GLYCYL-tRNA SYNTHETASE: EVIDENCE FOR TWO ENZYME FORMS AND SIGMOIDAL SATURATION KINETICS.

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Summary

Two forms of glycyl-tRNA synthetase from E. coli have been resolved by DEAE-Sephadex chromatography. The two forms display different kinetic responses to increasing tRNA concentration. One gives a sigmoidal response and a Hill coefficient of 2.2 ± 0.2 while the other shows Michaelis-Menten kinetics. It is suggested that these data may indicate a role for glycyl-tRNA synthetase in the regulation of protein biosynthesis. During DEAE-Sephadex chromatography and ammonium sulfate fractionation the addition of substrates (glycine, ATP, and $MgCl_2$) has proven to be of significant value in resolving the two enzyme forms from each other and from contaminating proteins.

Each aminoacyl-tRNA synthetase plays an essential role in protein biosynthesis by catalyzing the formation of specific aminoacyl-tRNAs. Despite the fact that these enzymes catalyze the same basic reaction, they display a wide diversity in quaternary structure (1). Purified synthetases from a variety of sources have been shown to exist as monomers as well as oligomers of either similar or dissimilar subunits.

Glycyl-tRNA synthetase from E. coli has an $\alpha_2\beta_2$ structure with subunits of 33,000 and 80,000 daltons (2). Although the presence of dissimilar subunits may be suggestive of a regulatory role by analogy to enzymes such as aspartate transcarbamylase (3), previous kinetic studies with glycyl-tRNA synthetase (4) yielded linear Lineweaver-Burk plots for glycine, ATP, and tRNA. Our studies of glycyl-tRNA synthetase were initiated with the aim of investigating the function of the subunits comprising this complex enzyme. Our isolation was based initially on the procedure described by Ostrem and Berg (4), but we introduced changes designed to decrease the possibility of proteolysis during isolation and to make purification steps more efficient. With these changes, two forms of glycyl-tRNA synthetase activity with different kinetic properties have been distinguished.

Methods

Escherichia coli B cells were purchased from Grain Processing Co. Unfractionated E. coli B tRNA and enzyme grade ammonium sulfate were purchased from Schwartz/Mann Inc. GF/C glass fiber filters were obtained from Whatman and [^{14}C]glycine (90 mCi/mmol) was purchased from California Bionuclear Corporation. Deoxyribonuclease was obtained from Worthington Biochemicals, DEAE-Sephadex A25 as well as phenylmethylsulfonylfluoride were supplied by Sigma Chemical Company.

Aminoacylation of tRNA at 37^{o} was monitored by determining incorporation of [^{14}C]glycine into acid-precipitable tRNA following the protocol of Folk and Berg (5). The standard assay solution (0.5 ml) contained 1 mM ATP, 1 mM [^{14}C]glycine (3600 cpm/nmol), 2.62 mg/ml unfractionated tRNA, 40 mM $MgCl_2$, 10 mM KCl, 200 µg/ml bovine serum albumin, 4 mM reduced glutathione, and 0.1 M sodium cacodylate (pH 7.1). For saturation experiments, the enzyme preparation was appropriately diluted in 0.1 M cacodylate buffer (pH 7.1) containing, in addition, 30% glycerol to stabilize the enzyme. Reactions were initiated by the addition of one-tenth volume of enzyme solution so that these reaction mixtures contained 3% glycerol. Initial velocities were determined routinely using eight time points.

The procedures described below were used in the purification of glycyl-tRNA synthetase. All manipulations were carried out at 0-4^{o}. Additions of solid $(NH_4)_2SO_4$ were made slowly over a period of 30 min with gentle stirring and the resulting suspension was allowed to stir for one hour before precipitates were separated by centrifugation. Centrifugation was routinely performed at 15,000 x g for 45 minutes.

