Güntner, C., & Holler, E. (1979) *Biochemistry 18*, 2028.
Gutfreund, H. (1972) in *Enzymes: Physical Principles*, Wiley-Interscience, New York.
Hanke, T., Bartmann, P., Hennecke, H., Kosakowski, H. M., Jaenicke, R., Holler, E., & Böck, A. (1974) *Eur. J. Biochem. 43*, 601.
Holler, E. (1976) *J. Biol. Chem. 251*, 7717.
Kosakowski, H. M., & Holler, E. (1973) *Eur. J. Biochem. 48*, 274.
Pimmer, J., & Holler, E. (1979) *Biochemistry 18*, 3714.
Pingoud, A., Boehme, D., Riesner, D., Kownatzki, R., & Maass, G. (1975) *Eur. J. Biochem. 56*, 617.
Riesner, D., Pingoud, A., Boehme, D., Peters, F., & Maass, G. (1976) *Eur. J. Biochem. 68*, 71.

Overproduction, Purification, and Subunit Structure of *Escherichia coli* Glycyl Transfer Ribonucleic Acid Synthetase†

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ABSTRACT: Glycyl-tRNA synthetase has been purified from an overproducing *Escherichia coli* strain carrying a hybrid *ColE1* plasmid containing the *glyS* locus. A novel scheme making extensive use of blue dextran–Sepharose affinity chromatography has yielded two forms of purified enzyme. Extensive physical and kinetic studies, however, have revealed no significant differences between them. The purified enzyme was found to have a molecular weight near 205 000 and is composed of two α and two β polypeptide chains with molecular weights near 40 000 and 65 000, respectively. Analyses of sedimentation velocity and sedimentation equilibrium data indicate that the enzyme is nonspherical and possesses considerable structural anisotropy. The native enzyme was found to dissociate in the presence of 0.5 M NaSCN, and the α and β subunits were readily separated by gel filtration. Following removal of NaSCN, hydrodynamic experiments showed that isolated β protein existed as a monomer in solution while isolated α protein displayed a reversible, self-association. Neither subunit showed significant catalytic activity. The subunits are not irreversibly denatured by NaSCN dissociation, since a complex indistinguishable from the native enzyme with virtually full catalytic activity is formed when the subunits are mixed in a 1:1 molar ratio. From these data, we infer that α–α interactions contribute to the stability of the native enzyme while β–β interactions do not. In addition, it appears that a functional catalytic site requires participation by both types of subunits, either directly via the contribution of amino acid residues by both subunits or indirectly via conformational stabilization.

Aminoacyl-tRNA synthetases catalyze the esterification of a specific amino acid to a cognate tRNA with the concomitant hydrolysis of ATP to AMP and pyrophosphate. Each of these enzymes, therefore, is essential to the synthesis of cellular proteins. Approximately 50 enzymes of this class, largely from procaryotes and yeast, have been isolated and studied from a number of viewpoints [for reviews, see Söll & Schimmel (1974) and Kisselev & Favorova (1974)]. Glycyl-tRNA synthetase from *Escherichia coli* was first isolated by Ostrem & Berg (1970, 1974), who described some of the enzyme's basic properties including an $\alpha_2\beta_2$ subunit structure, rare within this class of enzymes.

Work in our laboratory began with the aim of investigating the functional contribution made by each type of subunit to the native enzyme and to assess the enzyme's potential for regulation. Initial studies demonstrated the existence of two separable forms of enzymatic activity in partially purified preparations (Francis & Nagel, 1976). Significantly, one enzyme form showed a sigmoidal kinetic response to increasing tRNA concentration, suggesting it had the capacity to act as a regulatory element.

As is the case with many of these enzymes, the small amounts of enzyme available from wild type *E. coli* strains made rapid progress on this work difficult. The colony bank assembled by Clarke & Carbon (1976), however, has provided a solution to this problem. In particular, the isolation of a hybrid plasmid containing *glyS*, the structural gene for glycyl-tRNA synthetase, allowed the enzyme to be overproduced in *E. coli*. Some of our data which contributed to the characterization of the *glyS* plasmid are reported here.

Overproduction of the enzyme has allowed us to obtain sufficient quantities of the enzyme to investigate the molecular details of its structure and function. This paper describes the first results we have obtained with enzyme from the overproducing strain. A preliminary report of some of this work has been presented (McDonald et al., 1979).

Experimental Procedure

Materials. Unfractionated *E. coli* B tRNA was purchased from Plenum Scientific Research, Inc. [1-^{14}C]Glycine was obtained from ICN Pharmaceuticals, Inc., or Amersham Corp. Enzyme grade ammonium sulfate was purchased from Schwarz/Mann, Inc. Bovine pancreatic ribonuclease A, deoxyribonuclease I, bovine glutamic dehydrogenase, aldolase, bovine serum albumin, and blue dextran were purchased from Sigma Chemical Co. Sepharose 4B, Sepharose 6B, and Sephadex G-150 were products of Pharmacia Fine Chemicals, Inc. Ultrogel AcA44 was purchased from LKB. Cyanogen bromide and 1-methyl-2-pyrrolidinone were products of Eastman Kodak. Phosphorylase A and soybean trypsin inhibitor were obtained from Worthington Biochemicals Corp. Other chemicals were reagent or higher grade.

Bacterial Strains and Growth. Experiments were performed with the *E. coli* K12 derivative CH754 (*argH metE xyl trpA36 recA56*). This strain, as well as clones carrying four hybrid *ColE1* plasmids containing the *xyl* locus, was obtained

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from the colony bank of Clarke & Carbon (1976). Minimal media contained salts (Vogel & Bonner, 1956) supplemented with required amino acids (200 μg/mL) and xylose or glucose (0.2%) as a carbon source. Enriched medium, L broth, contained 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per L.

For large-scale growth, a 100-mL culture of CH754 (pLCl-3) grown overnight at 37 °C on xylose minimal medium was used to inoculate 18 L of the same medium in a 20-L carboy. This large culture was grown overnight at 37 °C with stirring and mild O_2 aeration and was used, in turn, as the inoculum for growth at the fermenter facility of the Molecular Biology Institute, University of California, Los Angeles. The inoculum was added to the fermenter containing xylose minimal medium to give a total volume of 150–170 L. The fermenter culture was grown overnight at 37 °C under 3 atm of air, and cells were harvested by centrifugation. This procedure yielded ~500 g of packed cells which were stored at −20 °C.

