Glutamine Active Site of Formylglycinamide Ribonucleotide Amidotransferase. 2. Amino Acid Sequence of Labeled Peptides†

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ABSTRACT: The purification and characterization of two related peptides making up the glutamine binding site of formylglycinamide ribonucleotide amidotransferase from chicken liver have been presented. An amino acid residue(s) involved in binding glutamine to the enzyme was selectively labeled with [^{14}C]iodoacetate. The labeled enzyme was reduced, carboxymethylated, and degraded by trypsin to a large radioactive peptide that yielded on acid hydrolysis only cysteine as a radioactive carboxymethylated derivative. The tryptic peptide was further digested with a protease from *Streptomyces griseus*. Two radioactive fractions (I and II) were obtained by gel filtration on Sephadex G-25. Furthermore, two ^{14}C-containing peptides have been isolated from fraction I by the aid of ion exchange chromatography on AG 1-X2, AG 50W-X2 and DEAE-cellulose. Upon acid hydrolysis both peptides yielded only carboxymethylcysteine (CMCys), cystine, glycine, valine, aspartic acid, and glutamic acid. The partial sequences of the amino residues in these peptides, which are located at the glutamine binding site, have been established by the dansyl–Edman method. The sequences of amino acids of peptides a and b are Gly-Val-Cys([^{14}C]CM)-Asp-Asx-Cys(CM)-Glx . . . and Gly-Val-Cys([^{14}C]CM)-Asx-Asx . . . , respectively. The two peptides are undoubtedly derived from the same segment of the original protein.

In previous studies with enzyme isolated from a bacterial source the sequence of amino acids on the amino-terminal side of the reactive cysteine of formylglycinamide ribonucleotide (FGAR)[1] amidotransferase has been identified as alanylleucylglycylvaline (Dawid et al., 1963; French et al., 1963). The purpose of this research has been to extend this investigation on enzyme from chicken liver to ascertain the composition and partial structure of a larger peptide containing the reactive cysteinyl residue. We will show in the present paper that the sulfhydryl group at the glutamine binding site of the enzyme can be selectively labeled with [^{14}C]iodoacetate under prescribed conditions (Ohnoki et al., 1977). After reduction and carboxymethylation the labeled protein was hydrolyzed with trypsin and bacterial protease. The present paper presents information on the sequence of amino acids around the cysteinyl residue involved in glutamine binding to the enzyme.

Experimental Procedure

Materials

FGAR amidotransferase was prepared by the method described in a previous paper (Ohnoki et al., 1977). Nine preparations of enzyme from chicken liver acetone powder (total amount, 2 kg) were individually labeled and used for structural analysis. Specific activities of most of the preparations were 400–500 units/mg. 2-Mercaptoethanol (Matheson Coleman and Bell) was purified by distillation in vacuo and the fraction distilling at 70 °C (26 mmHg) collected. The distilled reagent was stored at −15 °C in a sealed ampule under nitrogen. Iodoacetate (Eastman) and urea (Fisher) were recrystallized from hot chloroform and 80% ethanol, respectively. Pyridine, *N*-ethylmorpholine, and ethyl acetate were distilled and stored in the refrigerator. Tos-PheCH$_2$Cl-trypsin was purchased from Worthington Biochemical Corp., and protease (type VI from *Streptomyces griseus*), bacitracin, glutathione (oxidized form), oxytocin, bovine pancreatic insulin, and cytochrome *c* were obtained from Sigma Chemical Co. Human angiotensin I was provided by BACHEM Fine Chemicals, Inc. Thiodiglycol, phenyl isothiocyanate, trifluoroacetic acid, and constant boiling hydrochloric acid were purchased from Pierce Chemical Co., and dansyl chloride was from Sigma Chemical Co.

Sephadex G-25 and G-75 were products of Pharmacia Fine Chemicals, Inc., AG 1-X2, AG 50W-X2, Bio-Gel P-2 and P-4 were purchased from Bio-Rad Laboratories, and DEAE-cellulose was purchased from Schleicher and Schuell, Inc. Precoated thin-layer plates (cellulose) were purchased from Brinkmann Instruments, Inc., Chen-Chin polyamide layer sheets from Gallard-Schlesinger Chemical Mfg. Corp., and x-ray film was from Eastman Kodak Co. Distilled and deionized water was again passed through a Millipore filter and twice distilled before use. This purified water was used only in the last step of peptide purification and in the studies of sequence analysis.

Methods

Radioactive Counting. Radioactivity measurements were carried out with a Nuclear-Chicago liquid scintillation counter, Model 720. Ten- to fifty-microliter portions of the effluent from Sephadex or ion exchange columns were counted with 10 mL of Bray's solution (Bray, 1960).

Preparation of ^{14}C-Labeled Enzyme. The glutamine reactive site of FGAR amidotransferase was labeled with [^{14}C]iodoacetate by the method reported in the preceding paper (Ohnoki et al., 1977). Each preparation of enzyme (30–40 mg) was individually labeled and the reaction mixture dialyzed against 100 vol of 0.1 M potassium phosphate (pH 6.6) at 4 °C for 8 h and then against water for 2 days. The lyophilized material was stored in a freezer. The specific radioactivity of the [^{14}C]iodoacetate used to label the nine samples

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[1] Abbreviations used are: CM, carboxymethyl; Pth, phenylthiohydantoin; Dns, dansyl (5-dimethylamino-1-naphthalenesulfonate); FGAR, formylglycinamide ribonucleotide; Tos-PheCH$_2$Cl, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

varied somewhat, but the average specific activity weighted for amount and activity of enzyme was 10 000 cpm/nmol.

