Analysis of Truncated Forms of *Bombyx mori* Glycyl-tRNA Synthetase: Function of an N-Terminal Structure in RNA Binding†

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ABSTRACT: *Bombyx mori* glycyl-tRNA synthetase (GRS) was expressed as the full length protein and as N-terminally and C-terminally truncated forms. The intact enzyme and forms with deletions of 12, 27, 46, and 55 N-terminal residues were expressed, purified, and characterized. All were active, having 15−25% of both pyrophosphate exchange activity and aminoacyl-tRNA synthetase activity compared to wild type enzyme. Active site titration indicated that this difference in activity was not the result of production of inactive enzyme. Sedimentation and gel filtration experiments indicated that the N-terminally deleted forms and the wild type enzyme were dimers. Deletion of 55 N-terminal residues did not result in significant effects on the Michaelis constants for ATP, glycine, or tRNA, while deletion of 108 N-terminal residues and two internal 64- and 200-residue deletions generated inactive forms. Five forms with C-terminal deletions of 24, 37, 59, 162, and 327 amino acid residues were soluble and intact but lacked detectable pyrophosphate exchange activity or aminoacyl-tRNA synthetase activity. The C-terminal sequence may be required for catalysis or to maintain a stable structure. Zone electrophoresis demonstrated the wild type enzyme bound both $tRNA^{Gly}$ and noncognate $tRNA^{Ala}$. Deletion of 55 N-terminal residues resulted in altered binding of tRN^{Gly} and eliminated binding of $tRNA^{Ala}$. The first 55 N-terminal residues are not essential for catalysis, dimerization, or substrate binding in aminoacylation but are required for RNA binding not associated with aminoacylation.

Aminoacyl-tRNA synthetases are a group of ancient enzymes with diversity in both structure and mechanism, even though they catalyze the same overall two-step reaction sequence for charging tRNAs with their cognate amino acids (Freist, 1989; Mirande, 1987, 1991). On the basis of sequence and crystallographic studies, these enzymes were divided into two major classes with characteristic sequence and structural motifs, which form the catalytic and substrate binding domains (Burbaum et al., 1990; Ruff et al., 1991; Carter, 1993; Cusack et al., 1990; Eriani et al., 1990). In addition to their catalytic domains, many aminoacyl-tRNA synthetases contain N-terminal or C-terminal extensions. These extensions vary in structure and location and may represent additions to the catalytic domains that contribute to the specific tRNA binding or recognition (Cusack et al., 1991; Mirande & Waller, 1988). Studies on *Escherichia coli* seryl-tRNA synthetase (class II) showed that tRNA makes contacts on both subunits (Cusack et al., 1994; Vincent et al., 1995). Transfer RNA binds to *E. coli* seryl-tRNA synthetase in part through an extended N-terminal coiled-coil structure (Cusack et al., 1990). Some eukaryotic aminoacyl-tRNA synthetases contain amino or carboxyl terminal extensions that are absent in their prokaryotic counterparts and may reflect other functions besides the primary role of the enzymes in the tRNA aminoacylation. Examples of such functions are the splicing of mitochondrial RNAs in yeast and *Neurospora crassa* (Akins & Lambowitz, 1987; Herbert et al., 1986) and regulation of translation initiation (Clemens, 1990). Studies of the amino terminal extensions of several yeast aminoacyl-tRNA synthetases show that removal of residues from the N-terminal extension does not significantly affect catalytic activity (Mirande & Waller, 1988; Garret et al., 1991; Ludmerer & Schimmel, 1987; Escalante & Yang, 1993; Water et al., 1989), whereas in the case of human histidyl-tRNA synthetase, the amino terminal extension may be essential (Raben et al., 1994). The N-terminal extension of the human aspartyl-tRNA synthetase appears to mediate transfer of aminoacyl-tRNA to elongation factor 1 (Reed et al., 1994a,b).

Glycyl-tRNA synthetase (GRS)[1] from *Bombyx mori* contains an N-terminal motif which can be aligned with several other eukaryotic aminoacyl-tRNA synthetases (Nada et al., 1993). An alignment can also be made with the N-terminus of prokaryotic seryl-tRNA synthetase from *E. coli* (Raben et al., 1994). Recent crystallographic and enzymatic studies show that the N-terminal coiled-coil domain of seryl-tRNA synthetase is essential for tRNA binding (Borel et al., 1994). To identify the functional domains of glycyl-tRNA synthetase and to determine the function of an N-terminal motif, we examined truncated forms of the enzyme and the full length form. The results indicate that the first 55 residues from the N-terminus are not essential for catalytic activity and tRNA binding in aminoacylation but are important for RNA binding not directly associated with tRNA charging.

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[1] Abbreviations: GRS, glycyl-tRNA synthetase; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; TAME, tosylarginine methyl ester; BS3, bis(sulfosuccinimidyl) suberate; PEG, poly(ethylene glycol); SDS, sodium dodecyl sulfate; BD cellulose, benzoylated (diethylamino)ethyl cellulose; IPTG, isopropyl β-D-thiogalactopyranoside.

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EXPERIMENTAL PROCEDURES

Materials. Synthetic oligonucleotides were purchased from Operon Technologies, Inc., and Bio-synthesis, Inc. T4 DNA polymerase, modified T7 DNA polymerase, and DNA markers were from USB; T4 DNA ligase and restriction enzymes were from NEB. Electrophoresis supplies, protein molecular weight markers, goat anti-rabbit IgG alkaline phosphatase conjugate, BD cellulose (Cellex B. D.), and agarose were obtained from Bio-Rad. Q-Sepharose, Sephacryl S-300, and Sephacryl S-200 were from Pharmacia. Soybean trypsin inhibitor, phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT), ATP, tosylarginine methyl ester (TAME), histone, and bis(sulfosuccinimidyl) suberate (BS^3) were from Sigma. Supported nitrocellulose membranes (0.2 μm) were from Schleicher & Schuell. DEAE cellulose was from Whatman. [^{14}C]Glycine and acrylamide were from ICN. [^{32}P]Pyrophosphate and [α-^{35}S]dATP were from NEN; [γ-^{32}P]ATP was generously supplied by Dr. Herman A. J. Schut of the Department of Pathology of the Medical College of Ohio. The catalytic subunit of bovine heart cAMP dependent protein kinase used to determine the percentage of usable ATP in preparations of [γ-^{32}P]ATP was generously supplied by Dr. Erwin Reimann of the Department of Biochemistry and Molecular Biology of the Medical College of Ohio. Bovine liver tRNA was isolated and fractionated on benzoylated DEAE cellulose to enrich for $tRNA^{Gly}$ as described previously (Dignam & Dignam, 1984; Gillam & Tener, 1981). *B. mori* $tRNA^{Gly1}$ was produced in *E. coli* using a pBluescript (Stratagene)-based expression vector constructed in our laboratory. The $tRNA^{Gly1}$ was purified by successive chromatography on BD cellulose and phenyl-Sepharose and had an acceptor activity of 40 nmol mg^{-1}. The details of the construction of this vector and the purification of the RNA will be described elsewhere, but details will be made available from the authors on request. An expression vector encoding $tRNA^{Ala/CUA}$ (Hou & Schimmel, 1992) was generously provided by Dr. P. Schimmel of the Massachusetts Institute of Technology, and the tRNA was purified to an acceptor activity of 30 mol mg^{-1} by chromatography on BD cellulose.

