Glycyl Transfer Ribonucleic Acid Synthetase from *Escherichia coli;* Purification, Properties, and Substrate Binding†

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ABSTRACT: Glycyl-tRNA synthetase from *Escherichia coli* has been purified 875-fold to near homogeneity. The enzyme has a molecular weight of 225,000 and is a tetramer containing two dissimilar subunits (mol wt 33,000 and 80,000). The kinetic properties and amino acid composition have been determined. The native enzyme binds two molecules of glycyl-AMP per tetramer. The enzyme has eight sulfhydryl residues distributed equally among the four subunits. Each of the sulfhydryl groups reacts with *p*-hydroxymercuribenzoate but only two or three react with *N*-ethylmaleimide. Treatment of the native enzyme with *p*-hydroxymercuribenzoate dissociates the tetramer into inactive monomers which can reassociate to form active enzyme after removal of the mercurial. Reaction of the enzyme with *N*-ethylmaleimide eliminates tRNA acylating activity and increases the ATP–PP_i exchange activity two- to threefold.

Studies of the subunit structure of several of the purified aminoacyl-tRNA synthetases have revealed varying degrees of complexity (Loftfield, 1972), but three general classes exist: (1) single polypeptide chains of mol wt 70,000–115,000; (2) dimers and tetramers of apparently identical subunits each of mol wt 40,000–50,000; and (3) dimers or tetramers with nonidentical subunits. Several of the enzymes in classes 1 and 2 have been shown to have one active site per polypeptide chain (Muench and Joseph, 1971; Charlier and Grosjean, 1972; Gros *et al.*, 1972; Parfait and Grosjean, 1972; Rymo *et al.*, 1972; Boeker *et al.*, 1973; Chousterman and Chapeville, 1973). However, the methionyl-tRNA synthetase from *Escherichia coli*, a dimer of identical subunits,[1] appears to have four binding sites for ATP and only two for methionine and tRNA; the phenylalanyl-tRNA synthetase from *E. coli* apparently has only one active site per tetramer of identical subunits (Farrelly *et al.*, 1971; Kosakowski and Böck, 1971). Very little is known regarding substrate binding to class three enzymes. Glutamyl-tRNA synthetase from *E. coli* is probably a dimer of dissimilar subunits; but one subunit is active by itself and, therefore, can bind all the substrates (LaPointe and Söll, 1972). Befort *et al.* (1970) have reported that the yeast phenylalanyl-tRNA synthetase, an $\alpha_2\beta_2$-type enzyme, binds only one molecule of tRNA per tetramer.

We have reported earlier (Ostrem and Berg, 1970) that glycyl-tRNA synthetase (GlyRS)[2] of *E. coli* is in the third and most complex class of synthetases. It has a molecular weight of about 225,000 and an $\alpha_2\beta_2$ subunit structure (the molecular weights of the α and β subunits are 33,000 and 80,000, respectively). The fact that the two kinds of subunits can be separated and recombined to form active enzyme (Ostrem and Berg, 1970) makes GlyRS particularly attractive for use in studying aminoacyl-tRNA synthetase structure–function relationships. In this paper, we describe details of the purification procedure for GlyRS and report some of its physical, chemical, and catalytic properties.

Materials and Methods

Cells. Escherichia coli K12 cells (strain PB 127; Folk and Berg, 1970) were grown in a 100-l. Fermacell with vigorous aeration in a medium containing 10 g of sodium chloride, 10 g of yeast extract, 20 g of tryptone, and 10 g of glucose per l. The cells were harvested in late exponential phase, washed once with 0.15 M sodium chloride, and stored at −15°.

Chemicals. Acrylamide (electrophoresis grade) was obtained from Bio-Rad and sodium *p*-hydroxymercuribenzoate was purchased from Sigma Chemicals. Unfractionated *E. coli* tRNA was purchased from Schwarz. Aminoethyl-AMP (the phosphodiester formed from ethanolamine and 5′-AMP) was the kind gift of Dr. R. Symons. [^{14}C]HO-HgBzO[3] was purchased from Schwarz and [^{14}C]-*N*-ethylmaleimide was from Amersham-Searle. ^{32}P-Labeled sodium pyrophosphate and [^{14}C]glycine were obtained from New England Nuclear.

Proteins. Crystalline bovine serum albumin was obtained from Sigma Chemicals. The following enzymes were very generously donated by our colleagues in the Biochemistry Department: DNA polymerase from D. Brutlag, isoleucyl-tRNA synthetase from D. Arndt, valyl-tRNA synthetase from M. Yaniv, and aspartate transcarbamylase from G. Stark.

Assays. Glycyl-tRNA synthetase activity was measured either by aminoacyl-tRNA formation or by glycine-dependent ATP–PP_i exchange. Glycyl-tRNA synthesis was measured as described by Folk and Berg (1970); one unit of activity is defined as the formation of 1 nmol of glycyl-tRNA in 10 min at 37°. ATP–PP_i exchange activity was measured as described earlier (Calendar and Berg, 1966a,b); one unit of activity is equivalent to the incorporation of 1 nmol of [^{32}P]-pyrophosphate into ATP in 10 min at 37°.

Protein was determined colorimetrically by the method of Lowry *et al.* (1951), using bovine serum albumin as a reference standard. To eliminate buffer interference, the protein was first precipitated with 7% trichloroacetic acid.

† From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305. *Received October 23, 1973.* This work was supported by research grants from the U. S. Public Health Service (GM 13235-08) and from the American Cancer Society (VC 23B). D. L. O. is a U. S. Public Health Service trainee.

[1] Bruton, C. J. (1973), private communication.

[2] For the purposes of brevity and clarity, we shall refer to the individual aminoacyl-tRNA synthetases by the standard three-letter abbreviation for the amino acid followed by the capital letters RS, *e.g.*, GlyRS and IleRS refer to glycyl-tRNA synthetase and isoleucyl-tRNA synthetase, respectively.

[3] Abbreviations used are: HO-HgBzO, *p*-hydroxymercuribenzoate; Temed, *N,N,N′,N′*-tetramethylethylenediamine; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate).

TABLE I: Purification of Glycyl-tRNA Synthetase.[a]

Fraction	Total Protein (mg)	Total Units[b] ($\times 10^{-6}$)	Sp Act. (Units/mg)	Yield (%)	Purification
I. Crude extract	73,500	7.35	100	100	1.0
II. Autolysis	50,000	6.50	130	89	1.3
III. Ammonium sulfate	12,400	5.20	420	71	4.2
IV. DEAE-cellulose	765	2.44	3,200	33	32
V. Hydroxylapaptite	152	1.77	11,600	24	116
VI. Preparative electrophoresis	12	1.04	87,500	14	875

[a] The purification procedure is described under Results. [b] One unit of activity is equivalent to the formation of 1 nmol of glycyl-tRNA in 10 min at 37°.

Polyacrylamide Gel Electrophoresis. (a) Electrophoresis was carried out as described by Davis (1964), using a Tris–tricine buffer system (pH 8.1). The upper and lower gel buffers are described below in the section on preparative electrophoresis. When preelectrophoresis was used, the upper and lower buffers were solution A (see below) diluted fourfold. The resolving gels (6 × 70 mm) usually contained 5, 6, or 7.5% acrylamide. When a stacking gel was used (normally 0.30 ml for samples volumes of 0.20–0.25 ml) it was formed by combining one part of solution B (2.33 g of Tris, 14.0 ml of 1.0 M H_3PO_4, and 0.1 ml of Temed per 100 ml), two parts of solution D (5.0 g of acrylamide and 1.25 g of *N,N'*-methylenebisacrylamide per 100 ml), and one part of solution E (2 mg of riboflavine/100 ml). Electrophoresis was normally carried out at 2–3 mA/tube for about 2 hr. (b) Electrophoresis in the presence of 0.1% sodium dodecyl sulfate was done as described by Weber and Osborn (1969). (c) Gels were stained for 2 hr at 37° with Coomassie Brilliant Blue (0.25% in methanol–acetic acid–water, 5:1:5) and destained by soaking at 37° in 7.5% acetic acid–5% methanol. (d) Densitometer tracings were made using a Gilford 2000 spectrophotometer equipped with a gel-scanning device. (e) Gels to be analyzed for radioactivity were sliced into 1.5-mm pieces which were solubilized by heating for 2 hr at 50° with Amersham-Searle solubilizer (Basch, 1968). Counting solution (Bray, 1960) (2.5 ml) was then added to each solubilized sample, and the radioactivity was measured in a scintillation counter.

