Observations on the pH Dependence of the Glutaminase Activity of a Glutamine Amidotransferase, Carbamylphosphate Synthetase

(subunits/enzyme/dissociation/*E. coli*)

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ABSTRACT Glutamine-dependent carbamylphosphate synthetase (*Escherichia coli*) is composed of a heavy subunit (molecular weight about 130,000) and a light subunit (molecular weight about 42,000), which can be separated with retention of catalytic activities. The separated heavy subunit can catalyze activation of CO₂ by ATP and synthesis of carbamylphosphate from ammonia (but not from glutamine). The only catalytic activity exhibited by the separated light subunit is the ability to hydrolyze glutamine; the separated heavy subunit does not exhibit glutaminase activity. The pH-activity curve of the glutaminase activity of native carbamylphosphate synthetase exhibits maxima at about pH 4.2 and 9.5, while the glutaminase activity of the separated light subunit exhibits only a single optimum at about pH 6.7. When the light and heavy subunits are recombined, the two pH optima characteristic of native enzyme are restored. Glutaminase activities of native enzyme at both pH optima are similarly inhibited by the glutamine analog, L-2-amino-4-oxo-5-chloropentanoic acid, and also by dithiothreitol. Storage of native enzyme at pH 9 abolishes the glutaminase optimum at acid pH, but greatly increases the activity at alkaline pH. Treatment of native enzyme with *N*-ethylmaleimide increases the glutaminase activity dramatically and converts the pH profile to one that closely resembles that of the isolated light subunit. The data indicate that the same active site is involved in hydrolysis of glutamine at both acid and alkaline values of pH, and that this property of the enzyme depends upon interactions between the heavy and light subunits of native enzyme. The double-optima behavior of native enzyme seems to be related to participation of different catalytic groups of the enzyme which affect the maximum velocity rather than the binding of substrate. The findings offer additional evidence for occurrence of significant interactions between the subunits of carbamylphosphate synthetase, and may have significance in relation to other glutamine amidotransferases, including glutaminases.

The amide nitrogen atom of glutamine is used for biosynthesis of asparagine, carbamylphosphate, glucosamine-6-phosphate, diphosphopyridine nucleotide, guanosine-5'-phosphate, 5-phosphoribosylamine, formylglycinamide ribonucleotide, anthranilate, *p*-aminobenzoate, histidine, and cytidine triphosphate (1-3). These reactions are catalyzed by glutamine amidotransferases, enzymes that have a binding site for glutamine and a catalytic mechanism for cleavage of its amide bond, as well as a mechanism for using the liberated amide nitrogen in a particular reaction. Almost all of these enzymes can function with relatively high concentrations of NH₄⁺ in place of glutamine, and several amidotransferases exhibit glutaminase activity. There are also enzymes that seem to function only as glutaminases; these may be viewed as glutamine amidotransferases in which the amide nitrogen is

transferred to a hydrogen ion. It seems probable that there are structural, functional, and evolutionary relationships between the various glutamine amidotransferases. We report here observations on carbamylphosphate synthetase, which relate to the utilization of glutamine by this enzyme, and which may be of significance in relation to other glutamine amidotransferases, including the glutaminases.

Carbamylphosphate synthetase of *Escherichia coli*, catalyzes the following reactions (4):

and also several partial reactions that seem to reflect individual steps of the overall synthesis reactions (5-8):