Construction and Expression of Wild Type and Deletion Mutants of Glycyl-tRNA Synthetase. To construct and express the wild type *B. mori* glycyl-tRNA synthetase in *E. coli*, a *Hin*dIII fragment containing the entire coding sequence and some of the 3′- and 5′-untranslated regions was obtained by combining two overlapping genomic clones at a *Hin*cII site (Nada et al., 1993). This *Hin*dIII fragment was cloned into pBluescript SK(−) (Stratagene). Using this phagemid (pNADAH), single-stranded DNA was isolated and used as a template for introduction of mutations by oligonucleotide-directed mutagenesis (Kunkel, 1985; Kunkel et al., 1987). An *Nde*I site was created at the normal ATG start codon and at various positions to generate N-terminal deletions. The resulting *Nde*I-*Sal*I fragment was subcloned into pET-12a (Novagen) to generate pNADAHX. The enzyme was expressed in *E. coli* strain BL21DE3pLys S using YT medium containing 0.5% (w/v) glucose and 1 × M9 salts (Studier, 1991). Cells were grown at 22 °C to an OD of 0.4−0.5 at 595 nm, and IPTG was added to 0.2 mM. Expression of the enzyme was significantly higher (approximately 2-fold) in cells grown at lower temperatures (i.e. 22 vs 37 °C). After 12−15 h, cells were collected by centrifugation and the pellets stored at −80 °C. The optimal time for growth of the wild type and mutants was established empirically. Subcloning the *Nde*I-*Sal*I fragments with different *Nde*I sites generated deletions of 12, 27, 46, and 55 amino acid residues from the N-terminus. N-terminal deletion N108 was generated by *Nde*I-*Afl*II double digestion of pNADAHX, filling in the protruding ends using DNA polymerase I (Klenow fragment) and religation of the linear DNA. Two internal deletion mutants I<111−164> and I<110−309> were constructed by creation of a new *Afl*II site, deletion of the internal *Afl*II fragment, and religation of the resulting linear DNA. All inserts were sequenced to check for the creation of restriction sites and to exclude DNA polymerase-induced errors. To generate C-terminal deletions, pNADAHX was cleaved at a *Bam*HI site in the vector (pET-12a) and the ends were filled in using α-phosphorothioate nucleotide triphosphates (Putney et al., 1981); the resulting fragment was digested with *Nco*I to generate a recessed 3′-end, and the DNA was digested for various times with exonuclease III (Henikoff, 1984). An adaptor containing a stop codon and a *Bam*HI site was ligated into the plasmid after the ends were filled using Klenow DNA polymerase I (Klenow fragment). The *Bam*HI site was detected by restriction enzyme digestion. Boundaries of deletions and the presence of in-frame stop codons were determined by sequencing. Four carboxyl terminal deletions, C38, C60, C163, and C328, were generated in this fashion. C-terminal deletion C24 was constructed by introducing an in-frame stop codon by oligonucleotide-directed mutagenesis.

Purification of the Wild Type and Active N-Terminal Truncated Forms of Glycyl-tRNA Synthetase. The purification procedures were performed at 4 °C. Cells (80−100 g) stored at −80 °C were partially thawed, suspended gently with a glass rod in lysis buffer [50 mM Tris-HCl (pH 7.5), 10% glycerol, 0.5 mM DTT, 0.1% (v/v) Triton X100, 1 mM PMSF, 0.5 mM TAME, 5 mM ethylenediaminetetraacetic acid (EDTA), and 10 μg/mL soybean trypsin inhibitor], kept on ice until cells lysed (20 min), and then centrifuged for 1 h at 35 000 rpm in a Beckman type 35 rotor. The cell extract was brought to 1 mM glycine, 5 mM ATP, and 12 mM magnesium acetate and stirred for 30 min. A 50% (w/v) solution of poly(ethylene glycol) 8000 (PEG) was slowly added to 10% (w/v); magnesium ions were essential for this precipitation step. After the mixture was stirred for 30 min, the precipitate was collected by centrifugation for 30 min at 15 000 rpm, and then redissolved in 20 mM potassium phosphate (pH 7.5), 20% glycerol, 0.5 mM EDTA, and 0.5 mM DTT. The sample was applied to a 2.5 cm × 10 cm column of Q-Sepharose equilibrated in the same buffer, and the column was washed with 3−4 bed volumes of starting buffer and eluted with a linear gradient from 20 mM (150 mL) to 150 mM (150 mL) potassium phosphate buffer (pH 7.5), containing 20% glycerol, 0.5 mM EDTA, and 0.5 mM DTT. The wild type enzyme eluted at 85 mM potassium phosphate, while some N-terminal deletion mutants (e.g. N55) eluted at higher phosphate concentrations (up to 120 mM). The column was eluted at 50 mL/h and fractions of 5 mL were collected. Active fractions were pooled, concentrated 10-fold (to less than 10 mL) in an Amicon cell using a PM10 membrane, and applied to a 2.5 cm × 80 cm column of Sephacryl S-200 equilibrated with 50 mM potassium phosphate (pH 7.5), 20% glycerol, 0.5 mM EDTA, and 0.5 mM DTT. The S-200 column was eluted at 20 mL/

h, and fractions of 3.5 mL were collected. Samples taken at various steps were examined for aminoacyl-tRNA synthetase activity and by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Western blot analysis. The purified enzymes were also analyzed by active site titration. Using this procedure, 70−110 mg of the wild type enzyme and the N-terminal deletion mutants were isolated with overall yields in excess of 50%.

Enzyme Assays. Aminoacyl-tRNA synthetase assays were performed in 25 μL reaction mixtures as previously described (Dignam & Dignam, 1984), measuring the incorporation of [^{14}C]amino acid into acid insoluble aminoacyl-tRNA employing partially purified bovine liver tRNA^{Gly}. The pyrophosphate exchange assay was performed in 100 μL reaction mixtures at equilibrium as previously described (Eigner & Loftfield, 1974) by measuring the incorporation of [^{32}P]-pyrophosphate into Norit absorbable ATP. Active site titration assays were performed by a modification of a described method (Fersht et al., 1975) by measuring the release of [^{32}P]pyrophosphate from [γ-^{32}P]ATP at 4 °C. Reactions were performed at 4 °C due to the high rate of turnover of the enzyme−adenylate complex in the absence of tRNA at higher temperatures. Control reactions were performed in the absence of glycine to verify the dependence of the reaction on the amino acid. The [γ-^{32}P]ATP used was over 90% usable as determined by the phosphorylation of histone by cAMP dependent protein kinase (Reimann & Beham, 1983). A time course of release of [^{32}P]pyrophosphate was examined, and the data were fitted to an equation of the form

$$y = A(1 - e^{-Bx}) + Cx$$

The term $A = E_0[k_1'/(k_1' + k_2)]^2$, $B = k_1' + k_2$, and $C = E_0[k_1'k_2/(k_1' + k_2)]$. k_1' corresponds to the product of a rate constant and concentration terms for the formation of the E*AMP−Gly intermediate from free enzyme, glycine, and ATP; k_2 is the rate constant for the decomposition of the E*AMP−Gly complex. The variable y is the amount of pyrophosphate released, and x is time. The concentration of active enzyme was also estimated by extrapolation of the linear phase following the burst to the ordinate intercept as described by Fersht (1985).