Centrifugation. (a) Sedimentation equilibrium centrifugation was carried out in a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics. Centrifugation was at 12,000 rpm and the temperature of the rotor (AnH-4) was maintained at 25° by a rotor temperature indicator and control unit. The photographic plates were measured using a Gaertner microcomparator. (b) Sucrose density gradient velocity sedimentation was done in a Beckman L2-65B ultracentrifuge using the SW-56 rotor. The speed was 55,000 rpm and the temperature was maintained at 4°.

Amino Acid Analyses. Protein samples were hydrolyzed for 24 hr at 110° under reduced pressure, and the hydrolysates were analyzed on a Beckman 120 amino acid analyzer. Tryptophan was determined spectrophotometrically (Beaven and Holiday, 1952), and cysteine was determined by titration of reduced protein in 6 M guanidine hydrochloride with Nbs_2 (Ellman, 1959).

tRNA. (a) The major glycine-specific species of tRNA ($tRNA^{Gly}_{III}$) was purified by chromatography on DEAE-Sephadex, followed by chromatography on benzoylated DEAE-cellulose. The tRNA was then acylated with [^{14}C]-glycine (using purified glycyl-tRNA synthetase), modified with the naphthoxyacetyl ester of *N*-hydroxysuccinimide (Gilliam and Tener, 1971), and rechromatographed on benzoylated DEAE-cellulose. The purified tRNA accepted 1.25 nmol of glycine/A_{260}. (b) Other purified tRNA species were gifts of M. Yaniv. (c) Acceptor activity of tRNA was destroyed by oxidation with periodate. In a sample experiment, 50 mg of tRNA in 1.2 ml of 0.1 M sodium acetate (pH 4.6) was treated with 100 mg of sodium periodate. After 1-hr incubation at room temperature in the dark, the RNA was precipitated with an equal volume of cold ethanol and then centrifuged. The pellet was dissolved in 1.0 ml of sodium acetate (0.1 M, pH 4.6), containing 0.1 M ethylene glycol to destroy any remaining periodate. After incubation for 1 hr, the tRNA was again precipitated with ethanol and centrifuged. The pellet was dissolved in about 1 ml of water and dialyzed. No detectable glycine acceptor activity (<0.05%) remained after this treatment.

Results

A. Purification of Glycyl-tRNA Synthetase. Unless otherwise stated, all steps in the purification procedure were carried out at 0–4°. Following step III, glycerol was added to all fractions to stabilize the enzyme activity. Buffer A was 0.1 M potassium phosphate (pH 7.0) containing 0.01 M mercaptoethanol. Buffer B was 0.03 M potassium phosphate (pH 7.0) containing 0.01 M mercaptoethanol. The course of the purification procedure is summarized in Table I.

CRUDE EXTRACT. Frozen cells (500 g) were suspended in 750 ml of cold buffer A and the suspension was passed through a Manton-Gaulin laboratory homogenizer twice at a pressure of 6000–7000 psi. The extract was centrifuged for 30 min at 13,000*g* and the supernatant was saved (fraction I, 1000 ml).

AUTOLYSIS. Buffer A (2 l.) was added to fraction I and the temperature was raised to 37°. Incubation was continued at that temperature for about 3 hr at which time about 95% of the absorbance at 260 mμ was acid soluble (in 3.5% perchloric acid). The autolysate was cooled to 3° and after centrifugation for 30 min at 13,000*g*, the supernatant was removed (fraction II, 2800 ml).

AMMONIUM SULFATE FRACTIONATION. Fraction II was adjusted to 3450 ml with buffer A and solid ammonium sulfate (720 g) was added. After 30-min stirring, the precipitate was removed by centrifugation for 30 min at 13,000*g*. Additional ammonium sulfate (213 g) was added to the supernatant and after stirring for 30 min, followed by centrifugation as above, the precipitate was dissolved in 140 ml of buffer B and the solution was dialyzed twice against 4 l. of buffer B containing 25% glycerol (fraction III, 125 ml).

DEAE-CELLULOSE CHROMATOGRAPHY. Fraction III was applied to a column of DEAE-cellulose (8.0 × 40 cm) which had

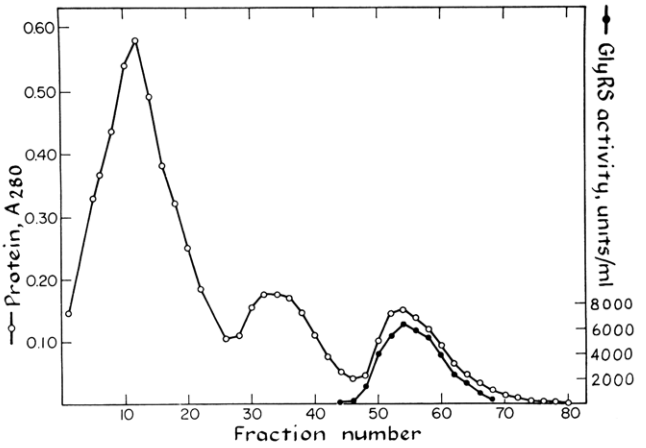

FIGURE 1: Preparative electrophoresis. The preparative electrophoresis was carried out as described in the text. The absorbance at 280 mμ was measured using fraction 90 as a reference blank. Each fraction was assayed for GlyRS activity and the most active fractions were combined.

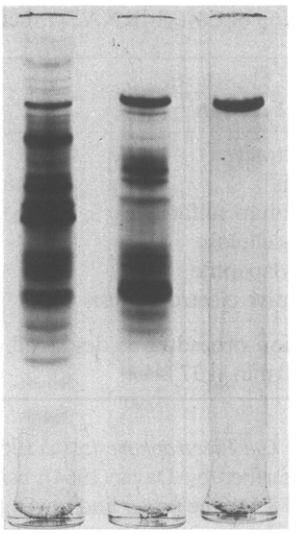

FIGURE 2: Polyacrylamide gel electrophoresis of fractions IV, V, and VI. The protein samples (about 30 μg of fraction IV, 20 μg of fraction V, and 10 μg of fraction VI) were applied to gels (7.5% acrylamide) using the Tris–tricine system described in the text.

been equilibrated with buffer B containing 20% glycerol. After washing with 1100 ml of the same buffer (at a flow rate of 200 ml/hr) the enzyme was eluted with linear gradient generated with 4 l. of 0.03 M potassium phosphate and 4 l. of 0.2 M potassium phosphate each at pH 7.0, and each containing 0.01 M mercaptoethanol and 20% glycerol. Fractions of 25 ml were collected, while the flow rate was maintained at 200 ml/hr with a peristaltic pump. After the fractions were assayed for GlyRS activity, the most active fractions were combined and concentrated to a volume of 40 ml by ultrafiltration (XM-50 membrane). The concentrate was then dialyzed twice against 2 l. of buffer C (0.005 M potassium phosphate (pH 7.0) containing 0.01 M mercaptoethanol and 30% glycerol (fraction IV, 25 ml).

HYDROXYLAPATITE CHROMATOGRAPHY. Fraction IV was applied to a column of hydroxylapatite (2.4 × 40 cm) which had been equilibrated with buffer C. The column was washed with 100 ml of buffer C at a flow rate of 50 ml/hr maintained with a peristaltic pump. The protein was eluted with a linear gradient formed with 1 l. of buffer C and 1 l. of 0.01 M potassium phosphate (pH 7.0) containing 0.01 M mercaptoethanol and 30% glycerol. Fractions of 20 ml each were collected and assayed for GlyRS activity, and the pooled fractions were concentrated by ultrafiltration (PM-30 membrane) to a volume of 30 ml (fraction V).

PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS. This procedure was carried out essentially as described by Jovin *et al.* (1964), substituting a Tris–tricine (pH 8.1) buffer system, and eliminating the stacking gel. The upper buffer contained 3.62 g of Tris and 6.02 g of tricine per l.; the lower buffer was 0.1 M Tris-HCl (pH 8.1). The resolving gel (5% acrylamide) was formed by combining 12 ml of solution C (30% acrylamide–0.8% *N,N'*-methylenebisacrylamide), 18 ml of solution A (4.26 g of Tris, 33.6 ml of 1.0 N HCl, and 0.1 ml of Temed per 100 ml), and 42 ml of solution P (0.3% ammonium persulfate). Fraction V was dialyzed against fivefold-diluted upper buffer containing 20% glycerol and 1 mM dithiothreitol and labeled fraction V-B (30 ml). After 1-hr preelectrophoresis, one-half of dialyzed fraction V (plus 0.1 ml of a 0.01% solution of Bromophenol Blue) was applied and electrophoresis was begun. The current was maintained at 50 mA during the run, while the voltage was 150–200 V. Elution buffer (0.1 M Tris-HCl, pH 8.1, containing 20% glycerol and 1 mM dithiothreitol) was pumped at 0.2 ml/min until the tracking dye reached the bottom of the gel (about 5–6 hr). Elution was then continued at 0.6 ml/min and fractions of 6 ml were collected. After a total of 20-hr operation, the fractions were analyzed for protein and enzyme activity. Figure 1 shows that the GlyRS activity eluted from the gel together with the third peak of protein. The most active fractions (fractions 49–68) were combined, concentrated to 18 ml by ultrafiltration (PM-30 membrane), dialyzed against 2 l. of buffer (0.02 M sodium phosphate, pH 7.0, containing 40% glycerol and 1 mM dithiothreitol), and stored at −15°. The procedure was repeated with the remainder of dialyzed fraction V, and the two recovered fractions were combined to yield fraction VI (16.2 ml).

