THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 251, No. 11, Issue of June 10, pp. 3254–3260, 1976
Printed in U.S.A.

Tryptophanyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli*

CHARACTER OF REQUIRED THIOL GROUP AND STRUCTURE OF THIOL PEPTIDES*

(Received for publication, August 6, 1975)

GARY V. KUEHL,‡ MING-LIANG LEE, AND KARL H. MUENCH§

From the Division of Genetic Medicine, Departments of Medicine and Biochemistry, University of Miami School of Medicine, Miami, Florida 33152

Native tryptophanyl-tRNA synthetase purified from *Escherichia coli* B has on each identical subunit a single thiol group which rapidly forms a mixed disulfide with a thionitrobenzoate moiety of 5,5′-dithiobis(2-nitrobenzoic acid). The reaction and the concomitant inactivation of the enzyme are both reversible by reductive removal of the thionitrobenzoate with dithiothreitol. Iodoacetamide and *N*-ethylmaleimide also react with the thiol group required for enzyme activity, but iodoacetic acid inactivates the enzyme through another mechanism. Three or 4 half-cystine residues/subunit were detected by amino acid analysis and by titration of the denatured enzyme with 5,5′-dithiobis(2-nitrobenzoic acid); no disulfide bonds were detected by borohydride reduction. Cleavage of the subunit (molecular weight 37,000) with 2-nitro-5-thiocyanobenzoic acid gave fragments of molecular weights 32,000, 27,000, and 9,500. Five carboxymethylated peptides were isolated from the trypsin products of the denatured enzyme after treatment with iodo[^{14}C]acetate. Three of these peptides represented unique sequences surrounding thiol groups in the enzyme. One cysteine-containing nonapeptide has a heptapeptide sequence homologous to a heptapeptide sequence in a cysteine-containing decapeptide from the tryptophanyl-tRNA synthetase of human placenta. The nonapeptide appears to bear the thiol group required for enzyme activity.

Although their role in catalysis has not been demonstrated, thiol groups are generally required for the function of the aminoacyl-tRNA synthetases (1). The tryptophanyl-tRNA synthetase of *Escherichia coli* is no exception and is of particular interest because the integrity of a single thiol group in the native enzyme is required for activity, and this thiol group is protected from 5,5′-dithiobis(2-nitrobenzoate) by the substrates (2). Here we report studies on the thiol groups of this enzyme and on the amino acid sequences of its thiol peptides. The sequences of some thiol peptides from *E. coli* isoleucyl-tRNA synthetase (3, 4) and methionyl-tRNA synthetase[1] and from human placental tryptophanyl-tRNA synthetase (5) are known. The required thiol group in *E. coli* tryptophanyl-tRNA synthetase may reside in a peptide homologous to one in the cognate enzyme from human placenta (6).

* This work was supported by United States Public Health Service, National Institutes of Health, Grants 5-P01-AM-09001, CA-14395, and G-K4-GM-13,399 and by National Science Foundation Grant GB042701.

‡ Predoctoral Fellow, partly supported by United States Public Health Service, National Institutes of Health, Grant GM-02011. The work represents partial fulfillment of the requirements for the degree of Doctor of Philosophy.

§ Leukemia Society of America Scholar and recipient of a Research Career Development Award.

[1] C. J. Bruton (1973) European Molecular Biology Organization Workshop on tRNA Structure and Function, Goteborg.

EXPERIMENTAL PROCEDURE

Materials—Escherichia coli B grown on minimal (Kornberg) medium and harvested at ¾ log phase was purchased from Grain Processing Corp. (Muscatine, Iowa). Transfer RNA from *E. coli* B, guanidine hydrochloride, and ribonuclease-free sucrose were from Schwarz/Mann Bioresearch. One A_{260} unit of tRNA has an A_{260} of 1.0 in a 1.0-cm optical path when dissolved in 1.0 ml of 5 mM KH_2PO_4/5 mM K_2HPO_4 (pH 6.9). Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone was from Worthington Biochemical Corp. The trypsin was dissolved (2.0 mg/ml) in 1 mM HCl and kept at 0° before use. Its concentration was determined spectrophotometrically. Iodoacetic acid from Aldrich Chemical Co. was dissolved in a minimal volume of warm chloroform and crystallized by addition of petroleum ether. Iodoacetamide recrystallized three times was a gift from Dr. P. Whitney. L-[3-^{14}C]Tryptophan, sodium [^{32}P]pyrophosphate, iodo[1-^{14}C]acetic acid, iodo[^{3}H]acetic acid, and iodo[^{3}H]acetamide were purchased from New England Nuclear. L-[3-^{14}C]Tryptophan was purified as previously described (7) and its concentration was determined spectrophotometrically. Fluorescamine, dansyl[2] chloride, dansyl amino acids, cyanogen bromide, *N*-ethylmaleimide and Sequanal grade sodium dodecyl sulfate, acetone, *N*-butyl acetate, and trifluoroacetic acid were from Pierce Chemical Co. 2,2′-Dithiodipyridine was from Aldrich Chemical Co. Cystamine dihydrochloride was from Sigma Chemical Co. Phenyl isothiocyanate purified by vacuum distillation and stored under nitrogen was from Eastman Organic Chemicals. Analytical reagent pyridine from Mallinckrodt Chemical Works was redistilled in the presence of ninhydrin. Chen-Chin polyamide thin layer sheets were from Gallard-Schlesinger Chemical Manufacturing Corp. Aminex A-5 resin was from Bio-Rad

[2] The abbreviation used is: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

Laboratories. All other materials used were the highest grade commercially available or were prepared as previously described (8, 9).