Twenty grams of E. coli B cell pack was suspended in 20 ml of 0.2 M potassium phosphate buffer (pH 7.0) containing 0.01 M 2-mercaptoethanol, 10% glycerol, 10^{-4} M phenylmethylsulfonylfluoride and 6000 Kunitz units of deoxyribonuclease. The suspension was sonicated with a Sonifier Cell Disruptor (Model W185 - Heat Systems Inc.) for 6 x 2 minutes so that the temperature remained between 2 and 24^{o}. After centrifugation, the supernatant was diluted to 80 ml with the same buffer and adjusted to pH 7.0 if necessary. The crude extract was made 1.37 M $(NH_4)_2SO_4$ (35% saturation) with solid ammonium sulfate. After centrifugation, the supernatant was made 2.34 M $(NH_4)_2SO_4$ (60% saturation), maintaining pH 7.0. The resulting precipitate was collected and dissolved in 80 ml 0.1 M potassium phosphate, 0.1 M Tris-HCl buffer (pH 8.5) containing 0.01 M 2-mercaptoethanol and 10% glycerol, as well as 5 mM each of ATP, glycine, and $MgCl_2$. This solution was made 1.76 M $(NH_4)_2SO_4$ (45% saturation) with solid ammonium sulfate while maintaining pH 8.5. After centrifugation the supernatant was adjusted to pH 7.0 with 2 N HCl containing 1.76 M $(NH_4)_2SO_4$ and 10% glycerol. The solution was then made 2.26 M $(NH_4)_2SO_4$ (58% saturation) while maintaining pH 7.0. The suspension was centrifuged and the precipitate dissolved in 10 ml of 5 mM potassium phosphate buffer (pH 7.0) containing 0.01 M 2-mercaptoethanol and 30% glycerol, and dialyzed against 3 x 1 liter of the same buffer.

Approximately 70 mg of dialyzed protein was applied to a DEAE-Sephadex column (0.6 x 30 cm) which had been equilibrated with 5 mM potassium phosphate buffer (pH 7.0) containing 0.01 M 2-mercaptoethanol and 30% glycerol. After washing with 100 ml of the same buffer, the column was washed with 0.03 M potassium phosphate buffer (pH 7.0) containing 0.01 M 2-mercaptoethanol and 30% glycerol. The column was eluted with a linear gradient between 150 ml of the 0.03 M potassium phosphate buffer and 150 ml of 0.3 M potassium phosphate (pH 7.0) containing 0.01 M 2-mercaptoethanol and 30% glycerol. Maintaining a 0.2 ml/minute flow rate, 2 ml fractions were collected. Columns were run both in the absence and presence of the substrates ATP, glycine, and $MgCl_2$ at a concentration of 5 mM. Conductivity measurements of the effluent were made using an Industrial Instruments Inc. Model RC-16B2 bridge.

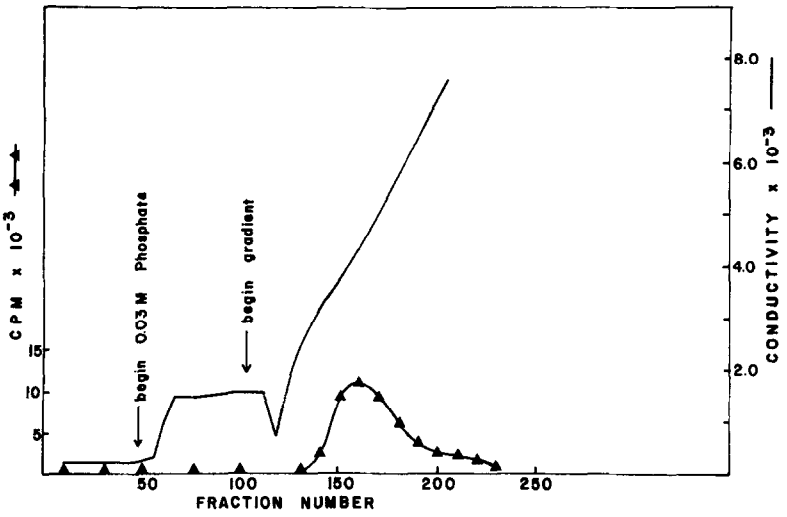

Figure 1: DEAE-Sephadex chromatography elution profile for solubility fractionation protein. No substrate is present in the buffers. Enzyme activity (Δ——Δ) and conductivity (——) of collected fractions were measured.