Cross-Linking Experiments. Protein cross-linking reactions were performed using bis(sulfosuccinimidyl) suberate (BS^3) as a cross-linking reagent. Reaction mixtures of 100 μL containing 0.2 μM enzyme and 0.6 mM BS^3 in 50 mM potassium phosphate (pH 7.5), 20% glycerol, 0.5 mM EDTA, and 0.5 mM DTT were incubated for 10 min at 25 °C; the reactions were stopped by the addition of SDS and mercaptoethanol, and the products were examined by SDS−PAGE.

Western Blot Analysis on Crude Extracts. To analyze the expression of the mutants, Western blot analysis was performed using affinity-purified antibody specific for the wild type enzyme purified as described (Smith & Fisher, 1984). Samples of cultures were taken at specific times following induction with IPTG; the cells were collected by centrifugation, frozen on dry ice, and stored at −80 °C. Crude extracts were prepared by thawing the cells on ice and lysing the cells in 5 volumes of the lysis buffer. After 10 min, the lysates were spun for 30 min at 13 000 rpm in a microcentrifuge, the supernatant fractions were treated with mercaptoethanol, and SDS and equivalent amounts of extract based on cell density were applied to an SDS−polyacrylamide gel in 10 μL. After electrophoresis, the samples were transferred electrophoretically to a nitrocellulose membrane and detected using affinity-purified antibody.

Determination of Molecular Parameters for N-Terminal Truncated Forms and the Wild Type Glycyl-tRNA Synthetase. Stokes radii were determined by gel filtration on Sephacryl S-300 using bovine serum albumin (35 Å), bovine catalase (52 Å), and ferritin (75 Å) as standards (Siegel & Monty, 1965). Sedimentation experiments were performed on 5−20% sucrose gradients using human hemoglobin (4.3×10^{13} s), yeast alcohol dehydrogenase (7.4×10^{13} s), and bovine catalase (11.3×10^{13} s) as standards (Martin & Ames, 1961). Frictional ratios and molecular weights were calculated on the basis of the Stokes radii and the sedimentation coefficients and a partial specific volume of 0.725 cm^3/g on the basis of amino acid composition.

Determination of Kinetic Parameters. Kinetic studies were performed using the aminoacyl-tRNA synthetase activity assay. Michaelis constants for ATP and glycine were determined at four concentrations of glycine from 0.05 to 0.5 mM and four concentrations of ATP from 0.01 to 0.2 mM at saturating tRNA (4.2 μM). The Michaelis constant for tRNA was determined at five concentrations of tRNA from 0.02 to 4.2 μM and three concentrations of glycine from 0.1 to 0.5 mM at saturating ATP (5 mM). The initial rates measured were verified by examination of time courses at the lowest and highest concentration of substrates. Turnover numbers (k_{cat}) were calculated from the double reciprocal plots of rate vs substrate concentration.

Zone-Interference Electrophoresis. Zone electrophoresis to examine tRNA binding to glycyl-tRNA synthetase was performed as described (Abrahams et al., 1988) using 8 cm × 6 cm × 1.5 mm 2% vertical agarose gels employing a Biorad minigel apparatus. Electrophoresis was performed at 200 V, and temperature was maintained at 6−10 °C by immersion of the electrophoresis chamber in ice−water. Enzyme (1 μg in 5 μL) in 20 mM Tris-acetate (pH 7.6), 3.5 mM $MgCl_2$, and 10% (v/v) glycerol was layered beneath a tRNA solution (50 μL) in the same solvent containing 4% (v/v) glycerol.

Gel Filtration of Wild Type Glycyl-tRNA Synthetase and Mutant N55 with tRNA. Gel filtration experiments (Figure 7) were performed to examine the association of wild type and mutant N55 enzyme with tRNA essentially as described (Kawakami & Nishio, 1985). Samples were applied to a column of Sephacryl S-300 (1.5 cm × 89 cm) equilibrated in 20 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 8 mM $MgCl_2$, 0.1 M KCl, and 2 mM DTT; the column was eluted at 11.6 mL/h, and fractions of 1.7 mL were collected. Samples of 0.4 mL containing wild type or N55 glycyl-tRNA synthetase (2 mg/mL) and tRNA^{Gly} or tRNA^{Ala} (0.38 mg/mL) were applied to the column.

RESULTS

Expression of Wild Type and Truncated Forms of Glycyl-tRNA Synthetases. Constructs encoding the wild type enzyme and the mutants were placed in an expression vector (pET 12a) that provides for IPTG inducible expression from a T7 phage promoter. The production of active wild type glycyl-tRNA synthetase reached the highest level 12−15 h after induction with IPTG. The expressed enzyme was

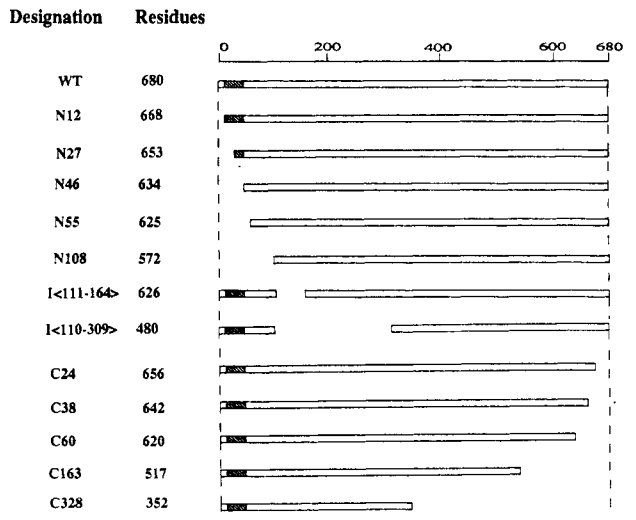

FIGURE 1: Truncated forms of glycyl-tRNA synthetases. The shaded area shows the N-terminal alignment sequence.

soluble and intact and constituted 2−3% of the soluble protein. No difference between the recombinant enzyme and the enzyme purified from silk gland was detected with respect to subunit and oligomeric molecular weight, immunological properties as detected by SDS−PAGE, and western blot analysis and specific activity. The wild type enzyme and mutants N12, N27, N46, and N55 were purified by successive 0−10% PEG precipitation, Q-Sepharose chromatography, and Sephacryl S-200 gel filtration. The scheme employed was based partly on the method used for silk gland enzyme (Dignam & Dignam, 1985). Q-Sepharose, which was used in place of DEAE Sephacel, gave a 13−15-fold purification. The purified enzymes were intact, with the expected subunit molecular weights, and were greater than 90% pure as estimated by SDS−PAGE (Figure 2). The wild type GRS had a specific activity of 7000−10000 units (nanomoles of [^{14}C]glycine−tRNA min^{-1}) mg^{-1} of protein, compared to 5000−7000 units mg^{-1} for the enzyme from silk glands.