Assay of Tryptophanyl-tRNA Formation—Aminoacylation activity of tryptophanyl-tRNA synthetase was measured as described by Joseph and Muench (8). The incubation period was 10 min unless otherwise stated. One unit of aminoacylation activity forms 1 nmol of tryptophanyl-tRNA in 10 min at 37°.

Assay of Tryptophan-Dependent ATP-PP_i Exchange—Exchange activity was assayed as described by Muench (10) without potassium fluoride.

Determination of Protein—The concentration of tryptophanyl-tRNA synthetase was taken as 91% (8) of that determined by the method of Lowry *et al.* (11) with bovine serum albumin as standard.

Purification of Enzyme—The early steps of the purification procedure were modified from Joseph and Muench (8) to accommodate large amounts of starting material. All steps were done at 2–4° except the autolysis. All phosphate buffers were at pH 6.9, composed of equimolar KH_2PO_4 and K_2HPO_4.

Approximately 3.2 kg of *E. coli* B frozen cell paste was broken into small pieces and placed in a large battery jar. A solution of 1 mM potassium phosphate buffer, 10 mM $MgCl_2$, and 20 mM 2-mercaptoethanol was added to give 7.6 liters of homogeneous suspension. The suspension was then passed through a Gaulin single-stage laboratory homogenizer at a pressure of 12,000 p.s.i. and collected directly in 250-ml centrifuge bottles. This treatment caused the temperature to increase from 2° to about 23°. The homogenate was centrifuged for 80 min at 16,000 × *g* to give 5400 ml of supernatant extract.

The extract was made 0.1 M in phosphate buffer and brought to 37° in a water bath over a period of about 1 hour and maintained at this temperature for 5 hours, when release of acid-soluble A_{260} units reached a plateau. At the end of this incubation the extract became turbid, and 51% of the total A_{260} became soluble. The autolysate was stored overnight on ice, then centrifuged at 16,000 × *g* for 1 hour to give 5200 ml of supernatant.

Ammonium sulfate (1123 g) was added slowly to the supernatant with constant stirring. After an additional 10 min the mixture was centrifuged at 16,000 × *g* for 30 min. To the supernatant (5270 ml) an additional 730 g of ammonium sulfate was added as previously described. After the turbid mixture had been stirred for 10 min it was centrifuged for 40 min at 16,000 × *g*. The pellets were dissolved in a minimal volume of 50 mM potassium phosphate buffer containing 20 mM 2-mercaptoethanol and dialyzed against several 24-liter volumes of 1 mM phosphate buffer containing 20 mM 2-mercaptoethanol.

At this point the enzyme was 1.5-fold purified in a yield of 68% equivalent to the $MnCl_2$ fraction of Joseph and Muench (8). The final pure enzyme (60 mg) was prepared by chromatography on DEAE-cellulose, hydroxylapatite, and Amberlite CG-50 as previously described (8). Specific activity and electrophoresis on sodium dodecyl sulfate-polyacrylamide gels were used as criteria of purity. A subsequent preparation by this method yielded 280 mg of pure enzyme from 18 kg of cells. The ammonium sulfate enzyme fraction may be stored at −20° without loss of activity.

Notes on Purification—In spite of the previous report (8) that autolysis destroyed activity we found no loss of activity during the autolysis described above. Perhaps the success or failure of this step depends on absence or presence of proteases in different batches of cells. In a preparation of the enzyme from 44 kg of *E. coli* the extract was repassed through the Gaulin homogenizer, and then both the autolysis and ammonium sulfate steps were omitted. Ribosomes were removed by continuous flow centrifugation at 100,000 × *g* on an Electronucleonics RK ultracentrifuge,[3] and the resultant supernatant was applied directly to the DE52 column. The yield was 1.0 g of pure enzyme.

Enzyme Storage—The pure enzyme at concentrations of about 2 mg/ml was stored frozen in enzyme diluent composed of 10 mM potassium phosphate buffer, pH 6.9/20 mM 2-mercaptoethanol/10% glycerol. For assay the enzyme was further diluted in this solution. In many experiments, for example, inactivation by *N*-ethylmaleimide, the enzyme was introduced in the diluent, and therefore, low concentrations of phosphate buffer, 2-mercaptoethanol, and glycerol were present.

Titration of Enzyme Thiol Groups with 5,5′-Dithiobis(2-nitrobenzoate)—In all cases thiol estimations were calculated on a basis of a molar absorbance of 13,600 for 5-carboxy-4-nitrothiophenol at 412 nm (12). Where not otherwise indicated the temperature was 23°. For the simultaneous titration of thiol groups and activity the enzyme was freed of exogenous thiol by passage over a calibrated Sephadex G-25 (fine) column equilibrated with 50 mM Tris-HCl buffer, pH 7.8, 0.5 mM EDTA, and 10% glycerol immediately before assay. To 0.75 ml of enzyme (246 μg) solution was added 0.015 ml of 15 mM 5,5′-dithiobis(2-nitrobenzoate) dissolved in 45 mM Tris base. The production of 5-carboxy-4-nitrothiophenol was measured with a Gilford model 240 spectrophotometer. At various times in the course of the reaction aliquots were withdrawn from the reaction vessel and diluted into 20 mM potassium phosphate buffer, pH 6.9, and 10% glycerol. One minute after dilution the enzyme was assayed for aminoacylation activity in the usual manner.

