The Amino Acid Sequence in the Vicinity of the Covalently Bound Adenylic Acid in Glutamine Synthetase from Escherichia coli

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SUMMARY

A single tryptic peptide containing adenylic acid has been isolated from Escherichia coli glutamine synthetase. The amino acid sequence of the peptide is

Asp-Leu-Tyr-Asp-Leu-Pro-Pro-Glu(Ala,Glu,Gly)-Lys

\[ \text{W-AMP} \]

The radioactivity of the \(^{14}\text{C-AMP}\) moiety was released from the peptide during the first Edman degradative cycle, and this was shown to be due to cleavage of the \(^{14}\text{C-adenine-ribose}\) bond by trifluoroacetic acid.

Under certain conditions of growth, the Escherichia coli glutamine synthetase is modified by the introduction into the enzyme molecule of covalently bound adenylic acid. This mechanism for modification of the preformed enzyme was discovered independently in this country by Kingdon and Stadtman (1, 2) and in Germany by Holzer et al. (3, 4). An enzyme was described by Kingdon, Shapiro, and Stadtman (5) and by Wulff, Mecke, and Holzer (6) which catalyzes the adenylylation of the glutamine synthetase in the presence of magnesium ions and glutamine, with ATP as the adenylic acid donor. Another enzyme, which catalyzes deacylhylation, has been studied by Shapiro and Stadtman (7), Shapiro (8), and Heihneyer, Battig, and Holzer (9). Kingdon et al. (5) showed that glutamine synthetase can be adenylylated to the extent of 12 5'-adenylyl groups per enzyme molecule. Since E. coli glutamine synthetase has been shown to have a molecular weight of 600,000, with 12 identical subunits (10), this corresponds to one 5'-adenylic acid per subunit. The 5'-adenyl group in naturally occurring glutamine synthetase was identified by Shapiro, Kingdon, and Stadtman (11), and Shapiro and Stadtman (12) have shown that this moiety is attached to the enzyme by a phosphodiester link to a tyrosine hydroxyl.

The present communication describes the isolation of a tryptic peptide containing covalently bound adenylic acid from fully adenylylated E. coli glutamine synthetase, and also reports the amino acid sequence in the vicinity of the AMP.

EXPERIMENTAL PROCEDURE

Materials—E. coli, strain W, was grown as described by Kingdon and Stadtman (2) on a glycerol-glutamate medium designed to yield glutamine synthetase with a high degree of substitution with adenylic acid (Synthetase II).

Glutamine synthetase was prepared from frozen lots of E. coli W by the procedure of Woolfolk, Shapiro, and Stadtman (10). A yield of 450 mg (0.75 pmole) of apparently homogenous enzyme was obtained from 1.5 kg of wet cells. A portion of the purified enzyme subjected to polyacrylamide gel electrophoresis at pH 8.5 (13) gave a single band. The ratio of the absorbance at 260 nm to that at 280 nm was 0.77, indicating an adenylic acid content of 9 moles per mole of enzyme (5). The specific enzyme activity of this preparation was consistent with that expected for pure enzyme labeled to a comparable degree with AMP (2, 5).

ATP-glutamine synthetase adenylyltransferase was partially purified as described by Mecke et al. (14) from the same cells as the glutamine synthetase.

The introduction of \(^{14}\text{C-labeled AMP}\) into the glutamine synthetase preparation was accomplished by methods reported earlier (5). Glutamine synthetase (450 mg), ATP-glutamine synthetase adenylyltransferase (177 mg), and \(^{14}\text{C-adenosine 5'-triphosphate}\) (Amersham/Searle, 50 \(\mu\text{Ci}, 250 \mu\text{moles}) were incubated for 90 min at 37° in a total volume of 50 ml in the presence of 10 mM Tris chloride buffer (pH 7.6), 50 mM MgCl\(_2\), and 1 mM L-glutamine. At the end of the incubation period, the reaction mixture was cooled to 0°, and the \(^{14}\text{C-adenyl glutamine synthetase}\) was repurified by repeated acid-ammonium sulfate precipitations, identical with Step VI in the standard enzyme preparation (10). The recovery of labeled protein after two reprecipitations was nearly quantitative (488 mg) and the specific radioactivity of the product was 480,000 cpm per pmole, consistent with the introduction of three to four labeled adenylyl groups per mole of enzyme. Polyacrylamide gel electrophoresis

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of the \(^{14}\)C-adenyllyl glutamine synthetase revealed a single band and this material provided the source of the adenylyl peptide.