To identify the functional domains of glycyl-tRNA synthetase, we constructed deletion mutants from both the N-terminus and C-terminus as shown in Figure 1. The N-terminal deletion mutants were designed to remove different amounts of structure in the N-terminal sequence that aligns with sequences in several tRNA synthetases (Table 1); the alignment is weakest with seryl-tRNA synthetase. Mutant N12 maintained the entire alignment sequence. Half of the alignment sequence was removed in mutant N27. N46 removed the entire alignment sequence, and an additional nine residues were removed in mutant N55. All of these deletion mutants were expressed at high levels relative to the wild type enzyme and were soluble and intact, with the expected subunit molecular weight based on SDS gel electrophoresis compared to that predicted from the sequence (Figure 2). Mutants N12, N27, N46, and N55 had both aminoacyl-tRNA synthetase activity and pyrophosphate exchange activity. Mutant N108, which lacked 53 residues relative to mutant N55, was inactive, as were two internal deletions, I<111−164> and I<110−309>, lacking 54 and 200 residues, respectively. The time course of expression of mutant N108 differed from that of the wild type enzyme and the other mutants as shown in the Western blot in Figure 3. Expression was highest at 4 h after induction with IPTG and then declined. N108 was partially purified by 0−10% PEG precipitation and Q-Sepharose chromatography; the protein was monitored by Western blot analysis. This preparation was approximately 50% pure based on SDS gel electrophoresis and Western blot analysis. No aminoacyl-tRNA synthetase activity or pyrophosphate exchange activity was detected in this preparation of the mutant, nor was activity detected in extracts prepared from cells expressing two internal deletions, I<111−164> and I<110−309>. Most of the C-terminal deletion mutants were not expressed

Table 1: Alignment of the N-Terminal Sequence of Glycyl-tRNA Synthetase with Similar Sequences in Other Aminoacyl-tRNA Synthetase

		N12[a] N27[a] N46[a] N55[a]
GRS[b]	*B. mori*	11-APLRANVKE--QGDLVRKLKEEKAPEIDIKKAVAELKTRKKILEDKE
GRS[b]	human	11-APLRLAVRQ--QGDLVRKLKEDKAPQVDVDKAVAELKARKRVL
WRS[b]	bovine	13-SPLELFHSIAAQGELVRDLKARNAAKDEIDSAVKMLLSLKTSY
HRS[b]	human	5-APLEELVKL--QGERVRGLKQQKASAELIEEEVAKLLKLKAQL
EPRS[b]	human	688-QGDVVRELKAKKAPKEDVDAAVKQLLSLKAEY
EPRS[b]	*Drosophila*	755-QGDLVRDLKSKKAAKDQIDVAVKKLLALKADY
SRS[b]	mouse	54-NKLKNLCSKTIGEKMKKKEAVGDDESVPENVL
SRS[b]	yeast	56-FNKLQKDIGLKFKNKEDASGLLAEKEKLTQQK
SRS[b]	*E. coli*	51-RNSRSKSIGQAKARGEDIEPLRLEVNKLGEEL

[a] Position of N-terminal deletions of *B. mori* glycyl-tRNA synthetase. [b] GRS, glycyl-tRNA synthetase (Nada et al., 1993); WRS, tryptophanyl-tRNA synthetase (Garret et al., 1991); HRS, histidyl-tRNA synthetase (Raben et al., 1992); EPRS, glutamylprolyl-tRNA synthetase [Cerini et al. (1991) for *Drosophila*; Fett and Knippers (1991) for mouse and human]; SRS, seryl-tRNA synthetase [Härtlein et al. (1987) for *E. coli*; Weygand-Durasevic et al. (1987) for yeast; Miseta et al. (1991) for mouse.

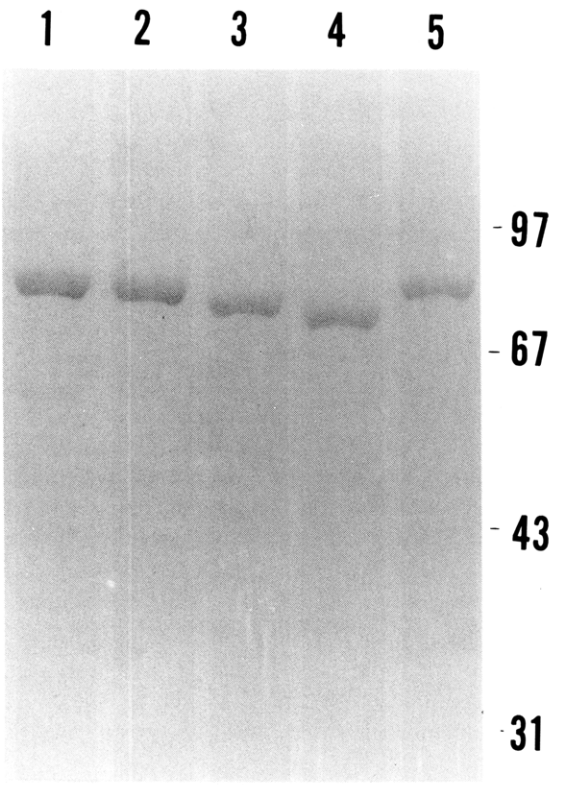

FIGURE 2: SDS−PAGE of purified wild type and N-terminal truncated glycyl-tRNA synthetases. The N-terminal mutants and wild type GRS were purified as described in Experimental Procedures. A sample (0.5 μg) of each protein was applied to a 10% SDS−PAGE. Samples are mutant N12 (lane 1), mutant N27 (lane 2), mutant N46 (lane 3), mutant N55 (lane 4), and wild type enzyme (lane 5). The molecular weight standards ($M_r \times 10^{-3}$) are indicated at the right side of the figure. Proteins were stained with Coomassie Brilliant Blue R 250.

at high levels, though the proteins were soluble and intact as shown by Western blot analysis (Figure 4); none had detectable aminoacyl-tRNA synthetase activity. Several expression conditions were tested to increase the level of expressed protein, including growth in various types of media and at different temperatures, but none of the conditions tested significantly improved expression. Several methods for purifying mutants C38, C60, and C328 which gave partial purification were tested. C38 and C328 remained associated with high-molecular weight nucleic acid throughout the purification steps and eluted in the void volume of Sephacryl S300; we did not establish whether they were bound to nucleic acid or ribosomes or were merely aggregated. The partially purified preparations of C38, C60, and C328 lacked aminoacyl-tRNA synthetase activity.

Aminoacyl-tRNA Synthetase Activity and Pyrophosphate Exchange Activity. We examined the purified wild type and N-terminal-deleted enzymes for both aminoacyl-tRNA synthetase activity and pyrophosphate exchange activity by standard methods described in Experimental Procedures. Mutants N12 through N55 had 15−25% of both activities relative to those of the wild type enzyme (Figure 5), while mutant N108 had no detectable activity in either assay. Active site titration experiments on the purified N12, N27, N46, and N55 enzymes indicated that 80−90% of the enzyme was active (data not shown); this analysis assumes that the initial burst in the release of pyrophosphate that occurs when the enzyme combines with ATP and glycine arises from the rapid formation of the aminoacyl adenylate followed by its slower decomposition. Hence, the differences in enzymatic activity for N12 through N55 are not the result of the production of enzyme that is inactive in the synthesis of the adenylate but represents an intrinsic property of the deletion mutants. Transfer RNA did not stimulate the ATP $\rightleftharpoons$ PP_i exchange reaction with the wild type enzyme or with the mutants. Extracts from cells harboring the vector (pET12a) had no detectable glycyl-tRNA synthetase activity under standard assay conditions since the silkworm and human enzymes (Shiba et al., 1994) either do not aminoacylate *E. coli* glycyl tRNAs or do so at a drastically lower rate. When such extracts were carried through the purification through the Q-Sepharose step, pyrophosphate exchange activity was not detectable at the dilutions equivalent to those used in the experiment shown in Figure 5. Hence, the *E. coli* glycyl-tRNA synthetase makes no significant contribution to the results. Further characterization of the N-terminal mutants was performed to explore the effect of the deletions on other enzymatic and physical properties.