Thiol groups in the native and denatured enzyme were determined with aliquots of enzyme freed of exogenous thiol by passage over a Sephadex G-25 (fine) column (14 × 1 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 8.1/1 mM EDTA. To one cuvette 1.0 ml of enzyme (105 μg) was added. To the control, 1.0 ml of protein-free Sephadex G-25 eluate was added. To each cuvette was added 0.075 ml of 10 mM 5,5′-dithiobis(2-nitrobenzoate). At 95 min 0.100 ml of 10% sodium dodecyl sulfate was added. The increase in A_{412} was recorded in a Cary model 14 spectrophotometer. In those experiments in which tryptophanyl-tRNA synthetase was modified with *N*-ethylmaleimide, iodoacetamide, or iodoacetic acid prior to titration with 5,5′-dithiobis(2-nitrobenzoate), 0.100 ml of 10 mM 5,5′-dithiobis(2-nitrobenzoate) in 100 mM Tris-HCl buffer, pH 8.1/1 mM EDTA was added to 0.900 ml of derivatized protein in buffer. The increase in A_{412} was recorded in a Cary model 14 spectrophotometer against an appropriate blank. After the initial reactions had reached completion, 0.100 ml of 10% aqueous sodium dodecyl sulfate was added, and the change in the A_{412} was recorded. Sodium dodecyl sulfate does not interfere with thiol determination by 5,5′-dithiobis(2-nitrobenzoate) (13).

To determine the total thiol group content of tryptophanyl-tRNA synthetase denatured in 8 M urea after sodium borohydride reduction, we used the method of Cavallini *et al.* (14) as modified by Lemaire *et al.* (15). To determine the thiol groups in tryptophanyl-tRNA synthetase denatured in 6 M guanidine hydrochloride the enzyme prepared as described by Lemaire *et al.* (15) was dissolved in 0.15 ml of 100 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA and 172 mg of guanidine hydrochloride was added. After its solution, 0.10 ml of 1 M KOH was added. The final concentration of guanidine hydrochloride was 6 M, and final pH was 8.5 in 0.300 ml. To 0.18 ml of this solution in a cuvette was added 0.020 ml of 10 mM 5,5′-dithiobis(2-nitrobenzoate) in 100 mM potassium phosphate buffer, pH 8.0. The A_{412} was read against the blank treated in parallel but with no protein.

Inactivation with N-Ethylmaleimide—The reaction of 330 μg of enzyme (0.02 mM subunit) with 20 mM *N*-ethylmaleimide in 253 mM triethanolamine hydrochloride buffer, pH 7.6, total volume 0.45 ml, proceeded for 1 hour at 22°. Aliquots of 0.005 ml were removed and mixed with enzyme diluent for assay at various times. The inactive enzyme was freed of reactants on the calibrated Sephadex G-25 (fine) column equilibrated with 0.1 M Tris-HCl buffer, pH 8.0/1 mM EDTA, prior to titration with 5,5′-dithiobis(2-nitrobenzoate).

Inactivation with Iodoacetamide or Iodoacetate—The reaction of 330 μg of enzyme (0.02 mM subunit) with 33 mM iodoacetamide or iodoacetate in 178 mM Tris-HCl buffer, pH 8.6 (total volume 0.45 ml), proceeded for 1 hour at 22°, with sampling for assay as above. The inactive enzyme was freed of reactant as above prior to titration with 5,5′-dithiobis(2-nitrobenzoate).

Amino Acid Analysis—The procedures used for amino acid analysis of protein and peptide samples were as described by Moore and Stein (16). Two amino acid analyzers were used, a Beckman 120 C with two columns and a Jeolco JLC-5AH with a single column and a stream splitter allowing fractions to be collected for further analysis.

Because of the low content of cysteic acid in hydrolysates of performate-oxidized tryptophanyl-tRNA synthetase, a correction factor for the color constant of cysteic acid was derived with hen egg lysozyme as a standard as described by Koch (17).

Prior to performic acid oxidation all protein samples were passed over a calibrated Sephadex G-50 (fine) column (1 × 24 cm) equilibrated with 0.1 M ammonium bicarbonate at 2°. The appropriate fractions were repeatedly freeze-dried before oxidation and subsequent amino acid analysis. Performic acid oxidation was done according to Hirs (18). Immediately after oxidation the samples were flash evaporated at 20°, dissolved in deionized water, and freeze-dried to remove traces of performic acid.

Cleavage with 2-Nitro-5-thiocyanobenzoate—2-Nitro-5-thi-

[3] Done in collaboration with Dr. Herbert Weissbach at the Roche Institute of Molecular Biology.

ocyanobenzoate was synthesized according to DeGani and Patchornik (19). Tryptophanyl-tRNA synthetase was cleaved with 2-nitro-5-thiocyanobenzoate by the two-step method of Jacobson *et al.* (20). The cleavage step was done in 25 mM sodium borate buffer, pH 9.1, containing 25 mM sodium acetate and 1% sodium dodecyl sulfate. After cleavage 2-mercaptoethanol was added, and the samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Reduction and Carboxymethylation with Iodo[^{14}C]acetic Acid—The procedure of Crestfield *et al.* (21) was followed. Fifty milligrams of pure tryptophanyl-tRNA synthetase was dialyzed against several 1-liter changes of 50 mM ammonia, freeze-dried, then dissolved in 9 ml of 178 mM Tris-HCl buffer, pH 8.4, containing 5.3 M guanidine hydrochloride and 0.45 mM EDTA. To this solution 0.020 ml of 1 M dithiothreitol was added, and the vessel was flushed with nitrogen and sealed with Parafilm. The protein solution was incubated for 3.4 hours at 23°. An additional 0.010 ml of dithiothreitol was added, and incubation was continued an additional 2.1 hours. Then 2.0 ml of solution containing 200 μmol of iodo[^{14}C]acetic acid, 2800 cpm/nmol, neutralized with sodium hydroxide, was added. After 2 hours in the dark at 23° the reaction was terminated by the addition of 1 ml of 2-mercaptoethanol. The reaction mixture was then dialyzed against 1 liter of 50 mM ammonium bicarbonate and 0.2% thiodiglycol at 2° for 15 hours. Dialysis was continued for two 16-hour periods against fresh 1-liter solutions of 50 mM ammonia containing 0.2% thiodiglycol. The dialyzed product was freeze-dried.