Enzyme Assays. Aminoacylation of tRNA was monitored by measuring the incorporation of [^{14}C]glycine into acid-precipitable tRNA using the procedure of Folk & Berg (1970) as modified by Francis & Nagel (1976). Saturation analysis was performed as described by Francis & Nagel (1976). Exchange activity measured the incorporation of [^{32}P]pyrophosphate into ATP in the presence of unlabeled glycine. The procedure used was that described by Calendar & Berg (1966), except that the standard assay (1 mL) contained 0.1 M sodium cacodylate, 5 mM $MgCl_2$, 10 mM KCl, 0.2 mg/mL bovine serum albumin, 4 mM glutathione, 2 mM ATP, 2 mM sodium [^{32}P]pyrophosphate (10^5 cpm/μmol), and 2 mM glycine (pH 7.1). Measurements of radioactive decay were made with a Beckman LS-230 liquid scintillation system. In both assays, a unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 nmol of product in 10 min at 37 °C.

Protein Determination, Ultrafiltration, and Dialysis. Protein concentrations were measured routinely by the procedure of Toennies & Feng (1965), using bovine serum albumin as a standard. Samples containing interfering substances such as glycerol were dialyzed vs. 0.1 M potassium phosphate buffer (pH 7.0) prior to measurement. For analytical measurements of purified glycyl-tRNA synthetase and its subunits, protein concentrations were determined refractometrically in the analytical ultracentrifuge using synthetic boundary cells and Rayleigh optics. Measurements of protein concentration as well as the relative concentrations of multiple components were also made during sedimentation velocity experiments by integration of Schlieren patterns and correction for radial dilution.

Protein samples were concentrated by ultrafiltration using an Amicon Diaflow apparatus (CDS-10) and PM 10 membranes under N_2 pressure. Dialysis membranes from VWR Scientific, Inc., were softened in hot dilute EDTA[1] solution and rinsed liberally with distilled water before use.

Electrophoresis. Analytical polyacrylamide disc gel electrophoresis was performed with a Tris–Tricine system as described by Ostrem & Berg (1974). Gels containing both 5 and 7.5% acrylamide were employed for analysis of protein purity; the higher acrylamide concentration gave better resolution of native glycyl-tRNA synthetase and its subunits. Mobilities relative to the tracking dye, bromphenol blue, for the native enzyme (0.16), α protein (0.55), and β protein (0.20) were observed at the higher acrylamide concentration. Protein bands were stained with Coomassie Brilliant Blue G-250 as described by Dizel et al. (1972).

Electrophoresis under denaturing conditions was performed in the presence of sodium dodecyl sulfate ($NaDodSO_4$) by the method of Weber & Osborn (1969) as modified by Clark & Switzer (1977). Protein samples were denatured by incubation at 100 °C for 10 min in the presence of 1% $NaDodSO_4$ and 1% β-mercaptoethanol prior to electrophoresis. For determination of polypeptide chain size, phosphorylase A (93 000 daltons), bovine serum albumin (68 000 daltons), bovine glutamate dehydrogenase (55 390 daltons), aldolase (40 000 daltons), and soybean trypsin inhibitor (21 000 daltons) were used as standards.

Analytical Ultracentrifugation. Measurements were made with a Beckman Model E ultracentrifuge equipped with Schlieren and Rayleigh optics. An AnD rotor, 12-mm centerpieces, and an adjustable Rayleigh mask mounted over the collimating lens (Richards et al., 1972) were used throughout. Prior to analysis, protein samples were dialyzed overnight at 4 °C vs. 0.1 M potassium phosphate and 10 mM β-mercaptoethanol (pH 7.0). A Model 6 Nikon profile projector was used to measure boundary positions and fringe displacements.

High-speed, meniscus-depletion sedimentation equilibrium (Yphantis, 1964) was employed to measure molecular weights of glycyl-tRNA synthetase and its subunits. Rotor temperatures between 6 and 13 °C and loading concentrations between 0.3 and 0.7 mg/mL were used routinely. The rotor velocities employed were 14 000 and 16 000 rpm for the native enzyme, 20 000 and 22 000 rpm for β protein, and 30 000 rpm for α protein.

Sedimentation velocity experiments were performed at 60 000 rpm between 15 and 21 °C with Schlieren optics. When two samples were measured simultaneously, one cell was fitted with a 1°, positively wedged quartz window. Sedimentation coefficients were corrected to a solvent having the density and viscosity of H_2O at 20 °C ($s_{20,w}$) as described by Schachman (1957). For the purpose of obtaining the sedimentation coefficient at infinite dilution ($s^0_{20,w}$), protein concentrations were varied systematically between 7 and 1 mg/mL and calculated values of $s_{20,w}$ were extrapolated linearly to zero concentration.

Enzyme Purification. Unless otherwise stated, procedures were performed at 4 °C and pH 7.0. Centrifugation was performed in 250-mL polyethylene cups in a Sorvall RC2B or Beckman J21 centrifuge at 8000 rpm for 45 min. Ammonium sulfate additions were made by using a standard saturation table (Green & Hughes, 1955) assuming that 100% saturation corresponded to 3.9 M at 4 °C. Blue dextran–Sepharose (BDS) was prepared as described by Moe & Piszkiewicz (1976).

(1) Crude Extract and Ammonium Sulfate Fractionation. Thawed cells (100 g) were suspended in 175 mL of cold 0.2 M potassium phosphate, 10% glycerol, 10 mM β-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 6000 Kunitz units each of deoxyribonuclease I and ribonuclease A. The suspension was sonicated in 90-mL portions with a Heat Systems Model W185 sonifier cell disruptor for 6 × 2 min, maintaining the temperature between 0 and 20 °C. After sonication, the sample was diluted to 400 mL with buffer and centrifuged. The supernatant (363 mL) was made 20% saturated by the addition of 41.3 g of $(NH_4)_2SO_4$ over a period of 45 min. The sample was allowed to stir for 1 h after salt addition and then was centrifuged. The supernatant (340 mL) was made 70% saturated by the addition of 115.6 g of $(NH_4)_2SO_4$ over a period of 45 min, and the sample was allowed

[1] Abbreviations used: BDS, blue dextran–Sepharose; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride.

to stir 1.5 h. After centrifugation, the precipitate was dissolved in 5 mM potassium phosphate, 30% glycerol, 10 mM β-mercaptoethanol, 5 mM $MgCl_2$, and 0.1 mM PMSF (pH 7.0) to a volume of 100 mL. The sample was then dialyzed vs. 3 × 1 L of the same buffer.