Physical Properties of the Active N-Terminal Deletion Forms and Wild Type Glycyl-tRNA Synthetases. For the active N-terminal deletion mutants and the wild type glycyl-tRNA synthetase, Stokes radii and sedimentation coefficients were determined and the molecular weight and frictional ratios were calculated (Table 2). Mutants N12, N27, N46,

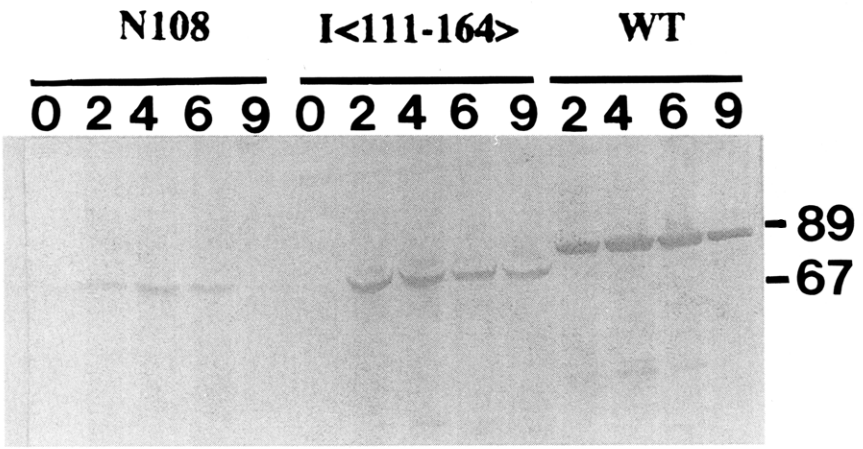

FIGURE 3: Western blot analysis of the time course of expression of mutants N108 and I<111−164> and wild type GRS. The samples (from 0 to 12 h) were taken after IPTG induction. Samples of 10 μL of the crude extracts for each mutant were applied to an SDS−polyacrylamide gel, transferred to a nitrocellulose membrane, and detected using affinity-purified anti-GRS antibody. Crude extracts were prepared as described in Experimental Procedures.

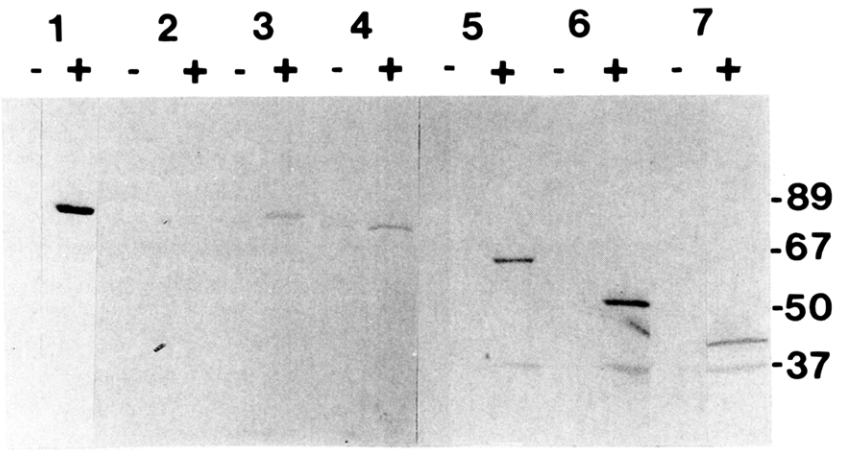

FIGURE 4: Expression of the C-terminal truncated forms of GRS. Samples of 20 μL of crude extract for C-terminal mutants and 2 μL for the wild type enzyme were applied to the gel; − indicates samples taken before IPTG induction, and + indicates samples taken 12 h after IPTG induction. Numbers at the top of the gel indicate wild type GRS (1), expression vector pET-12a only (2), mutant C24 (3), mutant C38 (4), mutant C60 (5), mutant C163 (6), and mutant C328 (7).

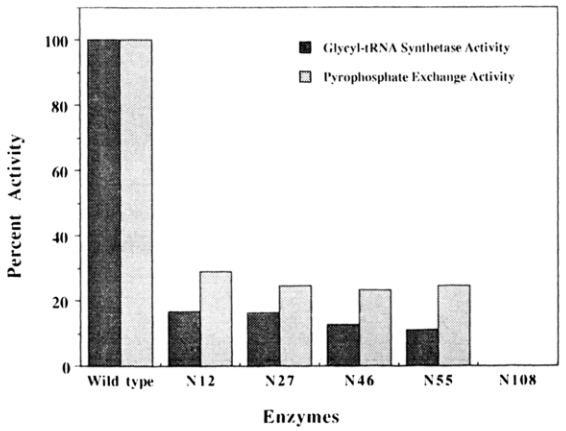

FIGURE 5: Glycyl-tRNA synthetase activity and pyrophosphate exchange activity of the N-terminal mutants and wild type GRS. The rate of aminoacylation of $tRNA^{Gly}$ (darkly shaded bars) was measured using the standard assay (Dignam & Dignam, 1984), with 0.01 pmol enzyme added to each reaction mixture. The rate of pyrophosphate exchange (lightly shaded bars) was measured as described in Eigener and Loftfield (1974); 0.323 pmol of each enzyme was added to each reaction mixture. Enzyme concentrations are based on molecular weights of 154 000 for the wild type enzyme and 141 200 for mutant N55. Results were expressed relative to the activity of the wild type enzyme (100%).

Table 2: Physical Properties of Wild Type (WT) and N-Terminal Deletion Mutants of Glycyl-tRNA Synthetase

enzyme	R_s[a] (Å)	$S_{20,w}$[a]	f/f_0[b]	$M_{r,oligomer}$[b]	$M_{r,subunit}$[c]
WT	55.5	6.93	1.57	155 000	76 919
N12	56.5	6.55	1.61	150 000	75 611
N27	53.5	6.17	1.59	134 000	73 890
N46	51.8	6.41	1.53	134 000	71 767
N55	49.8	5.95	1.51	120 000	70 627

[a] Values for R_s and $S_{20,w}$ were determined from gel filtration and sedimentation experiments as described in Experimental Procedures. [b] Values for f/f_0 and $M_{r,oligomer}$ were calculated from the Stokes radii and sedimentation constants using a value of 0.725 $cm^3\ g^{-1}$ for the partial specific volume. [c] Values for the subunit molecular weight were based on amino acid sequence.

and N55 and the wild type enzyme did not differ significantly in size as indicated by their Stokes radii or in shape as indicated by their sedimentation coefficients and frictional ratios. The molecular weights calculated indicate that wild type (Dignam & Dignam, 1984; Nishio & Kawakami, 1985) and N-terminal-truncated enzymes are dimeric. To corroborate the results of sedimentation and gel filtration experiments, cross-linking experiments were performed using bis(sulfosuccinimidyl) suberate. The N-terminal mutants exhibited the same behavior as the wild type enzyme in their patterns of cross-linked species seen on SDS−PAGE; the cross-linked species observed with the mutants were appropriately smaller than the wild type enzyme and were in accord with the molecular weights calculated from sedimentation and gel filtration data. Deletion of the N-terminal structure had no significant effect on the oligomeric state and did not significantly alter the shape of the molecule.