Tryptic Digestion of S-^{14}C-carboxymethylated Tryptophanyl-tRNA Synthetase—The S-^{14}C-carboxymethylated tryptophanyl-tRNA synthetase was suspended in 7 ml of 10 mM $CaCl_2$ at 23°. The pH of the suspension was adjusted to 8.2 by the addition of a small volume of 100 mM NaOH. The digestion was initiated by the addition of 0.5 mg of trypsin (see "Materials"). The pH of the suspension was monitored with a pH meter, and 100 mM NaOH was periodically added to maintain the pH of the suspension between pH 8.2 and 8.5. Additional 0.5-mg aliquots of trypsin were added at 30, 60, and 120 min during the digestion period (4 hours).

Ion Exchange Chromatography of S-^{14}C-carboxymethylated Tryptic Peptides—Chromatography was done at 55° on a jacketed Glenco high pressure liquid chromatography column (0.9 × 30 cm) packed to a height of 23 cm with sulfonated polystyrene (Bio-Rad Aminex A-5) resin. The resin was prepared and regenerated as described by Schroeder (22). Pools from gel filtration (Fig. 3) were concentrated and rendered salt-free by repeated freeze-drying. The dried peptides were then dissolved in 30% acetic acid and pumped over the column. The column was washed with equilibration buffer (first vessel) before starting of the gradient. The developing liquid was pumped at 200 to 300 p.s.i. with a Beckman Accuflo pump equipped with a sapphire piston and Teflon seals. Development was by a pH and pyridinium acetate buffer gradient generated in three 300-ml volumes connected in series. In the vessel leading into the column were 1.2 ml of pyridine and 90 ml of glacial acetic acid made up to 300 ml with water; in the middle 300 ml were 4.8 ml of pyridine and 84 ml of glacial acetic acid; in the final 300 ml were 48.3 ml of pyridine and 42.9 ml of glacial acetic acid.

Determination of Peptide Primary Amino Groups—Fraction aliquots taken to dryness at 90° were dissolved in 0.85 ml of 0.2 M sodium phosphate buffer, pH 8.0, and 0.300 ml of fluorescamine (0.20 mg/ml) dissolved in acetone was added. The relative fluorescence was determined according to Udenfriend *et al.* (23).

Determination of NH_2-terminal Amino Acid Residues in Peptides—The dansylation method described by Gros and Labouesse (24) was used.

Sequence Determination of Peptides—The Edman degradation was done as described by Gray (25). The dansylation reaction was done according to Gros and Labouesse (24). The dansyl-amino acids were chromatographed with the solvents described by Hartley (26) on polyamide thin layers (5 × 5 cm) as described by Woods and Wang (27). Identification of dansyl-amino acids was facilitated by the use of internal dansyl-amino acid standards as suggested by Hartley (26).

RESULTS AND DISCUSSION

Requirement of One Thiol Group for Enzyme Function—As can be seen in Fig. 1, 0.92 thiol group/subunit of native enzyme is titrated with 5,5′-dithiobis(2-nitrobenzoate). When the titration of 1.0 thiol group/subunit (on the *left ordinate*) is normalized to 100% loss of activity (*right ordinate*), then the rate and extent of the loss of aminoacylation activity are identical with the rate and extent of thiol modification. The pseudo-first order plot of the data, given in the *inset* of Fig. 1, shows a bend at the log $a/(a - x)$ value of 0.34, about the first half of the reaction. The two pseudo-first order rate constants are 12 and 9.4 × 10^{-4} s^{-1}. The break in the line is always observed (2) but remains unexplained. In the presence of saturating levels of L-tryptophan, ATP, and Mg^{2+}, 5,5′-dithiobis(2-nitrobenzoate) does not inactivate the enzyme nor release 5-carboxy-4-nitrothiophenol (2).

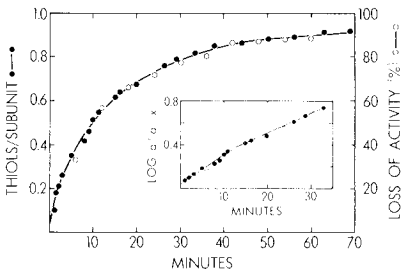

FIG. 1. Titration of the sulfhydryl groups and activity of tryptophanyl-tRNA synthetase with 5,5′-dithiobis(2-nitrobenzoate). Tryptophanyl-tRNA synthetase (0.25 mg or 3.4 nmol) was titrated with 0.3 mM 5,5′-dithiobis(2-nitrobenzoate) as described under "Experimental Procedure." Release of 5-carboxy-4-nitrothiophenol was followed by A_{412} and converted to thiol groups per subunit of enzyme (●). Aminoacylation activity (○) was measured as described under "Experimental Procedure." The *inset* is a first order plot of the data.

At pH 7.8, 5,5′-dithiobis(2-nitrobenzoate) is an anion. The possible effect of charge was investigated with 2,2′-dithiodipyridine (28) and with cystamine under similar conditions as those used for inactivation of the enzyme with 5,5′-dithiobis-(2-nitrobenzoate). Both reagents completely inactivate the enzyme, presumably by formation of mixed disulfides with the required thiol group.