(*2*) *Affinity Chromatography, BDS 1.* The dialyzed protein sample was applied to a 450-mL BDS column (4 × 36 cm) equilibrated with 5 mM potassium phosphate, 30% glycerol, 10 mM β-mercaptoethanol, 0.1 mM PMSF, and 5 mM $MgCl_2$ (pH 7.0). The column was eluted at 3 mL/min with 1 L of the same buffer, and 7-mL fractions were collected. Approximately 90% of the activity applied to the column did not adhere. Fractions containing enzyme activity were pooled and brought to 50 mM with solid $MgCl_2$ and then centrifuged. During $MgCl_2$ addition, the temperature was maintained between 0 and 5 °C and the pH was maintained at 7.0.

(*3*) *Affinity Chromatography, BDS 2.* The protein sample was applied to a 1.3-L BDS column (5.7 × 54 cm) equilibrated with 5 mM potassium phosphate, 30% glycerol, 10 mM β-mercaptoethanol, and 50 mM $MgCl_2$ (pH 7.0) and the column eluted at 4 mL/min with 2 L of the same buffer. More than 80% of the enzyme activity was bound. The column was then eluted with 3 L of 150 mM potassium phosphate, 30% glycerol, 10 mM β-mercaptoethanol, 5 mM EDTA, and 5 mM glycine (pH 7.0) and the eluant collected in 7-mL fractions. Fractions containing enzyme activity were pooled, dialyzed overnight vs. 2 × 1 L of 5 mM potassium phosphate, 30% glycerol, and 10 mM β-mercaptoethanol (pH 7.0), and concentrated to 29 mL by ultrafiltration.

(*4*) *Gel Filtration.* The protein sample was applied to a Sepharose 6B column (2.5 × 80 cm) equilibrated with 5 mM potassium phosphate, 30% glycerol, and 10 mM β-mercaptoethanol (pH 7.0) and eluted at 0.5 mL/min with the same buffer; fractions of 7 mL were collected. Fractions containing enzyme activity were pooled, and solid $MgCl_2$ was added to a final concentration of 50 mM as described above.

(*5*) *Affinity Chromatography, BDS 3.* A BDS column (2.5 × 41 cm) was equilibrated with 5 mM potassium phosphate, 30% glycerol, 10 mM β-mercaptoethanol, and 50 mM $MgCl_2$ (pH 7.0). The sample was applied to the column and eluted at 1 mL/min with 300 mL of the same buffer. The column was then eluted with a linear gradient (1 L of each buffer) from 5 to 150 mM potassium phosphate. Both buffers contained, in addition, 30% glycerol, 10 mM β-mercaptoethanol, 5 mM EDTA, and 5 mM glycine (pH 7.0). The eluant was collected in 7-mL fractions. Purified enzyme has been stored in 5 mM potassium phosphate, 10 mM β-mercaptoethanol, 50% glycerol, and 0.1 mM PMSF at −20 °C for 6 months with little loss of activity.

(*6*) *Subunit Separation.* In a typical separation, an 8-mL sample of glycyl-tRNA synthetase (5 mg/mL) in 0.1 M potassium phosphate and 10 mM β-mercaptoethanol (pH 7.0) was brought to 0.5 M NaSCN by the addition of 5 M NaSCN and 0.1 potassium phosphate (pH 7.0) at 4 °C. The sample was then applied to an Ultrogel AcA44 column (2.5 × 80 cm) equilibrated with the same buffer. The column was eluted at 4 °C with this buffer at a flow rate of 0.4 mL/min; 2-mL fractions were collected. Fractions containing α and β subunits were pooled separately and dialized vs. 5 mM potassium phosphate, 10 mM β-mercaptoethanol, and 30% glycerol (pH 7.0).

Results

Hybrid *ColE1* plasmids containing the *glyS* locus were obtained indirectly by selection for the nearby *xyl* locus as has been described by Clarke & Carbon (1976). Strains containing *xyl* plasmids were grown on enriched medium, and the specific activity of glycyl-tRNA synthetase in extracts was determined. As is shown in Table I, two of the four strains overproduced the enzyme five- to eightfold. Since it showed the highest level of enzyme activity, the strain containing the plasmid designated pLC1-3 was selected for use in all subsequent experiments.

Table I: Glycyl-tRNA Synthetase Activity in Extracts of *E. coli* CH754 Carrying *xyl* Plasmids

strains[a]	sp act. (units/mg of protein)
F⁻ [b]	37
pLC44-22	180
pLC1-3	230
pLC10-15	44
pLC32-9	32

[a] Strains were grown on L broth medium to an OD_{600nm} of 0.7–0.8. [b] Host strain lacking plasmid.

Table II: Glycyl-tRNA Synthetase Activity in Extracts of Cells Grown on Different Media

strain[a]	medium	sp act. (units/mg of protein)
CH754 (pLC1-3)	L broth	230
	glucose minimal	190
	xylose minimal	550
CH754 (F⁻)[b]	L broth	37
	glucose minimal	30

[a] Strains were grown to an OD_{600nm} of 0.7. [b] Host strain lacking plasmid.

Table II shows that a further elevation of enzyme activity was observed in extracts of plasmid-containing cells grown on xylose minimal medium as compared to the same cells grown on enriched medium or glucose minimal medium. For purposes of enzyme isolation, cells were grown on xylose minimal medium in a large-scale (170 L) fermenter. These fermenter-grown cells yielded extracts having glycyl-tRNA synthetase specific activities approximately 40-fold greater than small cultures of isogenic cells lacking the *glyS* plasmid.