Trypsin (type XI, diphenyl carbamyl chloride treated, crystallized, dialyzed salt-free and lyophilized; Lot 783-S70), carboxypeptidases A and B (treated with diisopropyl fluorophosphate), and dimethylaminonaphthalene-5-sulfonyl chloride were obtained from Sigma. Iodoacetic acid was purchased from Matheson and was used without further purification. Urea was recrystallized and deionized according to the procedures of Benech, Lardy, and Benesch (15). Pyridine, ethyl acetate, and phenylisothiocyanate (Matheson) were distilled before use. Tri-fluoroacetic acid was obtained from Aldrich. All other reagents were of the purest commercially available grade.

**Protein Determinations**—Protein was determined colorimetrically by a modification of the biuret reaction (16) with crystallized bovine albumin as a reference standard. Effluent fractions from column chromatographic procedures were monitored spectrophotometrically for protein content by measuring the absorbance of the solutions at 260 nm and 280 nm.

**Amino Acid Analyses**—Amino acids were determined by automated ion exchange chromatography according to the general procedures of Spackman, Stein, and Moore (17) with a Bio-Car HC-200 analyzer equipped with a microcuvette flow cell system and an electronic scale expander. These optional systems permitted quantitation of as little as 2 to 5 nmol of amino acid with an accuracy of ±5%. Peptide samples were hydrolyzed in 6 N HCl under reduced pressure for 22 hours at 110 ± 0.2°, special precautions being observed in the preparation and hydrolysis of samples (18, 19).

**Electrophoretic Separation of Peptides**—Fractionation of peptides by high voltage paper electrophoresis at pH 6.5 was performed in a Gibson Electrophorator (model D), following the general techniques outlined by Dreyer and Bynum (20). Peptide samples, together with leucine, aspartic acid, and lysine markers, were spotted on sheets of Whatman No. 3 chromatographic paper 57 cm in length, and electrophoresis was carried out in pyridine-acetate buffer, pH 6.5 (pyridine-acetic acid-H2O, 25:1:225) for 45 min at 3000 volts (53 volts per cm). The positions of the amino acids and peptides were detected by immersing the dry electrophoretograms in a solution of ninhydrin-CdCl\(_2\) reagent (20). After drying for 15 to 30 min at room temperature, the papers were placed in a forced air oven at 70° for 5 to 10 min in order to complete the color development. As little as 5 nmol of peptide could be visualized easily by this technique.

**Radioactivity Measurements**—The radioactivity of solutions containing \(^{14}\)C-labeled proteins and peptides was measured by counting 5- to 100-μl samples dried on aluminum planchets (1/4 inch) in a Nuclear-Chicago gas flow counter (model 181A) with an efficiency of about 85%. The radioactivity of peptides separated by high voltage paper electrophoresis was measured in a Packard Tri-Carb scintillation counter (model 574). Ninhydrin-positive spots from electrophoretograms were excised and immersed in vials (2.5 × 5 cm) containing 15 ml of the scintillation liquid (0.5% 2,5-diphenyloxazole and 0.025% p-bis(2'-5'-phenyloxazoyl)benzene in toluene). This procedure has an efficiency of approximately 65% for compounds containing \(^{14}\)C.

**Preparation of Reduced and Carboxymethylated Enzyme**—Approximately 100 mg of lyophilized \(^{14}\)C-adenyllyl glutamine synthetase (80,000 total counts per min) were reduced and carboxymethylated as described by Crestfield, Moore, and Stein (18). Separation of the modified protein from the other components of the reaction mixture was achieved by gel filtration of the solution on a column (4 × 52 cm) of Sephadex G-25 with 50% acetic acid as eluent. Protein appeared in the effluent at 310 ml and continued to emerge for 120 ml. The combined fractions containing protein were rotary-evaporated to a volume of 15 to 20 ml and the solution was lyophilized. Over-all recovery of protein was about 75%. Amino acid analysis of a 3-μg sample of reduced, carboxymethylated \(^{14}\)C-adenyllyl glutamine synthetase subjected to hydrolysis in 6 N HCl at 110° for 24 hours was in close agreement with the composition reported earlier (11). All of the cystine appeared as S-carboxymethyl cysteine and there was no indication that alkylation of methionine, histidine, or lysine residues had occurred.