The loss of L-tryptophan-dependent ATP-PP_i exchange and the loss of tryptophanyl-ATP ester (10) formation fall precisely on the curve for loss of aminoacylation activity during titration of the enzyme with 5,5′-dithiobis(2-nitrobenzoate). Whereas the native enzyme forms a complex with L-[^{14}C]-tryptophan and ATP isolable by gel filtration (2), enzyme inactivated with 5,5′-dithiobis(2-nitrobenzoate) fails to form such a complex. Examination of the ability of inactivated enzyme to bind $tRNA^{Trp}$ was investigated by sucrose density gradient centrifugation (29) and revealed that the inactive enzyme formed the characteristic complex with one $tRNA^{Trp}$/dimer.[4]

Bruton and Hartley (30) reported that modification of a specific thiol group in methionyl-tRNA synthetase causes dissociation of the enzyme into its component subunits with concomitant loss of activity. The possibility that modification of the required thiol group in tryptophanyl-tRNA synthetase results in dissociation of the subunits was investigated by sucrose density gradient centrifugation and by gel filtration, and both methods revealed that the enzyme inactivated by 5,5′-dithiobis(2-nitrobenzoate) remains a dimer.

Reversibility of Inactivation—The reversibility of the inactivation by 5,5′-dithiobis(2-nitrobenzoate) is shown in Fig. 2. The enzyme recovered from the experiment shown in Fig. 1 retained 8% of its original activity. As can be seen in Fig. 2, 1.1 molecules of 5-carboxy-4-nitrothiophenol are released per subunit of enzyme completely reduced with dithiothreitol. The reactivated enzyme had 88% of its initial specific activity

[4] K. H. Muench, manuscript in preparation.

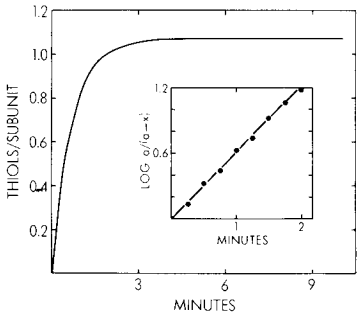

FIG. 2. Reactivation of tryptophanyl-tRNA synthetase-5-carboxy-4-nitrothiophenol by treatment with dithiothreitol. Immediately after tryptophanyl-tRNA synthetase was titrated with 5,5′-dithiobis(2-nitrobenzoate) as shown in Fig. 1, 0.5 ml of that reaction mixture was again passed over the calibrated Sephadex G-25 column described under "Experimental Procedure." An aliquot was withdrawn from the protein fraction and assayed for enzymatic activity. The 5,5′-dithiobis(2-nitrobenzoate)-free 5-carboxy-4-nitrothiophenol-mixed disulfide derivative of tryptophanyl-tRNA synthetase in 1.0 ml was placed in a cuvette. To another cuvette 1.0 ml of protein-free G-25 eluate was added. Both cuvettes were then placed in a Cary model 14 recording spectrophotometer. To each cuvette was added 0.010 ml of 100 mM dithiothreitol. The A_{412} was automatically recorded and converted to thiol groups per subunit. After the reaction ceased, the enzyme solution was again assayed for enzymatic activity. The *inset* is a first order plot of the recorded data.

in the aminoacylation reaction. The release of 5-carboxy-4-nitrothiophenol follows pseudo-first order kinetics with a rate constant of 2.3×10^{-2} s^{-1} as shown in the *inset* to Fig. 2. The experiment shows that during the titration of the enzyme with 5,5′-dithiobis(2-nitrobenzoate) the release of 1 molecule of 5-carboxy-4-nitrothiophenol/subunit is matched by a single 5-carboxy-4-nitrothiophenol residue covalently bound/subunit of inactive enzyme. The stoichiometry proves the requirement for one thiol per subunit and eliminates the possibility that the reaction of tryptophanyl-tRNA synthetase with 5,5′-dithiobis(2-nitrobenzoate) leads to formation of intramolecular disulfide bridges via the aryl disulfide-cysteine interchange reaction as observed by Connellan and Folk (31) with guinea pig liver transglutaminase. Enzyme inactivated by 2,2′-dithiodipyridine or by cystamine is also completely reactivated by treatment with dithiothreitol.

Other Thiol Residues—The 5,5′-dithiobis(2-nitrobenzoate) has free access only to the required thiol group in the native enzyme; for example, in one experiment 0.9 mM 5,5′-dithiobis(2-nitrobenzoate) titrated 1.3 thiol groups/subunit in a reaction complete at 45 min (pseudo-first order rate constant of 34×10^{-4} s^{-1}). Then denaturation of the enzyme with 1% sodium dodecyl sulfate immediately exposed 2.0 additional thiol groups/subunit, and these thiol groups reacted much more rapidly than the required thiol group in the native enzyme.

Inactivation by Other Reagents—*N*-Ethylmaleimide, iodoacetamide, and iodoacetate also inactivate the native enzyme. When treated as described under "Experimental Procedure," the enzyme is 90% inactivated in 60 min by 20 mM *N*-ethylmaleimide or 33 mM iodoacetate and 97% inactivated in 30 min by 33 mM iodoacetamide. In all cases the kinetics of inactivation are pseudo-first order. After such inactivation by *N*-ethylmaleimide or iodoacetamide only 0.2 thiol residue/subunit of enzyme reacts with 5,5′-dithiobis(2-nitrobenzoate) ("Experimental Procedure"), but denaturation with 1% sodium dodecyl sulfate results in immediate reaction of 2.0 and 2.1 additional thiol residues/subunit, respectively. These data indicate that *N*-ethylmaleimide and iodoacetamide react with the required thiol group identified by 5,5′-dithiobis(2-nitrobenzoate) in the native enzyme. However, iodoacetic acid is different in this respect, for enzyme inactivated by this reagent still contains 0.6 thiol residue/subunit reacting with 5,5′-dithiobis(2-nitrobenzoate). Upon denaturation with 1% sodium dodecyl sulfate, the additional 2.2 thiol residues/subunit are instantly titrated by 5,5′-dithiobis(2-nitrobenzoate). The tentative conclusion is that the inactivation of the native enzyme by iodoacetic acid does not result simply from modification of the required thiol residue which reacts with 5,5′-dithiobis-(2-nitrobenzoate), 2,2′-dithiodipyridine, cystamine, 2-nitro-5-thiocyanobenzoate (see below), iodoacetamide, and *N*-ethylmaleimide.