Table III summarizes the purification of glycyl-tRNA synthetase from these cells. Following ammonium sulfate fractionation, blue dextran–Sepharose (BDS) chromatography was used extensively in this isolation. We took advantage of the observation that the affinity of the enzyme for blue dextran was magnesium dependent. At low $MgCl_2$ concentration (5 mM) and low potassium phosphate concentration (5 mM), the enzyme activity did not adhere to the first column (BDS 1) and appeared in initial fractions with the bulk of the protein (Figure 1). A substantial purification was obtained, however, since many proteins were strongly bound to BDS under these conditions and since some fractionation of the eluted protein occurred. Fractions containing enzyme activity were pooled, and $MgCl_2$ was added to a final concentration of 50 mM. At this high concentration, the enzyme was tightly bound to a second column (BDS 2) and was, therefore, separated from proteins having a low affinity for BDS during elution of the column with application buffer. Glycyl-tRNA synthetase activity was then eluted from the column by raising the potassium phosphate concentration to 150 mM (Figure 2). Following concentration by ultrafiltration and chromatography on Sepharose 6B, the enzyme was obtained in pure form by gradient elution from a final affinity column (BDS 3). As shown in Figure 3, the enzyme activity was resolved into two components by this procedure. Substantial purity of the enzyme forms in all but the earliest fractions is indicated by the

Table III: Purification of Glycyl-tRNA Synthetase from *E. coli* CH754 (pLC1-3)

procedure	vol (mL)	enzyme act. (units × 10^{-6})	total protein (mg)	sp act. (units/mg)	recovery (%)	x-fold purifn
crude extract	380	15.9	11 900	1 340	100	1.0
$(NH_4)_2SO_4$ (20–70% satd)	106	11.3	9 690	1 170	71	0.87
BDS 1 (5 mM $MgCl_2$)	165	9.77	5 610	1 740	61	1.3
BDS 2 (50 mM $MgCl_2$)	146	5.75	369	15 600	36	11.6
Sepharose 6B	64	3.87	210	18 400	24	13.7
BDS 3 (phosphate gradient)						
form 1	20.9	1.02	21.3	47 900	6.4	35.7
form 2	34.7	1.15	21.3	54 000	7.2	40.3

Table IV: Physical Properties of Glycyl-tRNA Synthetase and Its Subunits

technique	quantity	protein	this work[a]	Ostrem & Berg (1970, 1974)
sedimentation velocity	sedimentation coeff	native enzyme	7.5 ± 0.2 S[b]	8.4 S[c]
		α	4 S, 8 S[d]	3.1 S[c]
		β	4.0 ± 0.2 S[b]	4.3 S[c]
sedimentation equilibrium	mol wt	native enzyme	205 000 ± 15 000	227 000
		α	43 000–55 000[e]	
		β	65 000 ± 3 000	
$NaDodSO_4$ gel electrophoresis	polypeptide chain mol wt	α chain	40 000 ± 3 000	33 000
		β chain	78 000 ± 3 000	80 000

[a] Uncertainties quoted reflect the range of values obtained in separate determinations. [b] Sedimentation coefficient, $s^0_{20,w}$, determined using the analytical ultracentrifuge and extrapolation of $s_{20,w}$ values to zero concentration. [c] Sedimentation coefficient determined using density gradient sedimentation. [d] Sedimentation coefficients, $s_{20,w}$, determined in the analytical ultracentrifuge at concentrations between 5 and 1 mg/mL. Boundaries were broad, and mass distribution was concentration dependent. [e] Molecular weight obtained from high-speed sedimentation equilibrium at 30 000 rpm. Plots of ln c vs. r^2 were curved, and the values quoted were obtained from tangents to the curve. Species of appreciably higher molecular weight would likely escape detection because of the high centrifugal fields employed in these experiments.

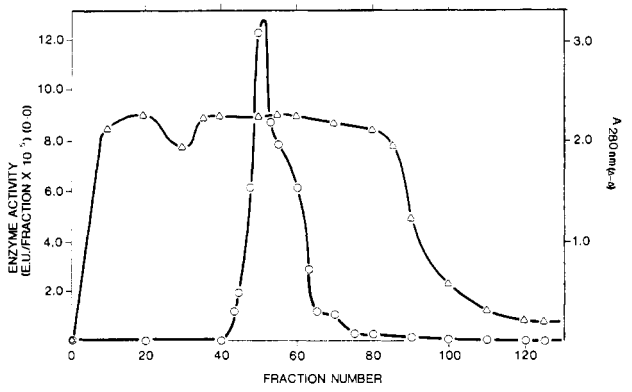

FIGURE 1: Elution profile of BDS 1. The dialyzed protein sample from the ammonium sulfate fractionation was purified by chromatography on blue dextran–Sepharose using buffers of low $MgCl_2$ (5 mM) and low potassium phosphate (5 mM) concentration. The total enzyme units in individual fractions (O) and the absorbance at 280 nm of fractions (Δ) were measured. The bulk of the enzyme activity did not adhere to the column and appeared in the initial eluant shown.

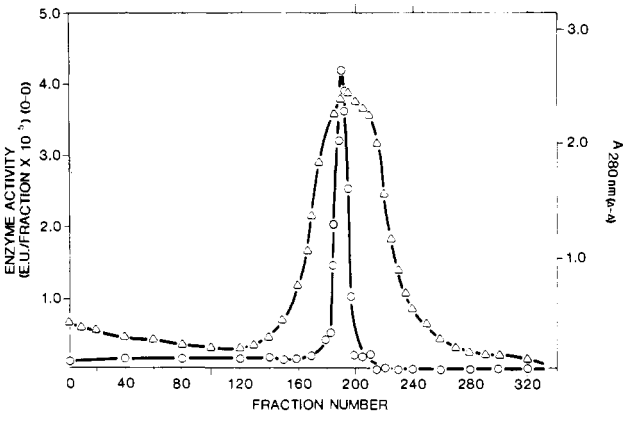

FIGURE 2: Elution profile of BDS 2. The protein sample from BDS 1 was purified by chromatography on blue dextran–Sepharose using buffers of high $MgCl_2$ concentration (50 mM). More than 80% of the enzyme activity was tightly bound and did not elute at low (5 mM) potassium phosphate concentration (not shown). The elution profile following application of buffer containing high (150 mM) potassium phosphate is shown. The total enzyme units in individual fractions (O) and the absorbance at 280 nm of fractions (Δ) were measured.

parallel changes in the A_{280nm} and enzyme activity profiles. Fractions containing the early-eluting form 1 (fractions 29–31) and the late-eluting form 2 (fractions 36–40) were pooled separately. The mixture of enzyme forms present in intermediate fractions could be resolved by rechromatography on a smaller column (BDS 4) under the same conditions, but the yield was modest (<10% of that from BDS 3). As shown in Table III, this procedure yielded a total of 42.6 mg of purified enzyme in 14% overall yield. The yield of each enzyme form was approximately equal.

The purified enzyme forms were studied separately by using a variety of techniques as described below. None of these experiments has shown a significant difference between the two forms. The results shown in Table IV for the native enzyme, therefore, are characteristic of both enzyme forms. Uncertainties quoted reflect the range of values obtained in different experiments. Values reported by Ostrem & Berg (1970, 1974) are presented for comparison.