Results

Initial experiments showed that the glycyl-tRNA synthetase activity in the crude extract precipitated over a wide range of ammonium sulfate concentration (35-60% saturation) with a minimal purification (1.6 fold). Subsequent experiments showed that the presence of substrates caused the enzyme to precipitate over a narrower range of ammonium sulfate concentration, and that the enzyme's solubility was increased at elevated pH. Using these facts, the solubility fractionation procedure as described in Methods was developed and gave a much improved purification (4.1 fold).

To investigate the effect of substrates further, equal portions of a dialized protein sample from the solubility fractionation were applied to identical DEAE-Sephadex columns. One column had no substrates added and the elution data are summarized in Figure 1. A broad activity profile can be seen. The other column had 5 mM ATP, glycine, and $MgCl_2$ in all buffers and the results are shown in Figure 2. The activity eluted from the second column in significantly fewer fractions and was separated into two distinct peaks. Thus,

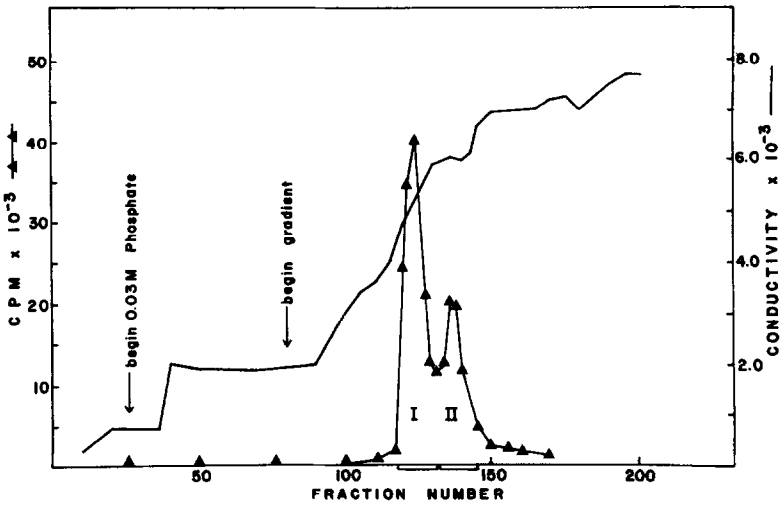

Figure 2: DEAE-Sephadex chromatography elution profile for a sample of the same solubility fractionation protein as was used for the elution shown in Figure 1. Present in all buffers are 5 mM ATP, glycine, and $MgCl_2$. Enzyme activity (Δ——Δ) and conductivity (——) of collected fractions were measured.

the presence of substrates led to increased chromatographic resolution. It is interesting to note, however, that the ionic strength eluting half of the activity was not shifted by substrates. The peak fractions were collected and designated peak I and peak II. Each peak had a specific activity twenty five-fold greater than that of the crude extract.

The results of kinetic measurements using these enzymes are shown in Figure 3. Peak I enzyme gave a sigmoidal saturation curve while peak II enzyme exhibited a hyperbolic dependence of velocity on tRNA concentration. Invarient substrates were maintained at standard (saturating) concentrations. Eadie-Hofstee plots of these data are also presented in Figure 3. Peak II activity gave a linear plot expected for Michaelis-Menten enzymes while peak I yielded a bell-shaped curve characteristic of many regulatory enzymes. A Hill plot of these data gave a value for the interaction coefficient, n, of 2.2 ± 0.2. Peak I enzyme showed Michaelis-Menten behavior, however, with respect to ATP and glycine. Double reciprocal plots were linear and gave K_m values of