**Hydrolysis of Reduced and Carboxymethylated Enzyme with Trypsin**—A suspension of 55 mg (42,000 total cpm) of reduced, carboxymethylated \(^{14}\)C-adenyllyl glutamine synthetase in 5 ml of 0.5 mm Tris chloride buffer, pH 8.0, was cooled to 0° and the pH was brought to 11.5 by the dropwise addition of chilled 1 N NaOH. After stirring for 5 to 10 min nearly all of the protein dissolved and the pH was immediately readjusted to 8.0 with cold 1 N HCl. The protein remained in solution under these conditions and precipitated only at pH values less than 7. The solution was placed in a vessel maintained at 40° in a pH-stat titrimeter (Radiometer Automatic Titrator, type TTT1) and 0.05 ml of trypsin (0.27 mg in 1 mm HCl) was added. The pH was kept at 8.0 by the addition, with the calibrated microcyrnose, of 0.1 N NaOH. Hydrolysis proceeded rapidly, and within 2 hours 80% of the theoretical amount of base required for the complete neutralization of the protons liberated during hydrolysis had been consumed. This calculation was based upon the number of lysine and arginine residues per mole of enzyme (11). A second 0.05-ml portion of trypsin was added and the reaction was allowed to proceed overnight at room temperature.

The digestion mixture was rotary-evaporated to dryness and dissolved in 2 ml of 0.1 N NH\(_4\)CO\(_3\). Insoluble material was removed by centrifugation and discarded. The supernatant solution, containing 95% of the original radioactivity, was passed through a column (1.5 × 193 cm) of Sephadex G-25 (fine) and eluted with 0.1 M NH\(_4\)HCO\(_3\) (cf. Fig. 1). Elution fractions were collected at a flow rate of 15 ml per hour, and the fractionation was monitored by determination of the absorbance at 260 nm and 280 nm and by measurement of radioactivity. As may be seen in Fig. 1, radioactivity emerged in two peaks, each of which coincided with peaks having high A\(_{280}\)/A\(_{260}\) ratios. The major component (Peak A) contained the adenylyl peptide and accounted for about 80% of the radioactivity of the sample added to the column. The elution behavior of Peak B suggested that it was of low molecular weight. High voltage paper electrophoresis of a portion of the pooled fractions comprising this peak revealed that the radioactivity was not coincident with ninhydrin-positive material. This would indicate that Peak B may consist in part of \(^{14}\)C-adenine liberated by mild acid hydrolysis of the labile adenine-ribose bond (12).

**Isolation of \(^{14}\)C-Adenyllyl Peptide**—The fractions containing Peak A (Fig. 1) were combined and lyophilized repeatedly to remove \(\text{NH}_4\)HCO\(_3\). The lyophilized material (32,000 cpm) was dissolved in 3 ml of 0.1 M acetic acid and the clear solution was added to a column (0.9 × 60 cm) of Dowex AG 1-X2 acetate (200 to 400 mesh) equilibrated with the same solvent. Peptides were eluted from the column with a linear gradient of increasing acetic acid concentration. The mixing chamber contained 250 ml of 0.1 M acetic acid and the reservoir contained an equal volume of 0.6 M acetic acid. Fractions of 5 ml were collected at a
flow rate of 15 ml per hour and these were analyzed for absorbance at 260 nm and radioactivity. As shown in Fig. 2, the 14C-adenyl peptide emerges late in the elution scheme, well separated from other ultraviolet-absorbing peptides. The yield of the adenylyl peptide emerges late in the elution scheme, well separated from other ultraviolet-absorbing peptides. The yield of the adenylyl peptide obtained in this step was more than 90%, and rated from other ultraviolet-absorbing peptides. The yield of the adenylyl peptide emerges late in the elution scheme, well separated from other ultraviolet-absorbing peptides.

RESULTS

A peptide containing covalently bound 14C-AMP was isolated in 70% yield from a tryptic digest of reduced and carboxymethylated 14C-adenyl glutamine synthetase by gel filtration on Sephadex (Fig. 1), and ion exchange chromatography on Dowex AG 1-X2 (Fig. 2). Paper electrophoresis of the purified adenylylated 14C-adenyl glutamine synthetase, was 70%. A portion of the combined radioactive fractions subjected to paper electrophoresis at pH 6.5 gave a single ninhydrin-positive spot coincident with radioactivity. The electrophoretic mobility of the adenylyl peptide indicated a net charge of -4 and amino acid analysis revealed it to be a dodecapeptide (12 amino acid residues).