The conclusion is supported by composition studies. When native enzyme was treated with iodoacetamide until less than 5% original activity remained and then subjected to amino acid analysis, 0.9 *S*-carboxymethylcysteine residue/subunit was found. When a similar experiment was done with tritiated iodoacetic acid, no radioactive *S*-carboxymethylcysteine was detectable. Enzyme inactivated with either iodoacetate or iodoacetamide contained no carboxymethyllysine nor carboxymethylhistidine residues detectable by amino acid analysis, but the detection of homoserine and homoserine lactone indicated that some *S*-carboxymethylmethionyl sulfonium residue had formed in both cases (32).

Number and Nature of Half-Cystine Residues—As already mentioned, 5,5′-dithiobis(2-nitrobenzoate) titration of the enzyme denatured in sodium dodecyl sulfate reveals 3.3 thiol groups/subunit. In two experiments when the enzyme was denatured in 6 M guanidine hydrochloride, 3.1 thiol groups/subunit were detected with 5,5′-dithiobis(2-nitrobenzoate). No disulfide bonds were detectable by 5,5′-dithiobis(2-nitrobenzoate) titration after sodium borohydride reduction of the enzyme in 8 M urea in two determinations, giving a value of 3.0 thiol groups/subunit. The molar absorbance of 5-carboxy-4-nitrothiophenol is not changed by the presence of guanidine hydrochloride (13) or urea (15). In four determinations of half-cystine as cysteic acid an average of 3.6 residues/subunit was found, and the same value emerged from three determinations of half-cystine residues as *S*-carboxymethylcysteine. As shown below three unique thiol peptides have been sequenced. The possibility of a fourth thiol peptide remains.

The absence of intersubunit disulfide bonding was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis done according to Weber and Osborn (33) but without reduction of the enzyme with 2-mercaptoethanol prior to the electrophoresis. Both the reduced and the unreduced control enzyme gave a subunit molecular weight of 35,000, in good agreement with the published value of 37,000 (2).

Cleavage at Thiol Residues—The cysteine-specific peptide cleavage reagent, 2-nitro-5-thiocyanobenzoate (20, 34) was used to determine positions of the thiol residues in tryptophanyl-tRNA synthetase. At 23° in 100 mM Tris-HCl buffer, pH 8.1/2.5 mM 2-nitro-5-thiocyanobenzoate (25 mM, prepared immediately before use and dissolved in the same buffer) gave 99.5% inactivation of native tryptophanyl-tRNA synthetase in 2 hours, the control enzyme without 2-nitro-5-thiocyanobenzoate remaining completely active. When the reagent was used to cleave tryptophanyl-tRNA synthetase, about 50% of the modified protein was cleaved into fragments of molecular weight 32,000, 27,000, and 9,500, as determined by polyacrylamide gel electrophoresis with sodium dodecyl sulfate.

Isolation and Sequencing of S-Carboxymethylcysteine Peptides—To initiate sequence studies on the enzyme we isolated and sequenced the thiol peptides. As described under "Experimental Procedure" 50 mg of tryptophanyl-tRNA synthetase was denatured in 5.3 M guanidinium chloride, reduced with dithiothreitol, and treated with iodo[^{14}C]acetic acid. According to the radioactivity of the dialyzed product 4.7 carboxymethyl residues/subunit were incorporated. When this material was subjected to amino acid analysis, 18% of the radioactivity appeared in the column breakthrough and was ninhydrin-negative. All of the remaining radioactivity was associated with the *S*-carboxymethylcysteine peak, 3.9 residues/subunit. No radioactivity was present in the positions of dicarboxymethyllysine, dicarboxymethylhistidine, 1′-carboxymethylhistidine, 3′-carboxymethylhistidine, monocarboxymethyllysine, homoserine, homoserine lactone, and *S*-carboxymethylhomocysteine, all of which could be resolved by the program used. The carboxymethylated protein was then digested with trypsin. During the digestion the uptake of NaOH was 47 μmol of a calculated 53 μmol for complete digestion.

The tryptic digest was partially resolved into four peaks by Sephadex G-25 (Superfine) gel filtration as described in Fig. 3. Each of the four peaks was then further resolved by ion exchange chromatography on the sulfonated polystyrene, Aminex A-5, as shown for Peaks I and III in Figs. 4 and 5. Five fractions were recovered for sequence determination, and their compositions, NH_2-terminals, and recoveries are given in Table I. Peptide IIA12a required a final purification step by thin layer chromatography on cellulose developed with butanol/acetic acid/water/pyridine (150/30/120/100, v/v).

Of the five peptides only three represent unique sequences around cysteine residues, for peptide IVA5 is clearly derived from peptide IIIA2 by chymotryptic cleavage, and peptide IIA12a seems to derive from peptide IA131, although the cleavage at a histidine residue is unexplained.