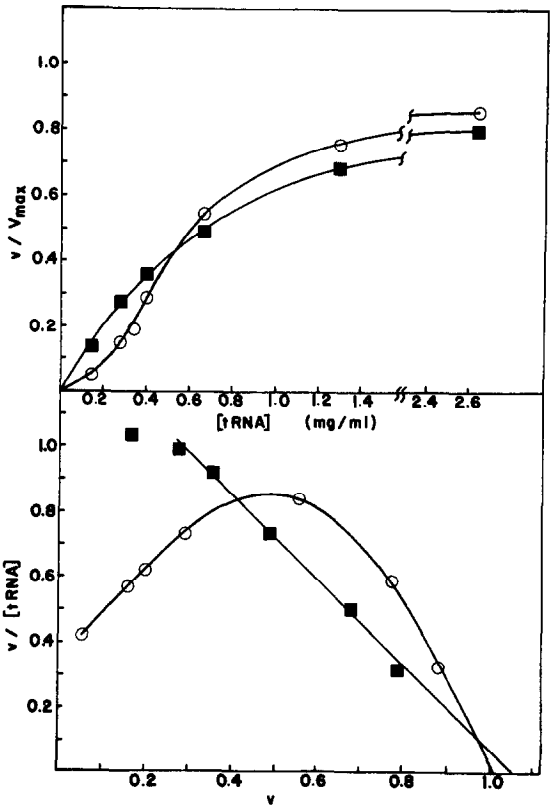

Figure 3: Kinetic saturation of glycyl-tRNA synthetase after DEAE-Sephadex chromatography; enzyme activity from Peak I (O-O) and Peak II (■—■) was determined as a function of tRNA concentration as described in Methods. The data are plotted in the upper panel as normalized initial velocity vs substrate concentration. Eadie-Hofstee plots of these data are shown in the lower panel; V_{max} values were determined from the x-intercepts of the Eadie-Hofstee plots.

8×10^{-5} M and 1.5×10^{-4} M for ATP and glycine respectively in good agreement with the values previously reported (4).

Discussion

Two forms of glycyl-tRNA synthetase have not been observed previously in E. coli extracts. It seems likely that the resolution of the enzyme activity into two forms is not an artifact of our purification procedure since the enzymes display different kinetic properties.

Our purification protocol which employed a protease inhibitor and elimi-

nated the "autolysis" step used by Ostrem and Berg (4) should minimize proteolysis in extracts. It is possible, however, that the two enzyme forms we have observed were generated by limited proteolysis as has been shown (6) for leucyl-tRNA synthetase. Final determination of the physical differences between these two forms, however, must await complete purification of substantial amounts of the enzymes reported here.

We regard the fact that one form of glycyl-tRNA synthetase shows sigmoidal saturation kinetics with limiting tRNA as a significant finding since kinetic properties of this type are unusual among the synthetases. This finding is not without precedent, however, since yeast seryl-tRNA synthetase has been shown to exhibit sigmoidal saturation with both limiting tRNA and serine (7). The value we have obtained for the Hill coefficient (2.2 $\pm$ 0.2) indicates a minimum of two interacting catalytic sites; however, a decision as to whether the enzyme displays true cooperative interactions between subunits or whether some other mechanism is responsible for the sigmoidal kinetics must await further study. Whatever the structural basis, it seems likely that the sigmoidal kinetics observed here have significance with regard to the formation of glycyl-tRNA *in vivo* and that they may contribute to the regulation of protein biosynthesis.

The combined use of three substrates during purification caused the glycyl-tRNA synthetase activity to precipitate over a more narrow range during ammonium sulfate fractionation and to elute more sharply from DEAE-Sephadex columns. The basis for this effect may be the conversion of the bulk of the enzyme population to the glycyl-AMP complex. Without addition of these substrates in relatively high concentrations, the total enzyme may exist as a mixture of free enzyme and enzyme bound to various substrates, intermediates, or products. Since aminoacyl-AMP is tightly bound to many synthetases and since this binding can lead to stabilization of labile synthetases, we suggest that this approach may prove useful in the purification of other synthetases as well.

The isolation of two glycyl-tRNA synthetase forms from Bacillus brevis has been reported recently (8). In this case, however, only one enzyme is capable of catalyzing tRNA aminoacylation. Since both enzyme forms reported here are fully active it appears unlikely that we have observed an identical phenomenon.

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