Sequential Analysis—Stepwise degradation of the adenylyl peptide was performed by the method of Edman (21) as modified by Gray (22). Amino acid analysis of the peptide was carried out after each stage of the degradative procedure. In some cases, end group analyses were performed by reaction of the peptides with DNS-Cl (22). The DNS-amino acids were separated and identified by two-dimensional thin layer chromatography on polyamide layers (Gallard-Schlesinger) as described by Woods and Wang (23).

The tryptic 14C-adenyl dodecapeptide is similar in composition to the decapeptide obtained after digestion of adenylylated glutamine synthetase with pepain and Pronase (12), except that it contains 1 residue less of proline and an additional residue each of alanine, leucine, and lysine.

The compositional analysis and the electrophoretic behavior of the peptide indicate the absence of amidated residues. Four negative charges and one positive charge are donated respectively by the β- and γ-carboxyl groups of the 4 aspartic and glutamic acid residues and the ε-amin group of lysine. The fifth negative charge is on the phosphoryl oxygen of the AMP moiety.

Sequence Analysis

The amino acid sequence of the tryptic adenylyl dodecapeptide and the steps used to establish this structure are summarized in Fig. 3. Details with regard to the degradative procedures are described in the following paragraphs.

Edman Degradation

Eight consecutive stages of the Edman degradation were performed in order to establish the sequence of the amino acid residues in the NH2-terminal region of the peptide chain. Portions of the residual peptides were subjected to amino acid analysis after each stage and the amino acids were identified by difference (24). In each amino acid analysis presented below, the missing amino acid is noted in boldface type. The results obtained by the subtractive method for the first 4 amino acid residues were confirmed by reaction of a portion of the residual peptide with DNS-Cl and identification of the amino terminal DNS-amino acids (Fig. 3). Analysis for lysine was omitted in all steps subsequent to the first.

First Step (83%)—Asp, 1.15; Leu, 2.12; Tyr, 0.90; Pro, 2.06;...
Glu, 2.00; Gly, 0.94; Ala, 1.00; Lys, 1.06. Paper electrophoresis of a portion of this peptide gave a single ninhydrin-positive spot corresponding to a peptide with a formal charge of -3. The peptide, however, no longer coincided with radioactivity, which appeared in the neutral band. These results suggested that, at some stage of the degradative procedure, part of the 14C-adenyl moiety had been cleaved from the original peptide. Since the altered electrophoretic mobility of the peptide was consistent with the removal of 1 residue of aspartic acid, it seemed likely that cleavage of the acid-labile adenine-ribose bond (12) may have occurred during the cyclisation step in trifluoroacetic acid. To test this hypothesis, 5 mg of 3'-AMP were dissolved in 1.0 ml of trifluoroacetic acid and the solution was incubated at 55° for 10 min. The trifluoroacetic acid was removed under vacuum and the residue was dissolved in 1 ml of butanol-1 saturated with water. A portion of this solution, together with adenosine, adenine, and 5'-AMP markers, was spotted on a sheet of Whatman No. 1 paper and subjected to ascending chromatography in butanol-1 saturated with water. Inspection of the chromatogram under ultraviolet light clearly revealed that the AMP had been completely converted to adenine. Another portion of the trifluoroacetic acid-treated nucleotide was chromatographed on Whatman No. 1 paper together with ribose and ribose 5-phosphate markers. The ascending solvent system was ethyl acetate-pyridine-H2O (40:10:5). Spots were detected by spraying the dried paper with a solution containing 0.8 g of phthalic acid, 0.5 ml of aniline, and 50 ml of butanol-1 saturated with water, and then drying the chromatogram in an oven at 80° for 10 to 15 min.

The only substance identified in the trifluoroacetic acid-treated nucleotide was ribose 5-phosphate. These findings suggest that during the Edman procedure the 14C-adenyl moiety is quantitatively converted to 14C-adenine and a peptide in which ribose is linked via a phosphodiester bond to tyrosine.