Fraction VI from this particular preparation had a specific activity of 87,500 units/mg of protein, but this value has varied somewhat from one preparation to the next, with a maximum of 96,000 units/mg. Purified enzyme preparations which have been stored at −15° in 40% glycerol at a protein concentration of 0.5 mg/ml retained at least 70% of their enzymatic activity after 8 months. Figure 2 shows the results of analytical polyacrylamide gel electrophoresis of samples from fractions IV, V, and VI. Although hydroxylapatite chromatography yielded only a threefold purification, that step removed several contaminating proteins which would have been difficult to eliminate by preparative electrophoresis under the particular conditions used.

Although extensive tests to validate the homogeneity of the enzyme preparation have not yet been carried out, we have previously reported (Ostrem and Berg, 1970) that polyacrylamide gel electrophoresis using gels of three different porosities (Hedrick and Smith, 1968) revealed that more than 95% of the protein migrated as a single band and the remainder appeared in two minor bands. We concluded that the minor bands were probably not contaminating proteins because they could be generated from the major band by treatment of the enzyme with urea or by storage of the enzyme in the absence of glycerol. In addition, we have found that the appearance of these bands can be reduced by preelectrophoresis of the gels or by thoroughly washing the topes of the gels with mercaptoethanol or dithiothreitol. When these precautions were taken, electrophoretic analysis of the purified enzyme

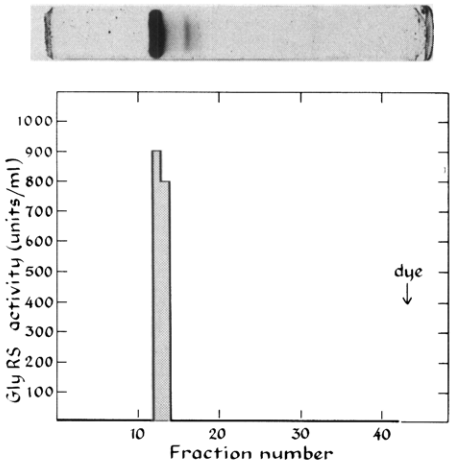

FIGURE 3: Polyacrylamide gel electrophoresis of fraction VI. About 30 μg of protein from fraction VI was applied to each of two Tris–tricine gels (6% acrylamide). After electrophoresis, one gel was stained and the other was sliced into 1.5-mm pieces. Each piece was placed in a separate tube and 0.10 ml of buffer (20 mM sodium phosphate, pH 7.0, 10 mM mercaptoethanol, containing 20% glycerol) was added. After elution for 12 hr at 4°, the fractions were assayed for GlyRS activity.

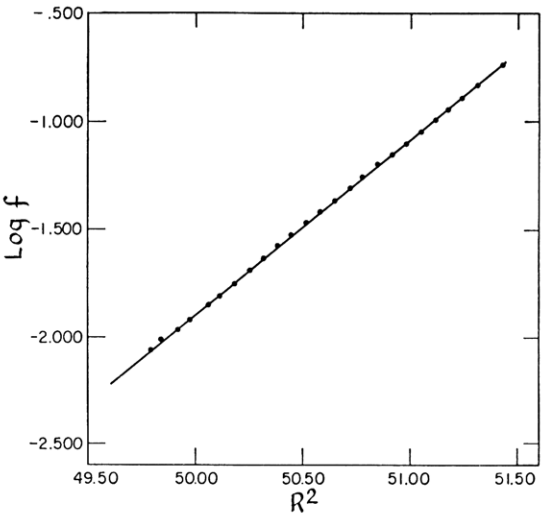

FIGURE 4: Sedimentation equilibrium ultracentrifugation. The centrifugation was carried out as described in Methods. The protein concentration was 0.5 mg/ml and the buffer was 10 mM potassium phosphate (pH 7.0), 100 mM KCl, 10 mM glycine, 10 mM $MgCl_2$, 2 mM ATP, and 1 mM dithiothreitol. Pictures were taken 2, 6, and 25 hr after attainment of equilibrium. Each point shown is the average of measurements on each of the three pictures.

showed only traces of the minor bands (Figure 3a); moreover, all of the enzymatic activity that could be recovered from the gel was coincident with the major band (Figure 3b). By this criterion, the enzyme preparation appears to be essentially free of contaminating protein.

Physical Properties of Glycyl-tRNA Synthetase. MOLECULAR WEIGHT. The molecular weight of the enzyme was determined by sedimentation equilibrium ultracentrifugation using the meniscus-depletion method described by Yphantis (1964). Figure 4 shows a plot of log f (fringe displacement) *vs.* the square of the radius. Using the slope of this line and a value of 0.740 cm^3/g for the partial specific volume of the protein (estimated from the amino acid composition (McMeekin and Marshall, 1952)), a weight-average molecular weight of 227,000 was calculated. In addition, the linearity of this plot indicates that there is no appreciable size heterogeneity in the enzyme preparation.

A second estimation of molecular weight was made using the polyacrylamide gel electrophoresis method described by Hedrick and Smith (1968) and Rodbard and Chrambach (1970). The method is based upon the relationship: $\log M = \log M_0 - K_r T$, where M is the electrophoretic mobility of the protein, M_0 is the free mobility, K_r is the retardation coefficient, and T is the total gel concentration. Determining the R_m for a protein (its mobility relative to that of a dye marking the electrophoretic front) at different gel concentrations, and then plotting $\log R_m$ *vs.* T, yields a line with a slope (K_r) which is characteristic of the molecular weight of the protein, a steeper slope indicating a greater molecular weight. The K_r values were determined in this way for GlyRS and several standard proteins, and Figure 5 shows a plot of the K_r for each protein *vs.* its molecular weight. The molecular weight for GlyRS obtained in this way was about 240,000.

SUBUNIT STRUCTURE. In an earlier paper (Ostrem and Berg, 1970) we reported that electrophoretic analysis of purified GlyRS in polyacrylamide gels containing sodium dodecyl sulfate revealed two protein bands, suggesting that the enzyme is an oligomeric protein with dissimilar subunits. The mobility of the slower moving component, β, was consistent with a molecular weight of 80,000 (with reference to several known proteins as markers) while the mobility of the other band, α, corresponded to a molecular weight of 33,000. Protein which had been reduced with mercaptoethanol and then carboxymethylated yielded the same two bands. Densitometer scans of stained gels show that the amount of protein in β is about 2.6 times that of α; and, therefore, the enzyme is probably a tetrameric protein with the empirical formula $\alpha_2\beta_2$. The molecular weight summed from the subunit weights is 226,000, which is nearly identical with that determined by equilibrium sedimentation.

We also reported that the native enzyme can be dissociated into its subunits by treatment with HO-HgBzO; subunits

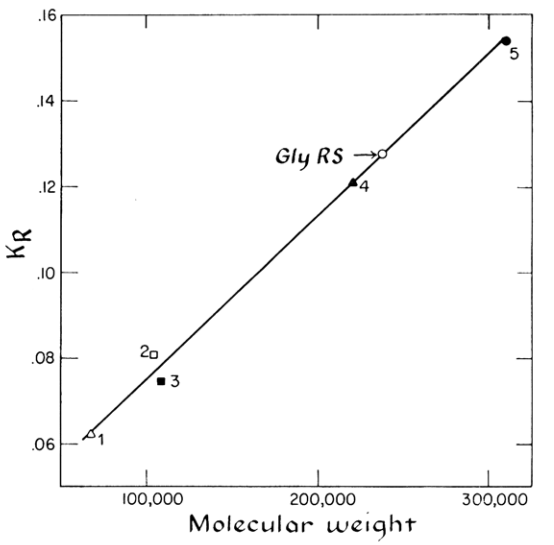

FIGURE 5: Determination of molecular weight of glycyl-tRNA synthetase by polyacrylamide gel electrophoresis. The electrophoresis was carried out using the Tris–tricine system. Gels varied in the total amount of acrylamide, but the ratio of acrylamide to bisacrylamide remained constant at 30:0.8. Electrophoretic mobilities were measured with reference to the migration of Bromophenol Blue. The reference proteins were (△) bovine serum albumin, (□) *E. coli* DNA polymerase (Richardson *et al.*, 1964), (■) *E. coli* isoleucyl-tRNA synthetase (Baldwin and Berg, 1966), (▲) isoleucyl-tRNA synthetase dimer, and (●) *E. coli* aspartate transcarbamylase (Gerhart and Schachman, 1965).