The enzyme is composed of two nonidentical subunits that can be separated without substantial loss of enzymatic activities (6, 8). The separated heavy subunit, which can catalyze reactions 2 and 3, has the binding sites for the allosteric effectors [IMP, UMP (9), ornithine (10, 11), and NH₃ (8; Trotta, P. P., Estis, L., Meister, A. & Haschemeyer, R. H., *J. Biol. Chem.*, in press)] of the enzyme. The only functions associated with the separated light subunit are the binding and cleavage of glutamine (6); in the intact enzyme, the latter reaction involves transfer of the amide nitrogen of glutamine to activated CO₂ on the heavy subunit, while the separated light subunit catalyzes the hydrolysis of glutamine. The separated heavy subunit can synthesize carbamylphosphate from ammonia (reaction 1b), but not from glutamine (reaction 1a); upon recombination of the heavy and light subunits, the capacity to utilize glutamine is restored (6). There is evidence for interactions between the subunits that facilitate synthesis of carbamylphosphate. Thus, binding of a glutamine analog to the light subunit of the enzyme increases the rate of ATP cleavage by the heavy subunit (8, 12). At-

FIG. 1. pH Dependence of the glutaminase activity of native carbamylphosphate synthetase. The reaction mixtures were prepared by addition of 0.01 ml of enzyme solution (2.2 mg/ml in 150 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA) to 0.09 ml of a solution containing 22.2 mM L-glutamine (final concentration) in: Δ , 0.1 M acetic acid adjusted by addition of 1 N HCl to give a final pH of 2–4; $\blacktriangle$, 0.1 M acetic acid adjusted by addition of 0.1 M K_2HPO_4 to give a final pH of 4.1–6.5; $\circ$, 0.1 M Veronal adjusted by addition of 0.1 M KH_2PO_4 to give a final pH of 6.8–8.8; or $\bullet$, 0.1 M Na_2CO_3 adjusted by addition of 0.1 M KH_2PO_4 to give final pH of 9–11.2. pH Values given here and in the other figures are those obtained at 37° under the assay conditions. The glutaminase reaction was stopped by addition of 0.05 ml of 1 N HCl; after neutralization with 0.05 ml of 1 M Tris, L-glutamate formation was determined.

FIG. 2. pH Dependence of the glutaminase activity of separated light subunit. Before the experiment the light subunit was activated by incubation at 37° for 30 min in a solution containing 0.45 mg/ml of the light subunit, 150 mM potassium phosphate (pH 7.8), 0.5 mM EDTA, and 25 mM dithiothreitol. The reaction mixtures were prepared by addition of 0.025 ml of this solution (11.3 μg of protein) to 0.125 ml of a solution containing 216 mM L-glutamine in: $\circ$, 0.15 M acetic acid adjusted by addition of 1 N HCl to give a final pH of 4.1–6; Δ , 0.15 M KH_2PO_4 adjusted by addition of 1 N KOH to give a final pH of 6.1–7.3; or $\bullet$, 0.15 M Veronal adjusted by addition of 1 N HCl or 1 N KOH to give a final pH of 8–10. Reactions were stopped by addition of 0.02 ml of 4 N HCl. After neutralization with 0.08 ml of 1 M Tris, glutamate formed was quantitated.

FIG. 3. pH Dependence of the glutaminase activity of reconstituted enzyme. Reconstituted enzyme was prepared by addition of 0.40 ml of a solution containing the heavy subunit (0.37 mg/ml in 150 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA and 5 mM dithiothreitol) to 0.44 ml of a solution containing the light subunit (0.47 mg/ml) prepared as described in Fig. 2. The reconstituted enzyme (0.084 ml) was added to 0.08

ml of a solution containing 40 mM L-glutamine in: 0.2 M acetic acid adjusted by addition of 4 N HCl to a final pH of 3.5–5.1; 0.2 M potassium phosphate adjusted by addition of 4 N KOH to a final pH of 6.0–7.9; or 0.2 M Na_2CO_3 adjusted by addition of 2 N HCl to obtain a final pH of 8.4–10.1. Reaction mixtures were then processed as described in Fig. 2.