Reduction and Carboxymethylation of Labeled Enzyme. The method of Crestfield et al. (1963) was employed with slight modification (procedure 1). The protein (40 mg) was dissolved in 7.5 mL of 0.6 M Tris buffer containing 9 M urea and 0.2% EDTA (pH 8.5) and incubated at 40 °C for 4 h under a nitrogen atmosphere in a sealed bottle. To the solution 315 μL of 2-mercaptoethanol (4482 μmol) was added and incubation was continued at 40 °C for 8 h. After reduction of the possible disulfide bonds, a freshly prepared solution of iodoacetate (837 mg in 4.5 mL of 1 N NaOH) was gradually added to the reaction mixture with the maintenance of pH at 8.3 by the addition of 1 N NaOH. The solution was stirred for 25 min at room temperature, transferred into a cellophane tube, dialyzed against running tap water for 2 days and then with deionized water for 2 days, and lyophilized.

As was later found, the above method did not result in the complete reduction and carboxymethylation of the labeled enzyme preparation representing the collection of the nine individual samples. In order to confirm that the labeled enzyme could be completely reacted an alternate procedure was used (procedure 2). A small sample (1.3 mg) was dissolved in 0.85 M Tris buffer (pH 8.6), containing 6 M guanidine·HCl, and 0.17% EDTA and 2 M 2-mercaptoethanol, and incubated at 40 °C for 18 h under nitrogen gas. Then 2.5 mmol of iodoacetic acid (463 mg) dissolved in 2.5 mL of 1 N NaOH was added gradually to the above. The pH was kept at 8.3 (Ferdinand et al., 1965).

Oxidization with Performic Acid. Samples of enzyme, labeled but otherwise untreated, as well as the isolated peptides were oxidized with performic acid by the method of Hirs (1956). Amino acid analysis of the hydrolyzed products of the oxidized enzyme sample showed that the oxidation of cystine or cysteinyl residues to cysteic acid had been complete.

Hydrolysis of Reduced, Carboxymethylated Enzyme by Trypsin. Reduced and carboxymethylated enzyme (55 mg) was dissolved in 0.1 M Tris–8 M urea buffer (pH 8.0). The clear protein solution was diluted with 3 vol of the Tris buffer containing 0.1% thiodiglycol in order to reduce the urea concentration to 2 M. The final protein concentration was 1.4%. The protein precipitated as an emulsion in 2 M urea solution. Forty microliters of 1% Tos-PheCH_2Cl-trypsin (0.4 mg) was added; the suspension cleared after 1 h. After 4 h at 25 °C another 0.4 mg of trypsin was added and hydrolysis was allowed to proceed at 25 °C for 17 to 22 h.

The digestion mixture was passed through a column (1.9 × 116 cm) of Sephadex G-75, which had been equilibrated with 0.1 M NH_4HCO_3 containing 0.1% thiodiglycol (cf. Figure 1). Effluent was collected at a rate of 60 mL/h in 6-mL fractions. The fractionation was monitored by determination of absorption at 280 nm and by measurement of radioactivity (50-μL samples). Radioactivity emerged in a single peak (Figure 1). The fractions containing radioactivity were combined and repeatedly lyophilized to remove NH_4HCO_3.

Hydrolysis of Radioactive Material Recovered from Sephadex G-75 by Protease. The radioactive material recovered from Sephadex G-75 (5×10^6 cpm) was dissolved in 8.3 mL of 0.1 M Tris buffer (pH 8.1). Protease (3.12 mg of type VI from *Streptomyces griseus*) was added and hydrolysis was allowed to proceed at 37 °C for 7 h and 40 min.

The digestion mixture was passed through a column (2.2 × 212 cm) of Sephadex G-25, which had been equilibrated with 0.1 M NH_4HCO_3 containing 0.1% thiodiglycol (Figure 2). Effluent was collected in 6-mL fractions. The fractionation was carried out in the same way as in the trypsin digestion. In a preliminary experiment the appearance of new radioactive peptides during protease digestion was monitored by thin-layer chromatography with detection of radioactive spots by x-ray film. The simplest pattern was obtained after a 7-h incubation.

Isolation of Labeled Peptide. The purification of labeled peptide was performed by column chromatography with AG 1-X2, AG 50W-X2, and DEAE-cellulose. The buffers employed in the chromatographic separation were prepared as follows (the quantities given are for 1 L of solution and buffers I–V contained 0.2% thiodiglycol): buffer I, pyridine–*N*-ethylmorpholine–0.05 M acetate (pH 7.5) (40 mL of pyridine, 10 mL of *N*-ethylmorpholine, and 50 mL of 1 N acetic acid); buffer II, pyridine–0.5 M acetate (pH 5.0) (2.87 mL of acetic acid and 40 mL of pyridine); buffer III, pyridine–2 M acetate (pH 3.95) (172 mL of acetic acid and 40 mL of pyridine); buffer IV, 0.005 M pyridine–formate (pH 2.4) (15 mL of 99% formic acid and 0.4 mL of pyridine); buffer V, 0.1 M pyridine–acetate (pH 4.1) (30 mL of acetic acid and 8.1 mL of pyridine); buffer VI, pyridine–0.05 M acetate (pH 6.3) (50 mL of 1 N acetic acid and 40 mL of pyridine); buffer VII, same as buffer II but without thiodiglycol. In the last step of purification (DEAE-cellulose), pure water described under Materials was used to make up buffers.