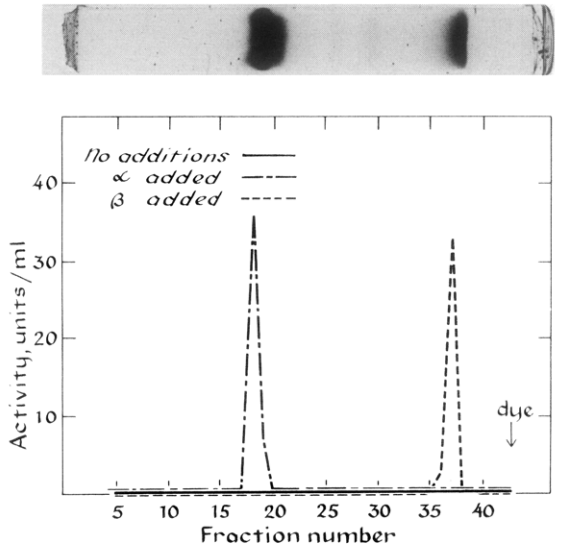

FIGURE 6: Electrophoresis of HO-HgBzO-treated glycyl-tRNA synthetase. A mixture of 40 μg of enzyme and HO-HgBzO (5 mM) was incubated at room temperature for 15 min. Half of the protein was applied to each of two Tris–tricine gels (6% acrylamide) and electrophoresis was carried out at 2.5 mA/gel. One gel was stained and the other was sliced into fractions (1.5 mm) and assayed for glycyl-tRNA synthesis activity. Complementation was done by combining aliquots of each fraction with an excess of one of the subunit proteins (removed from a similar gel) and preincubating for 45 min at 37° in the presence of glutathione, ATP, and Mg^{2+} (Ostrem and Berg, 1970). The remaining substrates were then added and the enzymatic activity was measured.

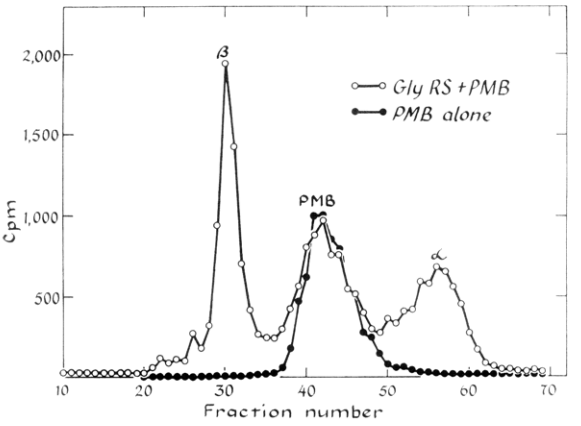

FIGURE 8: Electrophoresis of [^{14}C]HO-HgBzO-treated glycyl-tRNA synthetase (GlyRS). Samples of enzyme (78 pmol in 30 μl of 0.05 M potassium phosphate buffer, pH 7.0, containing 20% glycerol) were treated with [^{14}C]HO-HgBzO (1100 pmol in 0.05 M potassium phosphate, pH 7.0) and the mixtures were diluted fivefold with electrophoresis layering buffer (fivefold-diluted Tris–tricine upper buffer containing 10% glycerol). The samples were then applied to Tris–tricine gels (6% acrylamide, 6 × 80 mm) and electrophoresis was carried out at a constant current of 2 mA/gel. After electrophoresis, the gels were sliced into 1.5-mm pieces which were then soubilized and analyzed for radioactivity as described in Methods. Additional gels were run in parallel and stained for protein to determine the subunit positions, which were found to correspond with the first and last peaks of radioactivity. Control gels run using the enzyme sample buffer without protein showed that the middle peak corresponded to the position of unreacted HO-HgBzO.

isolated after this treatment, however, can reassociate to form active enzyme (Ostrem and Berg, 1970). Although preparations of α subunit were shown to be inactive for both ATP–PP_i exchange and glycyl-tRNA synthesis, fractions of β subunit had low activity in both assays. However, there was some question about whether this activity was intrinsic to the β subunit or due to contamination by α, since during the isolation of the β subunit the preparation could have been contaminated by some undissociated enzyme left after HO-HgBzO treatment. To resolve this issue, HO-HgBzO-treated enzyme was subjected to polyacrylamide gel electrophoresis, using conditions which would maximize the separation between β subunit and undissociated enzyme (stacking gel, longer resolving gel, smaller fractions). The gel was sliced into small sections, each of which was extracted and assayed for glycyl-tRNA synthesis. None of the fractions were active when assayed under the usual conditions (Figure 6). However, fractions corresponding in position to the β band on a stained gel were active when assayed in the presence of added α protein; and, similarly, the α fractions were active in the presence of added β. Furthermore, neither of the subunits isolated in this manner had any detectable activity in catalyzing glycine-dependent ATP–PP_i exchange. We conclude, therefore, that each subunit is enzymatically inactive in both the exchange and charging reactions (less than 0.1% activity would have been detected in either case).

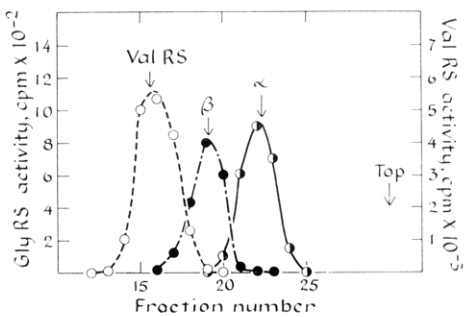

FIGURE 7: Sucrose density gradient sedimentation of isolated α and β subunits. Preparations of each subunit were sedimented in separate tubes (gradients of 5–20% sucrose in 0.1 M potassium phosphate, pH 7.0, containing 0.01 M mercaptoethanol) for 11 hr at 55,000 rpm. Fractions were collected and assayed by complementation as described in the legend to Figure 6. Valyl-tRNA synthetase (ValRS) was included in each tube as a marker.

To determine whether treatment of GlyRS with HO-HgBzO dissociates the enzyme into free α and β subunits or into α_2 and/or β_2 dimers, the separated subunits recovered from polyacrylamide gels (as in Figure 6) were sedimented in a sucrose density gradient (Figure 7) according to Martin and Ames (1961). Using valyl-tRNA synthetase (5.6 S, mol wt 1.1×10^5, Yaniv and Gros, 1969) as an internal marker, sedimentation coefficients for α and β were calculated to be 3.1 and 4.3, respectively. Assuming the proteins are globular, these values correspond to molecular weights of about 44,000 for α and 74,000 for β. These figures indicate that treatment of GlyRS with HO-HgBzO probably dissociates the enzyme into free monomers and not into α_2 or β_2 forms.

In order to determine how many of the enzyme's eight sulfhydryl residues react with HO-HgBzO, 65 pmol of purified enzyme was treated with an 80-fold excess of [^{14}C]HO-HgBzO, and the mixture was filtered through a Sephadex G-25 column. A total of 523 pmol of [^{14}C]HO-HgBzO eluted with the protein at the void volume (8.1 mol of HO-HgBzO/mol of enzyme), indicating that all of the sulfhydryl groups had reacted. Another experiment confirmed this result and allowed us to estimate the number of sulfhydryl residues which are on each subunit. Since reaction of GlyRS with HO-HgBzO causes the protein to dissociate into the individual subunits, enzyme which had been treated with [^{14}C]HO-HgBzO was analyzed by polyacrylamide gel electrophoresis (Figure 8). From several

TABLE II: Amino Acid Composition of Glycyl-tRNA Synthetase.[a]

Amino Acid	mol/225,000 g of Enzyme
Phenylalanine	92
Tyrosine	57
Leucine	222
Isoleucine	79
Methionine	36
Valine	134
Alanine	220
Glycine	138
Proline	108
Glutamic acid	259
Serine	75
Threonine	105
Aspartic acid	220
Arginine	123
Histidine	31
Lysine	112
Tryptophan	22
Cysteine	8

[a] The amino acid composition was determined as described under Methods.

