Direct Evidence for the Formation of an Acyl Phosphate by Glutamine Synthetase*

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ABSTRACT: Previous efforts to demonstrate the formation and utilization of γ-glutamyl phosphate—a hypothetical intermediate in the reaction catalyzed by glutamine synthetase—were unsuccessful because of the marked tendency of this acyl phosphate to undergo cyclization to pyrrolidonecarboxylate and to hydrolyze. In the present work, a glutamate analog, *cis*-1-amino-1,3-dicarboxycyclohexane (*cis*-cycloglutamate), a substrate of glutamine synthetase possessing a 5-carbon chain much more rigid than that of glutamate, was incubated with the enzyme, ATP, and Mn^{2+}, and an enzyme–substrate complex was isolated by gel filtration. An acidic compound was obtained from the enzyme that contained equal quantities of cycloglutamate and phosphate (from the γ-P of ATP); it reacted with hydroxylamine to give γ-cycloglutamyl hydroxamate.

Studies in this laboratory have provided evidence for the hypothesis that enzyme-bound γ-glutamyl phosphate is an intermediate in the reaction catalyzed by glutamine synthetase (Krishnaswamy *et al.*, 1962; Meister, 1968). The earlier finding, that the enzymatic synthesis of glutamine is accompanied by a transfer of oxygen from glutamate to inorganic phosphate (Kowalsky *et al.*, 1956; Boyer *et al.*, 1956), is consistent with a γ-glutamyl phosphate intermediate; however, other interpretations of this observation are not excluded (Kowalsky *et al.*, 1956; Meister, 1968). The ability of glutamine synthetase to catalyze the synthesis of β-aminoglutaramic acid from β-aminoglutaric acid and to utilize β-aminoglutaryl phosphate for the catalytic synthesis of ATP (Khedouri *et al.*, 1964), and the observation that the irreversible inhibitor methionine sulfoximine is phosphorylated when incubated with glutamine synthetase in the presence of ATP and metal ion (Ronzio and Meister, 1968; Ronzio *et al.*, 1969; Rowe *et al.*, 1969) provide strong evidence for the acyl phosphate hypothesis. Attempts to carry out studies with chemically synthesized γ-glutamyl phosphate (Levintow and Meister, 1956) were unsuccessful, as were a number of subsequent (unpublished) efforts to isolate this acyl phosphate, because of the marked tendency of γ-glutamyl phosphate to cyclize to pyrrolidonecarboxylic acid and to hydrolyze to glutamate. However, the recent finding that *cis*-cycloglutamic acid (*cis*-1-amino-1,3-dicarboxycyclohexane) is a good substrate of glutamine synthetase (Gass and Meister, 1970) suggested a direct experimental approach to the question of whether the enzyme catalyzes the formation of an acyl phosphate. Thus, *cis*-cycloglutamate, which possesses a relatively rigid 5-carbon chain (as compared to that of glutamate), cannot undergo a cyclization reaction analogous to the conversion of glutamate into pyrrolidonecarboxylate. We therefore reasoned that it should be possible to isolate γ-cycloglutamyl phosphate from glutamine synthetase after the enzyme was incubated with cycloglutamate, ATP, and metal ion. Such expectation has been realized and in this paper direct evidence is presented for the formation of enzyme-bound-γ-cycloglutamyl phosphate.

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Experimental Section

Materials

Glutamine synthetase was prepared from sheep brain as described by Rowe *et al.* (1970); preparations exhibiting specific activities of 175–190 units/mg were used, and a value of 400,000 for the molecular weight of the enzyme (Tate and Meister, 1971) was employed in the calculations. Unlabeled ATP was obtained from Sigma Chemical Co. Tritiated ATP was obtained from the New England Nuclear Corp. and [γ-^{32}P]ATP was obtained from Amersham-Searle.

