate that about 25% of the enzyme-bound radioactivity was in the form of glutamine, and that the remainder of the enzyme-bound radioactivity was glutamate. In the experiment described above an interval of 30 min elapsed between the addition of [^{14}C]glutamine to the enzyme and denaturation of the eluted enzyme by addition of trichloroacetic acid. In experiments in which this time interval was increased to 60 min or longer, the same total amount of radioactivity was found associated with the enzyme, but almost all of the radioactivity was recovered as glutamate.

In an effort to detect a covalent glutamyl enzyme complex, we carried out an experiment similar to that performed by Levitzki and Koshland (1971) on CTP synthetase. These workers incubated the latter enzyme with [^{14}C]glutamine and then added 5 mM I_3^- (which they found to inactivate the enzyme instantaneously). The inactivated enzyme was then subjected to gel filtration in 8 M urea; under these conditions, radioactivity equivalent to about 0.2 mol of [^{14}C]glutamine/mol of enzyme remained associated to the enzyme. We found that carbamyl phosphate synthetase is also rapidly (less than 1 min under our conditions) inactivated by 5 mM I_3^-. A mixture (0.2 ml) containing carbamyl phosphate synthetase (1.5 mg), potassium phosphate buffer (0.1 M), EDTA (0.5 mM), and L-[^{14}C]glutamine (5 mM, 600,000 cpm) was incubated for 5 min at 25° and then treated with 5 mM I_3^-. No radioactivity was found associated with the enzyme on subsequent gel filtration in the presence of 8 M urea, or in controls in which the treatment with I_3^- was omitted. However, when this experiment was repeated with 25 mM I_3^-, radioactivity equivalent to 0.3 mol of glutamine/170,000 g of enzyme was found to be associated with the enzyme after gel filtration in 8 M urea. In a control experiment performed under the same conditions, but in which gel filtration was carried out in the absence of 8 M urea, radioactivity equivalent to 1.1 mol of glutamine was found associated with the enzyme. We have made a number of attempts to determine the nature of the radioactive material associated with the enzyme after treatment of the [^{14}C]glutamine–enzyme complex with I_3^-. Thus, when the I_3^--treated complex recovered after gel filtration (either with or without 8 M urea) was dialyzed for 18 hr against 0.1 M potassium phosphate buffer (pH 7.6) or against water, all of the bound radioactivity was recovered in the dialysate; after concentration of this material it was subjected to paper electrophoresis at several values of pH and found to exhibit the properties of an uncharged molecule. In these studies, no radioactivity was found in the areas corresponding to glutamate, glutamine, and pyrrolidonecarboxylate. In another series of experiments, the I_3^--treated labeled enzyme was spotted directly on paper strips and subjected to electrophoresis at various values of pH; most of the radioactivity moved with a compound that exhibited behavior typical of an uncharged molecule, and no evidence for the presence of pyrrolidonecarboxylate, glutamate, or glutamine was obtained. These and a number of other studies have thus far failed to identify the bound radioactive material present in the I_3^--treated enzyme complex; however, it would appear that treatment with I_3^- leads to conversion of the bound glutamate and glutamine to a new compound which also binds

TABLE II: Binding of [^{14}C]Glutamine to the Enzyme.[a]

Expt No.	Carbamyl Phosphate Synthetase Act. (Units/mg)	Binding of ^{14}C: mol/170,000 g	Binding of ^{14}C: mol/mol of Active Enzyme
1	200	0.88	1.2
2	128	0.44	0.96
3	100	0.38	1.1
4	90	0.32	1.0
5[b]	0	0	0

[a] The experimental conditions were those described in Figure 1; the concentration of active enzyme was calculated by assuming that the active enzyme exhibits a specific activity of 280 units/mg. [b] Separated light subunit of the enzyme (Trotta *et al.*, 1971).

to the enzyme. Addition of hydroxylamine to the I_3^--treated [^{14}C]enzyme complex did not lead to formation of a hydroxamate as determined by the ferric chloride reagent. Until the chemical nature of the compound(s) bound to the I_3^--treated [^{14}C]glutamine-labeled complexes of carbamyl phosphate synthetase and CTP synthetase is established, definite conclusions concerning the significance of such binding would not seem to be in order. The binding of radioactivity under these conditions cannot therefore be taken as evidence for or as a measure of the covalent linkage of the glutamyl moiety to carbamyl phosphate synthetase; the same conclusion seems applicable also to the studies on CTP synthetase (Levitzki and Koshland, 1971).