Sedimentation velocity experiments performed on both enzyme forms between 7 and 1 mg/mL showed a single boundary and yielded a value for the sedimentation coefficient of 7.5 ± 0.2 S after linear extrapolation to zero concentration. High-speed sedimentation equilibrium experiments yielded linear plots of ln c vs. r^2 and a value for the anhydrous molecular weight of 205 000 ± 15 000. No systematic differences were observed at different rotor velocities or loading concen-

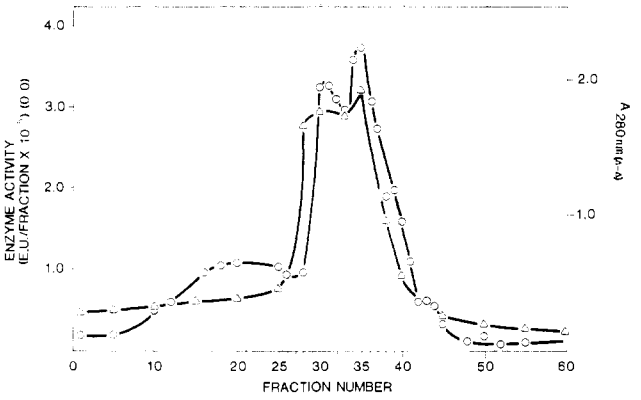

FIGURE 3: Elution profile of BDS 3. The protein sample from Sepharose 6B was purified by chromatography on blue dextran–Sepharose at high (50 mM) $MgCl_2$ concentration. The column was developed in the same manner described for BDS 2, except that the potassium phosphate concentration was increased by application of a linear gradient from 5 to 150 mM. Only the portion of the elution profile following application of the gradient to the column is shown.

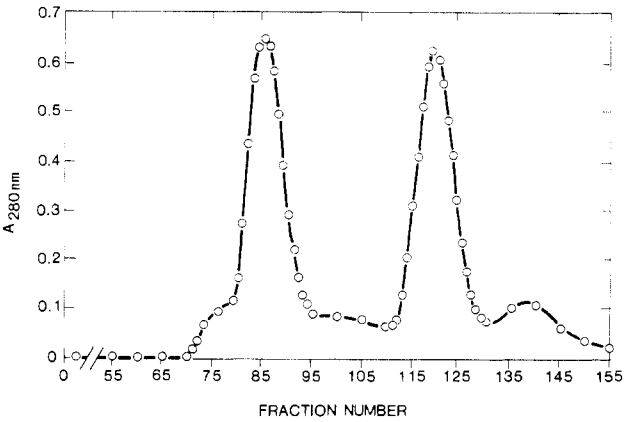

FIGURE 4: Subunit separation in the presence of NaSCN. Purified glycyl-tRNA synthetase was dissociated by the addition of NaSCN (0.5 M) and chromatographed on an Ultrogel AcA44 column equilibrated with the same buffer. The absorbance at 280 nm of fractions was measured. Subsequent analysis showed the early-eluting zone to contain β protein while the late-eluting zone contained α protein.

trations. The value of the partial specific volume, $\bar{v}$ = 0.74 cm^3/g, was calculated from the amino acid composition of the holoenzyme (L. Koski, G. M. Nagel, and B. H. Weber, unpublished experiments). The value of $\bar{v}$ was identical with that reported by Ostrem & Berg (1974).

Analysis of the sedimentation data by the method of Oncley (1941) yielded a value for the frictional ratio (f/f_0) of 1.55. With the assumption of a reasonable value for the hydration (w) of 0.6 g of H_2O per g of protein (Kuntz & Kauzmann, 1974), an axial ratio (a/b) for the enzyme of 5.5 was calculated. It should be noted that the value of the axial ratio is not particularly sensitive to the estimated value of w within reasonable limits. For w = 0.4 g/g, a/b = 6.5, and for w = 0.8 g/g, a/b = 4.6.

Electrophoresis of the purified enzyme on nondenaturing polyacrylamide gels showed a single major band in gels containing both 5% acrylamide (R_f 0.4) and 7.5% acrylamide (R_f 0.16). Minor bands seen in some preparations were estimated to be less than 5% of the total protein. Electrophoresis of the enzyme under denaturing conditions in $NaDodSO_4$–polyacrylamide gels (Table IV) showed two bands corresponding to 78 000 ± 3000 daltons for the β chain and 40 000 ± 3000 daltons for the α chain. Estimates of the relative mass of β and α protein in the enzyme were made from the peak areas calculated from absorbance profiles (A_{600nm} vs. distance) of these gels. The β/α ratio so determined (2.4), the polypeptide chain molecular weights, and the molecular weight of the holoenzyme indicate an $\alpha_2\beta_2$ subunit structure for the native enzyme.

The kinetic properties of the two enzyme forms were also indistinguishable. Saturation plots (v vs. [tRNA]) were hyperbolic, and double-reciprocal plots of the data were linear. The values obtained for the kinetic constants were K_m = 1.4 ± 0.4 mg/mL of unfractionated tRNA and V_{max} = (4.4 ± 0.5) × 10^4 nmol of Gly-tRNA per 10 min per mg of protein.

Addition of the chaotropic salt NaSCN (final concentration 0.5 M) just prior to analysis caused glycyl-tRNA synthetase to sediment as a broad boundary having a sedimentation coefficient ($s_{20,w}$) of ~4 S. The apparent dissociation of the enzyme was not an effect of ionic strength alone since a control sample containing 0.5 M NaCl gave $s_{20,w}$ = 7.3 S. No change in the centrifuge patterns was observed over a period of 48 h, indicating that the change brought about by NaSCN was complete in less than 1 h. The process was also fully reversible since, following dialysis vs. 0.1 M potassium phosphate and 10 mM β-mercaptoethanol to remove NaSCN, the sample gave a single, sharp boundary indistinguishable from that of the native enzyme ($s_{20,w}$ = 7.3 S).

Table V: Catalytic Activity of Isolated and Reconstituted Subunits of Glycyl-tRNA Synthetase

protein sample	aminoacylation (units/mg) × 10^{-3}	%	ATP–PP_i exchange act. (units/mg) × 10^{-3}	%
native enzyme	41.2	100	28.1	100
α	0.04	0.1	0	<0.2
β	0.02	0.05	0	<0.1
$\alpha + \beta^a$	38.9	94	25.8	92

[a] Subunits were mixed in a 1:1 molar ratio prior to enzyme assay.