Detection of peptides was performed by *o*-phthalaldehyde (Benson and Hare, 1975) without detergent. *o*-Phthalaldehyde dissolved in ethanol was treated with charcoal, and the colorless filtrate was used for preparation of reagents. The sample (0.2 mL) was mixed with 0.8 mL of 0.4 M borate buffer (pH 9.7) and then 1 mL of 0.08% *o*-phthalaldehyde reagent in 0.4 M borate buffer (pH 9.7) containing 0.2% 2-mercaptoethanol was added. The fluorescence was measured by a Farrand spectrofluorometer MK-I. Wavelengths of excitation and emission are 340 and 455 nm, respectively.

Amino Acid Analysis. Amino acids were determined by ion exchange chromatography with automatic recording equipment. For amino acid analyses of peptides eluted from columns, 5 nmol of sample was dried in a conical tube (15 × 100 mm), and 400 μL of constant boiling hydrocholoric acid was added. The test tube was drawn to a capillary near the neck and then immersed in a solid CO_2–acetone bath. The solution was thawed and deaerated under a pressure of 35 μmHg for 20 min and sealed. Hydrolysis was carried out for 24 h in a boiling toluene bath (110 °C). The hydrolysate was analyzed by a Durrum amino acid analyzer D500.

Sequence Analysis by the Dansyl–Edman Method. The combined dansyl–Edman procedure (Bruton and Hartley, 1970; Hartley, 1970; Gray and Smith, 1970) was used with modification. Approximately 2–4 nmol of peptide was dissolved in 25 μL of water in a 1-mL conical tube; 25 μL of 5% phenyl isothiocyanate (v/v) in pyridine was added and the tube flushed with nitrogen. The tube was sealed with parafilm and incubated at 45 °C for 1 h. The solution was dried in vacuo in a heated desiccator for 30 min at 70 °C. Fifty microliters of trifluoroacetic acid was added and the sample was left uncovered in the heated desiccator (70 °C). After 20 min the vacuum was applied and the sample was dried for 20 min. The residue was dissolved in 25 μL of water and extracted four times with 150 μL of water-saturated ethyl acetate. After the fourth extraction the aqueous phase was dried in vacuo and dissolved in 20–40 μL of water. A sample (approximately 0.2 nmol) was removed for dansylation and the remainder was treated with phenyl isothiocyanate exactly as before. In this way the sequence was determined from the N terminus. In

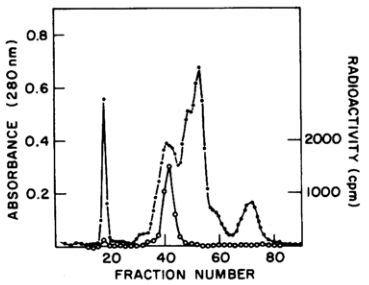


FIGURE 1: Fractionation of a tryptic hydrolysate of reduced and carboxymethylated ^{14}C-labeled FGAR amidotransferase on a column of Sephadex G-75 (1.9 × 116 cm) equilibrated with 0.1 M NH_4HCO_3 containing 0.1% thiodiglycol: rate, 60 mL/h; 6-mL effluent fractions collected; 88% of the radioactivity added to the column was recovered in a main peak; (●) absorbance at 280 nm; (○) radioactivity.

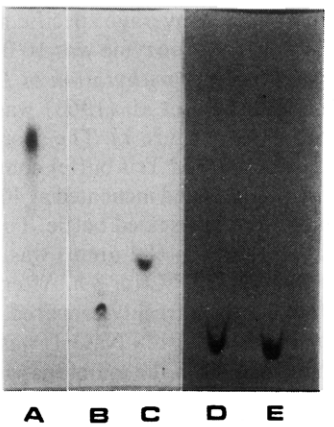


FIGURE 2: Thin-layer chromatography of radioactive peptides. The sample (500–1000 cpm) was spotted on a cellulose plate and run in the solvent of isoamyl alcohol–pyridine–water (35:35:30, v/v). Radioactive spots were detected by x-ray film: (A) radioactive fraction recovered from Sephadex G-75 of tryptic digest (Figure 1); (B) T-Pro I; (C) T-Pro-II; (D) T-Pro-I-2-a; (E) T-Pro-I-2-b. The R_f values for samples A, B, C, D, and E are 0.62, 0.19, 0.30, 0.11, and 0.09, respectively.

some cases the method for rapid sequence analysis described by Gray and Smith (1970) was used in which samples of 0.2 nmol each were taken in an appropriate number of tubes (2 × 50 mm) and degraded until the required number of cycles had been completed for each sample without extraction; then all extractions were performed together at one time rather than consecutively.

For dansylation the sample of peptide was transferred to a small tube (2 × 50 mm) and dried in vacuo over phosphorus pentoxide. Two microliters of 0.2 M sodium bicarbonate was added and the solution was dried again. Two microliters each of water and dansyl chloride solution (2.5 mg/mL of acetone) were added and the solution was incubated at 37 °C for 1 h. The sample was again dried and 20 μL of constant boiling hydrochloric acid was added. The tube was deaerated under a vacuum of 30 μmHg for 5 min and sealed, and the dansyl peptide was hydrolyzed at 105 °C for 17 h. The dansyl amino acid was determined by two-dimensional chromatography on polyamide layer sheets (7.5 × 7.5 cm) as described by Hartley (1970). The dried hydrolysate was dissolved in 5 μL of 50% pyridine, and 1 μL (about 40 pmol) was spotted carefully in as small an area as possible.