Studies on the Glutaminase Activity of Carbamyl Phosphate Synthetase. The studies described above showed that treatment of the labeled enzyme complex (obtained after incubation of the enzyme with [^{14}C]glutamine followed by gel filtration) with trichloroacetic acid yields only glutamine and glutamate; the relative amounts of these were found to depend on the time interval between addition of glutamine to the enzyme and denaturation with trichloroacetic acid. These observations led us to reinvestigate the possibility that carbamyl phosphate synthetase exhibits glutaminase activity. Such an activity was first observed during the early work in this laboratory (about 1965) on the purification of the enzyme. However, the possibility that the observed glutaminase activity was catalyzed by a contaminating protein could not then be definitely excluded. In subsequent studies, which have been cited elsewhere (Trotta *et al.*, 1971), it was shown that highly purified carbamyl phosphate synthetase catalyzes the hydrolysis of glutamine in the absence of other substrates at a low but measurable rate. As indicated in Table III, carbamyl phosphate synthetase exhibits glutaminase activity whose rate is equivalent to about 2% of that observed for glutamine-dependent carbamyl phosphate synthesis. We have also found that the enzyme catalyzes the hydrolysis of γ-glutamylhydroxamic acid (in the absence of ATP or added bicarbonate) at about the same rate that it catalyzes glutamine hydrolysis; these studies were carried out under the same conditions used here for the measurement of glutaminase activity. The effect of L-glutamine concentration (over the range 0.1–30 mM) on glutaminase activity gave a typical hyperbolic saturation curve. The data obtained indicate an apparent K_m value for L-glutamine of 0.42 mM. This value is close to that of 0.38 mM previously determined for the apparent K_m value for L-glutamine in the carbamyl phosphate synthetase reaction (Anderson and Meister, 1966). As described in Table III, treatment of the enzyme with L-2-amino-4-oxo-5-chloropentanoic acid led to substantial inhibition of glutaminase activity. Such inhibition of glutaminase parallels that of glutamine dependent carbamyl phosphate synthesis. When the enzyme was treated with the chloro ketone analog in the presence of L-glutamine (10 mM) no decrease in either glutaminase- or glutamine-dependent carbamyl phosphate synthetase activities was observed. In agreement with previous findings (Khedouri *et al.*, 1966), treatment with the chloro ketone led to a substantial increase in bicarbonate-dependent ATPase activity.

The enzyme also catalyzes the formation of γ-glutamylhydroxamate from glutamine and hydroxylamine at about the same rate as it catalyzes glutamine hydrolysis. The reaction was studied under the conditions given above (Methods) that were used for glutaminase assay, except that hydroxylamine (0.5–1.8 M) was added; the formation of hydroxamate was measured by the ferric chloride procedure (Lipmann and Tuttle, 1945), or by conversion of L-[γ-^{14}C]glutamylhydroxamate to pyrrolidonecarboxylate (Levintow *et al.*, 1955).[3]

TABLE III: Effect of L-2-Amino-4-oxo-5-chloropentanoate (Chloro Ketone) on the Glutaminase Activity of the Enzyme.

Reaction[a]	Control (Untreated) (μmol of Product/hr per mg)	Chloro Ketone Treated	
		μmol of Product/hr per mg	% Decrease
Glutaminase	1.6	0.10	93
HCO_3^--dependent ATPase	13	30	[130][b]
Carbamyl phosphate synthesis	96	0.64	99

[a] Carbamyl phosphate synthetase (glutamine-dependent), ATPase, and glutaminase activities were determined as described under methods using an enzyme preparation that was treated with 6×10^{-4} M chloro ketone for 30 min at pH 7.6 at 37° as described by Khedouri *et al.* (1966) and an untreated control; prior to study, the enzyme preparations were then dialyzed overnight at 5° against three changes of 0.2 M potassium phosphate buffer (pH 7.6) containing 5×10^{-4} M EDTA. [b] Increase.