The products of dissociation, however, could not be characterized in sedimentation experiments since the broad boundary observed in the presence of NaSCN could not be resolved. Gel filtration on Ultrogel AcA44 under the same conditions, however, gave the elution profile shown in Figure 4. Similar profiles were obtained with Sephadex G-150 columns, but the resolution of the two zones was less complete. Subsequent analysis of fractions by $NaDodSO_4$ gel electrophoresis showed that the early-eluting zone contained only β protein while the late-eluting zone contained only α protein.

For the purpose of determining if the isolated subunits or the reconstituted enzyme showed catalytic activity, peak fractions containing electrophoretically pure α and β protein were selected from the elution profile diagrammed in Figure 4. The samples were dialyzed separately vs. 0.1 M potassium phosphate, 10 mM β-mercaptoethanol, and 30% glycerol (pH 7.0) to remove NaSCN and analyzed as shown in Table V. Both subunits showed a greatly reduced capacity to catalyze the aminoacylation reaction and no detectable activity in the less-sensitive ATP–PP_i exchange assay. Even in the presence of 50 μg/mL tRNA, which increases the rate of the exchange reaction 2.5-fold for the native enzyme (Ostrem & Berg, 1974), no activity was detectable in this assay. When the subunits were mixed in a 1:1 molar ratio prior to the assay, virtually full activity was recovered in both enzyme assays.

For sedimentation analysis, fractions containing α and β protein were pooled separately, dialyzed vs. 5 mM potassium phosphate, 10 mM β-mercaptoethanol, and 30% glycerol (pH

7.0) to remove NaSCN, and concentrated by ultrafiltration. Prior to analysis, samples were dialyzed vs. 0.1 M potassium phosphate and 10 mM β-mercaptoethanol (pH 7.0) to remove glycerol and reconcentrated by ultrafiltration if necessary. The results of these experiments are summarized in Table IV.

Sedimentation equilibrium experiments for β protein yielded linear plots of ln c vs. r^2 and a molecular weight of 65 000 ± 3000. Sedimentation velocity experiments between 5 and 1 mg/mL showed a single boundary having a sedimentation coefficient, $s^0_{20,w}$, of 4.0 ± 0.2 S. These data indicate that isolated β exists as a monomer in solution. Addition of substrates (1 mM ATP and 5 mM $MgCl_2$ in the presence or absence of 1 mM glycine) had no appreciable effect on the sedimentation velocity results.

Samples of α protein yielded curved plots of ln c vs. r^2. Tangents to the curve gave molecular weight values ranging from 43 000 near the beginning of the gradient to 55 000 toward the cell bottom. Sedimentation velocity experiments showed two broad boundaries having sedimentation coefficients of approximately 4 and 8 S. At higher concentrations (5 mg/mL), the faster sedimenting species was prominent, comprising ~40% of the protein. At lower concentrations (1 to 2 mg/mL), the slower sedimenting species accounted for >90% of the mass. As was observed with β protein, the addition of substrates had no significant effect on the sedimentation velocity results. These data indicate a reversible self-association of α protein in solution.

Discussion

Approximately 40-fold overproduction of glycyl-tRNA synthetase in *E. coli* was accomplished via a hybrid *ColE1* plasmid containing the *glyS* locus. Since *ColE1* plasmids have been shown to exist in multiple copies in growing cells (Hershfield et al., 1974), enzyme overproduction can be attributed to a simple increase in the number of *glyS* genes in cells containing the hybrid plasmid. The basis for the specific effect of xylose-containing media in elevating further the enzyme levels may stem from the fact that the plasmids also contain the xylose fermentation marker, *xyl*. Since the host strain is unable to ferment xylose, increased glycyl-tRNA synthetase levels may result from the fact that selective pressure on the plasmid is maintained in these cells with xylose as the sole carbon source. Alternate explanations, however, such as increased plasmid transcription in the presence of xylose cannot be excluded.

The purification procedure described here differs substantially from that described by Ostrem & Berg (1974). In particular, the use of blue dextran–Sepharose (BDS) affinity chromatography has allowed us to avoid time-consuming and difficult procedures such as large-scale hydroxylapatite chromatography and preparative polyacrylamide gel electrophoresis which were used previously. Immobilized blue dextran has been used widely in the purification of dehydrogenases and kinases (Thompson et al., 1975) and was first applied to an aminoacyl-tRNA synthetase purification by Moe & Piszkiewicz (1976). The entire procedure described here can be completed in ~10 days by an individual. Recently, yields approaching 100 mg of purified enzyme per 100 g of packed cells have been possible. Since a purification of only 40-fold was necessary to obtain pure preparations, it appears that the plasmid-containing cells produce ~2% of the soluble protein as glycyl-tRNA synthetase.

Two enzyme forms were isolated by this procedure, but no physical differences between them have been found thus far. Although the serine protease inhibitor PMSF was present during much of the purification, it is possible that one form may have been generated from the other by limited proteolysis in view of the documented sensitivity of some aminoacyl-tRNA synthetases to protease action. Some specific examples from *E. coli* include the enzymes specific for lysine (Dimitrijevic, 1972), methionine (Cassio & Waller, 1971), leucine (Rouget & Chapeville, 1971), and isoleucine (Piszkiewicz & Goitein, 1974). It should be noted, however, that proteolytic modification in these cases either during purification or after protease addition to purified enzyme produced a large change in polypeptide chain size. In the case of methionyl-tRNA synthetase, for example, the native enzyme has an α_2 structure and a molecular weight of 180 000. The trypsinized enzyme, on the other hand, is a 64 000-dalton monomer. Since the physical techniques we have employed are capable of measuring differences of 5–10% in molecular weight and no differences have been observed in either the holoenzyme or its subunits, it is evident that any proteolytic modification in this system must be more subtle than that observed in the examples cited above. More sensitive techniques such as amino-terminal analysis and peptide mapping are currently being applied to help clarify this issue. Obviously, alternate explanations including enzyme modification, e.g., phosphorylation, and multiple polypeptide forms, perhaps of unique genetic origin, for the existence of multiple enzyme forms exist. The latter possibility is rendered somewhat more attractive by the fact that duplication of *glyS* has been documented in *E. coli* (Folk & Berg, 1971). Certainly, the hypothesis that multiple synthetase forms in *E. coli* exist in vivo should not be discarded; in at least two cases (Deobagkar & Gopinathan, 1976; Ohyama et al., 1977), two forms of a procaryotic aminoacyl-tRNA synthetase which appear not to have been generated as artifacts of isolation have been reported. In eucaryotes, the situation is rather different since the possibility of organelle-specific enzymes must be considered. Indeed, unique mitochondrial (Boguslawski et al., 1974) and chloroplast (Locy & Cherry, 1978) forms in addition to the cytoplasmic or microsomal forms have been identified in some cases. Previously, this laboratory reported the existence of two glycyl-tRNA synthetase forms in partially purified preparations from *E. coli* (Francis & Nagel, 1976). Although the present work corroborates the earlier results with regard to the number of enzyme forms, Francis & Nagel (1976) observed one form to display sigmoidal saturation kinetics with respect to tRNA concentration while the other form showed Michaelis–Menten behavior. Both forms reported here displayed Michaelis–Menten behavior and yielded indistinguishable values of K_m and V_{max}. No definite explanation for this discrepancy can be given at this time. A number of potential explanations remain to be investigated, including the possibilities that a sigmoidal enzyme form (a) was lost during purification, (b) is not present in the overproducing strain, or (c) requires participation by another factor and does not display sigmoidal kinetics in highly purified preparations. Interestingly, partially purified yeast glycyl-tRNA synthetase has been reported by Black & Hazel (1969) to display sensitivity to activators only under specific conditions, e.g., pretreatment with inorganic sulfide. The authors postulate that the regulatory properties of the enzyme were masked during isolation, perhaps via sulfhydryl oxidation.