FIG. 4. pH Dependence of the glutaminase activities of native carbamylphosphate synthetase treated with N-ethylmaleimide and stored at pH 9. Enzyme (5 mg/ml) was incubated at 37° for 1 hr in a solution containing 10 mM N-ethylmaleimide, 100 mM KH_2PO_4 , and 100 mM Tris (pH 7.5). After removal of excess N-ethylmaleimide by dialysis at 4° for 18 hr against the same buffer, glutaminase activity was assayed in a final volume of 0.1 ml containing 1–10 μg of enzyme, 10 mM L-[^{14}C]glutamine (80,000 cpm/ μmol), and either 75 mM potassium phosphate plus 75 mM sodium acetate (open symbols) or 75 mM potassium phosphate plus 75 mM Tris (closed symbols) adjusted to the indicated pH at 37° by addition of KOH. Formation of [^{14}C]glutamate was determined (*). The native enzyme (5 mg/ml) was also stored at 0° for 14 days in 0.1 M potassium phosphate and 0.1 M Tris, adjusted to pH 9 by addition of KOH. Glutaminase activity was determined as indicated above. Untreated enzyme catalyzed carbamylphosphate synthesis from glutamine at a rate of 115 $\mu\text{mol/mg per hr}$; the glutaminase activities of the untreated enzyme preparation at pH values of 4.5, 6, 7, 7.5, 8, 9.5, and 10.5 were, respectively, 2.7, 0.8, 1.0, 1.0, 2.0, 7.2, and 2.8.

EXPERIMENTAL

Materials. Carbamylphosphate synthetase was isolated from *E. coli* B as described (14; Trotta, P. P., Estis, L., Meister, A. & Haschemeyer, R. H., *J. Biol. Chem.*, in press). The subunits were separated by chromatography on Sephadex G-200 in 100 mM potassium phosphate buffer (pH 7.6) containing 1 M potassium thiocyanate and 0.5 mM EDTA (6, 8; Trotta, P. P., Pinkus, L., Meister, A. & Haschemeyer, R. H., *J. Biol. Chem.*, in press). DPN, dithiothreitol, L-glutamine, and L-glutamate dehydrogenase were obtained from Sigma. L-[^{14}C]Glutamine was obtained from New England Nuclear Corp. N-Ethylmaleimide was obtained from L. Light and Co. Ltd. (Colnbrook, England). L-2-Amino-4-oxo-5-chloropentanoic acid was synthesized as described (12, 15). Standard buffers were obtained from Fisher Chemical Co. and Mallinckrodt Chemical Works.

Methods. pH was determined under enzyme assay conditions at 37° with a Radiometer (type PHM 22 r) standardized with buffers that were within 1 pH unit of the unknown. A GK 2302 C type electrode (combined glass electrode and reference calomel electrode in one body) was used. Glutaminase activity was determined in various buffers by measurement of L-glutamate formation with glutamate dehydrogenase (16) or conversion of L-[¹⁴C]glutamine to [¹⁴C]glutamate (7). Assay conditions were chosen such that the L-glutamate formed was less than 15% of the initial glutamine concentration. The apparent K_m values were calculated from a linear least-squares analysis of a weighted Lineweaver-Burk plot with a computer program written by Dr. R. H. Haschemeyer.

RESULTS

The glutaminase activity of native enzyme exhibits two pH maxima, at about pH 4.2 and pH 9.5 (Fig. 1). Similar biphasic pH profiles were also found in studies with other buffers (e.g., sodium citrate, pyridine hydrochloride, Tris-phosphate).^{*} In contrast, the glutaminase activity of the isolated light subunit exhibits only one maximum, at about pH 6.7 (Fig. 2). The pH dependence of activity of the light subunit was not influenced by addition of dithiothreitol. [The light subunit exhibits a much higher K_m value for L-glutamine (130–180 mM) than does the native enzyme (about 1 mM) (Trotta, Pinkus, Meister & Haschemeyer, *J. Biol. Chem.*, in press)]. The separated heavy subunit exhibits no glutaminase activity over the pH range 4–9.5.