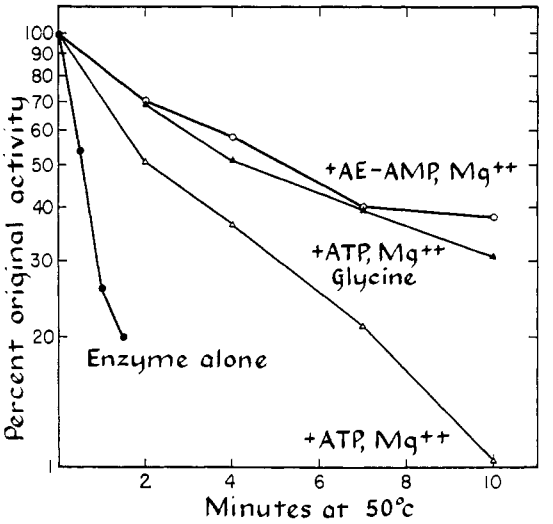

FIGURE 9: Thermal inactivation of glycyl-tRNA synthetase and the effect of substrates. Enzyme (about 10 μg) was incubated at 50° in 100 mM sodium cacodylate, pH 7.0, containing 1 mM dithiothreitol and 0.4 mg/ml of bovine serum albumin. At various times aliquots were removed, diluted tenfold, and assayed for glycyl-tRNA synthesis activity. When substrates were added the concentrations were 1 mM for ATP, 10 mM for $MgCl_2$, 1 mM for glycine, and 1 mM for aminoethyl-AMP (AE-AMP).

experiments, the total number of sulfhydryl groups which react with [^{14}C]HO-HgBzO was found to be 8.0 ± 0.4 per tetramer, and these are distributed nearly equally between the α and β subunits. We conclude, therefore, that GlyRS probably has two sulfhydryl groups on each of the four subunits, and that all the sulfhydryls are available for reaction with HO-HgBzO.

AMINO ACID ANALYSIS. The amino acid composition of GlyRS, analyzed as described in Methods, is presented in Table II. Except for the relatively few cysteine residues, the composition is generally unremarkable. As noted above, each of the cysteines exists as a free sulfhydryl residue. These sulfhydryl groups also react with *N*-ethylmaleimide (see later section), and this modification has profound effects on the catalytic properties of the enzyme.

STABILITY. GlyRS, like several other aminoacyl-tRNA synthetases, is unstable unless it is stored in the presence of glycerol. When a partially purified enzyme preparation (specific activity 860 units/mg, total protein 0.9 mg/ml, GlyRS about 8 μg/ml) was stored at 4° without glycerol (in 0.03 M potassium phosphate, pH 7.2, containing 0.01 M mercaptoethanol), the enzyme lost about 90% of its activity for glycyl-tRNA synthesis during a period of 2 weeks. However, when this inactivated enzyme was concentrated tenfold and dialyzed against a buffer containing glycerol (0.02 M potassium phosphate, pH 7.0, containing 0.01 M mercaptoethanol and 35% glycerol), there was a time-dependent recovery of the activity which reached nearly 70% of the original level after 1 week. Thus, the relatively rapid inactivation seen under the above conditions (low enzyme concentration, no glycerol) appears to be reversible. In contrast, when purified enzyme was stored at a concentration of 0.5 mg/ml under the conditions described earlier (−15°, in 40% glycerol), the slow decay of activity which we observed (30% loss in 8 months) could not be reversed.

THERMAL INACTIVATION. When GlyRS is heated at 50° in the absence of substrates, rapid inactivation occurs (Figure 9). The rate of inactivation is not significantly altered by the presence of glycine, tRNA, and Mg^{2+}, or glycine plus tRNA and Mg^{2+} in the incubation mixture.[4] However, appreciable protection of the enzyme is seen when the heating is carried out in the presence of ATP and Mg^{2+}; and even greater stabilization is seen with the combination of glycine, ATP, and Mg^{2+}. Significantly, aminoethyl-AMP, an analog of the glycyl-AMP intermediate which competitively inhibits the catalytic activity of GlyRS,[4] protects the enzyme to the same degree as the combination of substrates. This enzyme, therefore appears to resemble several other aminoacyl-tRNA synthetases which are more stable in the form of their enzyme–aminoacyl-AMP complexes (Baldwin and Berg, 1966; Iaccarino and Berg, 1969; Yaniv and Gross, 1969; Kosakowski and Böck, 1971).

Catalytic Properties. KINETIC PARAMETERS. The turnover number of GlyRS, based on a specific activity of 96,000 nmol of glycyl-tRNA synthesized in 10 min/mg of protein, is about 2200 min^{-1}. Assuming that enzyme in the original crude extract has the same specific activity as the purified enzyme, we calculate that there are about 1000 enzyme molecules/cell. By comparison, turnover numbers between 6 and 1200 and estimates of 1400–6000 enzyme molecules/cell have been made for other aminoacyl-tRNA synthetases (Fangman *et al.*, 1965; Calendar and Berg, 1966a,b; Joseph and Muench, 1971). Thus, while GlyRS has the highest turnover number yet reported, the number of enzyme molecules per cell appears to be on the low side.

When the kinetic properties of the enzyme are determined using the ATP–PP_i exchange assay, the results are different from those obtained from measurements of glycyl-tRNA synthesis. Under similar reaction conditions the ATP–PP_i exchange rate is only 40–45% of the rate of the aminoacylation reaction. This seems paradoxical since the exchange assay measures the rate of formation of glycyl-AMP, the putative intermediate in the synthesis of glycyl-tRNA. Since the major difference in the two reactions is the presence of tRNA in the charging assay, the ATP–PP_i exchange rate was measured in the presence of tRNA. Figure 10 shows that the rate of ATP–

[4] Ostrem, D. (1973), unpublished observations.

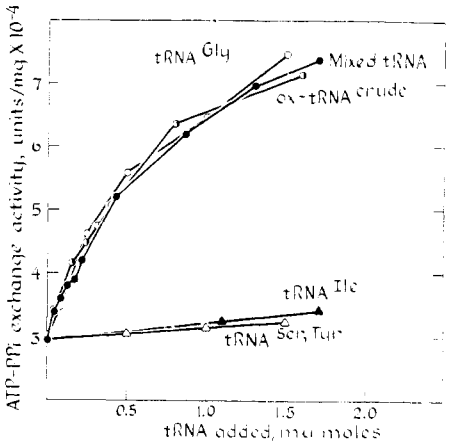

FIGURE 10: Effect of tRNA on the rate ATP–PP_i exchange. Enzyme activity was measured using the exchange assay conditions described in Methods except that varying amounts of tRNA were added. "Mixed" tRNA refers to unfractionated *E. coli* tRNA and "ox-tRNA" refers to periodate-oxidized tRNA (see Methods). When mixed tRNA was used, the amount added was based on the amount of $tRNA^{Gly}$ chains originally present.

PP_i exchange is increased by tRNA, the amount of stimulation being dependent upon the amount of tRNA added, and reaching a maximum of about 2.5-fold. The effect is due to the $tRNA^{Gly}$ chains in the tRNA preparation, since purified $tRNA^{Gly}$ stimulates as effectively as unfractionated tRNA, while neither $tRNA^{Ile}$, nor a mixture of $tRNA^{Ser}$ and $tRNA^{Tyr}$, has a significant effect. The stimulation occurs, however, whether or not the tRNA can be esterified with glycine, since periodate-oxidized tRNA is just as effective as native tRNA.

The effect of tRNA on the activity of GlyRS is also seen when the Michaelis constants for ATP and glycine are determined (Table III). The K_m values for ATP and glycine in the normal ATP–PP_i exchange reaction (no tRNA present) were significantly lower than those found for glycyl-tRNA synthesis. However, in the presence of tRNA, the K_m's for ATP and glycine in the exchange reaction were comparable to those obtained for glycyl-tRNA formation.

DETERMINATION OF THE NUMBER OF GLYCYL-AMP BINDING SITES. To determine the number of glycyl-AMP binding sites, the gel filtration method described by Norris and Berg (1964) was used for isolating the enzyme–glycyl adenylate complex (Figure 11). Enzyme was incubated at room temperature in buffer with [^{1}H]- or [^{3}H]ATP, $MgCl_2$, and [^{12}C]- or [^{14}C]glycine and applied to a Sephadex column for filtration at 4°.

TABLE III: Effect of $tRNA^{Gly}$ on the Michaelis Constants for ATP and Glycine in the ATP–PP_i Exchange Reaction and for Glycyl-tRNA Synthesis.[a]

	K_m (mol/l.)		
Substrate	Glycyl-tRNA Formation	ATP–PP_i Exchange − tRNA	ATP–PP_i Exchange + tRNA
Glycine	1.6×10^{-4}	0.3×10^{-4}	1.4×10^{-4}
ATP	4.2×10^{-5}	1.4×10^{-5}	4.0×10^{-5}
$tRNA^{Gly}$	2.0×10^{-7}		

[a] The values were determined by measuring initial reaction velocities with varying concentrations of one substrate and saturating amounts of the others. The data were analyzed according to Lineweaver and Burk (1934).