The data presented here verify the $\alpha_2\beta_2$ subunit structure reported by Ostrem & Berg (1970, 1974). This quaternary structure is rare among the aminoacyl-tRNA synthetases; only the glycine and phenylalanine enzymes have been shown to display this arrangement. Interestingly, the structure persists in enzymes isolated from different sources. The phenyl-

alanyl-tRNA synthetases from both yeast (Schmidt et al., 1971) and *E. coli* (Hanke et al., 1974) have been shown to be $\alpha_2\beta_2$ enzymes, as have the glycyl-tRNA synthetases from *E. coli* and *Bacillus brevis* (Surguchov & Surguchova, 1975). Although these data suggest a unique functional role for this quaternary structure, that role remains to be elucidated.

Our sedimentation measurements yielded a molecular weight of 205 000 ± 15 000 and a sedimentation coefficient of 7.5 ± 0.2 S for glycyl-tRNA synthetase. Both values are ~10% lower than those reported by Ostrem & Berg (1970, 1974). Taken together, these data may indicate that the enzyme we have isolated is somewhat smaller than that purified previously. The $NaDodSO_4$–polyacrylamide gel electrophoresis data, however, do not support this view. Certainly, the differences in the sedimentation coefficient can be ascribed to technique since Ostrem & Berg (1970, 1974) utilized density gradient sedimentation, inherently a less analytical method.

In combination, the sedimentation velocity and equilibrium data indicate that the overall structure of glycyl-tRNA synthetase is nonspherical. The frictional ratio ($f/f_0 = 1.55$) indicates an axial ratio (a/b) between 5 and 6, assuming a general shape approximating a prolate ellipsoid and a hydration (w) of 0.6 g of H_2O per g of protein. The values of M and s reported by Ostrem & Berg (1970, 1974) lead to virtually the same values of f/f_0 and a/b. Our estimate of w is probably high; the average value calculated by Kuntz & Kauzmann (1974) from sedimentation and diffusion data for proteins of known structure is closer to 0.5. Thus, our value of the axial ratio should be regarded as a low estimate. A similar finding for *E. coli* phenylalanyl-tRNA synthetase ($f/f_0 = 1.67$) has been reported by Hanke et al. (1974). These data suggest that molecular anisotropy may have functional significance for these enzymes. A simple possibility is that of tRNA recognition. For example, the data of Schoemaker & Schimmel (1974) indicate that the anticodon loop of $tRNA^{Tyr}$ is bound to tyrosyl-tRNA synthetase. Assuming that the 3′ terminus must be simultaneously bound to the enzyme and that the conformation of $tRNA^{Tyr}$ is similar to that of yeast $tRNA^{Phe}$ (Kim et al., 1973), Schoemaker & Schimmel (1974) concluded that the synthetase may encompass sections of tRNA which are separated by ~80 Å. Since the diameter of a spherical protein of 2×10^5 daltons and $\bar{v} = 0.74$ cm^3/g is just 78 Å (50 Å for 10^5 daltons), an elongated shape would be clearly advantageous to these enzymes in recognizing distal portions of a tRNA molecule without conformational rearrangement.

Sodium thiocyanate has provided an efficient means to reversibly dissociate glycyl-tRNA synthetase. This chaotropic salt generally destabilizes ordered structures in proteins (von Hippel & Schleich, 1969) and has been shown to dissociate reversibly the catalytic subunit of aspartate transcarbamylase (Burns & Schachman, 1979). Following separation by gel filtration and dialysis to remove NaSCN, the isolated subunits showed a greatly reduced capacity to catalyze both the aminoacylation and ATP–PP_i exchange reactions. Although electrophoresis revealed no contaminating subunits or reconstituted enzyme in either preparation, we cannot exclude the possibility that the aminoacylation activity observed was due to the presence of small amounts of reconstituted enzyme. We can conclude, however, that each subunit has less than 0.2% of the specific activity of the native enzyme. A similar conclusion was reached by Ostrem & Berg (1974) from experiments utilizing small amounts of electrophoretically separated subunits generated by mercurial dissociation of the enzyme. Their experiments, however, did not demonstrate that the isolated subunits were capable of regaining full activity upon reconstitution. We have observed, however, virtually full recovery of catalytic activity in both assays when the subunits are mixed in a 1:1 molar ratio. The presence of 30% glycerol in buffers following NaSCN removal appears to optimize the recovery of activity. These data and the sedimentation velocity experiments indicate that a complex indistinguishable from the native enzyme is formed upon reconstitution. Since reconstitution was complete, it is clear that the subunits were not irreversibly denatured by NaSCN treatment and that both subunits were fully competent to form the $\alpha_2\beta_2$ structure. In addition, since neither isolated subunit showed significant activity in either assay, it appears that both subunits are necessary for catalysis. It is not yet clear, however, whether both subunits participate directly in catalysis by contributing amino acid residues to the catalytic site or whether one subunit participates indirectly by stabilizing the "catalytic" conformation of the other.