[^{14}C]*cis*-Cycloglutamate (*cis*-1-amino-1,3-dicarboxycyclohexane) was synthesized by the procedure described by Gass and Meister (1970) using sodium [^{14}C]cyanide obtained from the New England Nuclear Corp. Sodium [^{14}C]cyanide (54.5 Ci/mole) was diluted 50-fold by mixing the radioactive material with 49 mg of unlabeled sodium cyanide; this was added together with 342 mg of ammonium carbonate monohydrate to 142 mg of 3-carboxycyclohexanone in 1.2 ml of 50% ethanol. The reaction was carried out as described by Gass and Meister (1970) except that the hydantoin was not isolated; the reaction mixture was made strongly alkaline by adding solid potassium hydroxide and then refluxed for 18 hr. After cooling, the pH was adjusted to 3.4 by addition of concentrated hydrochloric acid and the solvents were removed by flash evaporation; the remaining solid was obtained free of salts by adsorption on and elution (with 1 N ammonium hydroxide) from a column of Dowex 50 (H^+). The product exhibited a specific radioactivity of 2150 cpm/mμmole. [^{14}C]Cycloglutamine and [γ-^{14}C]cycloglutamyl hydroxamate were prepared from [^{14}C]cycloglutamate by the glutamine synthetase reaction (Gass and Meister, 1970).

Methods

Glutamine synthetase activity was determined by the γ-glutamyl hydroxamate assay procedure (Wellner and Meister, 1966). Paper electrophoresis was carried out at 0° on strips (4 cm wide) of Whatman No. 3MM paper. The samples were applied to the wet paper on the cooled migration plate. A potential gradient of 33 V/cm was used over an effective paper length of 90 cm. Electrophoresis was carried out in two systems: potassium phthalate buffer (25 mM, pH 6.1) and sodium citrate buffer (50 mM, pH 3.7).

Radioactivity (^{14}C, ^{32}P, and ^{3}H) was determined with a Nuclear-Chicago scintillation counter; in some experiments

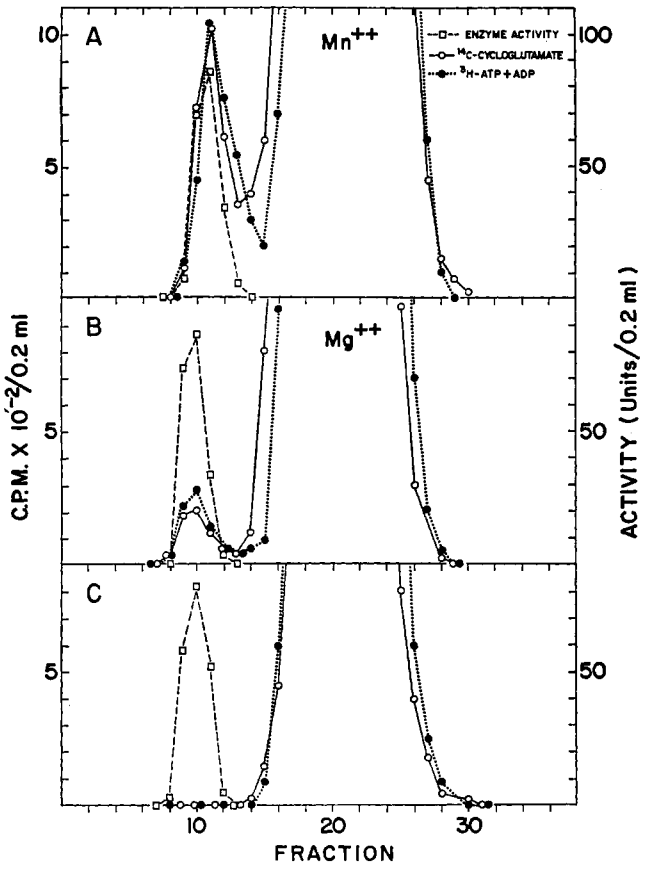


FIGURE 1: Isolation of an enzyme-[^{3}H]nucleotide-[^{14}C]cycloglutamate complex by gel filtration on Sephadex G-50. (A) A mixture (final volume, 0.5 ml) containing enzyme (3 mg), [^{14}C]cycloglutamate (10 mM), [^{3}H]ATP (5 mM), manganese chloride (3 mM), dithiothreitol (1.25 mM), and Tris-HCl buffer (50 mM, pH 7.2) was chromatographed on a Sephadex G-50 column as described under methods. (B) This was identical with A except that magnesium chloride was used in place of manganese chloride. (C) This was identical with A except that no metal ions were added.

double-isotope technique was used. The samples (0.2–0.3 ml) from the columns were mixed with 10 ml of scintillation fluid (Jeffay and Alvarez, 1961). The radioactivity present on 1-cm sections of the dried paper strips used for electrophoresis were determined by adding the papers directly to the scintillation fluid.