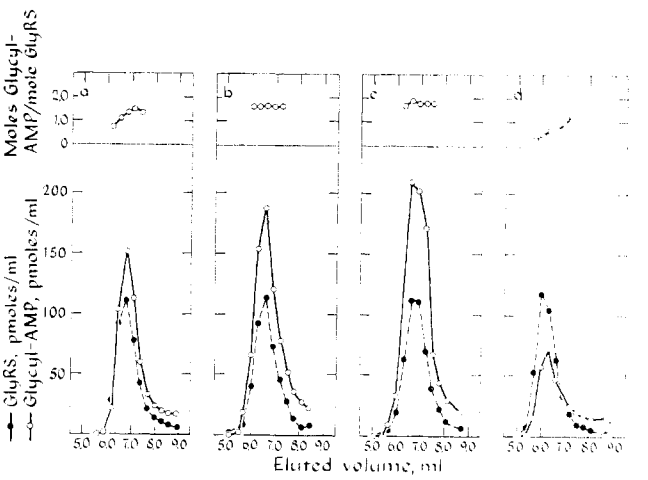

FIGURE 11: Isolation of the glycyl-tRNA synthetase–glycyl-AMP complex. Enzyme (about 250 pmol) was incubated for 20 min at room temperature in 100 mM sodium cacodylate (pH 7.0) containing 10 mM $MgCl_2$, 4 mM glutathione, 0.3 mg/ml of bovine serum albumin, 0.3 mM [^{14}C]glycine, and 1 mM ATP in a total volume of 0.20 ml. Glycerol (0.05 ml) was added and the mixture was cooled on ice and applied to a column (0.9 × 30 cm) of Sephadex G-75 which had been equilibriated with 50 mM sodium succinate (pH 6.0) containing 2 mM glutathione, 1 mM EDTA, and 25% glycerol. Fractions of 0.30 ml were collected at a flow rate of 0.1 ml/min. Separate aliquots of each fraction were assayed for GlyRS activity and counted for radioactivity using Bray's scintillation fluid (Bray, 1960). When tRNA was used, it was added to the incubation mixture and to the equilibration and elution buffers.

Fractions were collected, analyzed for radioactivity, and assayed for enzyme activity. Although the enzyme was relatively stable under the conditions used (recovery of activity was generally about 80%), the complex of enzyme with glycyl adenylate appeared to be unstable. The amount of glycyl adenylate appearing with the enzyme varied from a value of about 0.5 mol of glycyl adenylate/mol of enzyme tetramer at the leading edge of the peak to greater than 2.5 at the trailing edge. In separate experiments, the average value across the five most active fractions varied from 0.8 to 1.2 mol of glycyl adenylate per mol of tetramer. In contrast to the above results, when the Sephadex column was preequilibrated with periodate-oxidized tRNA, the enzyme–glycyl adenylate complex appeared to be stable during the isolation procedure (Figure 11b); and across the entire peak the binding ratio was constant at 1.8 mol of glycyl adenylate/mol of GlyRS tetramer. This effect of tRNA was specific for $tRNA^{Gly}$, since purified $tRNA^{Gly}$ stabilized the complex as effectively as mixed tRNA (Figure 11c), but a mixture of $tRNA^{Ser}$ and $tRNA^{Tyr}$ did not (Figure 11d). Since this particular method does not measure substrate binding under equilibrium conditions, the binding ratio determined in this way is a minimum value, and the true ratio in this case is probably two molecules of glycyl adenylate bound per enzyme tetramer.

The following controls show that the radioactivity can be attributed to enzyme-bound glycyl adenylate: (1) when ATP was omitted from the incubation mixture, no complex was formed; (2) in the absence of glycine, only a small amount of ATP was bound (this effect was also noted by Norris and Berg, 1964); (3) when both [^{3}H]ATP and [^{14}C]glycine were used as substrates, the ratio of bound ^{3}H to bound ^{14}C was close to unity (1.0–1.2); (4) the radioactivity appearing with the enzyme was not acid precipitable; and (5) greater than 70% of the labeled glycine could be transferred to tRNA in the usual glycyl-tRNA synthesis reaction.

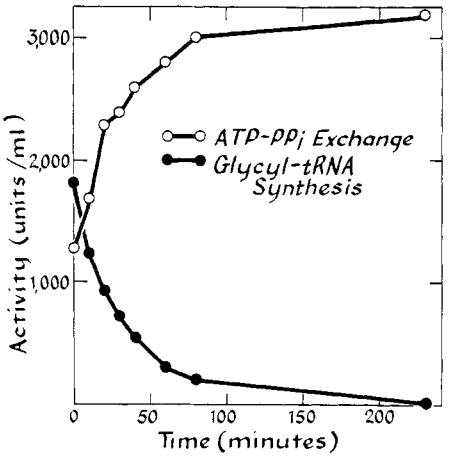

FIGURE 12: Effects of *N*-ethylmaleimide (NEM) treatment on glycyl-tRNA synthesis and ATP–PP_i exchange activities. About 20 μg of enzyme (from a solution containing 0.5 mg of protein/ml in 0.05 M potassium phosphate, pH 7.0, with 10^{-5} M dithiothreitol and 20% glycerol) was made 1.0 mM in NEM (by adding an equal volume of 2.0 mM NEM in 0.05 M potassium phosphate, pH 7.0) and the mixture was incubated at 21°. At various times, aliquots were taken, diluted tenfold (with 0.01 M potassium phosphate, pH 7.0, containing 1 mM dithiothreitol, 1 mg/ml of bovine serum albumin, and 10% glycerol) and assayed for ATP–PP_i exchange and glycyl-tRNA synthesis activities.

Effect of N-Ethylmaleimide on Glycyl-tRNA Synthetase. Treatment of GlyRS with the sulfhydryl reagent *N*-ethylmaleimide causes a progressive decrease in the capacity of the enzyme to synthesize glycyl-tRNA (Figure 12), although in this case polyacrylamide gel electrophoresis reveals that there is no concomitant dissociation of the protein into its subunits.[4] In a typical experiment, 10% of the glycyl-tRNA synthesis activity remained after 90-min *N*-ethylmaleimide treatment, and the enzyme was essentially inactive after 4 hr. On the other hand, when measured in the same experiment, the ATP–PP_i exchange activity was progressively elevated, the stimulation reaching nearly threefold after 90 min (Figure 12). Thus, following treatment with *N*-ethylmaleimide, the enzyme can still form the enzyme–glycyl-AMP intermediate (actually at a threefold increased turnover rate), but it cannot transfer the activated glycine residue to tRNA.

It is possible that reaction with *N*-ethylmaleimide eliminates the tRNA binding capacity of the enzyme; this seems plausible because the ATP–PP_i exchange activity of *N*-ethylmaleimide-treated enzyme is no longer stimulated by tRNA, as is untreated enzyme (Table IV). To test this possibility, the nitrocellulose filter assay described by Yarus and Berg (1967) was used to compare the binding of $tRNA^{Gly}$ by native and *N*-ethylmaleimide-modified GlyRS. *N*-Ethylmaleimide-treated enzyme, having less than 2% of the original activity for glycyl-tRNA synthesis (the ATP–PP_i exchange activity was stimulated 3.3-fold), still retained significant tRNA-binding capacity (Figure 13a). A double-reciprocal plot (Figure 13b) shows that while *N*-ethylmaleimide-modified enzyme has a threefold-reduced affinity for tRNA, its maximum binding capacity is only slightly less than that of untreated enzyme. This evidence indicates that *N*-ethylmaleimide-modified enzyme still binds tRNA under the normal charging reaction conditions ($tRNA^{Gly}$ about 4×10^{-6} M) and that the failure to form glycyl-tRNA has another cause.