Evacuation of the hydrolysis tube was absolutely necessary in order to identify dansyl carboxymethylcysteine, although most investigators so far have performed hydrolysis without evacuation in their reports on the use of the dansyl method. Otherwise, the dansyl derivative of carboxymethylcysteine was nearly completely destroyed during hydrolysis and consequently could not be detected, especially when the quantity of sample was very small.

Results

Isolation of ^{14}C-Labeled Peptides

Degradation of Labeled Enzyme by Trypsin. Labeled enzyme, which had been reduced and carboxymethylated, was digested by trypsin and the hydrolysate passed through a column of Sephadex G-75. A typical elution pattern is shown in Figure 1. Radioactivity emerged in a single peak except for a small amount of undigested material (2.3%). The total recovery of radioactivity was 90%. The ratio of elution volume of the main peak to void volume (v_e/v_0) was 2.14. When the tryptic digest was passed through a column of Sephadex G-25, the radioactivity appeared in nearly the same position as blue dextran (v_e/v_0 = 1.07). To estimate the molecular weight of the radioactive peptide recovered from Sephadex G-75, the radioactive fraction was passed through a column of Sephadex G-50 (1.0 × 115 cm), which had been equilibrated with 0.1 M NH_4HCO_3 containing 0.1% thiodiglycol. The ratio, v_e/v_0, of the peptide was 1.73, whereas the ratios of cytochrome *c* and bacitracin were 1.44 and 2.27, respectively, under the same conditions. Therefore, the apparent molecular weight of radioactive peptide obtained by tryptic digestion was roughly estimated to be about 5500.

Thin-layer chromatography of the peptide revealed a single radioactive spot (Figure 2, channel A). The material did not migrate during electrophoresis in a solution of pyridine–acetic acid–water (100:4:900, v/v, pH 6.4) probably because of its large molecular weight.

An aliquot of the radioactive material (1.6 mg) recovered from Sephadex G-75 was again reduced and carboxymethylated under the same conditions described in procedure 1 under Methods and passed through a column of Sephadex G-75 (1.0 × 112 cm). The radioactivity emerged in quite the same position as the untreated sample. The behavior of the twice reduced and carboxymethylated sample in thin-layer chromatography and electrophoresis was also quite the same as that of the original radioactive peptide isolated from the tryptic digest. Thus, further treatment by this procedure did not result in the production of smaller fragments through reduction of putative interchain disulfide bonds.

An aliquot of the radioactive tryptic peptide isolated from the Sephadex G-75 column was also subjected to hydrolysis in 0.5 mL of 6 N HCl at 110 °C for 24 h. The sample was dried in vacuo, dissolved in a small amount of water, and spotted on a thin-layer plate (cellulose MN 300, Brinkmann Instrument Co.) for chromatography with isoamyl alcohol–99% formic acid–water (20:1:5) or for electrophoresis in a solvent consisting of pyridine–acetic acid–water (1:10:289, pH 3.5). Radioautograms were developed from both plates. A single radioactive product corresponding to carboxymethylcysteine was identified in both cases. The procedure for labeling the enzyme at the glutamine site was thus specific for a cysteine residue.

Further Degradation of Radioactive Tryptic Peptide with Protease. In preliminary experiments we encountered difficulty in the purification of the tryptic peptide, since its solubility was low probably because of its large molecular weight. Therefore, the radioactive fraction from the tryptic digest was again hydrolyzed by protease as described under Methods to

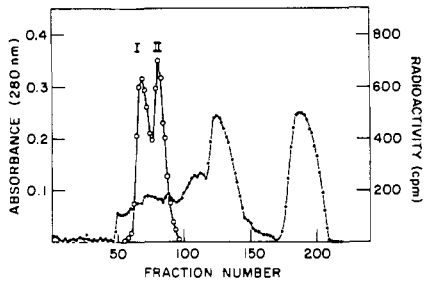


FIGURE 3: Fractionation of a protease digest of the radioactive material recovered from Sephadex G-75 (Figure 1) on a column of Sephadex G-25 (2.2 × 212 cm). Six-milliliter effluent fractions were collected. Twenty-microliter samples of every other tube were counted: (●) absorbance at 280 nm; (○) radioactivity.

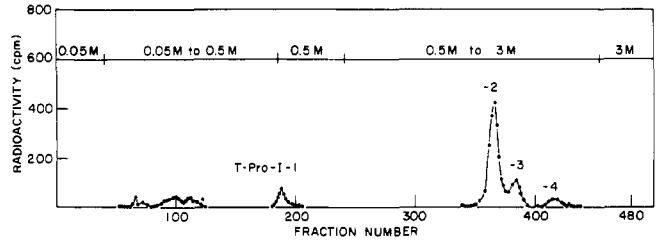


FIGURE 4: Chromatography of the radioactive fraction, T-Pro-I, on a column of Dowex AG 1-X2. The column (1.0 × 115 cm) was equilibrated with a pyridine-*N*-ethylmorpholine-acetate buffer (pH 7.5 (buffer I). The rate of elution was 30–50 mL/h; 6-mL effluent fractions were collected. Twenty microliters of effluent of every other tube was counted. The concentration of acetate ion of the eluting buffer is shown at the top of chromatogram.

yield smaller peptides. Figure 3 shows one example of gel filtration of the protease digest. Two major radioactive peaks were obtained with a total recovery of 93% of the radioactivity applied. The ratio of elution volume to void volume is 1.24 for peak I and 1.45 for peak II. The ratio of the two fractions, T-Pro-I and T-Pro-II, was nearly 50:50 on the basis of radioactivity. The patterns obtained by thin-layer chromatography of T-Pro-I and T-Pro-II are shown in Figure 2, channels B and C, respectively.