Gel filtration was carried out on Sephadex G-50 as follows. The reaction mixture, usually containing enzyme, cycloglutamate, ATP, metal ion, and buffer, was incubated at 37° for 5 min and then cooled in ice. The mixture was added to the top of a column (0.9 × 12 cm) of Sephadex G-50 previously equilibrated with 40 mM Tris-HCl buffer (pH 7.2) which contained 1 mM dithiothreitol; elution was carried out with the same buffer at 4°. Fractions (usually 0.2 ml) were collected at a flow rate of 0.5 ml/min. The concentration of enzyme in each fraction was determined by measurement of glutamine synthetase activity. Determinations of radioactivity were carried out as described above.

Results

Isolation of Enzyme–Substrate Complex by Gel Filtration. When a mixture containing glutamine synthetase, [^{14}C]-cycloglutamate, [^{3}H]ATP, and Mn^{2+} was subjected to gel filtration on Sephadex G-50, considerable radioactivity

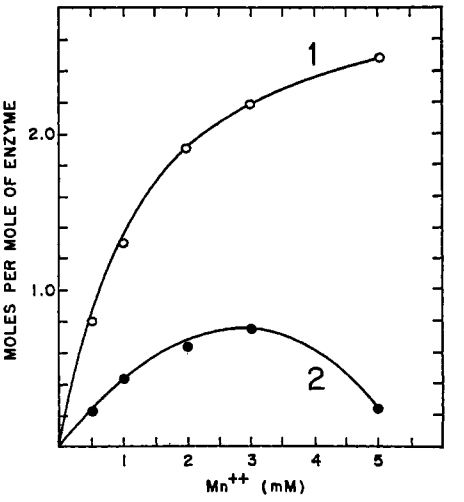


FIGURE 2: Effect of Mn^{2+} concentration on the binding of cycloglutamate and of nucleotide to the enzyme. The experimental procedure was the same as given in Figure 1 except that the concentration of manganese chloride was varied. Curve 1, nucleotide; curve 2, cycloglutamate.

derived from the nucleotide and the amino acid remains associated with the enzyme (Figure 1A). In this experiment the values for the binding of nucleotide and cycloglutamate to the enzyme were, respectively, 2.2 and 0.77 moles per mole of enzyme. Binding was also observed when Mn^{2+} was replaced by Mg^{2+} (Figure 1B); in this experiment the values for the binding of nucleotide and cycloglutamate were, respectively, 0.57 and 0.15 mole per mole of enzyme. There was tailing of the radioactivity in both experiments suggesting that there is some breakdown of the complex during gel filtration. In the experiment described in Figure 1C, in which no metal ion was added, there was no significant binding of either nucleotide or cycloglutamate. In experiments similar to those described in Figure 1A,B, in which ATP was omitted there was no binding of cycloglutamate. In an experiment similar to that described in Figure 1A, in which Mn^{2+} (1 mM) was added to the eluting buffer, the values for the binding of nucleotide and cycloglutamate, were, respectively, 4.5 and 0.42 moles per mole of enzyme. In an experiment analogous to that