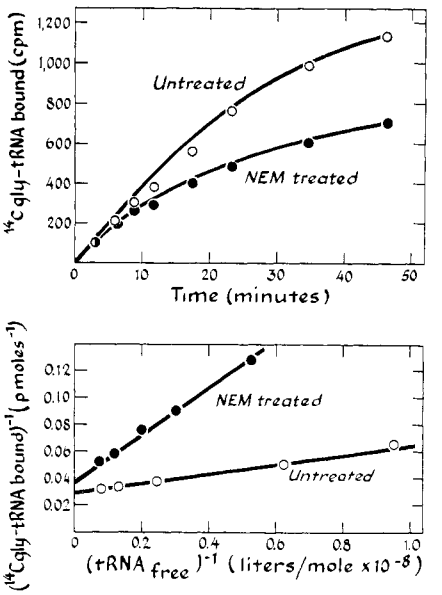

FIGURE 13: Binding of $tRNA^{Gly}$ by untreated and NEM-modified glycyl-tRNA synthetase. The binding of [^{14}C]glycyl-tRNA to the enzyme was measured using the nitrocellulose filter assay described by Yarus and Berg (1967). The particular conditions used in the experiment (binding buffer–50 mM sodium cacodylate, pH 5.6, containing 6 mM $MgCl_2$, 1 mM dithiothreitol, and 50 μg/ml of bovine serum albumin; wash buffer–50 mM sodium cacodylate, pH 5.6) yielded a calculated binding efficiency of 21%. NEM modification was carried out by treating a total of 380 pmol of enzyme with NEM (at a final concentration of 4 mM) for 60 min at 21°. The resulting modified enzyme had less than 2% of its initial activity for glycyl-tRNA synthesis, while the ATP–PP_i exchange activity was increased 3.3-fold.

TABLE IV: Effect of tRNA on the ATP–PP_i Exchange Activity of *N*-Ethylmaleimide-Modified Glycyl-tRNA Synthetase.[a]

Time for Reaction of Enzyme with NEM[b] (min)	ATP–PP_i Exchange Act. (Units/ml)	
	− tRNA	+ tRNA
0	1140	2120
40	2600	2860
80	3000	3000
230	3170	2980

[a] Enzyme was treated with *N*-ethylmaleimide as described in the legend to Figure 12. At various times aliquots were removed and assayed for ATP–PP_i exchange activity with and without added tRNA. The concentration of $tRNA^{Gly}$ in the assay mixture was about 4×10^{-6} mol/l. [b] NEM = *N*-ethylmaleimide.

How many sulfhydryl groups must be modified by *N*-ethylmaleimide to inactivate the aminoacyl-transfer reaction? After incubation of GlyRS with [^{14}C]*N*-ethylmaleimide for 120 min, by which time the enzyme is inactivated more than 90%, less than three molecules of *N*-ethylmaleimide have been incorporated per enzyme tetramer (Figure 14). To localize the site of *N*-ethylmaleimide incorporation, enzyme was reacted with unlabeled *N*-ethylmaleimide, and the remaining sulfhydryl residues were titrated with [^{14}C]HO-HgBzO. The modified enzyme, labeled and dissociated in this manner (*N*-ethylmaleimide treatment does not interfere with the ability of HO-HgBzO to dissociate the enzyme), was subjected to polyacrylamide gel electrophoresis to separate the subunits. Table V shows that with the untreated enzyme, 7.8 sulfhydryl groups reacted with HO-HgBzO, but with the *N*-ethylmaleimide-treated enzyme, only 5.0 sulfhydryl groups could react with HO-HgBzO. Of the 2.8 sulfhydryl residues thus blocked

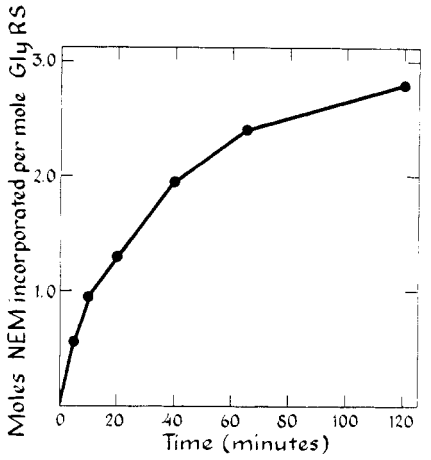

FIGURE 14: Kinetics of [^{14}C]NEM incorporation into glycyl-tRNA synthetase. Enzyme (1500 pmol in 0.65 ml) was treated with [^{14}C]-NEM (1.0 mM final concentration) as described in the legend to Figure 12. At various times 0.10-ml aliquots were withdrawn and diluted 1:2 with a mixture of mercaptoethanol (0.02 M) and bovine serum albumin (0.1 mg/ml). The protein in each tube was precipitated and the radioactivity was analyzed as described by Iaccarino and Berg (1969).

by *N*-ethylmaleimide, 2.6 were on the β subunit and 0.2 were on α. These results indicate that the changes in the catalytic properties of GlyRS caused by *N*-ethylmaleimide treatment probably result from the modification of one sulfhydryl group per β subunit.

Discussion

The isolation of pure glycyl-tRNA synthetase (GlyRS) has enabled us to detect and study several interesting features of the enzyme's structure and catalytic activity. The purified enzyme is a tetramer, mol wt 225,000, constructed from two small (α subunit, mol wt 33,000) and two large (β subunit, mol wt 80,000) polypeptide chains. Under certain conditions, but most efficiently by reaction of the two free sulfhydryl groups of each subunit with HO-HgBzO, the enzyme is dissociated into its constituent polypeptide chains; the tetrameric structure, and fully active enzyme, can be reconstituted by removal of the mercurial from a mixture of purified, derivatized α and β subunits (Ostrem and Berg, 1970). Although the data strongly support our conclusion that purified GlyRS is an $\alpha_2\beta_2$-type tetramer, it is difficult to prove beyond question that this is the true structure *in vivo*, particularly since it has been reported that proteolysis of certain aminoacyl-tRNA synthetases may occur during their purification (Lawrence *et al.*, 1973; Rouget and Chapeville, 1971b). We do not believe that the structure of GlyRS is altered during purification for the following reasons: (1) if there is proteolysis, for example during the autolysis procedure, it must not affect the activity of the enzyme, since all of the activity is recovered; (2) there would have to be a total conversion, since only one species of GlyRS is found; and (3) when GlyRS in a fresh sonic extract (which had been maintained at 0–4° during preparation was treated with HO-HgBzO, subsequent velocity sedimentation in a sucrose density gradient revealed only α and β subunits, and no species of higher molecular weight, such as a covalently connected $\alpha\beta$ unit, were seen.[4]

From the fact that the native enzyme binds 2 equiv of glycyl adenylate/tetramer, we infer that there are two active sites per enzyme molecule. Quite clearly, neither purified α nor β alone is able to catalyze either the overall reaction, or the glycine-dependent ATP–PP$_i$ exchange; but in some way the two subunits complement each other. Each subunit might contribute directly to the structure and workings of the active sites, or one subunit might induce the catalytically active conformation in the other. Our earlier finding that mutations in either the α or β subunit of GlyRS (Folk and Berg, 1970; Ostrem and Berg, 1970) can alter the kinetic parameters of the enzyme does not distinguish between these possibilities. It may be that the minimal catalytically active structure is the $\alpha\beta$ dimer, but further studies are needed to explore this possibility.

The amino acid dependent ATP–PP$_i$ exchange catalyzed by most aminoacyl-tRNA synthetases is unaffected by the presence of their cognate tRNAs. However, some are absolutely dependent on tRNA (Ravel *et al.*, 1965; Deutscher, 1967; Mehler and Mitra, 1967; LaPointe and Söll, 1972; Parfait and Grosjean, 1972), while others are stimulated (Loftfield and Eigner, 1965; James *et al.*, 1968; James and Bucovaz, 1969; Charlier, 1972) and a few are inhibited (Yarus and Berg, 1969; Buonocore and Schlesinger, 1972). With GlyRS the rate of exchange increases two- to threefold on binding tRNA^{Gly}; and since periodate-oxidized tRNA^{Gly} is just as efficient in this regard, the stimulation is clearly independent of aminoacylation. Besides increasing the rate of the ATP–PP$_i$ exchange reaction, the binding of tRNA^{Gly} to the enzyme increases the Michaelis constants for ATP and glycine. At this time it is not known whether the effect of tRNA^{Gly} on these kinetic parameters is related to the stabilization of the enzyme–glycyl adenylate intermediate. Other aminoacyl-tRNA synthetases may also have their Michaelis constants affected by interaction with cognate tRNA; for example, the K_m for isoleucine and that for tyrosine are increased two- to threefold when their respective aminoacyl-tRNA synthetases bind the corresponding tRNAs (Yarus and Berg, 1969; Buonocore and Schlesinger, 1972). The molecular basis for these affects is also unknown.