Kinetic Properties of the Active N-Terminal Deletion Forms and the Wild Type Glycyl-tRNA Synthetases. To determine the effect of the N-terminal structure on the kinetic properties of the enzyme, mutant N55 and the wild type enzyme were examined. Mutant N55 was chosen as a representative of the active N-terminal mutants, since all had similar characteristics with respect to enzymatic activity and physical properties. In one set of experiments, the K_m values for ATP and glycine were determined by varying the concentrations of ATP and glycine at saturating tRNA (Figure 6A, wild type; Figure 6C, N55). In a second set of experiments, the concentrations of tRNA and glycine were varied at saturating ATP (Figure 6B, wild type; Figure 6D, N55). Values for Michaelis constants and k_{cat} for the wild type enzyme and mutant N55 from several experiments are summarized in Table 3. The double reciprocal plots shown in Figure 6A for the wild type and Figure 6C for mutant N55 indicate that addition of glycine and ATP to the enzyme is sequential. The pattern of parallel lines in Figure 6B,C when tRNA is varied at fixed concentrations of glycine and the absence of a requirement for tRNA in the pyrophosphate−ATP exchange reaction indicate that pyrophosphate is released before tRNA addition in a classic ping−pong type mechanism (Cleland, 1963) as has been observed for many aminoacyl-tRNA synthetases (Freisht, 1989). The K_m values for ATP, glycine, and tRNA were not significantly different between mutant N55 and the wild type enzyme. The wild type enzyme and N55 also had similar magnesium optima. The k_{cat} value for mutant N55 was reduced 5−10-fold relative to that of the wild type enzyme. The results indicate that deletion of 55 residues from the N-terminus does not result

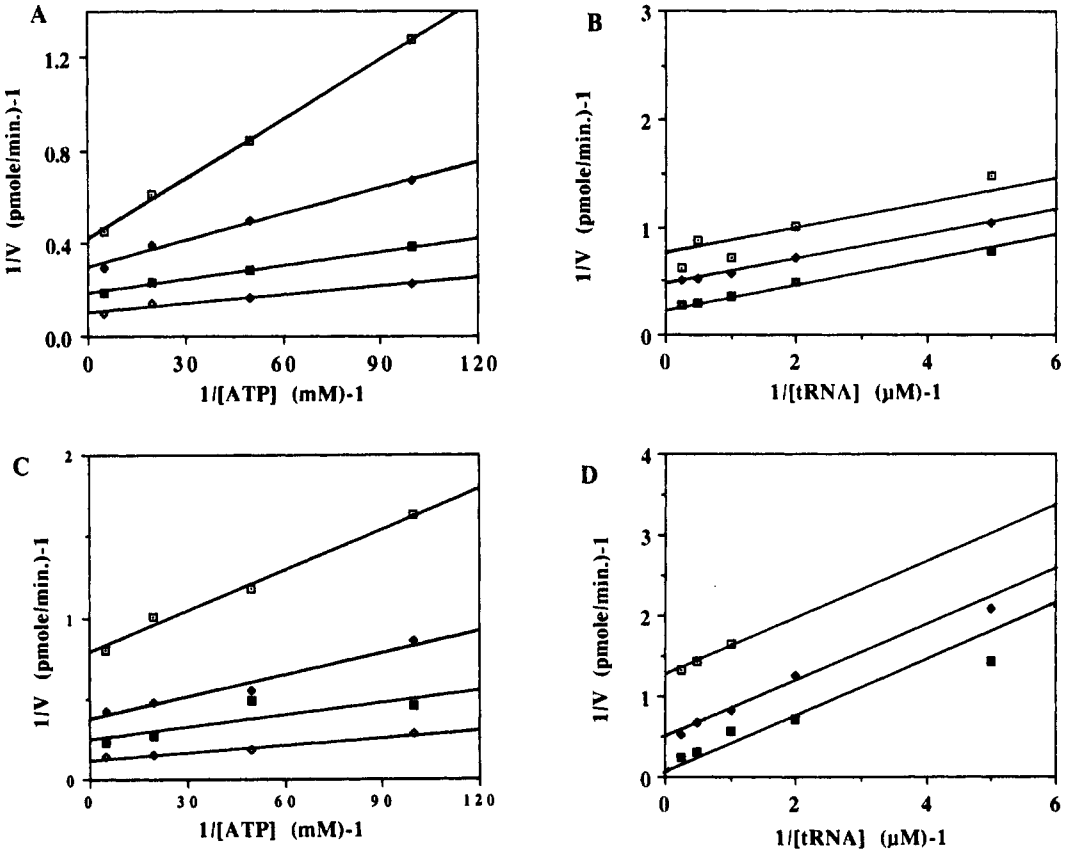

FIGURE 6: Substrate saturation experiments for ATP, glycine, and $tRNA^{Gly}$ with wild type glycyl-tRNA synthetase and the N-terminal mutant N55. Panels A and B show results obtained with wild type glycyl-tRNA synthetase, and panels C and D show those obtained with mutant N55. A and C show plots of $1/v$ vs 1/[ATP] at saturating tRNA (4.2 μM), respectively. The concentrations of glycine in A and C were 0.05 mM (⊡), 0.1 mM (◆), 0.2 mM (■), and 0.5 mM (◇). Each incubation mixture contained 0.0168 pmol of wild type GRS (A) and 0.0408 pmol of mutant N55 (C). B and D show plots of $1/v$ vs 1/[tRNA] at saturating [ATP] (5.0 mM). The concentrations of glycine in B and D were 0.1 mM (⊡), 0.2 mM (◆), and 0.5 mM (◇). Each reaction mixture contained 0.001 68 pmol of wild type GRS (B) and 0.0206 pmol of mutant N55 (D). Other experimental details are described under Experimental Procedures.

Table 3: Kinetic Parameters for Wild type and N55 Glycyl-tRNA Synthetase

enzyme	$K_{ATP}{}^a$ μM	$K_{gly}{}^a$ mM	$K_{tRNA}{}^b$ (μM)	$k_{cat}{}^a$
wild type	13 ± 6.1	0.35 ± 0.05	1.2 ± 0.8	29 ± 4.8
N55	14 ± 1.2	0.55 ± 0.25	1.0 ± 0.3	5.1 ± 1.0

[a] K_{ATP}, K_{gly}, and k_{cat} values are the mean of four determinations ± standard error. [b] K_{tRNA} values are the mean of three determinations ± standard error.

in significant effects on the Michaelis constants for substrates but does reduce k_{cat} significantly.