Reconstitution of the enzyme by mixing the separated heavy and light subunits led to reestablishment of the glutaminase optima at acid and alkaline values of pH (Fig. 3). The high glutaminase activity observed at about pH 6.7 in this pH profile reflects the addition of about a 4-fold molar excess of light subunit; an excess of light subunit must be added to achieve full reconstitution (6, 8; Trotta, Pinkus, Meister & Haschemeyer, *J. Biol. Chem.*, in press). These findings indicate that the double-optima pH profile is an authentic property of carbamylphosphate synthetase and render unlikely the possibility that the maxima of glutaminase activity observed at about pH 4.2 and pH 9.5 are associated with contaminating glutaminases. Preparations of native enzyme often exhibit a shoulder in the region of pH 7–8 (Fig. 1); this may be due to a tendency of the light subunit to dissociate.[†]

Treatment of native enzyme with *N*-ethylmaleimide inactivates synthetase activity (Wellner & Meister, in preparation), but increases greatly the glutaminase activity at pH 7.5 and thus leads to a rate of glutamine utilization similar to that observed with native enzyme in the glutamine-dependent synthesis reaction. Treatment of the enzyme with *N*-ethylmaleimide leads to loss of the glutaminase maximum at pH 4.2 (Fig. 4); indeed, the pH profile of the treated enzyme is very similar to that of the separated light subunit (Fig. 2) in that both exhibit a single maximum at about pH 6.7. A similar pH profile was obtained when the enzyme was treated with *N*-ethylmaleimide in the presence of ATP and Mg⁺⁺; Anderson and Marvin (17) reported that under these condi-

tions a specific sulfhydryl group on the light subunit reacts. Treatment of the separated light subunit with *N*-ethylmaleimide leads to inhibition of its glutaminase activity.

When native enzyme is stored at pH 9 at 0° for 14 days there is stimulation of the glutaminase activity in the alkaline range, and synthetase activity is destroyed. This result, similar to that observed with *N*-ethylmaleimide, may be related to sulfhydryl group oxidation. However, in distinction to the enzyme treated with *N*-ethylmaleimide, the enzyme stored at pH 9 exhibits an optimum at pH 9 (Fig. 4); under these conditions, the optimum in the acid range is also lost.

Apparent K_m values for L-glutamine in the glutaminase reaction catalyzed by native enzyme were determined at pH values of 4.30, 5.00, 6.35, 7.75, and 9.50; the corresponding K_m values were 1.2, 1.1, 1.2, 1.2, and 5.0 mM. Since the substrate concentration used here (20 mM) is saturating over this pH range, we may conclude that the trough of activity between the two maxima is not related to a decrease in affinity for substrate. The double-optima behavior of the native enzyme therefore seems to be associated with participation of catalytic groups of the enzyme that affect the maximum velocity rather than the binding of substrate.

The glutaminase activity of native enzyme was determined after the enzyme was preincubated with L-2-amino-4-oxo-5-chloropentanoic acid, an analog that inactivates glutamine-dependent synthetase activity (12, 15). The activity, which was determined after various periods of preincubation, was similarly inhibited at pH values of 9.10, 7.85, and 4.15 (Fig. 5). Much less inhibition was observed when the enzyme was treated with the chloroketone in the presence of either L-glutamine or L-albizziin, indicating that these compounds protect against the inhibition. The glutaminase activity of native enzyme is also inhibited by 1 mM dithiothreitol, and L-glutamine and L-albizziin protect against such inhibition (Trotta, Pinkus & Meister, *J. Biol. Chem.*, in press). In the present work the enzyme was preincubated with 1 mM dithiothreitol for various periods; the glutaminase activity was then measured at pH values of 9.40, 7.76, and 4.20 (Fig. 6). A similar time course of inhibition was observed at each pH value; inhibition was greatly reduced when either L-glutamine or L-albizziin was added during preincubation. These findings on inhibition by the chloroketone and dithiothreitol support the conclusion that the glutaminase activities exhibited by native enzyme at pH 4.2 and pH 9.5 are properties of the synthetase itself.