Turning to the effects of sulfhydryl modification on the properties of the enzyme, we have shown that when the enzyme's eight sulfhydryl residues, which are distributed equally among the four subunits, react with HO-HgBzO, the enzyme dissociates into α and β monomers. *N*-Ethylmaleimide, on the other hand, probably reacts readily with only one of the

TABLE V: Location of Sulfhydryl Residues Reacted with *N*-Ethylmaleimide.[a]

	Total	β	α
Sulfhydryl residues reacting with HO-HgBzO before NEM treatment	7.8	4.0	3.8
Sulfhydryl residues reacting with HO-HgBzO after NEM treatment	5.0	1.4	3.6
Sulfhydryl residues reacted with NEM	2.8	2.6	0.2

[a] Enzyme (76 pmol in 45 μl of 0.05 M potassium phosphate, pH 7.0, containing 10^{-5} M dithiothreitol and 20% glycerol) was made 1.0 mM in *N*-ethylmaleimide (by adding 15 μl of a 4.0 mM solution in 0.05 M potassium phosphate, pH 7.0) and the mixture was incubated at 21°. After 100 min, [^{14}C]HO-HgBzO (1650 pmol in 15 μl) and Tris–tricine electrophoresis layering buffer (75 μl) were added and the mixture was analyzed electrophoretically as described in the legend to Figure 11.

sulfhydryl residues of each β subunit. The consequence of this irreversible covalent modification is not dissociation of the tetramer, but rather a drastic alteration in the catalytic behavior of the enzyme. The modified enzyme fails to catalyze the formation of glycyl-tRNA, but it carries out ATP–PP_i exchange at a two- to threefold-increased rate. Thus, *N*-ethylmaleimide modification of one sulfhydryl residue per β subunit "uncouples" the activation and acylation steps of GlyRS. The effect of the *N*-ethylmaleimide modification on the ATP–PP_i exchange reaction is similar to that caused by $tRNA^{Gly}$ binding to the unmodified enzyme; significantly, in the *N*-ethylmaleimide-modified enzyme, binding of $tRNA^{Gly}$ produces no further increase in the rate of ATP–PP_i exchange. Perhaps both the *N*-ethylmaleimide modification and $tRNA^{Gly}$ binding alter the behavior or location of a sulfhydryl residue which interferes in some way with the glycine-dependent ATP–PP_i exchange reaction. Such a sulfhydryl residue might actually participate in the catalytic events (as a transient acceptor–donor of the aminoacyl group?); or alternatively, modification of the β-subunit sulfhydryl residue could influence the conformational parameters of the catalytic site. These and other possibilities need further study. It is of interest that modification of sulfhydryl residues on other aminoacyl-tRNA synthetases also causes selective alterations in their catalytic activities. For example, modification of a single sulfhydryl residue of leucyl-tRNA synthetase (LeuRS) (Rouget and Chapeville, 1971a) causes an apparent uncoupling of the acylating and exchange sites similar to that reported here. The modified LeuRS still activates leucine (at a slightly reduced rate), but the activated amino acid cannot be transferred to $tRNA^{Leu}$, even though the tRNA still binds effectively. The modification also prevents another type of interaction between the binding site for $tRNA^{Leu}$ and the sites for leucine and ATP. While the rate constants for the binding of $tRNA^{Leu}$ by native LeuRS are increased in the presence of leucine and ATP, these substrates have no effect on tRNA binding by the modified enzyme. Similarly, Iaccarino and Berg (1969) have shown that *N*-ethylmaleimide modification of isoleucyl-tRNA synthetase eliminates tRNA acylating activity without preventing the binding of $tRNA^{Ile}$. In this case, however, the isoleucine-dependent ATP–PP_i exchange rate of the modified enzyme is markedly reduced.

In view of our results showing that tRNA binding and *N*-ethylmaleimide modification have similar effects on the exchange activity of GlyRS, it would be especially interesting to test whether any of the other aminoacyl-tRNA synthetases which are stimulated by tRNA, particularly those with absolute requirements for tRNA, would be affected (lose their tRNA requirement?) following *N*-ethylmaleimide treatment.

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Characterization of the Peptidyltransferase Reaction Catalyzed by Rat Liver 60S Ribosomal Subunits†

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ABSTRACT: The formation of acetylphenylalanyl-puromycin from acetyl[^{3}H]phenylalanyl-tRNA and puromycin was catalyzed by isolated rat liver 60S subunits in the presence of 4 mM $MgCl_2$, 0.3 M KCl, and 33% alcohol, at pH 7.5. Poly(U) stimulated significantly the reaction with acetylphenylalanyl-tRNA, especially if allowed to react with 60S subunits prior to the peptidyltransferase assay, but appeared to inhibit the reaction with other acetylated aminoacyl-tRNAs; peptidyltransferase with acetylated lysyl-tRNA was stimulated slightly by preincubation of the 60S particle with poly(A). Preincubation of 60S subunits with 40S particles markedly inhibited the peptidyltransferase reaction.

The peptidyltransferase reaction represents a ribosomal activity that catalyzes the synthesis of peptide bonds between peptidyl-tRNA and aminoacyl-tRNA on adjacent ribosomal sites (see review by Lucas-Lenard and Lipmann (1971)). This activity also appears to participate in the release of the completed polypeptide chain, from peptidyl-tRNA, in the process of termination (Caskey *et al.*, 1971). Studies in both bacterial (Monro, 1967; Maden *et al.*, 1968; Monro *et al.*, 1969; Ballesta *et al.*, 1971; Nierhaus and Montejo, 1973) and mammalian (Vazquez *et al.*, 1969; Falvey and Staehelin, 1970) systems indicate that only the large subunit of the ribosome is required for transpeptidation. Using ribosomes, or the large ribosomal subunit (50 S or 60 S) and relatively high concentrations of alcohol, peptidyltransferase can be assayed by the reaction between an acylaminoacyl-tRNA or acylaminoacyl-oligonucleotide donor and puromycin (Monro and Marcker, 1967; Monro *et al.*, 1969; Pestka, 1970; Monro, 1971; Lessard and Pestka, 1972; Nierhaus and Montejo, 1973). The binding to ribosomes and activity of aminoacylated oligonucleotides of varying composition and chain length have also been reported (Hussain and Ofengand, 1972).

Previous studies from this laboratory and others described the synthesis of peptide bonds, from 80S ribosome-bound peptidyl-tRNA and exogenous aminoacyl-tRNA or puromycin (Skogerson and Moldave, 1968a, 1968b; Pestka *et al.*, 1972; Pestka, 1972; Schneider and Maxwell, 1973) and from exogenous acetylphenylalanyl-tRNA and puromycin with stripped 80S or 70S ribosomes (Siler and Moldave, 1969b; Pestka, 1970). This communication describes the characteristics of the peptidyltransferase reaction with isolated rat liver ribosomal 60S subunits and the effects of some components of the translational system on this reaction.

Materials and Methods

Rat liver ribosomes were prepared from microsomes, purified by centrifugation through discontinuous sucrose gradients containing 0.5 M NH_4Cl (Skogerson and Moldave, 1967, 1968a), stripped of endogenous peptidyl-tRNA with puromycin (Gasior and Moldave, 1972), and dissociated into subunits with 0.88 M KCl (Martin and Wool, 1968; Gasior and Moldave, 1972). The dissociated subunits were resolved by ultracentrifugation in a linear-with-radius 20–45% sucrose gradient using a Beckman Ti-15 zonal rotor; the solutions used for the gradient zonal centrifugation contained 0.88 M KCl, 0.05 M Tris-HCl (pH 7.6), 12.5 mM $MgCl_2$, 6 mM mercaptoethanol, and 1 μg/ml of poly(vinyl sulfate).

Isotopically labeled *Escherichia coli* phenylalanyl-tRNA was prepared as described (Siler and Moldave, 1969a) and acetylated with acetic anhydride (Haenni and Chapeville, 1966); the specific radioactivity of the acetylphenylalanyl-tRNA preparations used varied between 1500 and 3000 cpm/μg of tRNA, and the specific activity of the tRNA-bound [^{3}H]phenylalanine was 5350 cpm/pmol of phenylalanine. The specific radioactivity of acetyl[^{3}H]lysyl-tRNA, prepared by similar procedures, was 1600 cpm/μg of tRNA and the specific activity of the tRNA-bound [^{3}H]lysine was 5000 cpm/pmol of lysine. Acetyl[^{3}H]methionyl-tRNA was prepared by aminoacylation of rat liver $tRNA_i$ with [^{3}H]methionine and acetylation of the Met-$tRNA_i^{Met}$ resolved on BD-cellulose (Smith and Marcker, 1970); the specific radioactivity was 1400 cpm/μg of tRNA and the tRNA-bound [^{3}H]methionine had a specific activity of 1550 cpm/pmol of amino acid.

Incubations for peptidyltransferase activity were, unless otherwise specified, carried out with 60 or 120 pmol of 60S subunits, acetylphenylalanyl-tRNA containing 25–30 pmol of tritium-labeled phenylalanine, 33% methanol, and 0.8 mM puromycin in buffered salts–dithiothreitol solution (40 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 0.3 M KCl, and 1.4 mM dithiothreitol). Some incubations, with 60 pmol of 60S sub-

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