RNA Binding Properties of Wild Type and N55 Glycyl-tRNA Synthetase. RNA binding properties of glycyl-tRNA synthetase were examined by zone electrophoresis and gel filtration as shown in Figures 7 and 8. Though not readily discernable in Figure 7 (top left), at least two species were detected by zone electrophoresis in a number of experiments with wild type enzyme when the binding of *B. mori* $tRNA^{Gly1}$ was examined [Figure 7 (top left)]. The two species may represent enyzme with one and two tRNAs bound. Binding of the wild type enzyme to *B. mori* $tRNA^{Ala/CAU}$ was also detected [Figure 7 (top right)]. Under the same conditions, binding of alanyl-tRNA synthetase to $tRNA^{Ala/CAU}$ was detected, but binding of alanyl-tRNA synthetase to $tRNA^{Gly1}$ was not (data not shown). Mutant N55 bound to $tRNA^{Gly1}$ [Figure 7 (bottom left)], though, with reduced affinity and binding to $tRNA^{Ala/CUA}$, was not detected. These results were confirmed by gel filtration experiments (Figure 8) which indicated that the wild type enzyme formed an isolatable complex with $tRNA^{Gly1}$ (Figure 8A) but did not form a complex with $tRNA^{Ala/CUA}$ sufficiently stable (Figure 8B) to permit its isolation; the elutions of both the tRNA and the enzyme were shifted toward larger species. No binding of N55 to $tRNA^{Gly1}$ was detectable by this approach (Figure 8C); enzyme and tRNA eluted at the positions of free enzyme and free tRNA. Results of zone electrophoresis using unfractionated tRNA suggest that wild type enzyme binds to a number of noncognate tRNAs (data not shown).

DISCUSSION

B. mori glycyl-tRNA synthetase expressed in bacteria was purified to homogeneity with a specific activity equal to or greater than that of the enzyme from silk glands. The expressed enzyme and the enzyme from silk glands had the same subunit and oligomeric molecular weight and similar values for kinetic constants. The N-terminus of glycyl-tRNA synthetase contains a 34-amino acid motif that aligns with sequences in other aminoacyl-tRNA synthetases (Fett & Knippers, 1991; Garret et al., 1991; Raben et al., 1992). Truncated forms of the enzyme, with the N-terminal motif interrupted (N12 and N27) or with the motif deleted (N46 and N55), were active with 15−25% of aminoacyl-tRNA synthetase activity and pyrophosphate exchange activity compared to those of the wild type enzyme. Active site titrations indicated that the difference in activity was not the result of production of inactive enzyme. The hydrodynamic

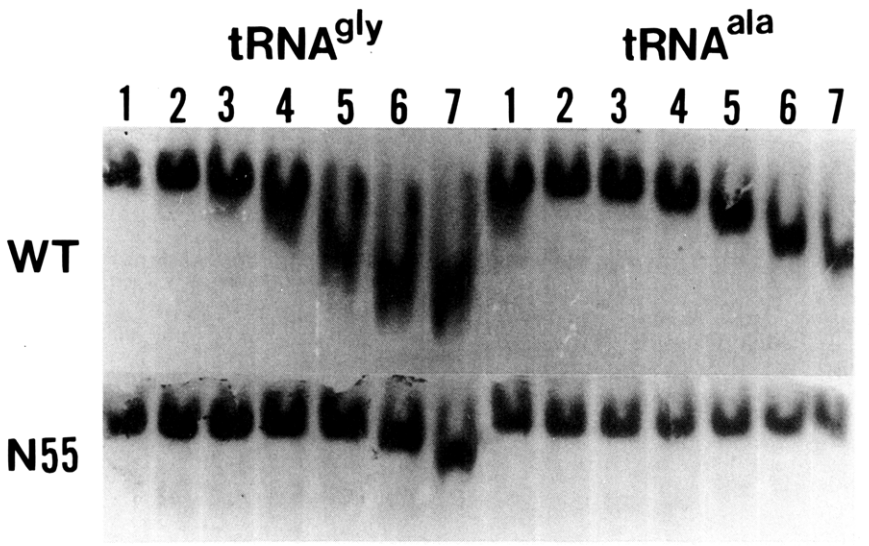

FIGURE 7: Zone electrophoresis of wild type and N55 glycyl-tRNA synthetase. Samples of wild type glycyl-tRNA synthetase (WT) or deletion mutant N55 (N55) were subjected to zone electrophoresis with the indicated concentrations of $tRNA^{Gly}$ or $tRNA^{Ala}$: lane 1, 0; lane 2, 0.25 μM; lane 3, 0.5 μM; lane 4, 1.0 μM; lane 5, 2 μM; lane 6, 5 μM; and lane 7, 10 μM.

properties of the N-terminal deletions and chemical cross-linking experiments indicated that the mutants and the wild type enzyme are dimers. Deletion of 108 residues (mutant N108) resulted in a protein lacking enzymatic activity, as did the two internal deletions (I<111−164> and I<110−309>) which retain the N-terminal motif. By comparison to the structure of the *Thermus thermophilus* enzyme (Logan et al., 1995), mutant N108 removes part of motif 1, I<111−164> removes motif 1 entirely, and I<110−309> removes motif 1 and part of motif 2. The first 55 N-terminal residues are not essential for catalysis, while deletion of additional structures (e.g. N108, I<111−164>, and I<110−309>) either removes essential features of the active site or results in an unstable structure. Removal of a similar N-terminal motif in the human histidyl-tRNA synthetase appears to abolish aminoacyl-tRNA synthetase activity (Raben et al., 1994), though pyrophosphate exchange was not examined. The authors suggested that this structure functions in tRNA binding as does a similar structure in *E. coli* seryl-tRNA synthetase. Deletion of the N-terminal arm of the seryl-tRNA synthetase reduced aminoacylation activity and increased $K_{tRNA^{Ser}}$ 30-fold but did not affect the amino acid activation step (Borel et al., 1994). Glycyl-tRNA synthetase differs from the serine and histidine enzymes since deletion of the first 55 N-terminal residues did not have significant effects on the Michaelis constants for ATP, glycine, or tRNA but reduced k_{cat} 5−10-fold. The result is consistent with an altered rate of adenylate formation or pyrophosphate release, since both amino acid activation and aminoacylation were affected. The eukaryotic aminoacyl-tRNA synthetases that have this motif charge different amino acids, and except for seryl-tRNA synthetase, an equivalent sequence is absent in their prokaryotic counterparts. This structure is located outside the core catalytic domains formed from the three sequence motifs characteristic of class II aminoacyl-tRNA synthetases. While this structure is not essential for the formation of the active site, the reduction in the rate of pyrophosphate exchange and a similar effect on the rate of aminoacylation suggest that the N-terminal domain has an effect on the structure of the active site that is manifested in the reduced value for k_{cat}. The N-terminal structure is not involved in amino acid or ATP binding, and the unaltered $K_{tRNA^{Gly}}$ value suggests that the structure is not crucial for tRNA binding in aminoacylation, which is consistent with its absence in the yeast glycyl-tRNA synthetase (Shiba et al., 1994) and the *T. thermophilus* enzyme (Logan et al., 1995); both the yeast and *T. thermophilus* enzymes have obvious similarities with the human and insect enyzmes in other regions of the polypeptide. Analysis of the N-terminal structure (Borel et al., 1994) in seryl-tRNA synthetase prompted Raben et al. (1994) to extend the comparison of the N-terminal motif found in several aminoacyl-tRNA synthetases to the N-terminal coiled-coil structure of *E. coli* seryl-tRNA synthetase. They noted limited sequence similarity between histidyl-tRNA synthetase and several other aminoacyl-tRNA synthetases (including glycyl-tRNA synthetase) and the *E. coli* seryl-tRNA synthetase over a 32-amino acid residue region. The similarity of the N-terminal motifs in *B. mori* and human (Ge et al., 1994; Shiba et al., 1994; Williams et al., 1995) glycyl-tRNA synthetase to the corresponding sequence in the human histidine enzyme and conformational studies of synthetic peptides based on the sequence of the histidine enzyme suggest that the N-terminal structure forms an antiparallel coiled coil, similar to the N-terminus of *E. coli* seryl-tRNA synthetase (Cusack et al., 1990). The absence of detectable effects of its removal on the Michaelis constant for tRNA may indicate that the Michaelis constant does not reflect binding but is a complex kinetic constant. An alternate explanation is that there are two RNA binding sites, one for the aminoacylation of tRNA and a second effector site that accommodates either the cognate $tRNA^{Gly}$ or noncognate tRNAs, such $tRNA^{Ala}$. While $tRNA^{Gly}$ is a logical candidate for the ligand which would bind to such a site, it may not be the physiologically relevant ligand.