Purification of Labeled Peptides from T-Pro-I. The lyophilized fraction, T-Pro-I (2.91×10^6 cpm), was dissolved in 6 mL of starting buffer or buffer I and applied on a column of AG 1-X2 (200–400 mesh, 1.0 × 115 cm) equilibrated with this buffer. After the column was washed with 120 mL of the same buffer, linear gradient elution was begun by allowing buffer II (500 mL) to flow into a mixing chamber of 500 mL of the starting buffer. Six-milliliter fractions were collected with a flow rate of 30 to 50 mL per h. After the gradient elution was completed, the column was washed with 340 mL of buffer II (pH 5.1), and a second gradient was applied in the same way using this buffer (600 mL) and 600 mL of buffer III.

Radioactive peptides were located by measurement of the radioactivity of 20-μL samples from alternate tubes. One main radioactive peak in addition to some other minor ones were found as shown in Figure 4. The yield of the main peak (peak 2) was 44% in terms of the radioactivity added to the column; the total recovery from the column was 77%. The migration patterns of the materials in peaks 2, 3, and 4 in thin-layer chromatography were very similar; each consisted of one main radioactive spot and some minor ones. This result suggested that the components of peaks 3 and 4 were derived from the materials of peak 2 by slight modification.

For further purification, the main radioactive fraction, T-Pro-I-2 (9.5×10^5 cpm) recovered from AG 1-X2, was dissolved in 1.5% formic acid (pH 2.2) and applied on a column of AG 50W-X2 (200–400 mesh, 1.0 × 113 cm) equilibrated with buffer IV. A linear gradient was developed from 300 mL of buffer IV and 300 mL of buffer V; 5.5-mL fractions were collected. When the column was washed with buffer V after the gradient elution, the radioactivity emerged in a very sharp single peak in the region of 594 to 609 mL of the effluent with recovery of 70%. Thin-layer chromatography of the radioactive fraction showed that it consisted of one main radioactive spot and one minor, and still contained nonradioactive but ninhydrin-positive spots. Electrophoresis at pH 6.4 indicated that both of these radioactive peptides are very acidic with the major one less acidic than the minor one.

Finally, these two radioactive peptides were purified by column chromatography with DEAE-cellulose. The radioactive material (5.5×10^5 cpm) recovered from AG50W-X2 was dissolved in 1 mL of buffer VI and applied on a column of DEAE-cellulose (0.3 × 100 cm), which had been equilibrated with this buffer. After a wash with 18 mL of the same buffer, a linear gradient was performed by use of 35 mL of buffer VI and 35 mL of buffer VII. The effluent was collected at a flow rate of 6.4 mL/h in 0.45-mL fractions. Two radioactive peptides, T-Pro-I-2-a and -2-b, were obtained with a recovery of 42 and 21%, respectively, as shown in Figure 5. Thin-layer chromatography confirmed that each of these fractions consisted of a single radioactive spot, which coincided with a single ninhydrin spot (Figure 2, channels D and E, respectively). N-Terminal analyses by the dansylation method also showed that both of these peptides have only glycine as N-terminal residues. The purification procedure of the labeled peptides is summarized in Chart I.

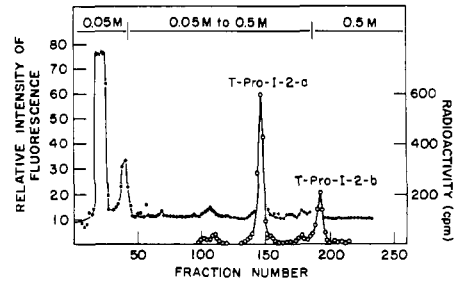


FIGURE 5: Chromatography on DEAE-cellulose of the radioactive material from AG 50W-X2. The column (0.3 × 100 cm) was equilibrated with a pyridine-acetic acid buffer (pH 6.3) (buffer VI). For gradient elution, see the text. Fractions of 0.45 mL were collected. Radioactivity (10 μL, ○) and relative intensity of fluorescence by reaction with *o*-phthalaldehyde (0.2 mL, ●) are shown. The fluorescence of the fractions in the region where main radioactive peaks emerged was not measured to save samples. The concentration of acetate ion of the eluting buffer is shown at the top of the chromatogram.