The five peptides were sequenced by the Edman procedure coupled with identification of the NH_2-terminal residue at each step as the dansyl derivative (26). Although the *S*-carboxymethylcysteine residues in peptides IIA12a and IVA5 were not detected by amino acid analysis (Table I), they were detected as the dansyl derivative in the Edman degradation, and the radioactivity of each was released into the solvent at the proper point of the Edman degradation. No ambiguities were encountered in the sequence analyses, which were always carried through the COOH-terminal residue. The sequences confirmed the compositional data given in Table I and are:

IA91	Leu-Ala-Cys-Gly-Ile-Asx-Glx-Pro-Lys
IA131	Cys-Ile-Val-Asx-Glx-His-Ala-Ile-Thr-Val-Arg
IIA12a	Cys-Ile-Val-Asx-Glx-His
IIIA2	Ala-Leu-Asn-Cys-Tyr-Thr-Tyr-Phe-Gly-Glu-Leu-Ser-Arg
IVA5	Ala-Leu-Asx-Cys-Tyr

The assignments of asparagine in the third position and of glutamic acid in the tenth position of peptide IIIA2 were made according to electrophoretic mobilities and molecular weights of the whole peptide and of the residual COOH-terminal tetrapeptide (35).

In summary the three or four thiol groups present in the enzyme are matched by three unique thiol peptide sequences. The data do not exclude the possibility of a fourth unique

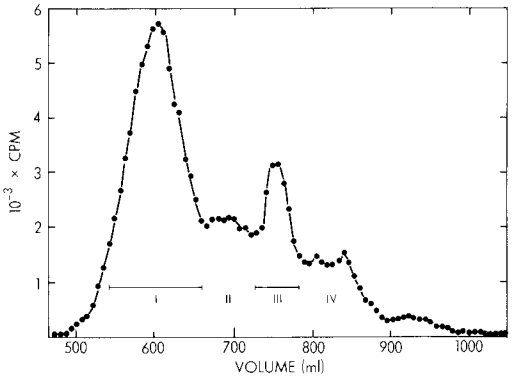

FIG. 3. Gel filtration of *S*-[^{14}C]carboxymethylated tryptic peptides. The digest was centrifuged at 23° for 10 min at 1050 × *g*. To the supernatant was added 1 ml of 1 M ammonium bicarbonate, and the mixture was clarified by centrifugation again. The clear digest was then placed directly over a Sephadex G-25 (superfine) column (2.6 × 260 cm) equilibrated with 0.1 M ammonium bicarbonate, and 6.9-ml fractions were collected at 28 ml/hour. An aliquot from each fraction was placed on a GF/C filter, dried, immersed in liquid scintillator, and counted. The *heavy black lines* indicate pooled fractions of *Peaks I* and *III*. The recovery of radioactivity was 95%.

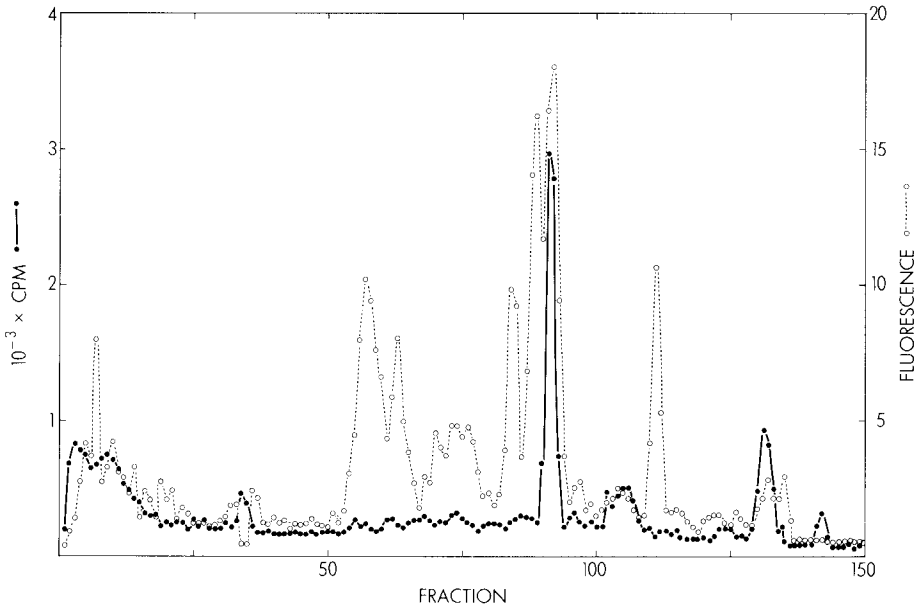

FIG. 4. Ion exchange chromatography of the pool I peptides (Fig. 3) on a sulfonated polystyrene column. The Pool I tryptic peptides were prepared and chromatographed as described under "Experimental Procedure." The gradient was started at Fraction 33. The column was developed at 30 ml/hour, and 6-min fractions were collected. An aliquot from each fraction was withdrawn and placed on a GF/C filter, dried, immersed in liquid scintillator, and counted (●). The recovery of radioactivity was 100%. The presence of primary amino groups in each fraction (○) was determined with fluorescamine as described under "Experimental Procedure."