Discussion

These studies show that carbamyl phosphate synthetase can interact with L-glutamine in the absence of other substrates, and that the glutamine is bound to the enzyme in a form which does not equilibrate with unlabeled L-glutamine subsequently added. No binding was observed with enzyme that had been previously treated with L-2-amino-4-oxo-5-chloropentanoic acid. The studies in which the labeled enzyme complex was subjected to gel filtration on a column containing unlabeled glutamine, ATP, magnesium ions and bicarbonate (Figure 2) indicate that the bound [^{14}C]glutamine is effectively utilized and released from the enzyme as [^{14}C]glutamate. Studies on the nature of the bound material indicate that all of the radioactivity can be accounted for as glutamine and glutamate; the finding that the amount of glutamine bound decreases as the time between addition of labeled glutamine to the enzyme and analysis increases is consistent with enzymatic cleavage of the bound glutamine to glutamate; glutamate formed in this reaction remains bound to the enzyme. The findings are consistent with the view that some of the bound glutamate is covalently linked to the enzyme, *i.e.*, as γ-glutamyl enzyme. However, while it is reasonable to suppose that a γ-glutamyl-enzyme intermediate is formed in both the glutaminase and the glutamine-dependent carbamyl phosphate synthetase reactions catalyzed by the enzyme, direct evidence for such an intermediate has not been obtained. The finding that the enzyme catalyzes γ-glutamylhydroxamate formation from

[3] γ-Glutamyl hydroxamate cyclizes to form pyrrolidonecarboxylate (5-oxoproline) much more rapidly than does glutamine. Thus, in experiments in which the formation of γ-glutamylhydroxamate is not very large and in which long incubation periods are used, the γ-glutamylhydroxamate formed may not be readily detected by the ferric chloride procedure.

glutamine and hydroxylamine is analogous to findings on other enzymes that catalyze amide hydrolysis (Meister *et al.*, 1955; Ehrenfeld *et al.*, 1963), and is also consistent with intermediate formation of a γ-glutamyl enzyme.

The enzyme exhibits relatively low glutaminase activity, which is equivalent to about 2% of the rate of glutamine-dependent carbamyl phosphate synthesis. Thus, the rate of glutamine cleavage is increased about 50-fold when ATP, Mg^{2+}, and bicarbonate are added. Similarly, the rate of hydrolysis of γ-glutamylhydroxamate is increased about 20-fold when ATP, Mg^{2+}, and bicarbonate are added. It has been shown in other studies in this laboratory that carbamyl phosphate synthetase consists of a protomeric unit containing 1 heavy subunit (molecular weight, about 130,000) and 1 light subunit (molecular weight, about 42,000) (Trotta *et al.*, 1971). It was also established that the only catalytic activity exhibited by the light subunit is the hydrolysis of glutamine, and that the heavy subunit contains the sites for the binding of ATP and bicarbonate. The present findings are in accord with the conclusion that the light subunit of the enzyme functions to bind glutamine and to catalyze its hydrolysis at a slow rate. However, the affinity of the separated light subunit for glutamine is apparently much lower than that of the light subunit–heavy subunit complex; thus, no evidence was obtained for the binding of glutamine to the separated light subunit by the gel filtration procedure (Table II).

The marked stimulation of glutamine and γ-glutamylhydroxamate utilization in the presence of bicarbonate, ATP and Mg^{2+} seems to reflect a significant intersubunit interaction. Such interaction is also indicated by the finding that the binding of the chloro ketone analog of glutamine to the glutamine binding site on the light subunit decreases the apparent K_m value for ammonia in the ammonia-dependent carbamyl phosphate synthetase reaction catalyzed by the heavy subunit (Pinkus and Meister, 1972). Furthermore, the chloro ketone treated enzyme exhibits a substantially greater bicarbonate-dependent ATPase activity than does the untreated enzyme. It thus appears that, although the light subunit is responsible for the binding of glutamine, there is an intersubunit mechanism which functions in a manner such that the binding of glutamine to the light subunit facilitates both the utilization of the amide moiety and also the formation or utilization of activated carbon dioxide. The linkage between the two subunits is apparently such as to largely exclude water from the region of the glutamine binding site; thus, the enzyme exhibits only relatively low glutaminase activity. When the intersubunit linkage is altered by treatment of the enzyme with certain sulfhydryl reagents, there is a dramatic increase of glutaminase activity; studies on this phenomenon will be described in a subsequent communication.

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