DISCUSSION

The evidence indicates that the two pH optima observed for the glutaminase activity of carbamylphosphate synthetase are functions of the same active site. The pH profile for glutaminase is markedly different from that of the glutamine-dependent synthetase activity, which exhibits a broad optimum between pH 7.5 and 8.4. Although the glutaminase activity of the enzyme is catalyzed by the light subunit, interaction between the light and heavy subunits evidently influences the activity significantly; thus, the isolated light subunit exhibits only one pH optimum (at about pH 6.7) and exhibits a much higher apparent K_m value for glutamine than does the native enzyme. The findings suggest that certain amino-acid side chains of the heavy subunit may function directly in catalysis of glutamine hydrolysis or in binding of glutamine; another possibility is that interaction of the heavy subunit with the light subunit alters the conformation of the

* Glutamine-dependent carbamylphosphate synthetase of *Enterobacter aerogenes* also exhibits two pH optima, at about 4.5 and 9 (unpublished work in this laboratory).

† Spontaneous dissociation of the subunits of anthranilate synthetase has been observed (33).

glutamine binding site. As suggested previously (8), the heavy subunit may play a role in binding of the $-\text{NH}_2$ moiety of the amide group of glutamine; the presence of functional groups of the heavy subunit in the neighborhood of the amide group of glutamine would be consistent with the observation that the amide nitrogen released from the light subunit is efficiently transferred to the heavy subunit for use in the synthesis reaction. The effects of *N*-ethylmaleimide and of storage at pH 9 on the native enzyme seem to reflect changes in the subunit

FIG. 5. Effect of preincubation with L-2-amino-4-oxo-5-chloropentanoic acid on the glutaminase activity of native enzyme at several values of pH. The enzyme (2.1 mg/ml) was preincubated at 37° with 0.25 mM chloroketone in 150 mM potassium phosphate (pH 7.8) containing 0.5 mM EDTA. At the indicated time intervals, samples (0.02 ml) were withdrawn and assayed by addition to 0.08 ml of a solution containing 25 mM L-glutamine in: $\oplus$, 100 mM potassium phosphate (final pH, 7.85); $\circ$, 100 mM Na_2CO_3 adjusted to a final pH of 9.10 by addition of 1 N HCl; or $\bullet$, 0.1 M acetic acid adjusted to a final pH of 4.15 by addition of 1 N KOH. Under the conditions used, chloroketone did not inhibit during assay. Reaction mixtures were processed as described in Fig. 1.

FIG. 6. Effect of preincubation with dithiothreitol on the glutaminase activity of native enzyme. Enzyme (2.0 mg/ml) was preincubated at 37° in a solution containing 1 mM dithiothreitol, 150 mM potassium phosphate, 25 mM Veronal, and 0.5 mM EDTA (pH 8.6). At the indicated intervals, samples (0.01 ml) were removed and assayed by addition to 0.09 ml of a solution containing 22.2 mM L-glutamine (final concentration) in: $\bullet$, 0.1 M acetic acid adjusted to a final pH of 4.20 by addition of 1 M KOH; $\oplus$, 0.1 M KH_2PO_4 adjusted to a final pH of 7.76 by addition of 1 M KOH; or $\circ$, 0.1 M Na_2CO_3 adjusted to a final pH of 9.40 by addition of 0.5 N HCl. Dithiothreitol did not inhibit during the assay. Reaction mixtures were processed as described in Fig. 1.

interactions that facilitate access of water to the region of the glutamine-binding site.

The data suggest that the two pH optima observed for the glutaminase activity of native enzyme are functions of the titration of protein side chains involved in catalysis rather than in substrate binding. It is tempting to speculate that the amino-acid residue titrated in the alkaline range may be the sulfhydryl group at the glutamine-binding site that reacts with the chloroketone. This sulfhydryl must still be intact in the enzyme stored at pH 9 since this enzyme can interact with the chloroketone; however, many of the other sulfhydryl groups are oxidized (Wellner & Meister, in preparation). A glutamyl or aspartyl side chain may be involved in the glutaminase activity observed at pH 4.2.