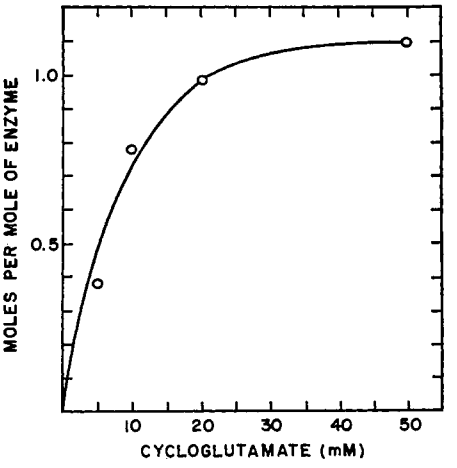
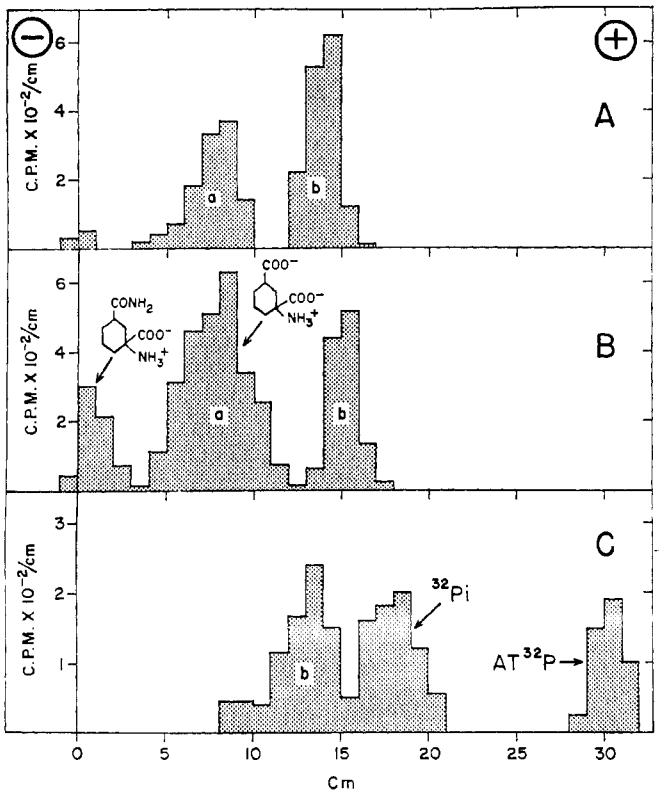


FIGURE 3: Effect of cycloglutamate concentration on the binding of cycloglutamate to the enzyme. The conditions were as given in Figure 1 except that the concentration of cycloglutamate was varied.