Some indication of other cellular functions for aminoacyl-tRNA synthetases may be seen in the participation of mammalian seryl-tRNA synthetase in protein synthesis initiation (Miseta et al., 1991), in the involvement of mitochondrial tyrosyl-tRNA synthetase in splicing class I introns (Mohr & Lambowitz, 1991; Roland et al., 1995), and in the involvement of tryptophanyl-tRNA synthetase and

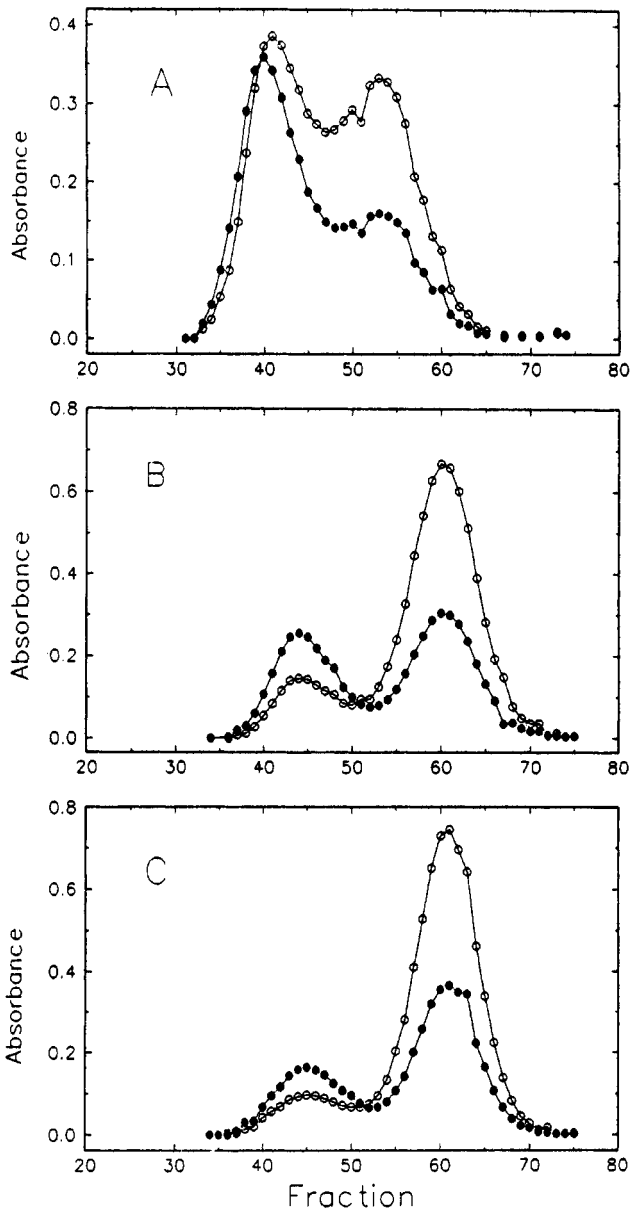

FIGURE 8: Gel filtration of wild type and N55 glycyl-tRNA synthetase with $tRNA^{Gly}$ and $tRNA^{Ala}$. Samples were prepared as described in Experimental Procedures and applied to a column of Sephacryl S-300: (A) wild type glycyl-tRNA synthetase with glycyl-tRNA, (B) wild type glycyl-tRNA synthetase with alanyl-tRNA, and (C) glycyl-tRNA synthetase deletion mutant N-55 with glycyl-tRNA. Solid circle: absorbance at 280 nm; open circles, absorbance at 260 nm.

other aminoacyl-tRNA synthetases in the synthesis of diadenosine 5′,5″-P^1,P^4-tetraphosphate (Varshavsky, 1983; Zamecnik, 1983). Some N-terminal extensions can be removed without affecting aminoacyl-tRNA synthetase activity; examples are the yeast glutaminyl- (Ludmerer & Schimmel, 1987), lysyl- (Mirande & Waller, 1988), and methionyl-tRNA synthetases (Walter et al., 1989) and both yeast (Lorber et al., 1988) and human (Escalante & Yang, 1993) aspartyl-tRNA synthetases. The N-terminal structure in glycyl-tRNA synthetase (and other tRNA synthetases) may be involved in tRNA binding in some cases, such as seryl-tRNA synthetase and perhaps histidyl-tRNA synthetase, but might serve other functions in enzymes such as glycyl-tRNA synthetase and the bifunctional glutamyl-prolyl-tRNA synthetase (Fett & Knippers, 1991; Cerini et al., 1991). Fragments of the human glutamyl-prolyl-tRNA synthetase containing the repeated structure bind to the 3′-end of its mRNA *in vitro* (Schray & Knippers, 1991), suggesting a role in regulation of translation or mRNA stability. Given the similarity of the N-terminal structure in eukarotic glycyl-tRNA synthetases to structures in the glutamyl-prolyl-tRNA synthetase, it is likely they serve similar functions. Since this structure is not found in the yeast or *T. thermophilus* glycyl-tRNA synthetase (Logan et al., 1995), it presumably is required for a function restricted to higher eukaryotes.

Deletions from the C-terminus of glycyl-tRNA synthetase lacking 24, 38, 60, 163, and 328 residues had no detectable activity. That partially purified C328 lacked activity would be expected since motif 3 has been removed. However, one might have expected the smallest deletion mutants, C24, C38, and C60, to at least have pyrophosphate exchange activity since structures that form the adenylate binding site have been preserved. The partially purified C38 and C60 mutants had no detectable pyrophosphate exchange activity or aminoacyl-tRNA synthetase activity. Results obtained with the smallest C-terminal deletions must be viewed cautiously since the absence of detectable activity may reflect inherent instability of these structures and may imply that the C-terminal sequence is required for proper folding or stability of the protein. A sequence comparison (Cusack, 1993) indicated that *B. mori* glycyl-tRNA synthetase contains a C-terminal extension (residues 558−680) which is similar to the C-terminal extensions of the threonyl-, prolyl-, and histidyl-tRNA synthetases. This domain is thought to be involved in tRNA anticodon recognition.

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