**Second Step (68%)—Asp, 1.06; Leu, 1.20; Tyr, 0.91; Pro, 2.10; Glu, 2.05; Gly, 0.59; Ala, 0.98.**

**Third Step (80%)—Asp, 1.02; Leu, 1.18; Tyr, 0.21; Pro, 2.02; Glu, 2.10; Gly, 0.94; Ala, 0.96.**

**Fourth Step (78%)—Asp, 0.28; Leu, 1.10; Tyr, 0.20; Pro, 2.15; Glu, 1.94; Gly, 1.01; Ala, 1.06.**

**Fifth Step (73%)—Asp, 0.25; Leu, 0.30; Tyr, 0.20; Pro, 1.97; Glu, 2.04; Gly, 1.01; Ala, 0.99.**

**Sixth Step (70%)—Asp, 0.26; Leu, 0.32; Tyr, 0.15; Pro, 1.33; Glu, 2.01; Gly, 0.98, Ala, 1.05.**

**Seventh Step (88%)—Asp, 0.20; Leu, 0.25; Tyr, 0.18; Pro, 0.62; Glu, 2.09; Gly, 0.87; Ala, 1.10.**

**Eighth Step (67%)—Asp, 0.22; Leu, 0.27; Tyr, 0.16; Pro, 0.55; Glu, 1.42; Gly, 0.88; Ala, 1.05.**

**Treatment with Carboxypeptidases A and B.**

Lysine was placed at the carboxyl terminus of the 14C-adenyl peptide on the basis of the specificity of trypsin. Proof for this assignment was obtained by hydrolysis of the peptide with carboxypeptidase B. A solution of the peptide (0.02 μ mole) and 10 μg of carboxypeptidase B in a total volume of 200 μl of 0.2 ml N-ethylmorpholine acetate buffer, pH 8.5, was incubated for 2 hours at 37°. Short column amino acid analysis of 50 μl of the reaction mixture gave 0.0048 μ mole of lysine, indicating a release of over 95% of the COOH-terminal lysine. Analysis of a blank control containing no peptide revealed no basic amino acids.

After 2 hours of reaction with carboxypeptidase B, 10 μg of carboxypeptidase A were added and aliquots were removed for long column amino acid analysis at 45, 90, and 180 min of reaction at 37°. No further release of amino acids was observed. Similar reaction mixtures were prepared containing the synthetic peptides Gly-Gly-Ala, Ala-Gly-Gly and Gly-Gly-Leu. In 3 hours at 37°, carboxypeptidase A catalyzed the nearly quantitative removal of both COOH-terminal alanine and COOH-terminal leucine but was almost totally inactive toward a glycine residue in this position. These findings are in keeping with the known specificity of carboxypeptidase A (26). Thus it would appear as if the penultimate residue in the adenyl peptide is glycine or glutamic acid and not alanine.

**DISCUSSION**

The E. coli glutamine synthetase is subject to a unique form of metabolic regulation which can be summarized by the following equations:

\[
\text{ATP, glutamine, Mg}^{2+}, \text{adenyllyating enzyme} \rightarrow \text{Glutamine synthetase} \rightarrow \text{Adenyl glutamine synthetase} \rightarrow \alpha\text{-Ketoglutarate, deadenyllyating enzyme}
\]

From these equations, and from corroborating experiments in vivo (2), it is apparent that this mechanism provides the organism with a method of rapid, reversible change in the net glutamine-synthesizing capacity of the cell. Nitrogen surfeit favors the adenylylated form; nitrogen starvation favors the unadenylated form (4). Since the adenylylated form has a lower specific activity in the presence of Mg++ (2, 5), and since it is more sensitive to feedback inhibition by a number of feedback inhibitors (1, 5) than is the unadenylated form, it seems likely that there is less glutamine-synthesizing capacity under conditions of nitrogen surfeit. Experiments in vivo have shown that when the environment of the organism is suddenly changed from nitrogen surfeit to nitrogen starvation the return to the more active enzyme is very rapid (2).

A recent review by Holzer is available which deals with the broad topic of regulation of enzymes by enzyme-catalyzed chemical modifications, including the glutamine synthetase example (26).

The present report delineates the amino acid sequence of the glutamine synthetase in the region of the covalently bound adenyl group. The partial sequence of a tryptic, adenylylated decapeptide isolated from the enzyme is

Asp-Leu-Tyr-Asp-Leu-Pro-Pro-Glu(Ala, Glu, Gly)-Lys

\[
\text{14C-AMP}
\]

Since after quantitative removal of lysine by carboxypeptidase B, no further amino acids were released from the peptide by carboxypeptidase A, one would expect the penultimate residue to be either glutamic acid or glycine. The fact that a single AMP-containing peptide was obtained by tryptic digestion further strengthens the argument that all 12 subunits of the enzyme are identical (10). It should be emphasized, however, that both in the identification of tyrosine as the AMP-binding moiety by Shapiro and Stadtman (12) and in the current study radioactive AMP was introduced into the glutamine synthetase beginning with an enzyme preparation that already contained...
some AMP. If, therefore, early labeled AMP sites were different from late labeled AMP sites, the current studies could still have overlooked one or two nonidentical subunits among the 12. This possibility is being investigated further.

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