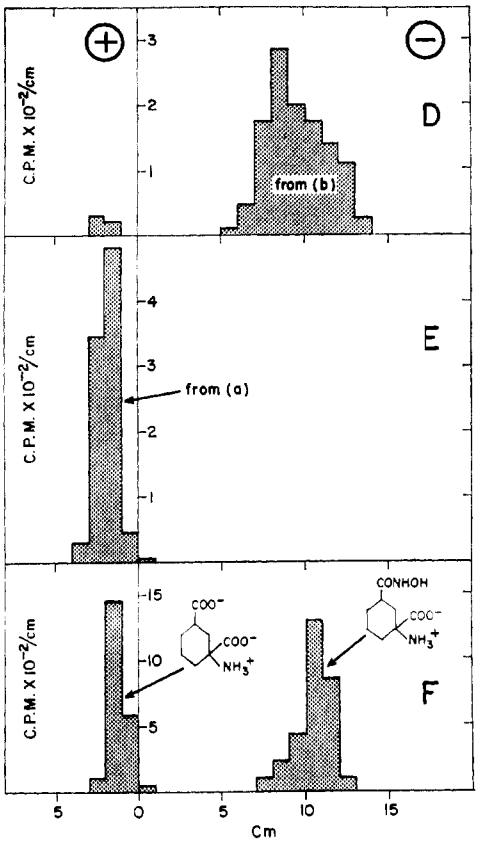


FIGURE 4: Demonstration of an intermediate containing cycloglutamate and phosphate. A mixture (final volume, 7 ml) containing enzyme (25 mg), ATP (5 mM), [^{14}C]cycloglutamate (10 mM, 2150 cpm/mμmole), dithiothreitol (1.25 mM), manganese chloride (3 mM), and Tris-HCl buffer (50 mM, pH 7.2) was incubated at 37° for 5 min and then chromatographed on a column (2.5 × 12 cm) of Sephadex G-50 at 4°. The column was eluted with Tris-HCl buffer (40 mM, pH 7.2) with a flow rate of 2.5 ml/min; fractions of 2.4 ml were collected. A separation of the enzyme complex was obtained that was virtually identical to that described in Figure 1A. The fractions containing the enzyme complex were combined and lyophilized and the resulting dry powder was dissolved in 0.5 ml of water. (A) 0.1 ml of the solution obtained above was subjected to paper electrophoresis in potassium phthalate buffer (pH 6.1) for 120 min. After electrophoresis the radioactivity present was located as described under Methods. (B) Same as part A except that [^{14}C]cycloglutamine (2480 cpm) and [^{14}C]cycloglutamate (2480 cpm) were added to the sample prior to electrophoresis. (C) A mixture comparable to that described above was prepared in which the unlabeled ATP was replaced with [γ-^{32}P]ATP (2560 cpm/mμmole) and the labeled cycloglutamate was replaced by unlabeled cycloglutamate. Gel filtration and lyophilization were carried out as described above and the dried material was dissolved and subjected to paper electrophoresis in potassium phthalate buffer (pH 6.1; for 120 min); the results obtained are given in part C. D and E. Paper strips identical with that shown in part A were sprayed with 1 M hydroxylamine (pH 7.2) on the cooled migration chamber immediately after electrophoresis was terminated. The radioactive bands a and b were separately extracted from the paper with three portions (15 ml each) of water. Each aqueous extract was concentrated to dryness in a flash evaporator and the residue was dissolved in 0.2 ml of water. These solutions were subjected to paper electrophoresis in sodium citrate buffer (pH 3.7) for 90 min. The distribution of radioactivity was determined after electrophoresis as described under Methods. (D) Electrophoresis after hydroxylamine treatment of band b. (E) Electrophoresis after hydroxylamine treatment of band a. (F) Paper electrophoresis of [^{14}C]cycloglutamate (2480 cpm) and [^{14}C]cycloglutamyl hydroxamate (2480 cpm) in sodium citrate buffer (pH 3.7) for 90 min.

described in Figure 1B, with Mg^{2+} (1 mM) in the eluting buffer, only 1.3 moles of nucleotide was bound per mole of enzyme and no cycloglutamate was bound.

The effect of Mn^{2+} concentration in the reaction mixture on the binding of nucleotide and of cycloglutamate to the enzyme is described in Figure 2. Under the particular conditions of gel filtration employed, maximal binding of cycloglutamate was observed with an Mn^{2+} concentration of 3 mM, and less cycloglutamate was bound to the enzyme with 5 mM Mn^{2+}. It is possible that Mn^{2+} complexes with the enzyme or with the amino acid and that such a reaction decreases the binding of amino acid. In contrast, the binding of nucleotide to the enzyme increased with increasing concentrations of Mn^{2+} under these conditions. Studies of the binding of cycloglutamate to the enzyme as a function of cycloglutamate concentration are described in Figure 3. Binding reaches an apparent maximum at about 50 mM under the conditions employed (3 mM, Mn^{2+}, 5 mM ATP). Under the conditions of the studies described in Figure 3, the maximum binding of cycloglutamate to the enzyme was about 1.1 moles of cycloglutamate/mole of enzyme. In experiments with 100 mM cycloglutamate and 10 mM ATP, we observed binding of 1.4 moles of cycloglutamate per mole of enzyme.[1]

Evidence that the Enzyme-Bound Intermediate Is γ-Cycloglutamyl Phosphate. In an effort to investigate the chemical nature of the enzyme-bound cycloglutamate, a relatively large amount of enzyme was incubated with ATP, [^{14}C]cycloglutamate, and Mn^{2+} and the enzyme–nucleotide–cycloglutamate complex was isolated by gel filtration on Sephadex G-50, essentially as described in Figure 1A. The fractions

[1] It should be emphasized that the data on binding reported here apply specifically to the particular conditions employed, and that they are not necessarily applicable to binding under equilibrium conditions.