Sedimentation measurements of isolated β protein gave a molecular weight of 65 000 ± 3000 and a sedimentation coefficient of 4.0 ± 0.2 S. In combination, these data yield a frictional ratio near 1.3, indicating that β is more spherical than the holoenzyme. The fact that the molecular weight obtained is near that obtained from $NaDodSO_4$–polyacrylamide gel electrophoresis indicates that isolated β protein exists as a monomer in solution. Thus, we can infer that β–β interactions do not contribute to the stabilization of the $\alpha_2\beta_2$ structure of the native enzyme. This conclusion must remain tentative, however, since conformational changes accompanying α–β association could unmask β–β bonding domains. The rather large discrepancy between the molecular weight values from sedimentation equilibrium (65 000) and $NaDodSO_4$–polyacrylamide gel electrophoresis (78 000) was surprising. Since the electrophoretic technique does not measure molecular weight directly, we presently favor the lower value. However, it should be noted that the partial specific volume of both subunits was assumed to be that of the native enzyme. If the true values differ markedly, the molecular weights from sedimentation equilibrium could change by as much as 10% from those we have reported.

The sedimentation data for isolated α protein indicate self-association of α-polypeptide chains. Since higher protein concentrations favored the faster sedimenting component and vice versa, this associative process appears reversible. At low protein concentrations, sedimentation equilibrium experiments gave molecular weight values (43 000) near those obtained from electrophoresis under denaturing conditions (40 000), but values up to 55 000 were obtained at higher concentrations near the cell bottom. It should be emphasized that these sedimentation equilibrium experiments were designed to estimate more accurately the molecular weight of the monomer. At the high centrifugal fields employed, the contribution of associated species to the molecular weights measured is minimized. Although a simple monomer–dimer equilibrium is suggested by the data, the sedimentation coefficients observed (4 and 8 S) are difficult to reconcile with reasonable molecular weight values (40 000 and 80 000) for the monomer and dimer. The fact that a sedimentation coefficient (8 S) near that of the holoenzyme was observed indicates that the self-association of α proceeds beyond the dimer stage. Obviously, a good deal of additional work will be required to determine accurate values for the sedimentation coefficients and the limiting size of complexes formed as well as the influence of a variety of experimental parameters (e.g., buffer composition, pH, and

temperature) on the equilibria. Interactions between α subunits obviously exist, however, and we can infer that α–α interactions also contribute to the stability of the native enzyme.

References

Black, S., & Hazel, B. (1969) *J. Biol. Chem. 244*, 1656.

Boguslawski, G., Vodkin, M. H., Finkelstein, D. B., & Fink, G. R. (1974) *Biochemistry 13*, 4659.

Burns, D., & Schachman, H. K. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 2169 Abstr.

Calendar, R., & Berg, P. (1966) *Biochemistry 5*, 1681.

Cassio, D., & Waller, J. P. (1971) *Eur. J. Biochem. 20*, 283.

Clark, J. M., & Switzer, R. L. (1977) in *Experimental Biochemistry*, pp 53–54, W. H. Freeman, San Francisco, CA.

Clarke, L., & Carbon, J. (1976) *Cell 9*, 91.

Deobagkar, D. N., & Gopinathan, K. P. (1976) *Biochem. Biophys. Res. Commun. 71*, 939.

Dimitrijevic, L. (1972) *FEBS Lett. 25*, 170.

Dizel, W., Kopperschläger, G., & Hofmann, E. (1972) *Anal. Biochem. 48*, 617.

Folk, W. R., & Berg, P. (1970) *J. Bacteriol. 102*, 139.

Folk, W. R., & Berg, P. (1971) *J. Mol. Biol. 58*, 595.

Francis, T. A., & Nagel, G. M. (1976) *Biochem. Biophys. Res. Commun. 70*, 862.

Green, A. A., & Hughes, W. L. (1955) *Methods Enzymol. 1*, 67.

Hanke, T., Bartmann, P. Hennecke, H., Kosakowski, H. M., Jaenicke, R., Holler, E., & Böck, A. (1974) *Eur. J. Biochem. 43*, 601.

Hershfield, V., Boyer, H. W., Yanofsky, C., Lovett, M. A., & Helinski, D. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3455.

Kim, S. H., Quigley, G. J., Suddath, F. L., McPherson, A., Sneden, D., Kim, J. J., Weinzierl, J., & Rich, A. (1973) *Science 179*, 285.

Kisselev, L. L., & Favorova, O. O. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 40*, 141.

Kuntz, I. D., Jr., & Kauzmann, W. (1974) *Adv. Protein Chem. 28*, 239.

Locy, R. D., & Cherry, J. H. (1978) *Phytochemistry 17*, 19.

McDonald, T., Wadey, K. L. W., Scott, M., Hom, S., & Nagel, G. M. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 3107 Abstr.

Moe, J. G., & Piszkiewicz, D. (1976) *FEBS Lett. 72*, 147.

Ohyama, K., Kaneko, I., Yamakawa, T., & Wantanabe, T. (1977) *J. Biochem. (Tokyo) 81*, 1571.

Oncley, J. L. (1941) *Ann. N.Y. Acad. Sci. 41*, 121.

Ostrem, D. L., & Berg, P. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1967.

Ostrem, D. L., & Berg, P. (1974) *Biochemistry 13*, 1338.

Piszkiewicz, D., & Goitein, R. K. (1974) *Biochemistry 13*, 2505.

Richards, E. G., Bell-Clark, J., Rosenthal, A., Kirschner, M., & Schachman, H. K. (1972) *Anal. Biochem. 46*, 295.

Rouget, P., & Chapeville, F. (1971) *Eur. J. Biochem. 23*, 452.

Schachman, H. K. (1957) *Methods Enzymol. 4*, 32.

Schmidt, J., Wang, R., Stanfield, S., & Reid, B. R. (1971) *Biochemistry 10*, 3264.

Schoemaker, H. J. P., & Schimmel, P. R. (1974) *J. Mol. Biol. 84*, 503.

Söll, D., & Schimmel, P. R. (1974) *Enzymes, 3rd Ed. 10*, 489.

Surguchov, A. P., & Surguchova, I. (1975) *Eur. J. Biochem. 54*, 175.

Thompson, S. T., Cass, K. H., & Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 669.

Toennies, G., & Feng, F. (1965) *Anal. Biochem. 9*, 411.

Vogel, H. J., & Bonner, D. M. (1956) *J. Biol. Chem. 218*, 97.

von Hippel, P. H., & Schleich, T. (1969) *Acc. Chem. Res. 2*, 258.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Yphantis, D. A. (1964) *Biochemistry 3*, 297.