Structure of ^{14}C-Labeled Peptides

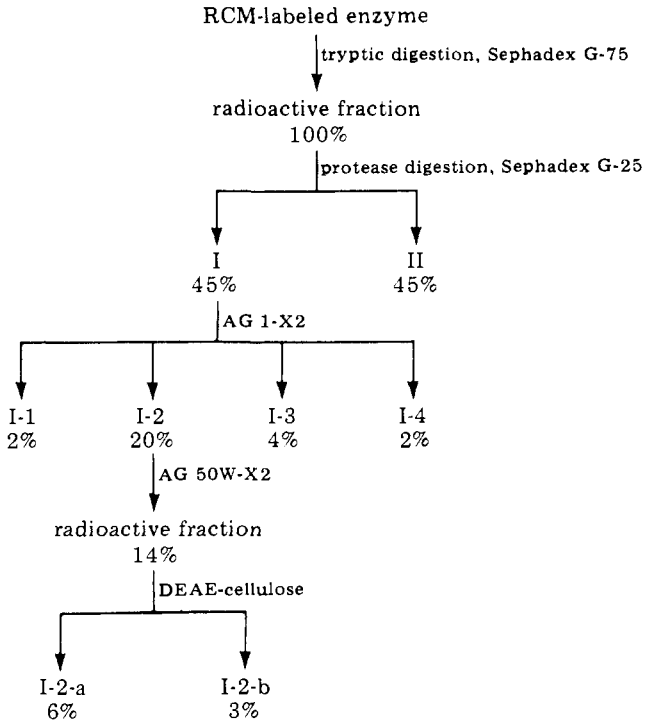
Amino Acid Composition. The amino acid compositions of the peptides T-Pro-I-2-a and T-Pro-I-2-b are shown in Table I. Only the six amino acids listed were found in either case. No other amino acid was detected except cysteic acid for which the values were 0.36 mol/3 mol of valine in T-Pro-I-2-a and 0.22 mol/2 mol of valine in T-Pro-I-2-b. We have estimated the molecular weight of each peptide on the basis of the radioactivity of one labeled carboxymethylcysteine (see following section), by equating all amino acids relative to the composition of valine, and by filtration of the peptides on Bio-Gel P-4. The average specific radioactivity of the iodoacetate used to label the enzyme preparations was 10 000 cpm per nmol. Either

TABLE I: Amino Acid Composition of the Purified Peptides of Fraction I.[a]

Amino Acid	T-Pro-I-2-a nmol per Sample[b] 1	Residues per [^{14}C]CMCys 2	Residues Normalized to Val = 3 3		T-Pro-I-2-b nmol per Sample[c] 4	Residues per [^{14}C]CMCys 5	Residues Normalized to Val = 2 6	
Carboxymethylcysteine	11.00	3.04	2.70	(3)	6.67	1.82	1.51	(2)
Aspartic acid	15.40	4.25	3.79	(4)	9.37	2.56	2.12	(2)
Glutamic acid	13.39	3.70	3.29	(3)	10.28	2.81	2.33	(2)
Glycine	23.19	6.41	5.70	(6)	17.56	4.80	3.98	(4)
Valine	12.21	3.37	3.00	(3)	8.82	2.41	2.00	(2)
Half-cystine	9.21	2.54	2.26	(2)	8.11	2.22	1.84	(2)
Total residue				(21)				(14)

[a] The molar ratios calculated on a basis of radioactivity of valine content are listed. Nearest integral numbers are shown in parentheses. The value of half-cystine is not corrected for loss in hydrolysis. [b] Based on radioactivity, sample contained 3.62 nmol of peptide. [c] Based on radioactivity, sample contained 3.66 nmol of peptide.

CHART I: Flow Sheet for the Purification of the Peptide Involved in Glutamine Binding from Reduced and Carboxymethylated (RCM) Labeled Enzyme by Tryptic and Protease Digestion.[a]



[a] Percentages refer to the starting material for Sephadex G-25 chromatography.

sample of peptide used for amino acid analysis contained approximately 36 000 cpm of ^{14}C. On the basis of one labeled carboxymethylcysteine per peptide we find approximately 3 mol of total carboxymethylcysteine for peptide 2-a and two for peptide 2-b. The compositions of the other amino acids are then calculated as shown in columns 2 and 5, Table I. Peptide 2-a consisting of 21 residues has a molecular weight of 2193 and peptide 2-b with approximately 14 residues is assigned a molecular weight of 1459. A similar composition is arrived at by assigning value integral numbers of 3 and 2, respectively (columns 3 and 6, Table I).

Filtration on Bio-Gel P-4. In view of the unusual composition of amino acids in either peptide, an additional method of estimating molecular weight was desirable, i.e. by filtration on a column (0.6 × 115 cm) of Bio-Gel P-4 equilibrated with 0.05 M $NaHCO_3$. The location of the peptide was determined by measurement of absorption at 280 nm or by the *o*-phthaldehyde method (Benson and Hare, 1975). A standard curve for partition coefficient vs. molecular weight was made by use of several peptides, the molecular weights of which are known, i.e. bacitracin, human angiotensin I, oxidized gluthathione, oxytocin, and insulin oxidized by performic acid. Under the same conditions the partition coefficients of the unknown peptides were 0.29 for peptide T-Pro-I-2-a and 0.23 for peptide T-Pro-I-2-b. Therefore, the molecular weights of these peptides were estimated from the standard curve to be 2150 for 2-a and 2700 for 2-b. The molecular weight of peptide 2-a as determined by gel filtration agrees well with that obtained by radioactivity. On the other hand, the molecular weight of peptide 2-b as determined by radioactivity was one-half that as determined by gel filtration. Since the molecular weight of peptide 2-b as determined by gel filtration and radioactivity differed by a factor of 2, we have considered the possibility of dimerization of this peptide in the column either by ionic or hydrophobic forces. This possibility does not seem likely, however, since one would expect both peptides 2-a and 2-b to exhibit the same properties by virtue of their nearly identical compositions.