containing the complex were concentrated and a sample of this material was subjected to high-voltage electrophoresis at pH 6.1. As indicated in Figure 4A, two major radioactive bands separated; one of these, band b, moved about 14 cm toward the anode and separated completely from the other band, a. Under these conditions the enzyme remained at the origin. Band a moves with authentic cycloglutamate; this is evident from Figure 4B, which shows electrophoresis of the sample used in Figure 4A after authentic samples of [^{14}C]-cycloglutamate and [^{14}C]cycloglutamine were added. In an experiment in which unlabeled cycloglutamate and [^{32}P]ATP were used (Figure 4C) it was found that considerable amounts of ^{32}P moved in the position of band b and that this material exhibited a mobility different from that of P_i. In this experiment 6.1 mμmoles of [^{32}P]P_i were found in band b, while 5.5 and 4.7 mμmoles of ^{32}P were present in the inorganic phosphate and ATP areas, respectively. In a comparable experiment with [^{14}C]cycloglutamate and unlabeled ATP, 5.9 and 3.9 mμmoles of ^{14}C were found in bands b and a, respectively. Thus, the ^{14}C to ^{32}P ratio for band b was 5.9/6.1 or 0.97. The presence of free [^{14}C]cycloglutamate and of [^{32}P]P_i seems to reflect partial breakdown of the enzyme-bound product; the finding of somewhat more [^{32}P]P_i (5.5 mμmoles) than [^{14}C]cycloglutamate (3.9 mμmoles) may indicate some hydrolysis of ATP under the conditions of these studies. The findings are in accord with the formation of a relatively unstable intermediate containing equimolar amounts of cycloglutamate and phosphate derived from the terminal phosphate moiety of ATP.

When bands b and a (from the strip shown in Figure 4A) were separately treated with hydroxylamine, and then subjected to electrophoresis in citrate buffer (pH 3.7), the results shown in Figure 4D,E were obtained. Treatment of band b with hydroxylamine gave a ^{14}C product that moved in the position of cycloglutamyl hydroxamate (D), while similar treatment of band a gave a ^{14}C compound that moved with cycloglutamate (E).

Discussion

The data presented here indicate that cycloglutamate binds to glutamine synthetase only in the presence of ATP and Mn^{2+} and to a much lesser extent, with Mg^{2+}. This is in accord with earlier findings that ATP and metal ions are required for the binding of glutamate (Krishnaswamy *et al.*, 1962) and of methionine sulfoximine (Ronzio and Meister, 1968; Ronzio *et al.*, 1969). Under the present conditions, saturation of the enzyme with cycloglutamate was not attained.[1] Nevertheless, the findings indicate that a large fraction of the cycloglutamate which is bound is in a form which moves on electrophoresis as a molecule which is more negatively charged than cycloglutamate, and which reacts with hydroxylamine to give a derivative which migrates on electrophoresis with γ-cycloglutamyl hydroxamate. The observation that the activated cycloglutamate compound (Figure 4A, band b) contains, within experimental error, equimolar amounts of ^{32}P from [γ-^{32}P]ATP and ^{14}C from [^{14}C]cycloglutamate, gives additional and direct support to the conclusion that the intermediate is the corresponding acyl phosphate, *i.e.*, γ-cycloglutamyl phosphate.

Although the enzyme contains 8 subunits (Haschemeyer, 1970), we observed here a maximum binding of about 1.4 moles of cycloglutamate/mole of enzyme. It is notable that, under the conditions of gel filtration used, there was evidence that dissociation of the complex occurred. The possibility must be considered that the binding of amino acid to the enzyme may be promoted by ammonia (or hydroxylamine). The present findings do not exclude the possibility that the enzyme is phosphorylated at some stage in catalysis, and that the mechanism is thus similar to that of the reaction catalyzed by succinyl thiokinase, in which both succinyl phosphate and phosphorylated enzyme are formed (Nishimura and Meister, 1965; Nishimura, 1967; Grinnell and Nishimura, 1969; Kreil and Boyer, 1964; Ramaley *et al.*, 1967).

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