Previous work has led to the finding of two glutaminases in *E. coli*, which have recently been designated as glutaminase A (18–20) and glutaminase B (21, 22). The functions of these enzymes are not known, but it has been suggested that glutaminase B, an allosterically regulated enzyme, may play a role in cellular energetics (21, 22). Glutaminases A and B have been thought to be isoenzymes and are considered to be readily distinguishable by their differences in pH optimum; glutaminase A exhibits a pH optimum at about 5 while glutaminase B is active above pH 7. While there are data that seem to support the view that these activities are catalyzed by different proteins, the present studies suggest that the pH optimum of a particular glutaminase activity may not always be a reliable criterion for distinguishing isoenzymes. This point is emphasized by the present findings, which show that the glutaminase activity of carbamylphosphate synthetase exhibits optima at about pH 4, 7, and 9 depending on conditions as described above. Several glutamine amidotransferases exhibit glutaminase activity, and it is reasonable to expect that the others, after further study, will also be found to exhibit glutaminase activity. Certain glutamine amidotransferases can be modified during isolation (1). Thus, CTP synthetase was first isolated in a form that utilized only ammonia (23), but later work showed that this enzyme can utilize either ammonia or glutamine (24). Procedures used during isolation of enzymes may not only inactivate the synthetase functions of glutamine amidotransferases, but may also stimulate their glutaminase activities. Examples of such effects on carbamylphosphate synthetase are shown in Fig. 4. It is interesting that glutamine-dependent asparagine synthetase was isolated from RADA 1 tumor cells in a form that exhibits very high glutaminase activity (25). It is also notable that the glutaminase activity of anthranilate synthetase is stimulated by the substrate chorismate (26, 27), and that of CTP synthetase by an analog of ATP (28). Similar effects have been found with formylglycinamide ribonucleotide synthetase (29). It appears that the glutaminase activity of a glutamine-amidotransferases may normally be present in a "latent" form since it is tightly coupled to a particular synthetase reaction, and that uncoupling may occur under certain conditions.

As previously suggested (6, 8), carbamylphosphate synthetase may have evolved by combination of a primitive ammonia-dependent synthetase with a glutaminase; this may have been associated with the replacement of ammonia by glutamine as the principal nitrogen source for the organism, or possibly because there was some other evolutionary advantage to the use of glutamine rather than ammonia. A similar hy-

pothesis has been considered in relation to formylglycinamide ribonucleotide synthetase (29); indeed this idea may be generally applicable to the glutamine amidotransferases, and would be consistent with the finding that most of these enzymes can also use ammonia. If utilization of glutamine for carbamylphosphate synthesis and other metabolic functions evolved at about the same time, one might expect to find considerable structural similarity between the glutamine-binding subunits of various glutamine amidotransferases (6). The recent finding (30) that the same glutamine-binding subunit of *B. subtilis* can react with both anthranilate and *p*-aminobenzoate synthetases is of interest in this connection. However the glutamine-binding subunit of *B. subtilis* exhibits a molecular weight of 16,000 as compared to about 42,000 for that of carbamylphosphate synthetase from *E. coli*, and the glutamine-binding sites of certain glutamine amidotransferases appear to be covalently linked to the portion of the molecule that is responsible for the synthetase reactions (31, 32). The presently available data therefore suggest that the glutamine-binding sites of the various glutamine amidotransferases have been modified through evolution in various ways; nevertheless, the finding that most of these enzymes are inhibited by the same glutamine analogs (e.g., azaserine, 2-amino-4-oxo-5-chloropentanoic acid, 6-diazo-5-oxonorleucine), which in many instances bind to a specific sulfhydryl group, suggests that the glutamine-binding sites of these enzymes may exhibit a significant degree of homologous structure.

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