Disulfide Bridges. One other possibility for dimerization is the presence of interchain disulfide bridges possibly formed during chromatography. Cystine is present in both peptides because of the incomplete reduction and carboxymethylation of the labeled enzyme by procedure 1 (Methods). This problem could have been avoided had we used, as later shown, more forceful conditions (procedure 2).

However, aliquots of peptides 2-a and 2-b were themselves subjected to further reduction and carboxymethylation by procedure 1 and then applied to columns of Bio-Gel P-2. Since both peptides were eluted in the void volume of this column, their molecular weights were estimated to be greater than 1800. As an independent check that all disulfide bonds had been rendered in samples used for gel filtration, we then oxidized aliquots of both peptides with performic acid by the method of Hirs (1956) and subjected the products to filtration on Bio-Gel P-4. The untreated and oxidized samples eluted with approximately the same partition coefficients, 0.29 and 0.28, respectively, for peptide 2-a and 0.23 and 0.21 for peptide

2-b. The results indicate that the disulfide bonds formed from cysteine residues other than those at positions 3 and 6 are intrachain in both peptides and cannot account for dimerization by interchain linkages of the same peptide.

The possibility that more than one cysteine of peptide 2-b is labeled by [^{14}C]iodoacetate requires further exploration when greater quantities of peptide become available. Although this explanation would account for the difference in molecular weight as determined by the two methods, our preliminary results would not seem to support this hypothesis. We are presently unable to explain the agreement in the estimation of the molecular weight of peptide 2-a by the two methods and the divergence in the corresponding estimates for peptide 2-b.

Sequence of T-Pro-I-2-a. The sequence of amino acid residues in the N-terminal region of the peptides was determined by combining the Edman degradation with the dansyl method for N-terminal analysis. The degradation results of peptide T-Pro-I-2-a are given in detail in the paragraphs to follow:

N-terminal analysis: Gly

Edman degradation:

Gly-Val-Cys([^{14}C]CM)-Asp-Asx-Cys(CM)-Glx . . .

In the first step, only valine was detected except for a trace amount of glycine, which was carried over from the N terminus. In the second and third steps, distinct carboxymethylcysteine and aspartic acid residues were identified, respectively. In the fourth step, aspartic acid and a trace amount of glycine were detected. Dominant spots found in the third and fourth steps were also confirmed as Dns-aspartic acid by mixing the sample with standard Dns-aspartic acid. In the fifth step, carboxymethylcysteine and aspartic acid, which was less but not negligible, were detected. The latter spot may be due to the summation of aspartic acid carried over from the third and fourth steps. In the sixth step, glutamic acid and a small amount of aspartic acid were detected. In the seventh step, equal amounts of glutamic acid and aspartic acid were found. Therefore, determination of the eighth amino acid could not be made.

In a separate analysis the mixture of ethyl acetate extracts (Pth-CMCys and Pth-Asx) after the third and fourth degradations was taken to dryness, spotted on a polyamide plate (5 × 5 cm), and subjected to two-dimensional chromatography according to the method of Summers et al. (1973). Phenylthiohydantoin derivatives of carboxymethylcysteine and aspartic acid were detected on a chromatogram. Therefore, the fourth amino acid was aspartic acid rather than asparagine.

Location of Radioactivity. From a count of the ethyl acetate extracts after each degradation from the first to ninth steps, 67% of the radioactivity used for degradation was found in the extract of the third step and only 2–5% in each of the others. In a manual procedure for the Edman degradation, the yield of phenylthiohydantoin derivatives of the amino acid produced in the over-all reaction of one cycle may be less than 100%, as suggested by the existence of trace amounts of other spots that are carried over from previous steps. Therefore, after several degradation steps have occurred, one would not expect to recover 100% of radioactivity in a single fraction, even though only one particular residue in the whole sequence was radioactive. For example, if the overall yield in each cycle is assumed to be 90%, the theoretical yield of the phenylthiohydantoin (Pth) derivative of the amino acid after the third degradation is 73%. Taking this consideration into account, we have concluded that only the third amino acid, i.e. carboxymethylcysteine, is labeled in the entire sequence, and that the radioactivity does not distribute among the carboxymethylcysteine in the third position and other carboxymethylcysteines in the ratio of 67 to 33.

C-Terminal Analysis with Carboxypeptidase. The peptide (0.1 nmol) was incubated at 25 °C with carboxypeptidase A (0.9 μg) in a total volume of 2 μL and the liberated amino acid was identified by the dansyl method. Glutamine was faintly detected as its derivative after 30 min, and a definite glutamine residue, but no other amino acid, was detected after 20 h. There are two possible assignments for the location of glutamine in the carboxyl-terminal position; the most probable one is . . . Gln; however, another, . . . Gln-Cys(S), cannot be excluded, since a half-cystine present in the terminal position would not be released as a free amino acid if linked to another half-cystine by an S–S bond.

Sequence of T-Pro-I-2-b. Application of the dansyl–Edman method to this peptide revealed that the sequence of the first five amino acid residues is Gly-Val-Cys([^{14}C]CM)-Asx-Asx . . . . Again, the radioactivity was found in the ethyl acetate extract of the third step. The sequence of the N-terminal part of this peptide is therefore quite the same as that of T-Pro-I-2-a. Undoubtedly, the two peptides were derived from the same part of the original protein since they have the same sequence for the five amino acids that have been examined.

The following tentative structure is proposed for T-Pro-I-2-a, although we should note that this assignment is still ambiguous for the C-terminal residue. Because of our uncertainty about the size of T-Pro-I-2-b we prefer not to commit ourselves about its possible structure other than to point out that regardless of its actual length it shares with peptide 2-a the unusual composition of five amino acids.