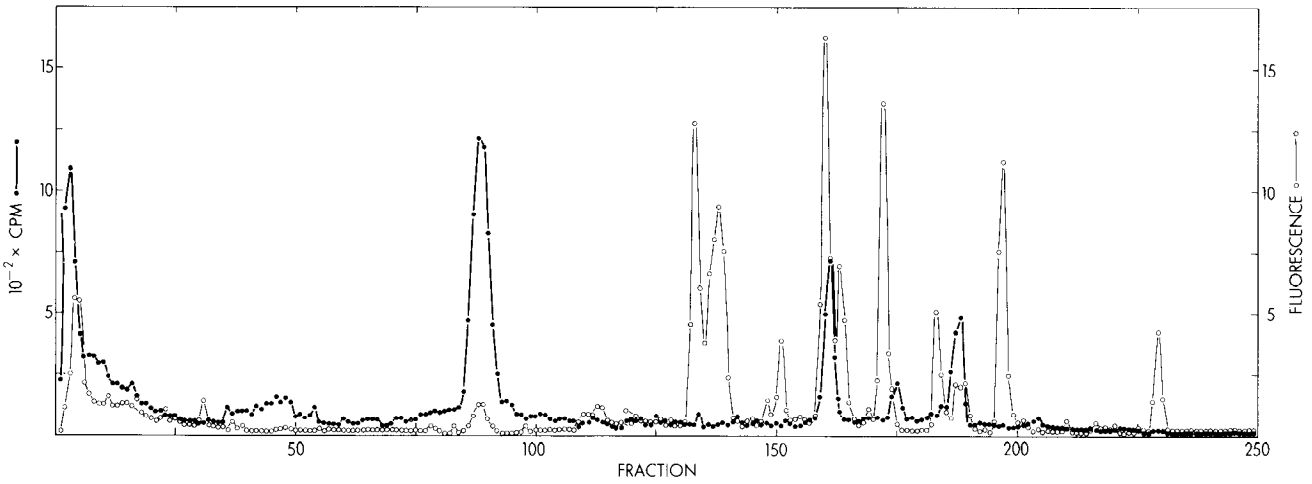

FIG. 5. Ion exchange chromatography of the Pool III peptides (Fig. 3) on a sulfonated polystyrene column. The Pool III peptides were prepared and chromatographed as described under "Experimental Procedure." The gradient was started at Fraction 16. The column was developed at 26 ml/hour with collection of 6-min fractions. Radioactivity (●) and primary amines (○) were determined as in Fig. 4. Recovery of radioactivity was 83%.

TABLE I

Compositions, NH-terminals, and recoveries of S-[^{14}C]carboxymethylcysteine-containing tryptic peptides

Peptides were hydrolyzed for 24 or 72 hours (IIA12a) and analyzed as under "Experimental Procedure." The values in parentheses indicate the number of assigned residues. The NH_2-terminal amino acids as determined by the dansylation method are indicated by asterisks. Recoveries from the original 1350 nmol of enzyme subunit denatured and carboxymethylated are given under the name of each peptide.

Peptide recovery	IA91, 32%	IA131, 9%	IIA12a, 2%	IIIA2, 14%	IVA5, 2%
			residues/peptide		
Lysine	1.00 (1)	0.16	0	0	0
Histidine	0.07	0.85 (1)	1.01 (1)	0.17	0
Arginine	0.12	0.94 (1)	0	0.88 (1)	0
S-Carboxymethylcysteine	0.75 (1)	0.68 (1)*	0 (1)*	0.51 (1)	0 (1)
Aspartic acid	1.10 (1)	1.08 (1)	1.00 (1)	1.04 (1)	1.14 (1)
Threonine	0.17	0.86 (1)	0.06	0.88 (1)	0.16
Serine	0.27	0.27	0.13	1.00 (1)	0.42
Glutamic acid	1.23 (1)	1.13 (1)	1.21 (1)	1.18 (1)	0.47
Proline	1.02 (1)	0	0	0	0
Glycine	1.07 (1)	0.36	0.38	1.16 (1)	0.48
Alanine	0.87 (1)	1.00 (1)	0.08	0.99 (1)*	1.00 (1)*
Valine	0.34	1.55 (2)	0.78 (1)	0.23	0
Isoleucine	0.88 (1)	1.37 (2)	0.63 (1)	0.16	0
Leucine	1.02 (1)*	0.14	0	1.79 (2)	0.78 (1)
Tyrosine	0	0	0	1.54 (2)	0.51 (1)
Phenylalanine	0.08	0	0	0.85 (1)	0

thiol peptide. Repeating sequences containing cysteine are not present in the enzyme.

Because the derivatization with iodoacetic acid was done under denaturing conditions, these data do not distinguish the peptide in which the thiol group required for enzyme activity resides. As shown above iodoacetate does not react with the required thiol group in the native enzyme. Therefore, to determine which of the three peptides contains the required thiol group we could not use the direct approach of carboxymethylating the thiol in native enzyme. An alternate experiment was done as follows: the native enzyme was first treated with 5,5′-dithiobis(2-nitrobenzoate) until completely inactive. The 5-carboxy-4-nitrothiophenol-enzyme mixed disulfide was then denatured in 5.3 M guanidinium chloride containing 20 mM unlabeled iodoacetate in order to carboxymethylate the thiol groups inaccessible in the native enzyme. The partially carboxymethylated derivative was then reduced with dithiothreitol to remove the 5-carboxy-4-nitrothiophenol residue from the required thiol group, which was now carboxymethylated with iodo[^{3}H]acetic acid. The derivatized enzyme was then subjected to trypsin digestion as previously described, and the tryptic digest was mixed with ^{14}C-labeled peptide IA91 as a marker prior to gel filtration as described in Fig. 3 and ion exchange chromatography as described in Fig. 4. Of the ^{3}H radioactivity recovered in positions of the thiol peptides at least 68% was found in the peak co-chromatographing with the ^{14}C-labeled peptide IA91 marker. The maximum for any other peptide was 24%. This evidence points to peptide IA91 as that containing the cysteine residue required for enzyme activity. However, a definitive answer awaits derivatization of the native enzyme with iodo[^{14}C]acetamide, trypsin digestion, peptide purification, and composition and sequence studies. The answer is of enhanced interest since the discovery that peptide IA91 is homologous to a thiol peptide in the tryptopha-

nyl-tRNA synthetase of human placenta (6). The existence of homologous peptides in cognate aminoacyl-tRNA synthetases from the extremes of the phylogenetic scale lead us to believe that homologies will be discovered among other sets of cognate aminoacyl-tRNA synthetases.

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G V Kuehl, M L Lee and K H Muench

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