Gly-Val-Cys([^{14}C]CM)-Asp-Asx-Cys(CM)-Glx
× (Asx_2,Glx,Cys(CM),Gly_5, Cys,Val_2,Cys)(Gln)
S——S

Discussion

In a preceding report (Ohnoki et al., 1977) we have developed a method for specific labeling of the glutamine binding site of FGAR amidotransferase with [^{14}C]iodoacetate. This method has several advantages compared to those in which the active site was labeled with glutamine analogues such as azaserine or 6-diazo-5-oxonorleucine. In the latter instances rearrangements occur that complicate the isolation of specific peptide products from proteolytic or acid hydrolysates. However, with none of the procedures is the difficulty of working with microquantities of a sulfur-containing product overcome. Even so. we have been able to isolate in pure form radioactive peptides from labeled chicken liver enzyme and to determine their composition and partial sequences.

The radioactive tryptic peptide isolated in one of the first steps of the proteolytic digestion had an approximate molecular weight of 5500 and was, therefore, too large for direct structural analysis. This unusually large fragment does not result from incompleteness of digestion nor from remaining interdisulfide bridges, since any attempts to reduce the size by repetition of carboxymethylation of the radioactive tryptic peptide or by its oxidation with performic acid were unsuccessful. The analysis of the tryptic peptide for radioactive amino acid derivatives revealed that only carboxymethylcysteine could be detected among the hydrolysis products of the labeled enzyme.

We, therefore, turned to use of a second procedure to fragment further this tryptic peptide. We did not succeed in obtaining discrete smaller products in satisfactory yield by chemical degradation with cyanogen bromide or dilute acid hydrolysis or by enzymatic digestion with chymotrypsin, pepsin, Nargarse, or α-protease. However, digestion of the tryptic peptide with a protease from *S. griseus* did yield two smaller radioactive products (fractions I and II) in good yield and in approximately equal amounts. Fraction II has not yet been sufficiently purified to permit analysis of the composition and sequence of the radioactive peptide. However, fraction I has been resolved into two closely related peptides. The more abundant of the two peptides, T-Pro-I-2-a, consists of approximately 21 amino acids as determined by two independent methods. The size of the second peptide, T-Pro-I-2-b, has not been conclusively established. The identification of both peptides with the true binding site of glutamine has been obtained by comparison of the initial amino-terminal residues with the sequence of one of the peptides isolated after proteolytic digestion of a bacterial FGAR amidotransferase (Dawid et al., 1963; French et al., 1963) labeled with [^{14}C]azaserine. In the latter case a radioactive product derived from the tripeptide, glycylvalylcysteine, was isolated. Both peptides of fraction I in the current experiments begin with this sequence.

The amino acid analysis of the two peptides of fraction I revealed a similar and surprisingly simple composition. Both peptides yield only six amino acid residues upon acid hydrolysis, namely carboxymethylcysteine, cystine, glutamic acid, aspartic acid, valine, and glycine. The presence of cystine in our amino acid hydrolysates resulted from the incomplete reduction and carboxymethylation of the enzyme by the procedure used for the preparative samples. Complete reaction could be obtained, however, by use of more forceful conditions. The disulfide bridges are intra- rather than interchain.

We have reported here a partial sequence of the peptides. The sequence of peptide a is Gly-Val-Cys-([^{14}C]CM)-Asp-Asx-Cys(CM)-Glx . . . and that of peptide b is Gly-Val-Cys-([^{14}C]CM)-Asx-Asx . . . . Undoubtedly both peptides are fragments from the same section of the enzyme, i.e. the glutamine binding site.

One purpose in undertaking the present sequence studies was the hope of elucidating some features of the enzymatic transfer of the amide group of glutamine to the nucleotide acceptor and the specific role of amino acid residues at the active site in this reaction. Our perception of a possible mechanism has been stimulated by a recent publication by Dixon et al. (1976) in which, on the basis of their finding of Ni ion in urease (Dixon et al., 1975), they propose the involvement of metal ions in several reactions of nitrogen metabolism, including amide transfer. The participation of a metal ion in amide transfer from glutamine to FGAR would have several attractive features, principally in maintaining the amide group in a highly nucleophilic state as a metal ligand formed from native enzyme and glutamine. The aspartic and cysteine residues immediately adjacent to the reactive sulfhydryl group could ligate the metal ion.

We, therefore, undertook a search for metal cofactors in FGAR amidotransferase. The enzyme was examined for Zn^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, and Cu^{2+} by microwave emission spectroscopy (Kawaguchi and Auld, 1975) with the generous assistance of Dr. David Auld and Dr. Bert Vallee. None of these metals, however, was found in significant quantities above the background levels. Analysis of other metals is underway.

A second goal of examining the amino acid composition of an amidotransferase at the glutamine active site has been the documentation and comparison of several glutamine utilizing enzymes. It would be of interest to determine whether they have had a common or divergent evolutionary origin with respect to the composition and sequence of amino acids at the active site for glutamine. Since many of the amidotransferases utilize ammonia in place of glutamine but at lower rates and exhibit glutaminase activity in the absence of cosubstrates, we would propose as a working hypothesis that the primitive enzymes were originally designed for reaction with ammonia. However, as requirements for complicated and faster reactions emerged, particularly at physiological pH, a finer tuning of the structures took place in which glutamine became the preferred substrate.

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